Universidade do Minho
Escola de Ciências

Ana Margarida Loureiro Piloto Cerqueira

Fluorescent photolabile protecting groups based on O and N heterocycles

outubro de 2013

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Fluorescent photolabile protecting groups based on O and N heterocycles

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Professora Doutora Maria do Sameiro Torres Gonçalves

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"Usa a capacidade que tens.

A floresta ficaria silenciosa se

só o melhor pássaro cantasse."

(Oscar Wilde)

Ao Nuno,

à nossa família.

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A todos a minha sincera gratidão!

ABSTRACT

Fluorescent photolabile protecting groups based on O and N heterocycles

The choice of specific protecting groups remains of crucial importance in the success of many steps of organic synthesis and manipulation of polyfunctional molecules, since they prevent the formation of undesired side products and reactions.

The use of light of appropriate wavelength can liberate in a spatial and temporal controlled release, synthetic or biologically relevant molecules from its light-sensitive conjugated inactive precursor, covalently bonded to the functional group.

These photoremovable protecting groups (PPGs), known as phototriggers or cages in biochemistry, have been widely applied in recent years, as an alternative to classical acid- and base-labile protecting groups, including in phosphates, thiols, amines, alcohols and carboxylic acids. The use of fluorescent photolabile protecting groups allows the visualization, quantification, and the follow-up of spatial distribution, localization, and depletion of the active compound through the monitoring of its fluorescent caged precursor using fluorescence techniques. This broad range of applications supports the development of new photolabile groups with enhanced properties to be suitable for biological purposes, such as large molar extinction coefficients and high photolysis efficiency above 350 nm, which will allow fast cleavage and release of the corresponding functionality and the use of longer wavelengths to minimise side reactions or cell damage. Nevertheless, among the considerable number of light-sensitive groups that have been reported, only few cleave in a practical time by irradiation at wavelengths longer than 400 nm.

As part of our research on the design, synthesis and evaluation of new fluorescent (hetero)aromatics, different fluorescent skeletons, such as nitrobenzyl, naphtho-oxazoles, acridines, benzocoumarins as well as their thionated, oxazole- and julolidine-fused analogues, were synthesized, to provide a new contribution to the collection of photoremovable protecting groups.

Their application as photosensitive groups for the protection of the carboxylic acid function was studied with amino acids, such as valine and phenylalanine; the

neurotransmitter amino acids, glycine, alanine, glutamic acid, β-alanine and γ-aminobutyric acid, as well as with the short chain fatty acid, butyric acid, as model molecules, by coupling through ester bonds to the carboxylic acid terminal.

The behaviour of these conjugates was evaluated under different photolysis conditions, namely the wavelength of irradiation and the solvent system (mixtures of organic solvents and aqueous buffer solutions) in a photochemical reactor equipped with lamps of 254, 300 and 350 nm. Given the nature of the bioactive molecules under study and considering the prospective photorelease of this type of compounds in biological media, it is desirable that the photocleavage occurs in short irradiation times at such a wavelength that is compatible with the media to minimize potential side effects. As a result, cleavage with lamps of 419 nm was tested for those conjugates that had short irradiation times at 350 nm. A cleavage kinetic study was undertaken, by monitoring the irradiated reaction mixture by high performance liquid chromatography (HPLC) with UV spectrophotometry detection. In some cases the monitoring was also done by ^{1}H NMR spectroscopy.

For some compounds, time-resolved fluorescence measurements were made to elucidate the dynamics of the photolysis process and determine the decay kinetics.

The characterization of all new synthesized compounds was made by the usual spectroscopic techniques (UV/vis absorption, FT-IR and ^{1}H and ^{13}C NMR) and analytical techniques (mass spectrometry). Considering the fluorescence properties of the obtained compounds, the fluorescence emission parameters (wavelength of maximum emission, relative fluorescence quantum yield and Stokes´s shift) were also determined.

RESUMO

Grupos protetores fotocliváveis fluorescentes baseados em heterociclos de O e N

A escolha criteriosa de grupos protetores reveste-se de enorme importância no sucesso da síntese orgânica envolvendo vários passos, bem como na manipulação de moléculas polifuncionais, uma vez que previnem a formação de muitos produtos e reações secundárias indesejáveis

O uso de radiação com o comprimento de onda adequado permite a libertação controlada espacial e temporal de moléculas sintéticas e biológicas relevantes, a partir dos respetivos precursores inativos, covalentemente ligados e sensíveis à luz.

Estes grupos protetores fotocliváveis, conhecidos como "phototriggers" ou "cages" em bioquímica, têm sido amplamente aplicados nos últimos anos em alternativa aos grupos protetores clássicos cliváveis por ação de ácidos ou bases, na proteção de fosfatos, tióis, aminas, álcoois e ácidos carboxílicos. Além disso, o facto dos grupos protetores fotocliváveis serem fluorescentes, permite a visualização, quantificação e localização do composto ativo, através da monitorização da distribuição espacial e desaparecimento do precursor por técnicas de fluorescência. Esta vasta gama de aplicações impulsiona o desenvolvimento de novos grupos fotolábeis com propriedades melhoradas para fins biológicos, designadamente possuindo coeficientes de extinção molar e eficiência de fotólise acima dos 350 nm elevados. Estas características possibilitam a clivagem e libertação rápida do composto ativo em comprimentos de onda mais elevados, menos prejudiciais para as células e minimizam possíveis reações secundárias. No entanto, entre o vasto número de grupos fotossensíveis que têm surgido, apenas um número reduzido clivam com tempos de irradiação praticáveis a comprimentos de onda superiores a 400 nm.

Atendendo aos interesses de investigação da equipa em que este trabalho se inseriu e que incluem o design, a síntese e avaliação de novos compostos (hetero)aromáticos, foram sintetizadas diversas estruturas fluorescentes derivadas de nafto-oxazole, acridina, benzocumarina e respetivos tionados, bem como análogos de

anéis fundidos de benzocumarinas com oxazole e julolidina, de modo a contribuir para o aumento da diversidade de grupos protetores fotocliváveis disponíveis.

A sua aplicação como grupos fotossensíveis para a proteção da função ácido carboxílico foi estudada usando os aminoácidos valina e fenilalanina, os neurotransmissores glicina, alanina, ácido glutâmico, β-alanina e o ácido γ-amino butírico, bem como o ácido butírico, como representante de ácidos carboxílicos de cadeia curta, por acoplamento através de uma ligação éster ao terminal ácido carboxílico.

Foi avaliado o comportamento destes conjugados face a diferentes condições de fotólise, nomeadamente o comprimento de onda de irradiação e sistemas de solvente (mistura de solventes orgânicos e soluções tampão aquosas), num reator fotoquímico equipado com lâmpadas de comprimento de onda de 254, 300 e 350 nm. Dada a natureza das moléculas bioativas em estudo e de forma a considerar possíveis aplicações de fotolibertação em meio biológico, é desejável que a fotoclivagem ocorra com tempos de irradiação curtos a um comprimento de onda que seja compatível com o meio para minimizar potenciais efeitos indesejáveis. Dessa forma, testou-se a clivagem ao comprimento de onda de 419 nm, para os conjugados com tempos de irradiação curtos a 350 nm. Foi realizado o estudo cinético da fotoclivagem por monitorização da mistura reacional irradiada por cromatografia líquida de elevado desempenho (HPLC) com deteção espetrofotométrica na zona do UV. Em alguns casos, foi também realizada a monitorização através de espetroscopia de RMN de ^{1}H.

Para alguns compostos foram efetuadas medições de fluorescência resolvidas no tempo para melhor elucidar o processo de fotólise e determinar a cinética de decaimento.

Foi feita a caraterização de todos os compostos novos sintetizados, pelas técnicas espetroscópicas usuais (absorção no UV/vis, IV e RMN de ^{1}H e ^{13}C) e por técnicas analíticas (espetrometria de massa). Dadas as propriedades de fluorescência dos compostos obtidos, foram também determinados os parâmetros de emissão de fluorescência (comprimentos de onda de emissão máxima, rendimento quântico relativo de fluorescência e desvio de Stokes).

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CHAPTER 1 - Introduction

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SYMBOLS AND ABBREVIATIONS LIST

A	adenine
abs	absorption
Ac	acetyl group
Acm	(acridin-9-yl)methyl group
Acm-OH	(acridin-9-yl)methyl alcohol
ACM	(7-acetoxycoumarin-4-yl)methyl group
ACN	acetonitrile
AcOEt	ethyl acetate
AcOH	acetic acid
aeg-PNA	*N*-(2-aminoethyl)glycine peptide nucleic acids
Ala	alanine
m-AMSA	*N*-(4-(acridin-9-ylamino)-3-methoxyphenyl)methanesulfonamide
Aqmoc	(anthraquinon-2-yl)methoxycarbonyl group
Arg	arginine
Asn	asparagine
Asp	aspartic acid
BA	butyric acid
Bba	(9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl) methylene or (9-methoxy-3*H*-naphtho[2,1-*b*]pyran-3-one) methylene
BBHC	[8-[bis(carboxymethyl)aminomethyl]-6-bromo-7-hydroxycoumarin-4-yl] group
BBHCM	[8-[bis(carboxymethyl)aminomethyl]-6-bromo-7-hydroxycoumarin-4-yl]methyl group
BBHCMOC	[8-[bis(carboxymethyl)aminomethyl]-6-bromo-7-hydroxycoumarin-4-yl]methoxycarbonyl group
Bbp	(6-methoxy-2-oxo-2*H*-benzo[*f*]benzopyran-4-yl) methyl group or (6-methoxy-2*H*-naphto[1,2-*b*]pyran-2-one)methyl group
BCMACM	[7-[bis(carboxymethyl)amino]coumarin-4-yl]methyl group
BCMCM	[6,7-bis(carbonylmethoxy)coumarin-4-yl)]methyl group

BECMCM	[6,7-bis(ethoxycarbonylmethoxy)coumarin-4-yl)]methyl group
BHC	6-bromo-7-hydroxycoumarin
BHCM	(6-bromo-7-hydroxycoumarin-4-yl)methyl group
BhC/Ac	(6-bromo-7-acetoxycoumarin-4-yl)methyl group
BHCMOC	(6-bromo-7-hydroxycoumarin-4-yl)methoxycarbonyl group
BHIA	4,5-bis(*N*,*N*-di(2-hydroxyethyl)iminomethyl)acridine
BHQ	(8-bromo-7-hydroxyquinoline-2-yl)methyl group
BHQ-OAc	(8-bromo-7-hydroxyquinolin-2-yl)methyl acetate
BMCM	6-bromo-7-methoxycoumarin
Bn	benzyl group
Boc	*tert*-butyloxycarbonyl group
*t*Bu	*tert*-butyl group
C	cytosine
cAMP	cyclic adenosine monophosphate
CANBP	2-(4'(bis(carboxymethyl)amino)-4-nitro-[1,1'-biphenyl]-3-yl)propan-1-ol
CDI	*N,N'*-carbonyldiimidazole
CDNI-Glu	4-carboxymethyl-5,7-dinitroindolinylglutamate
CMCM	(7-carboxymethoxycoumarin-4-yl)methyl group
CHQ-OAc	(8-chloro-7-hydroxyquinolin-2-yl)methyl acetate
CNS	central nervous system
CyHQ	(8-cyano-7-hydroxyquinolin-2-yl)methyl group
CT	charge transfer
δ	chemical shift in nuclear magnetic resonance
d	days
d	doublet
δ_a	two-photon absorption cross-section
δ_{unc}	uncaging cross-section
dA	deoxyadenosine
DAS	decay associated spectra
DBHCMOC	[6-bromo-8-(diethylaminomethyl)-7-hydroxycoumarin-4-

	yl]methoxy-carbonyl group
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
dC	deoxycytidine
DCC	*N,N'*-dicyclohexylcarbodiimide
DCDHF	dicyanomethylenedihydrofuran
DCM	dichloromethane
dd	double doublet
Ddz	α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl group
DEACM	[7-(diethylamino)coumarin-4-yl]methyl group
dG^{DEACM}	O^6- DEACM-caged deoxyguanosine phosphoramidite
DMACM	7-dimethylamino(coumarin-4-yl)methyl group
DMAP	4-*N,N'*-dimethylaminopyridine
DMB	3,5-dimethoxybenzoin
DMCM	(6,7-dimethoxycoumarin-4-yl)methyl group
DMF	*N,N*-dimethylformamide
DMNB	4,5-dimethoxy-2-nitrobenzyl group
DMNPB	(3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl group
DMSO-d_6	hexadeuterated dimethylsulfoxide
DNA	deoxyribonucleic acid
DNB	2,6-dinitrobenzyl group
DNBOC	di(nitrobenzyl)oxycarbonyl group
DNPM	2,2'-dinitrophenylmethyl group
DOPA	L-3,4-dihydroxyphenylethylamine
dt	double triplet
ε	molar extinction coefficient
EAA	excitatory amino acids
EANBP	2-(4'-(bis((2-methoxyethoxy)ethyl)amino)-4-nitro-[1,1'-biphenyl]-3-yl)propan-1-ol
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
equiv.	equivalent
ESI	electrospray ionization

Et	ethyl group
eT	electron transfer
EtOH	ethanol
FAB	fast atom bombardment
Flu	fluorophore
Fm	9-fluorenylmethyl group
Fmoc	9-fluorenylmethoxycarbonyl group
fs-TA	femtosecond transient absorption
Φ	quantum yield
Φ_F	relative fluorescence quantum yield
Φ_{Phot}	photochemical quantum yield
Φ_{unc}	uncaging quantum yield
G	guanine
GABA	γ-aminobutyric acid
GC	gas chromatography
GIT	gastrointestinal tract
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GM	Göppert-Mayer
h	hour
HAT	photo-induced hydrogen atom transfer
o-HC	*o*-hydroxycinnamate
HCM	(7-hydroxycoumarin-4-yl)methyl group
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
His	histidine
HMBC	heteronuclear multiple bond coherence
HMQC	heteronuclear multiple quantum coherence
hν	radiation
HOBt	1-hydroxybenzotriazole
HOMO	highest occupied molecular orbital

HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
5-HT	5-hydroxytryptamine
Hz	hertz
p-HP	*p*-hydroxyphenacyl group
p-HPAA	*p* hydroxyphenylacetic acid
IAA	inhibitory amino acids
ICAM-1	intercellular adhesion molecule-1
I_0	incident photon flux
Ile	isoleucine
i-PrOH	isopropylalcohol
IR	infrared
IRF	instrumental response function
ISC	intersystem crossing
J	coupling constant
Jul	11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl)methyl group
k	rate constant
k_c	rate constant for the initial bond cleavage
k_r	radiative rate constant
k_{nr}	non-radiative rate constant
λ	wavelength
λ_{abs}	wavelenght of maximum absorption
λ_{exc}	wavelength of excitation
λ_{em}	wavelength of maximum emission
$\Delta\lambda$	Stokes´s shift
LG	leaving group
LR	Lawesson`s reagent
LUMO	lowest unoccupied molecular orbital
m	multiplet
M	molar

M^+	molecular ion
MCM	(7-methoxycoumarin-4-yl)methyl group
MDNI-Glu	4-methoxy-5,7-dinitroindolinylglutamate
Me	methyl group
MeNPOC	3,4-(methylenedioxy)-6-nitrophenylethoxycarbonyl group
MeNPOM	α-methyl-(6-nitropiperonyloxymethyl) group
MeNV	methyl-6-nitroveratryl group
MeNVOC	methyl-6-nitroveratryloxycarbonyl group
MeOH	methanol
Me_4Si	tetramethylsilane
μL	microliter
min	minute
MNI	4-methoxy-7-nitroindolinyl group
MNNM	7-methoxy-3-nitro-2-naphthalenemethanol group
Mp	melting point
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
o-NB	*o*-nitrobenzyl group
p-NB	*p*-nitrobenzyl group
NBS	*N*-bromosuccinimide
ND	not determined
NDBF	nitrodibenzofuran group
NI	7-nitroindolinyl group
nm	nanometer
NMR	nuclear magnetic resonance
1-NPE	1-(2-nitrophenyl)ethyl group
NPEOC	1-(2-nitrophenyl)ethyloxycarbonyl group
NPm	naphthalene group
NPP	2-(*o*-nitrophenyl)-propyl group
NPPOC	1-(2-nitrophenyl)propyloxycarbonyl group
NV	6-nitroveratryl group

NVOC	6-nitroveratryloxycarbonyl group
υ	wavenumber
ns	nanosecond
Nu	nucleophiles
OPE	one photon excitation
PBHCMOC	{6-bromo-7-hydroxy-8-[(piperazin-1-yl)methyl]coumarin-4-yl}methoxycarbonyl group
PBS	phosphate-buffered solution
PCM	(7-propionyloxycoumarin-4-yl)methyl group
PENB	(4′-tris-ethoxymethoxy-4-nitrobiphenyl-3-yleth-2-yl)methyl group
Ph	phenyl group
Phe	phenylalanine
pJ	picojoule
PMNB	(4′-methoxy-4-nitrobiphenyl-3-yleth-2-yl)methyl group
PPA	polyphosphoric acid
PPE	polyphosphate ester
PPGs	photoremovable protecting groups
ppm	parts per million
ps	picosecond
Px	9-phenylxanthyl group
S-Px	9-phenylthioxanthyl group
Pym	pyrene group
RP	reverse-phase
RNA	ribonucleic acid
R_f	retention factor
rt	room temperature
RuBi	ruthenium-bipyridine
s	singlet
S_0	singlet ground state
S_1	first excited singlet state

SCFA	short chain fatty acid
Ser	serine
S_N1	unimolecular nucleophilic substitution
T	thymine
τ	lifetime
t	triplet
$t_{1/2}$	half-lives
T_1	first excited triplet state
Tba	(9-methoxy-3-thioxo-3*H*-benzo[*f*]benzopyran-1-yl)methyl group
TBS	*tert*-butyldimethylsilyl group
*t*Bu	*tert*-butyl group
TCSPC	time-correlated single-photon counting
THF	tetrahydrofuran
THQ	1,2,3,4-tetrahydroquinoline
t_{irr}	irradiation time
TLC	thin layer chromatography
$TMSCHN_2$	trimethylsilyldiazomethane
TNF-α	tumor necrosis factor-α
TPA	two-photon absorption
TPE	two-photon excitation
TRES	time resolved emission spectra
TRIS	tris(hydroxymethyl)aminomethane
UV	ultraviolet
UV/vis	ultraviolet/visible
UV/LED	ultraviolet/light emission diode
Vac	9-acrylamidoacridine
Val	valine
W	watt
Z	benzyloxycarbonyl group

THESIS OUTLINE

The present dissertation is divided into four chapters and a description of each chapter is outlined.

Chapter 1 is a brief introduction comprising general aspects of carboxylic acid targets used as models of (bi)functional molecules, as well as the O-, N- and S-heteroaromatic units, which constitute the skeleton of the photoremovable protecting groups developed in the research work performed. An overview of photoremovable protecting groups focusing on the chemical structures, release mechanism, scope, limitations and applications concerning the release of organic molecules of biological relevance will be given. Based on a literature review of recent years, it includes selected examples of one-photon and two-photon excitation groups, as well as a discussion of the possibility of selective-wavelenght deprotection-chromatic orthogonality. This chapter is intended to better contextualize the obtained experimental results and act as a complement to the articles introductions.

Chapter 2 comprises a compilation of articles, published or submitted to international scientific journals, in the ambit of this doctoral thesis. Each article is presented as a sub-chapter and the formatting has been uniformed, and does not correspond to the journal formatting where it was published or submitted to. The organization of this second chapter is as follows:

2.1 "Photorelease of amino acids from novel thioxobenzo[*f*]benzopyran ester conjugates", *Amino Acids*, **2012**, *42*, 2275-2282.

2.2 "Long-wavelength photolysis of amino acid 6-(methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl)methyl esters", *European Journal of Organic Chemistry*, **2011**, 5447-5451.

2.3 “Photolytic release of butyric acid from oxygen- and nitrogen-based heteroaromatic cages”, *European Journal of Organic Chemistry*, **2012**, 922-930.

2.4 “Acridinyl methyl esters as photoactive precursors in the release of neurotransmitter amino acids”, *Photochemical and Photobiological Sciences*, **2013**, *12*, 339-347.

2.5 “Photoinduced release of neurotransmitter amino acids from novel coumarin fused julolidine ester cages”, *European Journal of Organic Chemistry*, DOI: 10.1002/ejoc.201300730.

2.6 "Photoactivable heterocyclic cages in a comparative release study of butyric acid as a model drug", submitted to *European Journal of Organic Chemistry*.

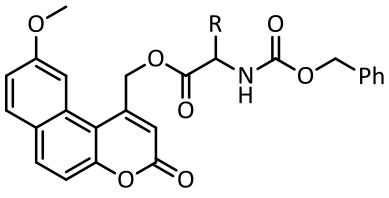
2.7 “Wavelength-selective cleavage of *o*-nitrobenzyl and polyheteroaromatic benzyl protecting groups”, accepted for publication in *Tetrahedron*.

Chapter 3 presents a general overview of results by comparison of experimental results reported in the articles in chapter 2.

Chapter 4 presents the conclusions of the research reported in this dissertation and considerations for future work.

COMPOUNDS LIST

Chapter 2.1 "Photorelease of amino acids from novel thioxobenzo[*f*]benzopyran ester conjugates", *Amino Acids*, **2012**, *42*, 2275-2282.



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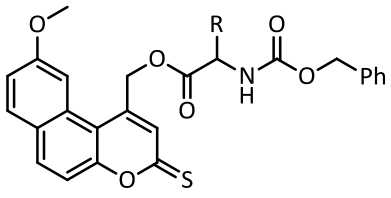

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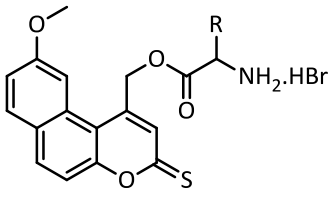

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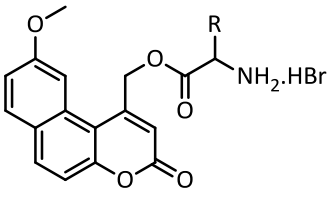

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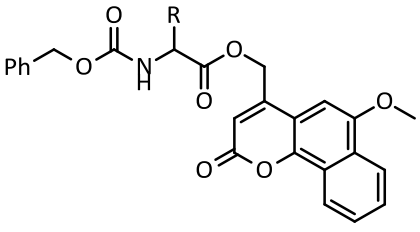
a R = $CH(CH_3)_2$
b R = CH_2Ph

Chapter 2.2 "Long-wavelength photolysis of amino acid 6-(methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl)methyl esters", *European Journal of Organic Chemistry*, **2011**, 5447-5451.



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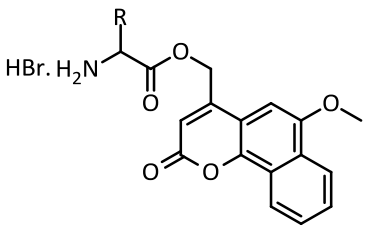

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a R = $CH(CH_3)_2$
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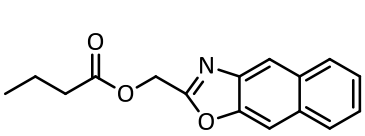
Chapter 2.3 "Photolytic release of butyric acid from oxygen- and nitrogen-based heteroaromatic cages", *European Journal of Organic Chemistry*, **2012**, 922-930.



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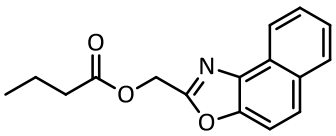

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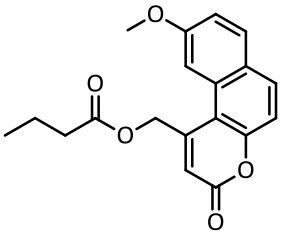

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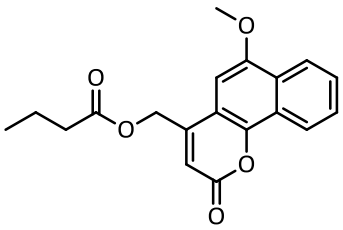

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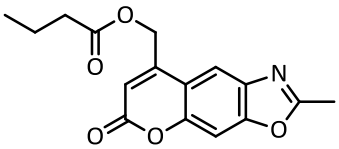

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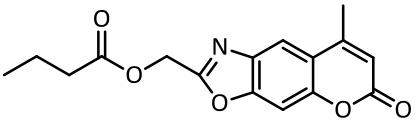

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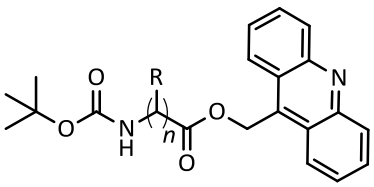
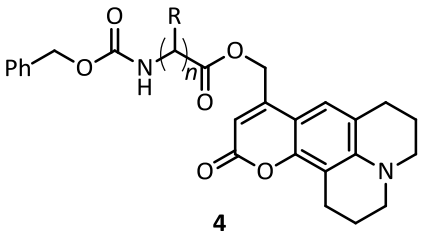
Chapter 2.4 "Acridinyl methyl esters as photoactive precursors in the release of neurotransmitter amino acids", *Photochemical and Photobiological Sciences*, **2013**, *12*, 339-347.



3

a *n* = 1, R = H
b *n* = 1, R = Me
c *n* = 1, R = $(CH_2)_2CO_2Me$
d *n* = 2, R = H
e *n* = 3, R = H

Chapter 2.5 “Photoinduced release of neurotransmitter amino acids from novel coumarin fused julolidine ester cages”, *European Journal of Organic Chemistry*, DOI: 10.1002/ejoc.201300730.



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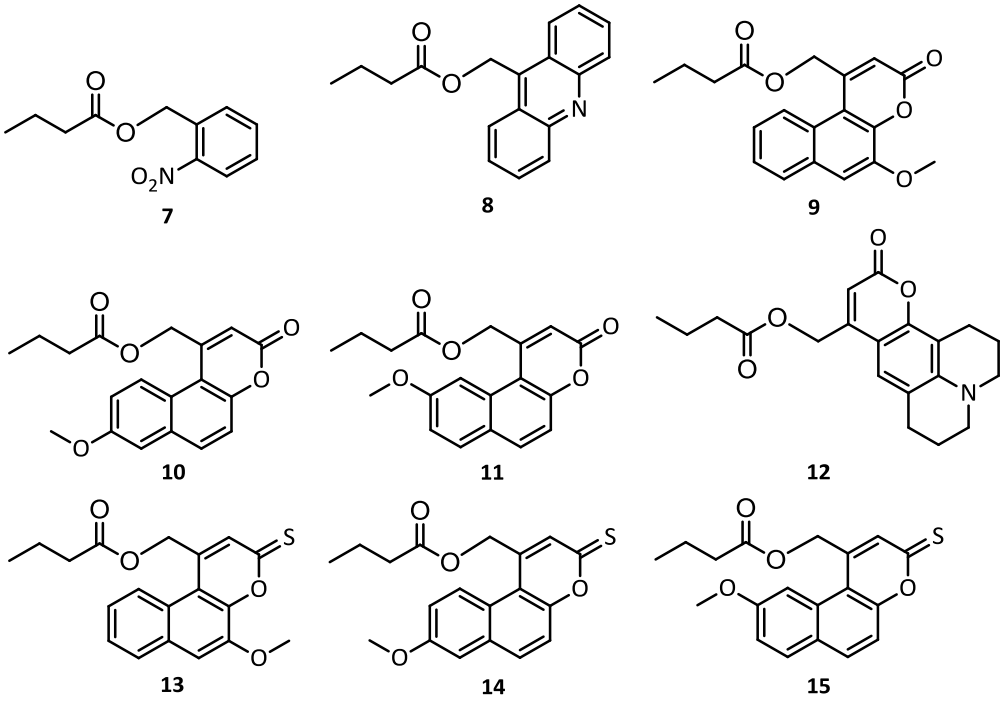
a *n* = 1, R = H
b *n* = 1, R = Me
c *n* = 1, R = $(CH_2)_2CO_2Me$
d *n* = 2, R = H
e *n* = 3, R = H

Chapter 2.6 "Photoactivable heterocyclic cages in a comparative release study of butyric acid as a model drug" submitted to *European Journal of Organic Chemistry.*

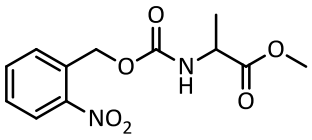


7 **8** **9**

10 **11** **12**

13 **14** **15**

Chapter 2.7 “Wavelength-selective cleavage of *o*-nitrobenzyl and polyheteroaromatic benzyl protecting groups”, accepted for publication in *Tetrahedron*.



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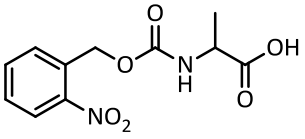

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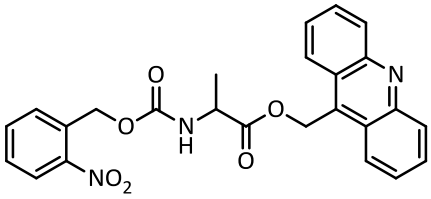

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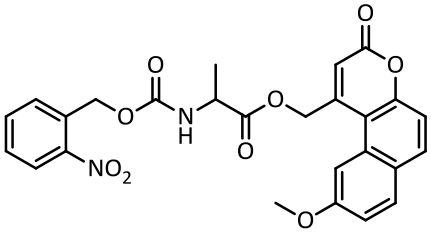

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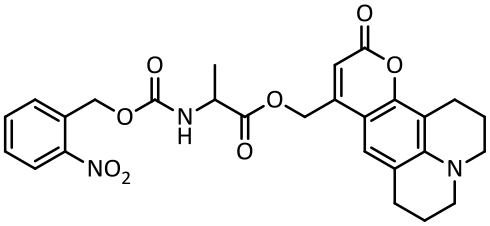

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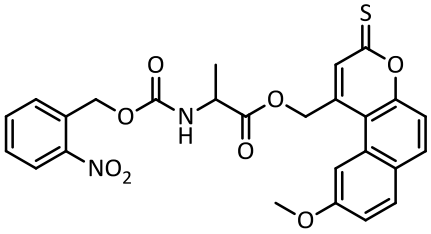


9

Chapter 1

Introduction

1. General aspects

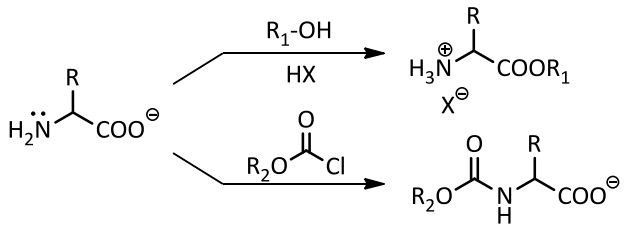
1.1. Carboxylic acid targets

1.1.1. Amino acids

The term amino acid is almost always used to refer to an α-amino carboxylic acid. Amino acids undergo many of the standard reactions of both amines and carboxylic acids. However, conditions for some of these reactions must be carefully selected, so that the amino group does not interfere with a carboxyl group reaction, and *vice versa*. These reactions are often used to protect either the carboxyl group or the amino group while the other function is being modified or coupled to another amino acid. The requirement for protecting groups represents a shortcoming from the concept of an ideal organic synthesis, which should be a simple, safe and effective process that proceeds quickly and in quantitative yield from readily available, inexpensive starting materials. Despite some interesting examples of protecting group-free syntheses reported in recent years, most of the synthetic work is still accomplished by using classical protecting group chemistry, with its inherent drawbacks.[1]

Amino acids can be esterified by treatment with a large excess of an alcohol and an acidic catalyst (often gaseous hydrochloric acid). Under these acidic conditions, the amino group is present in its protonated form ($-NH_3^+$), so it does not interfere with esterification. Aqueous acid hydrolyses the ester and regenerates the free amino acid.

Acylation of the amino group is also done to protect it from undesirable nucleophilic reactions. The amino group is often protected with the *N*-benzyloxycarbonyl group, or with the *tert*-butyloxycarbonyl group, the amide half of a carbamate ester (a urethane), which is more easily hydrolyzed than most other amides (Scheme 1).



Scheme 1. Protection at the C- and N-*terminus* of amino acids.

Several other types of protecting groups for the α-amino and carboxylic acid functions associated to different methodologies of protection/deprotection have been developed and recently revised.[2,3] Within the present work, protecting groups that can be cleaved by irradiation at a specific wavelength without chemical reagents will be outlined.

Over the last years evidences have been accumulating to indicate that amino acids are the most widely distributed neurotransmitters in the central nervous system (CNS). These small molecules are present at high concentrations in all cells probably because of their key role in protein synthesis and metabolism.[4]

Concentration changes in these molecules due to misformations in their synthesis, receptor's expression or errors in their metabolism, have been shown to influence cognitive processes, like learning and memory and to relate to some neurological disorders, such as schizophrenia, anxiety, epilepsy as well as Alzheimer's or Parkinson's diseases. Brain chemistry research uses analysis methods of neurotransmitters that may provide means for the diagnosis and treatment strategies for neurological disorders. The low concentration of the neurotransmitters set high demands on the matrices and on the analysis methods, which often are biological or contain high concentrations of salts.[5]

From a simplistic point of view, two major effects of neurotransmitters conduct changes in the membrane potencial, inducing depolarization and hiperpolarization states. As a consequence, when a selective ion channel is opened, the membrane conductance to specific ions like Na^+, K^+, Ca^{2+} and Cl^-, which typically flow down their electrochemical gradients, is modified. This two opposing alterations in neuron membrane potentials are commonly referred to excitatory or inhibitory action, depending on whether it produces depolarization or hyperpolarization of the neural membrane, respectively.[6]

Glutamate is present in nearly all functions of the CNS (which includes the brain and the spinal cord), specially those related to metabolism and transmittion (Figure 1). Also, glutamate acts in neurons as a precursor for γ-aminobutyric acid (GABA) and in astrocytes as a precursor for glutamine.[7] In addition, glutamate is a fundamental molecule in neural processes, namely synapse formation, dendrite pruning, cell migration, differentiation and death.

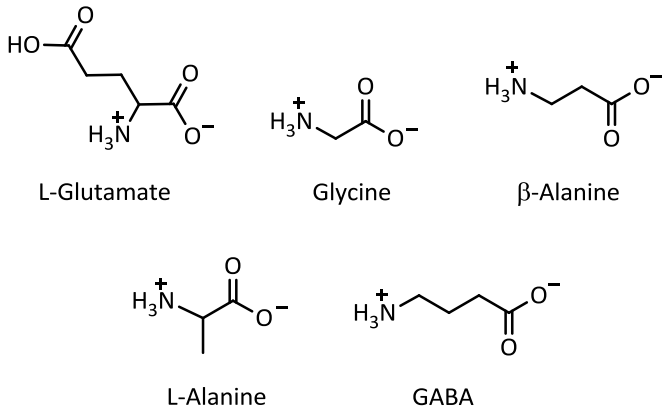


Figure 1. Structures of amino acid neurotransmitters.

Glycine is consigned mostly to the spinal cord and medullary parts of the brain stem. Its capacity to inhibit neural action by increasing their membrane permeability to chloride and potassium ions, acting as a postsynaptic inhibitory transmitter, causes a hyperpolarization, mainly at the spinal cord synapses. This postsynaptic inhibitory action is generally more marked on spinal neurons than on supraspinal neurons.[8]

β-Alanine (β-Ala), the only occurring beta amino acid acting as a physiological transmitter, is the rate-limiting precursor of carnosine, which is a β-alanine-histidine dipeptide present in muscle and brain tissues.[9] Alanine (Ala) is also an inhibitory neurotransmitter and acts as an agonist of ionotropic glycinergic and GABA receptors.[10]

γ-Aminobutyric acid, GABA is the precursor of glutamate and the major inhibitory neurotransmitter found within the CNS of vertebrates, having widespread involvement in numerous processes that include maintenance of homeostatic synaptic plasticity and circadian rhythms. GABA has been identified as the postsynaptic inhibitory transmitter of glutamate, acting by a membrane mechanism indistinguishable from that of glycine. Other functions assigned to GABA like mediator of the presynaptic inhibition are still subject of some controversy.[8]

Neurotransmitter amino acids are examples of biomolecules where the caging strategy (temporary suppression of activity through a protecting group) has been largely applied, in this case through an ester linkage of a suitable photoreleasable group (including coumarins) to their carboxylic terminal.[11-13] Due to their importance, in the present research work, neurotransmitter amino acids were used as model of heterobifunctional molecules in the evaluation of some newly developed

photoremovable protecting groups (PPGs). This also aims to give a contribution for the variety of protecting groups that can be suitable for neurotransmitter molecules.

1.1.2. Butyric acid

The short chain fatty acids (SCFAs) are weak acids, containing up to seven carbon atoms in a linear or branched-chain, with a $pK_a \leq 4.8$. They are mainly originated from the bacterial fermentation of plant materials, like celluloses, fibres, starches and sugars at the intestines of mamals, because they lack the necessary enzymes for their degradation. Among the SCFAs, acetic, propionic and butyric acid (BA) exist in higher amounts in the fermentation chambers of the gastrointestinal tract (GIT) and 90-99% as anions rather than as free acids, once the pH there is nearly neutral.[14]

Butiric acid has received much attention relating to its anticancerous properties. The effects of BA, however, depend on the experimental model (*in vivo*, *in vitro*) and the state of the cells (normal or cancerous),[15] the degree of inflammation[16] and the doses used.[17] In addition to the recognised effects on intestinal metabolism, BA shows indirect effects that contribute to the general metabolism of animals. In all tissues BA is a natural component of cellular metabolism. It may also act as a growth promoter when added to diets at low doses (0.1-0.5 g/kg).[18]

Apoptosis is a programmed death process in normal cells. However, cancer cells do not undergo apoptosis, and continue to multiply. In experiments with lab animals, BA has been found to inhibit multiplication of cancerous colonocytes and stimulate apoptosis in the cells.[19]

Despite several strategies to overcome common drawbacks on the use of prodrugs - pharmacologically inert chemical derivatives, likely to be converted into active drug molecules, some limitations still remain, such as low oral drug absorption, lack of site specificity, chemical instability, toxicity and poor patient acceptance.[20,21]

Prodrugs based on butyric acid, upon intracellular hydrolytic degradation, release acids and aldehydes, being formaldehyde - a specific anticancer actuator and BA - a histone deacetylase inhibitor, the main responsibles for the anticancer action.[23] Prodrugs that release formaldehyde increase apoptosis of cancer cells, at concentrations about 10-fold lower than butyric acid and 100 times faster. The formaldehyde released

from these prodrugs has been shown to be a critical antiproliferative factor that induces differentiation and cell death.[24]

Considering the interest in the development of alternative prodrugs of butyric acid, new light sensitive prodrugs were synthesized and the release of butyric acid evaluated at different wavelengths of irradiation, within our research work.

1.2. O-, N-, and S-heteroaromatics

1.2.1. Coumarin derivatives

Coumarins (trivial designation of 2-oxo-2*H*-benzopyrans) consist of a large class of compounds made of fused benzene and α-pyranone rings (Figure 2).

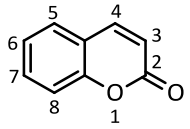


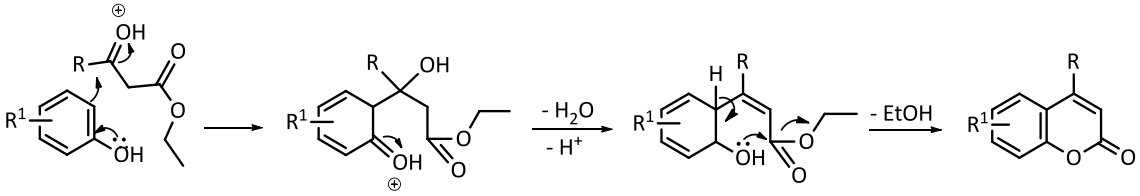
Figure 2. Basic structure of coumarins.

These compounds are widely used as additives in food, cosmetics, pharmaceuticals, dyes, insecticides and in chemosensores.[25-30] Several natural coumarins possess particular biological relevance, being associated to antitumoral,[31] anti-HIV,[32] antioxidative,[33] anticancerous[34] and antimicrobial activities.[35]

As further mentioned in this dissertation, coumarins have also been used in the last three decades as PPGs for various functionalities on target molecules. The present research work also aims to give a contribution by increasing the variety of coumarin derivatives that can be suitable as alternative PPGs for carboxylic acids.

Regarding the synthesis, these compounds can be obtained by several methods, such as Pechmann, Perkin, Knoevenagel, Reformatsky, Witting and Claisen. However, the Pechmann reaction is one of the simplest and more direct method since it proceeds from very simple starting materials, namely phenols, β-ketoesters and malonates utilizing various catalysts, mineral acids like sulfuric acid, trifluoroacetic acid, phosphorus pentoxide and Lewis acids.[36]

In the presence of catalytic acids, the carbonyl group of the β-ketoester is protonated and undergoes intermolecular nucleophilic attack by the phenol derivative. The intermediate structure loses one molecule of water and a proton; a new intramolecular nucleophilic attack by the hydroxyl group at the ester carbonyl group leads to the closure of the pyran ring (Scheme 2).[37]



Scheme 2. Pechmann mechanism for coumarin synthesis.

1.2.2. Thiocoumarin derivatives

Organosulfur compounds are valued for their interesting and diverse chemistry that opens the possibility for acting as versatile reagents in organic synthesis and provide useful properties in a broad range of applications. Among them are thiocoumarins, the thio-analog of coumarins which, despite their importance, have received relatively little attention.[38]

A simple substitution of the oxygen on the carbonyl group by a sulfur atom, frequently imparts a significant change in the properties of the resulting compound. This was proved, for example, in the work of Kumar *et al.* where coumarins **1**-**4** and thiocoumarins **6**-**10** were evaluated for their ability to modulate TNF-α induced ICAM-1 expression and for the inhibition and termination of initiation and propagation steps of NADPH-catalyzed microsomal lipid peroxidation, respectively, in order to examine their anti-oxidant property (Figure 3).[38] The authors verified that dihydroxy and diacetoxy derivatives of thiocoumarin were more potent in comparison to the corresponding coumarin derivatives in inhibiting TNF-α-induced expression of ICAM-1. Nevertheless, coumarin derivatives revealed more powerful in comparison to the corresponding thiocoumarins in inhibiting microsomal lipid peroxidation. Heterocycles **5** and **7** were also evaluated for their inhibitory activity on TNF-α-induced ICMA-1 expression and the

results established that thiocoumarin **7** is better than coumarin **5**.

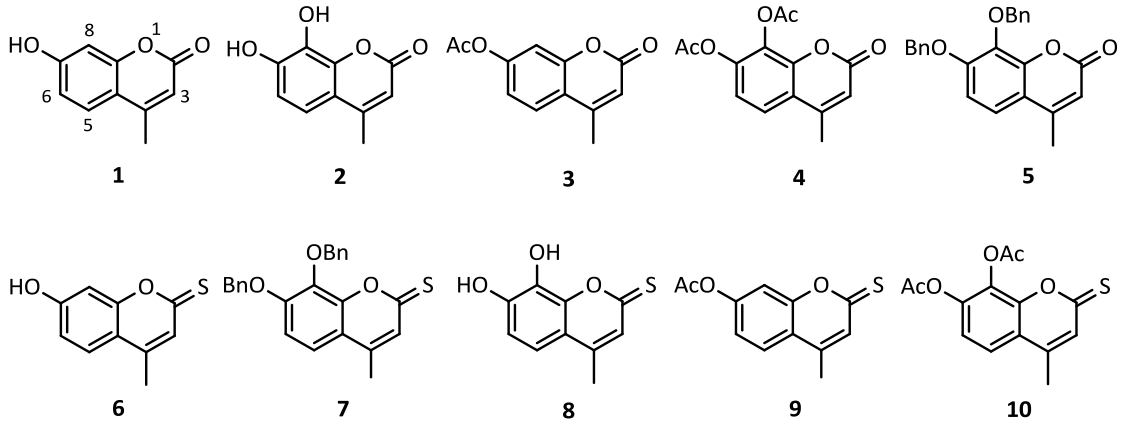
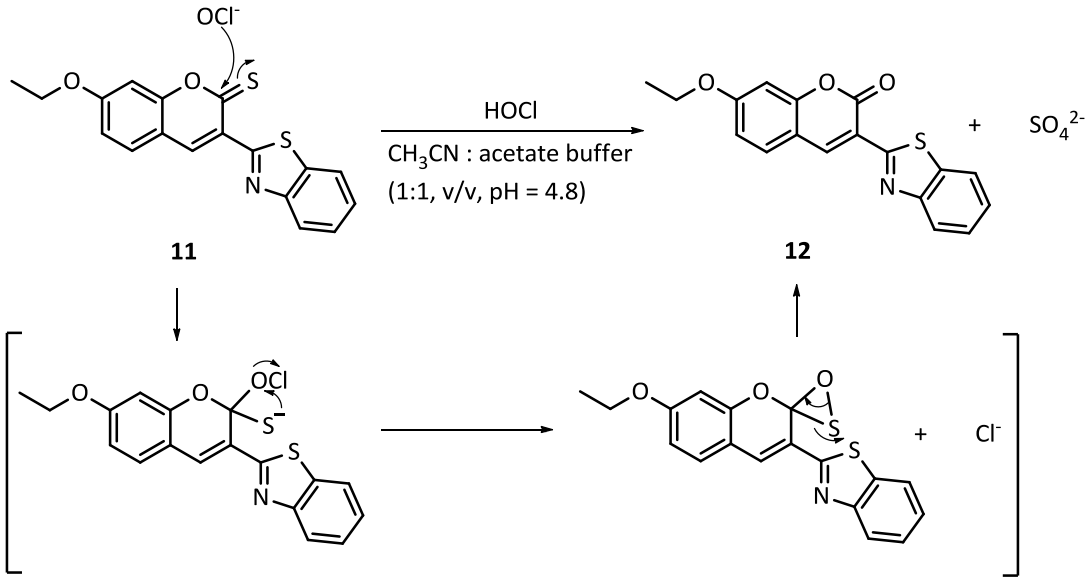


Figure 3. Structures of coumarins **1**-**5** and thiocoumarin derivatives **6**-**10**.

In recent years, desulfurization of thiocarbonyl function has been used in the design of probes for the signaling of metallic cations[39] and commonly used oxidants, such as *m*-chloroperbenzoic acid.[40] Transformation of thiocarbonyl to carbonyl function has advantages in the design of probe systems, such as pronounced chromogenic behavior, which could be used for colorimetric analysis and marked “off-on” type fluorescence changes.

Thiocoumarins are among the compounds used for this purpose and have been recently investigated as hypochlorous acid-selective signaling system.[41] The thio derivative of ethoxycoumarin **11** showed selective and efficient colorimetric and fluorogenic signaling behavior over other commonly used oxidants in an aqueous environment (Scheme 3). The signaling is due to the oxidative desulfurization of the thiocarbonyl moiety by HOCl to yield oxocarbonyl function. The interference from Hg^{2+} ions was successfully removed by using bromide ions as a masking agent. HOCl signaling was possible, with a detection limit of 8.3×10^{-7} M in the presence of common metal ions and anions as background. As suggested by the authors, the designed probe could be useful for the determination of hypochlorous acid in chemical and environmental samples.



Scheme 3. Signaling of hypochlorous acid by thicoumarin **11**.

Taking advantage of the photophysical and photochemical properties of thio(benzo)coumarin derivatives, another interesting application is related to their use as PPGs, which has been recently explored by our research group and within the present dissertation and will be further discussed.

Thionation is a suitable and efficient method towards the synthesis of precursors of various active molecules from biological/industrial point of view. Several methods are reported in the literature for the thionation of organic compounds which make use of various thionating agents (either alone or in combination) such as phosphorus pentasulfide, elemental sulfur and 2,4-bis(*p*-methoxyphenyl)-1,3-dithiaphosphetane 2,4-disulfide, known as Lawesson's reagent (LR) (Figure 4).[42] LR was made popular in 1978 by Lawesson, who used it for the efficient conversion of oxygen functionalities into their thio analogues. It can be obtained by reaction of phosphorus pentasulfide with methoxybenzene and is nowadays commercially available.[43]

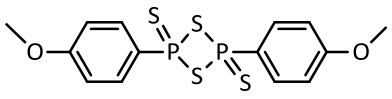


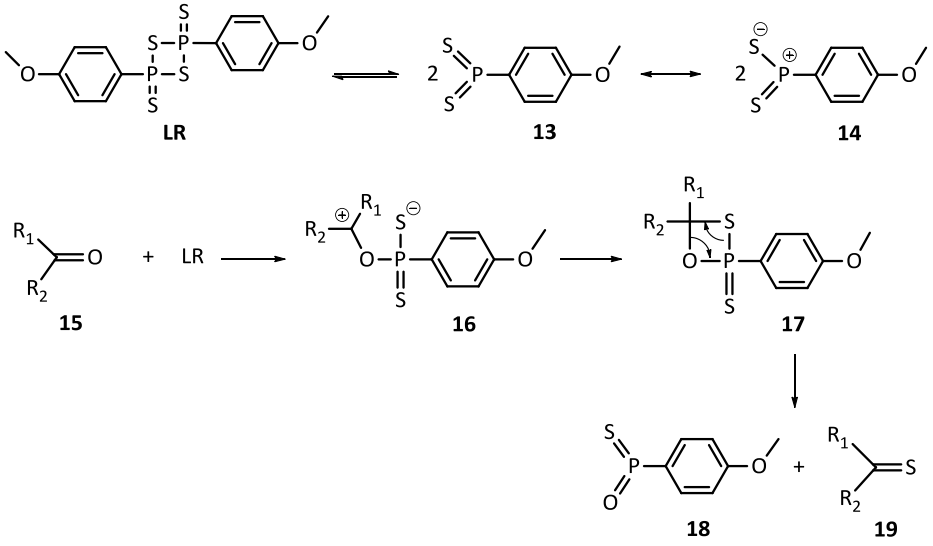
Figure 4. Structure of Lawesson's reagent.

High yield, convenient handling, easy work up, commercial availability, and use of

mild reaction conditions made LR popular among synthetic chemists. Since 1978, increasing numbers of reports on thionation using LR have been published on the synthesis of thio analogues of ketones, flavones, isoflavones, lactones, amides, and esters.[44]

Thiocoumarins can also be obtained from thionation of the corresponding coumarins using Lawesson's reagent and it was the selected method used in the synthesis of the thiobenzocoumarins obtained in the present dissertation. However, sometimes prolonged reaction times in refluxing toluene or xylene are required, since the conversion of esters to thio-esters is difficult because of the generally low reactivity of the ester carbonyl group.

The mechanism of thionation reaction by LR has been established in the literature and is described below (Scheme 4).[43]



Scheme 4. Mechanism of thionation reaction by LR.

The highly reactive dithiophosphine ylide **13**, **14** can be in equilibrium with LR. Both mesomeric structures shown in scheme 4 can react with carbonyl compounds to form thio-oxophosphonate **16**, which decomposes into Wittig-analogs **17** to the corresponding thiocarbonyl compounds **19**.[43]

1.2.3. Oxazole derivatives

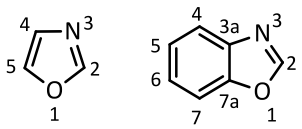
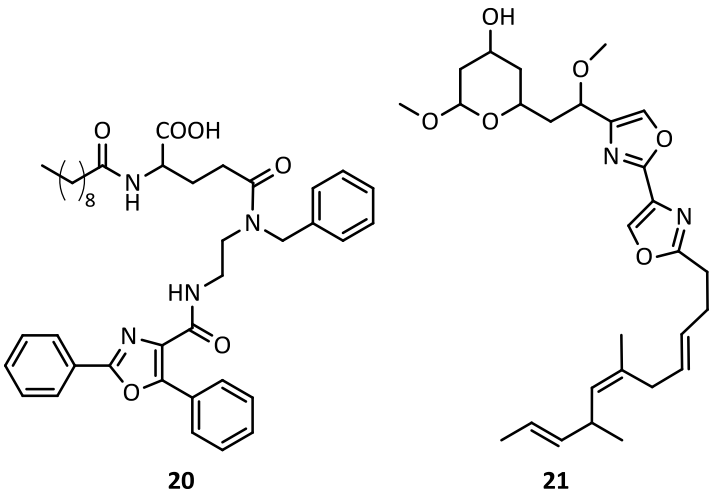


Figure 5. Oxazole and benzoxazole basic structures.

Oxazoles (Figure 5) are a class of five-membered heterocyclic compounds that are believed to occur in nature from post-translational modification of serine and threonine residues in peptides. They are the key building blocks of natural products, pharmaceuticals, and synthetic intermediates. The oxazole ring is associated with various activities such as hypoglycemic, analgesic, anti-inflammatory and antibacterial.[45] Besides, oxazoles show antiproliferative activity against many cancer cells, especially human prostate cancer and human epidermoid carcinoma.[46] For example, compound **20**, an oxazole-containing small molecule, was found to be a potent CDC25 phosphatase inhibitor. The natural product hennoxazole A **21**, which was first isolated from the marine sponge, displays predominant antiviral activity against herpes simplex type I (Figure 6).[47,48]



20 **21**

Figure 6. Examples of oxazole-containing bioactive molecules **20** and **21**.

Furthermore, the oxazole ring is also present in the structure of dyes possessing favorable photophysical properties, namely, strong absorption and luminescence in the

UV/vis spectrum. 2,5-Diaryloxazole derivatives have been the focus of intensive work for the synthesis of *p*-conjugated materials[49] and have also been used in the determination of biological processes such as protein activity and measurement of important ions, namely H^+.[50,51] Their two-photon properties have also been lately reported for applications in multi-photon fluorescence microscopy.[52]

Recently, Mahuteau-Betzer and Piguel[53] prepared a new series of fluorescent dyes that incorporated π-conjugated spacers at the 2- or 5-positions of a 2,5-disubstituted aryloxazole (see as example compounds **22**-**24**) (Figure 7). These compounds showed emissions from visible to 700 nm along with significant Stokes´s shifts up to 208 nm and a strong solvatochromic fluorescence.

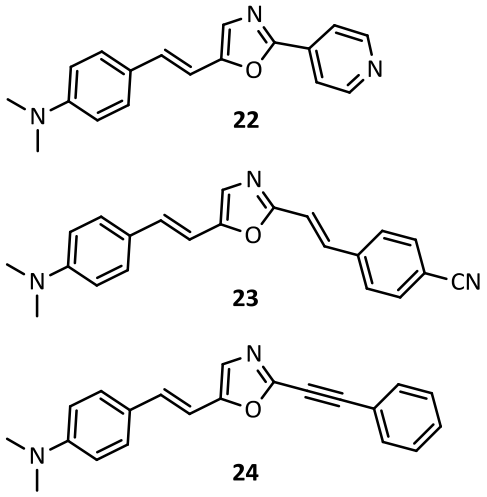
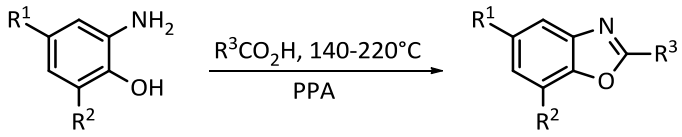


Figure 7. 2,5-Disubstituted aryloxazoles with π-conjugated spacers **22**-**24**.

Moreover, the evaluation of oxazole fused derivatives as PPGs was initiated by our research group in 2010,[54] and owing to the promising results, it was continued in the research work conducted during this dissertation, as it is adressed in Chapters 1 and 2.

Concerning the synthesis of oxazole derivatives, up to date a variety of protocols have been reported. 2-Aminophenols can cyclize to benzoxazoles in the presence of carboxylic acids upon heating to high temperatures, either neat or in a solvent, in the presence of a dehydrating agent (e.g. polyphosphoric acid PPA, polyphosphate ester PPE or boric acid). When equimolar amounts of 2-aminophenols **25** and aryl- or alkylcarboxylic acids are heated, temperatures of 140-220 °C are required for the formation of the corresponding 2-aryl- or 2-alkylbenzoxazoles **26** (Scheme 5).[55] This

strategy is relevant and was also used in the synthesis of oxazole derivatives described in the present dissertation.



25 **26**

Scheme 5. Synthesis of benzoxazoles **26** from 2-aminophenols **25** and carboxylic acids.

1.2.4. Acridine derivatives

Acridine was first isolated in 1870 from the high boiling fraction of coal tar by Carl Grabe and Heinrich Caro, in Germany. Chemically, acridine is an alkaloid from anthracene which is also designated by the names dibenzopyridine, 2,3,5,6-dibenzopyridine, 2,3-benzoquinoline or 10-azaanthracene (Figure 8). Acridine and its homologues are stable compounds of weakly basic character, with a pK_a of 5.6, similar to that of pyridine.

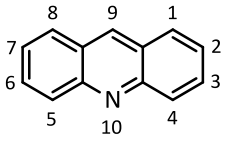
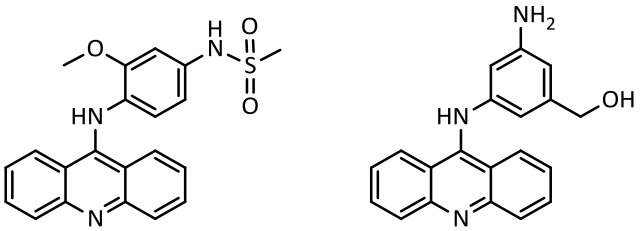


Figure 8. Structure of acridine.

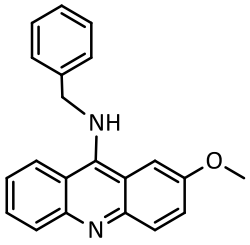
Acridine is one of the most extensively explored DNA intercalating skeletons for developing anticancer, antiviral, antimalarial, and antibacterial agents, and for biological fluorescent probes.[56] The 9-anilinoacridine derivative, *m*-amsacrine (*m*-AMSA, **27**, Figure 9) has attracted much attention for its DNA-binding capability. It exhibits potent cytotoxic activity and has been found to be clinically useful. Amsacrine is active in the treatment of acute leukaemias and lymphomas but is ineffective in solid tumours. Various analogues, such as 3-(9-acridinylamino)-5-hydroxymethylaniline (AHMA, **28**, Figure 9) have been designed and tested for anticancer activities.[57,58]



27 **28**

Figure 9. 9-Anilinoacridine derivatives **27** and **28**.

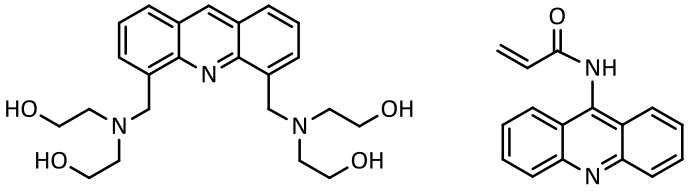
Recently, a series of novel acridine derivatives were synthesized by modifying previously unexplored linkers between the acridine and benzene groups, and evaluated for their antiproliferative activity and DNA-binding ability.[59] Among them, compound **29** demonstrated DNA-binding capability and topoisomerase I inhibitory activity (Figure 10). In K562 cell lines, **29** induced apoptosis through mitochondria-dependent intrinsic pathways.



29

Figure 10. Structure of 2-methoxy-*N*-benzylacridin-9-amine **29**.

On the other hand, acridine derivatives are found to be efficient fluorescent chemosensors for the recognition of transition metal ions such as Cd^{2+}, 4,5-bis(*N*,*N*-di(2-hydroxyethyl)iminomethyl)acridine (BHIA, **30**),[60] and Hg^{2+}, 9-acrylamidoacridine (Vac, **31**),[61] (Figure 11).



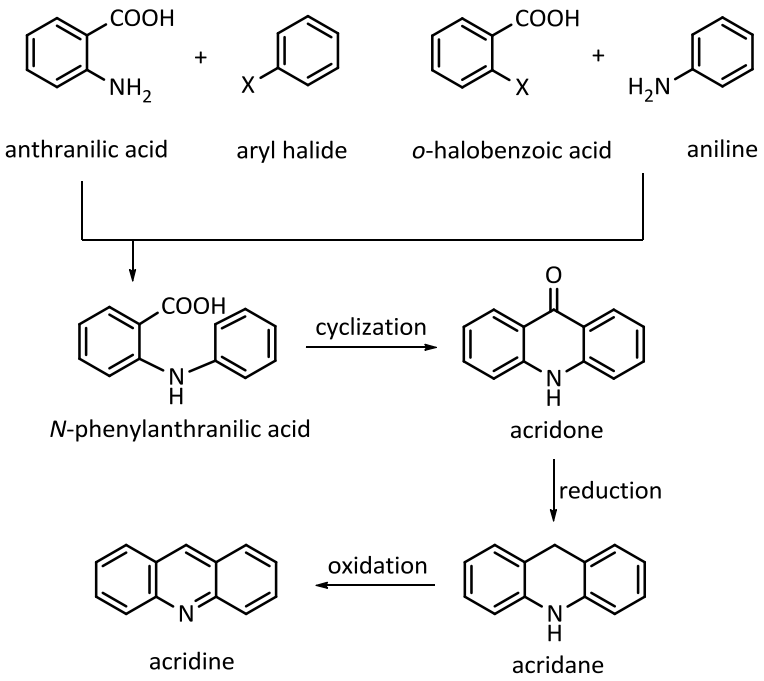
30 **31**

Figure 11. Structures of BHIA **30** and Vac **31**.

In addition, the development of acridine derivatives as PPGs was carried out in our research work, as described in the present dissertation (Chapter 2), and it can represent another area to be further exploited on the application of these heterocycles

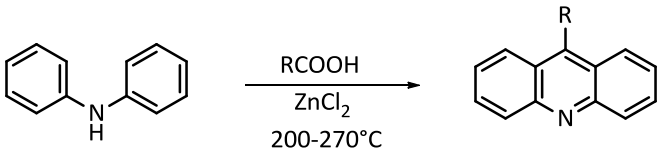
The synthesis of acridine and analogues has attracted considerable attention from organic and medicinal chemists for many years, given the large number of methods that have been reported. The acridine system is susceptible to electrophilic substitution, reaction towards nucleophiles, reduction, oxidation, reductive alkylation and photoalkylation. As a result, several acridine derivatives have been prepared from the acridine moiety through these reaction types.

The condensation of primary amines with aromatic aldehydes/carboxylic acids in the presence of strong mineral acids (sulfuric acid/hydrochloric acid), followed by dehydrogenation, yields acridines (Scheme 6).[62]



Scheme 6. Ullmann synthesis for acridine.

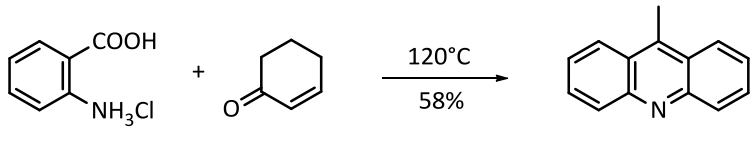
Bernthsen synthesis involves the reaction of diphenylamine **32** with carboxylic acids in the presence of zinc chloride, resulting in the formation of acridine (Scheme 7).



32

Scheme 7. Bernthsen synthesis for acridine.

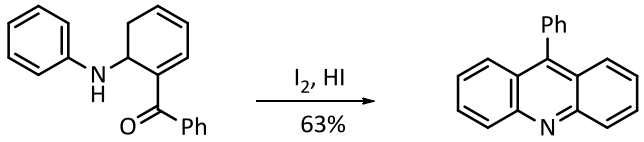
In Friedlander synthesis, the salt of anthranilic acid **33** is treated with cyclohex-2-enone **34** at 120 °C to give 9-methylacridine **35** (Scheme 8).



33 **34** **35**

Scheme 8. Friedlander synthesis for 9-methylacridine **35**.

Another possibility is the reaction of phenyl-(6-phenylaminocyclohexa-1,3-dienyl)methanone **36** in the presence of iodine/hydroiodic acid under heating conditions, leading to 9-phenylacridine **37** (Scheme 9).

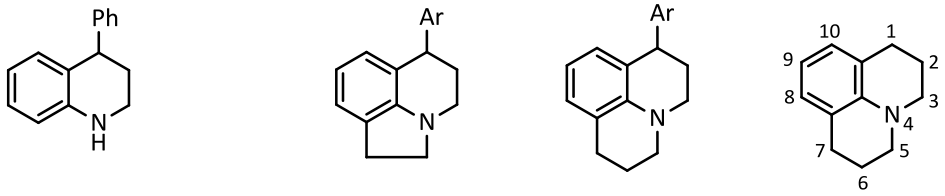


36 **37**

Scheme 9. Synthesis of acridine from C-acylated diphenylamines **36**.

1.2.5. Julolidine derivatives

1,2,3,4-Tetrahydroquinoline (THQ) derivatives constitute key structural elements in several natural products,[63] and find themselves in important applications, for example, as thrombin inhibitors,[64] anticoagulants and multidrug-resistant agents.[65] Various fused tricyclic compounds have in their constitution the THQ moiety, such as 1,2,5,6-tetrahydro-4*H*-pyrrolo[3,2,1-*ij*]quinoline (lilolidine) and 2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*ij*]quinoline (julolidine) (Figure 12).[66]



4-Phenyl-1,2,3,4-tetrahydroquinoline 6-Aryl-lilolidine 1-Aryl-julolidine Julolidine

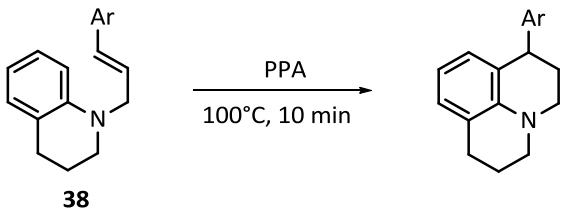
Figure 12. Structures of THQ derivatives including julolidines.

Julolidine, also named 2,3,6,7-tetrahydro-1*H*,5*H*-benzo[*ij*]-quinolizine[67] has been known for over a century and compounds based on its skeleton are extremely valuable. They find applications in industry and also in biological systems, such as photoconductive materials,[68] nonlinear optical materials,[69] highly fluorescent molecular rotors,[70] and chemiluminescent dyes.[71]

However, the use of julolidine derivatives as photoremovable protecting groups is scarce and it will be also discussed here. The present dissertation aims to give a contribution in this field by the evaluation for the first time of a chloromethyl coumarin, built on the julolidine nucleous as PPG (Chapter 2).

Concerning the synthesis, julolidine can be obtained by different procedures. Katritzky *et al.* reported julolidine synthesis based on benzotriazole intermediates. The method is especially useful for the preparation of symmetrically and asymmetrically 1,7-disubstituted julolidines as well as for the introduction of one, two, or three substituents, in any combination, at C-1, C-2, and C-3 of the julolidine system.[67]

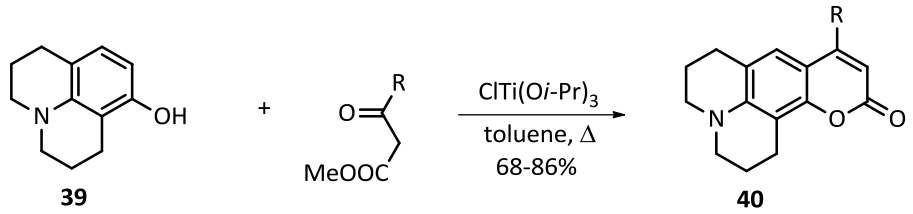
Lingman *et al.* described a straightforward procedure for the synthesis of 1-aryl-julolidines by cyclization of *N*-aryl allyl anilines with polyphosphoric acid.[66] Accordingly, *N*-allyl-1,2,3,4-tetrahydroquiniline derivatives **38** were subjected to cyclization in the presence of polyphosphoric acid at 100-110 °C for 10-15min, affording the corresponding 1-aryl-julolidine in fairly good yields (Scheme 10).



38

Scheme 10. Synthesis of 1-aryl-julolidines.

Recently, Wirtz *et al.* reported the Pechmann condensation of hydroxyjulodine **39** with a range of functionalized β-oxoesters to afford amino-substituted coumarins (julolidine derivatives) **40**. Although Pechmann condensation is suitable for a wide range of phenols, it is critical for aminophenols, probably due to amine protonation and deactivation of the aromatic ring by the strong acid. Therefore, the authors developed a very mild procedure using chlorotriisopropoxytitanium (IV) chloride, which was found to be especially suitable to catalyze the synthesis of 4-substituted coumarins **40** (Scheme 11).[72]

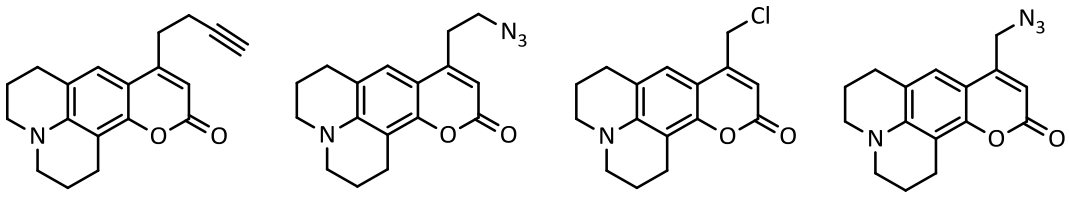


39 **40**

R = CH_2NHZ; $(CH_2)_3NHZ$; $CH_2C(CH_3)_2NHZ$; $CH_2NHCOC(CH_3)NHZ$; CH_3; $(CH_2)_2CH_3$; C_6H_4-4-NO_2

Scheme 11. Synthesis of 4-substituted coumarins **40** *via* modified Pechmann condensation.

Bearing in mind this straightforward protocol, later the authors synthesized several new alkynyl- and azido-substituted coumarins (**41**-**44**), which could be coupled to amino acid and peptide derivatives (Figure 13).[73]



41 **42** **43** **44**

Figure 13. Coumarin derivatives bearing the julolidine moiety **41**-**44**.

2. Photoremovable protecting groups

Photoremovable (also named photolabile, photocleavable or photoreleasable) protecting groups more specifically designated as phototriggers or cages in a biological

perspective, allow the spatial and temporal controlled release, based on sensitivity to light, of numerous chemical and biologically relevant entities. Examples include small inorganic species and ions (NO,[74] CO,[75] Ca^{2+},[76-] Zn^{2+},[77] Cd^{2+},[78] or Cu^{+}),[79] amino acids,[80] nucleotides,[81] peptides,[82] proteins,[83] neurotransmitters,[84] and other cell-signalling molecules.[85] The conversion of prodrugs, pharmacologically inactive derivatives of active agents, through light, as an alternative stimulus to chemical and/or enzymatic triggers, can also be considered in a certain way a particularly innovative application of PPGs.[86-89]

Considering (bio)organic molecules, the use of PPGs include the caging of several functional groups (e.g. phosphates,[81] thiols,[90] amines,[81] alcohols,[91,92] and carboxylic acids[92]).

The requisites of a photolabile protecting group are dependent on the particular application, and no single group needs to satisfy all the following characteristics. A PPG should strongly absorb at wavelengths above 300 nm, where irradiation is less damaging for biological molecules. The photolysis should be clean with efficient release, or high quantum yield. The PPG must be pure, reveal low intrinsic activity, and be stable in media after and during photolysis. It should possess solubility in targeted media and affinity to specific elements e.g., binding sites on cancer cells or the active site of an enzyme. The photolysis byproducts obtained besides the desire active molecule, should preferably be transparent at the irradiation wavelength, in order to eliminate competitive absorption of the excitation wavelengths, as well as non reactive with the system studied.

In order to achieve the required criteria for the various increasingly ambitious applications, researchers have proposed PPGs possessing a large variety of chemical structures. Among the most relevant PPGs are arylcarbonylmethyl groups: *p*-hydroxyphenacyl groups and benzoin groups; nitroaryl groups: *o*-nitrobenzyl groups, *o*-nitro-2-phenethyloxycarbonyl groups and nitroindoline; benzyl groups: simple benzyl groups, polyaromatics and polyheteroaromatics, including coumarin-4-ylmethyl groups (Figure 14).

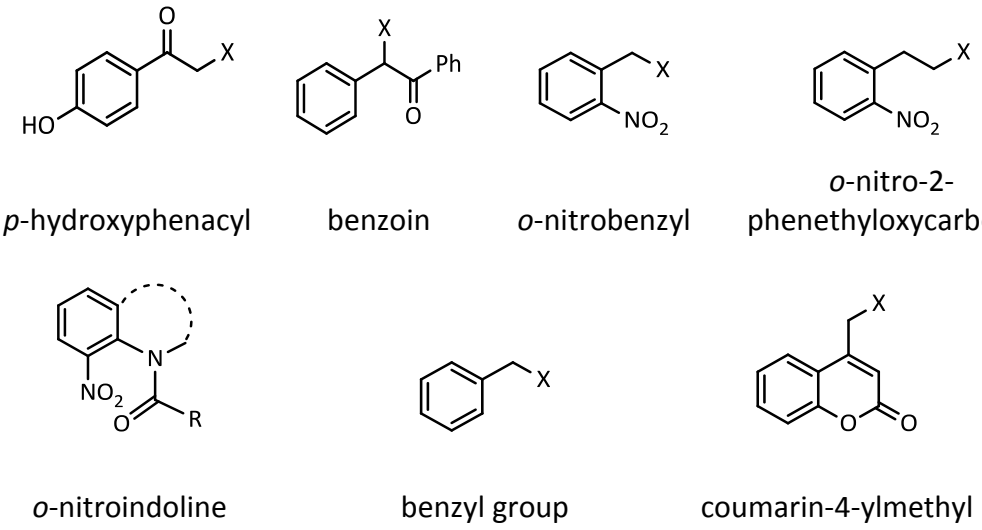


Figure 14. General structures of photoremovable protecting groups.

As a result of the interest in the last decade in the development of PPGs, it is possible to find a considerable amount of research papers, and some revision works published as perspectives and reviews papers in scientific recognised journals, as well as book chapters covering the various applications of PPGs in synthesis and life sciences.[3,93-97]

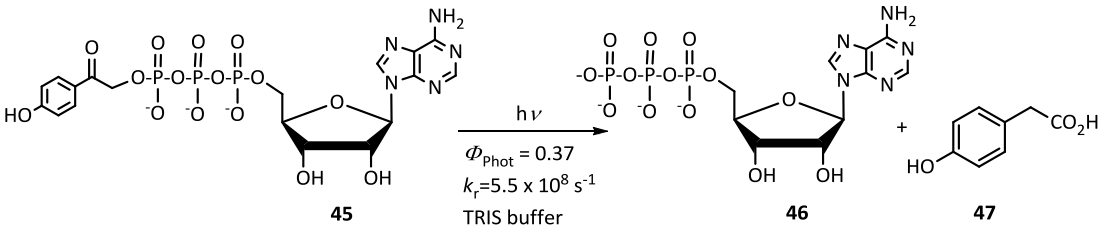
In order to contextualise the research work described in the present thesis, a brief overview of the PPGs focusing on the chemical structures, release mechanisms, scope, limitations and applications concerning the release of organic molecules with biological relevance (amino acids including neurotransmitters, peptides, nucleosides, among others) will be given.

Considering the aim of the present work, polyheteroaromatic analogues of the benzyl group were emphasised. In addition to one-photon, two-photon excitation groups were also considered. The possibility of selective-wavelenght deprotection – a type of orthogonality referred to chromatic orthogonally closes this overview.

2.1. *p*-Hydroxyphenacyl groups

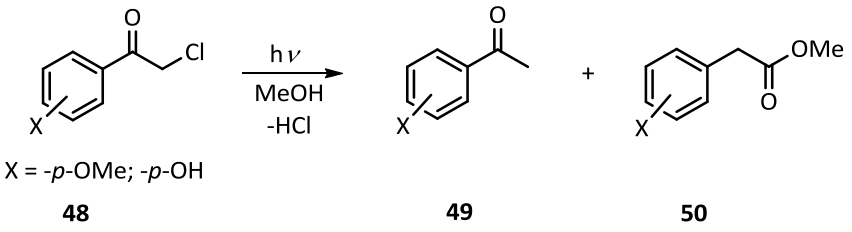
Among the most relevant photoremovable protecting groups is the *p*-hydroxyphenacyl (*p*-HP) moiety, which was used by Givens and Park,[98,99] for the first

time, in the caging of bioactive compounds, namely in the release of ATP **46** from *p*-HP-ATP **45** (Scheme 12).



Scheme 12. Photorelease of ATP from *p*-hydroxyphenacyl-ATP cage.

The initial proposal concerning the use of phenacyl derivatives as photoremovable protecting groups was carried out by Sheehan and Umezawa, being the *p*-methoxyphenacyl used in the release of glycine derivatives. A previous important contribution was made from Anderson and Reese who reported that photoreactions of *p*-methoxy- and *p*-hydroxyphenacyl chlorides **48** rearranged, in part, to the corresponding methyl phenylacetates **50** when irradiated in methanol (Scheme 13). Nevertheless none of earlier researchers exploited the potential of this chromophore as a photoremovable protecting group.[3]



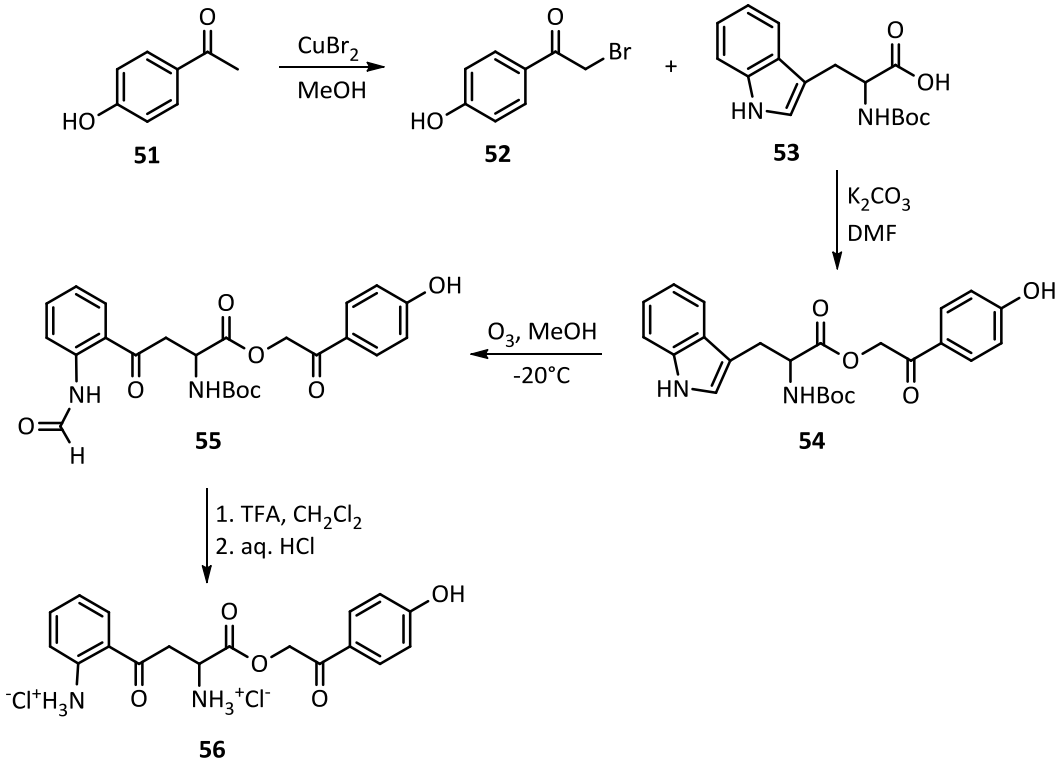
Scheme 13. Photolysis of *p*-methoxy and *p*-hydroxyphenacyl chlorides.

Givens and collaborators continued this research and later other research groups have recognized the importance of this chromophore and have also contributed to the development of the field resulting in several applications of *p*-hydroxyphenacyl as a light-triggered protecting group in neurobiology,[93,100] enzyme catalysis,[96,100] and synthetic organic chemistry.[101]

The most important features of this protecting group include the skeletal rearrangement that occurs together with the release of a substrate, the quantitative

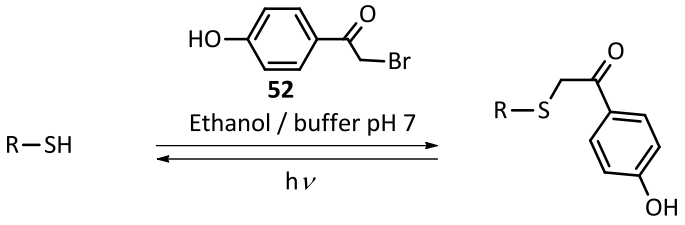
chemical yield of released product, and the necessary role of water.[93,96,98-100,102] Furthermore, the hydrophilicity of the *p*-HP ligand, the high quantum yields, and the unusual clean photolytic reaction that produces only one significant byproduct with an absorption spectrum different from the initial cage, are also remarkable capabilities.

The various applications of the *p*-hydroxyphenacyl group as PPG include the caging of carboxylic acids, thiols and phosphates.[103-105] Recently, Maitrani and collaborators[106] reported the use of *p*-HP in the preparation of the ester conjugate of L-kynurenine **56**, an intermediate in the eponymous metabolic pathway of tryptophan in mammals and some fungi and bacteria. Reaction of *N*-*tert*-butyloxycarbonyl-L-tryptophan **53** with 4-hydroxyphenacyl bromide **52** under basic conditions, followed by ozonolysis resulted in the Boc-protected *N*-formyl kynurenine ester **55**. Subsequent cleavage of *N*-protecting groups and lyophilisation yielded the caged kynurenine as the hygroscopic dihydrochloride salt **56** (Scheme 14). Photolytic studies revealed that L-kynurenine 4-hydroxyphenacyl ester **56** can be uncaged in microseconds with 355 nm laser pulses.



Scheme 14. Synthesis of L-kynurenine 4-hydroxyphenacyl ester dihydrochloride **56**.

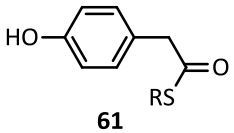
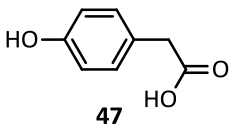
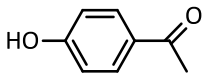
The suitability of *p*-hydroxyphenacyl group in caging the thiol function was demonstrated by Specht and co-workers[104] with a study concerning the synthesis and evaluation towards irradiation (λ = 312 nm) of *p*-hydroxyphenacyl *S*-substituted *N*-Bz-cysteine-OMe **57**, 3-thio-2-deoxythymidine phosphate **58** and glutathione **59** (Scheme 15). The results revealed that over 70% of the free thiol biomolecules were released, together with *p*-hydroxyphenylacetic thioesters **61** derived from the corresponding thiols, as sulfur-containing byproducts (Table 1).



RSH = *N*-Bz-cysteine-OMe **57**
RSH = 3'-Thio-deoxythymidine phosphate **58**
RSH = Glutathione **59**

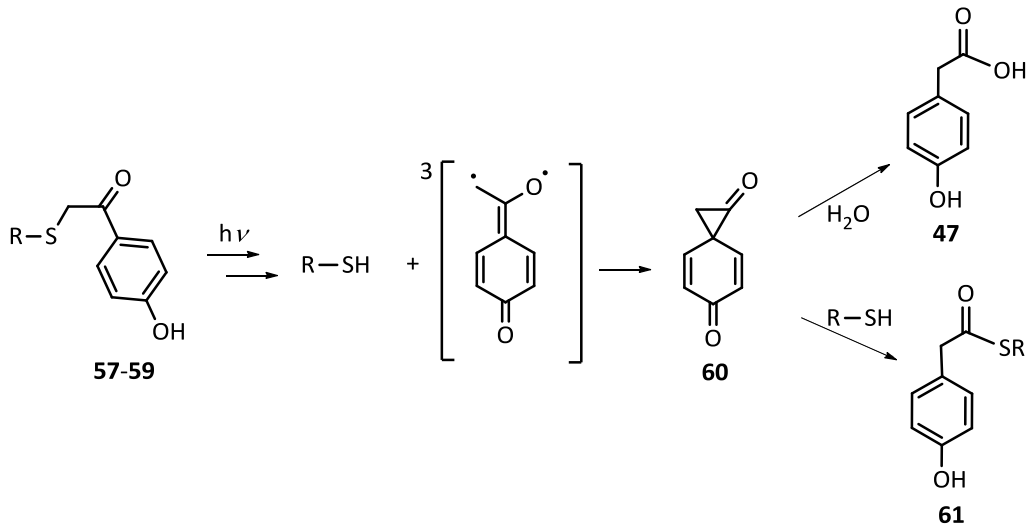
Scheme 15. *p*-Hydroxyphenacyl bromide **52** as photoreversible thiol label.

Table 1. Product yields generated by 312 nm irradiation of 0.1 mM solutions of **57**-**59**, in 100 mM Tris-HCl buffer pH 7.2, 1 mM.

Compound	**57**	**58**	**59**
Photolytic products (%)			
RSH	60	71	ND
 **61**	30	29	23
 **47**	60	62	65
 **51**	10	9	12

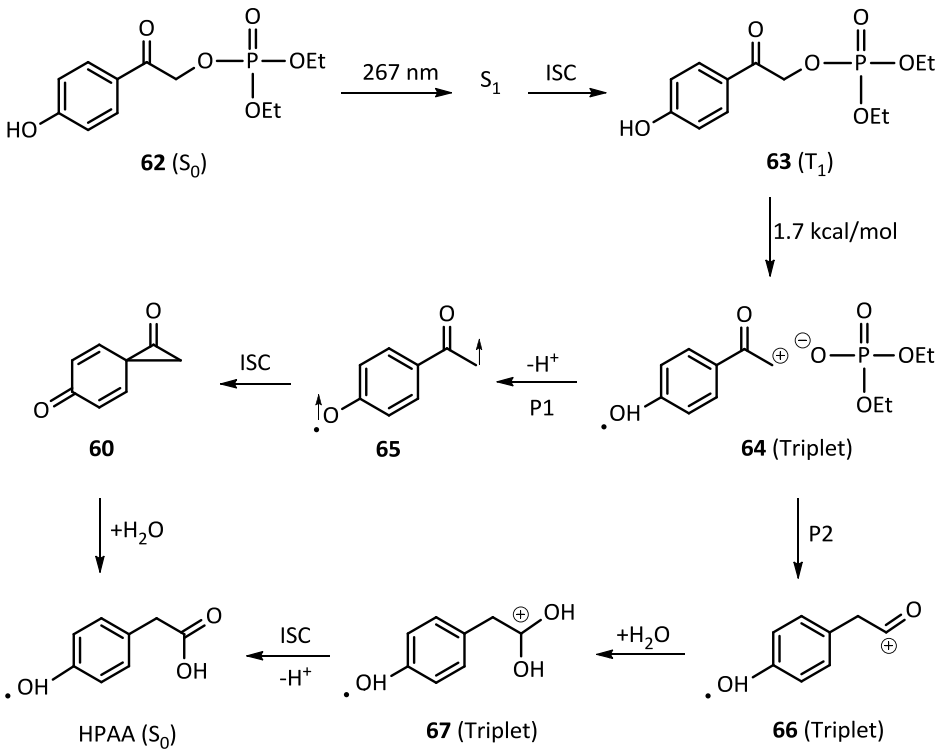
ND - not determined

Although not fully established, the authors suggested a photorelease mechanism of *p*-hydroxyphenacyl cages. A triplet diradical intermediate has been proposed to generate a hypothetical spirocyclopropanone intermediate **60** whose hydrolysis does explain the formation of the *p*-hydroxyphenylacetic acid **47** (Scheme 16).[93,99] Furthermore, the acidic character of thiols (pK_a about 8.4 in water) can allow fragmentations to occur while their strong nucleophilic properties may possibly explain the formation of the thioesters by nucleophilic opening of the spirocyclopropanone.



Scheme 16. Hypothetical photochemical fragmentation mechanism of *p*-hydroxyphenacyl thioethers **57**-**59**.

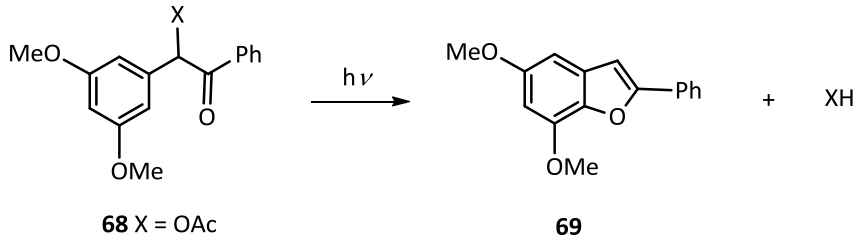
Concerning the application of *p*-HP group in the caging of phosphates, in the last years several studies have been carried out in order to clarify the photochemical mechanism involved in the release of the free active molecule. Recently, Cao *et al.*[107] profiting from gathered knowledge published[108] and based on their experimental and computational results, proposed a detailed reaction mechanism shown in Scheme 17. The authors studied the *p*-hydroxyphenacyl diethylphosphate **62** in aqueous solution investigating the deprotection reaction and subsequent steps including the two probable pathways, P1 and P2, to produce the *p*-hydroxyphenylacetic acid (*p*-HPAA) rearrangement product.



Scheme 17. The reaction pathway for the photo-deprotection and rearrangement reaction of conjugate **62**.

2.2. Benzoin groups

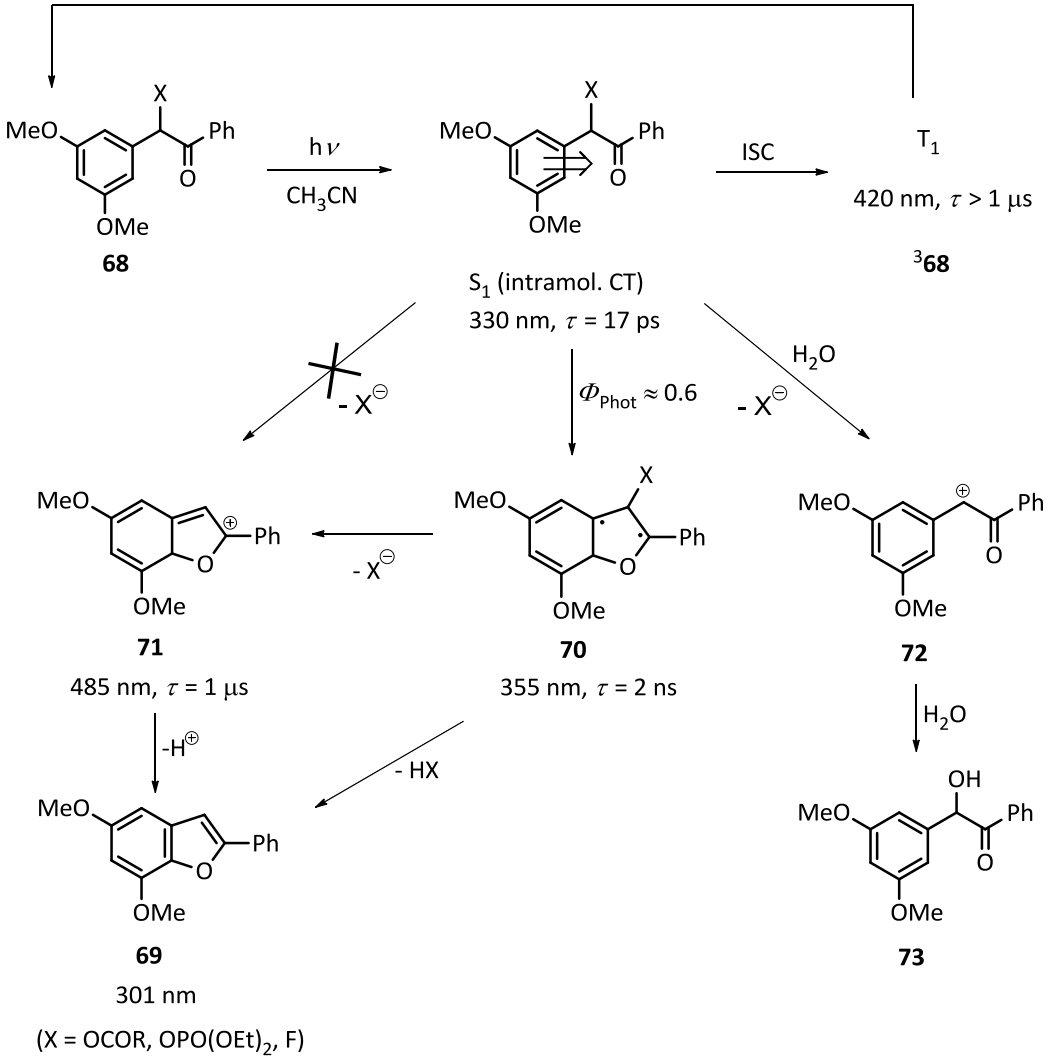
The studies concerning the application of benzoin derivatives in the photorelease of substrates started with the work of Sheehan and Wilson in 1964.[3] The authors verified that the release of acetate occurred along with the formation of 2-phenylbenzofuran from cyclization of the benzoin moiety during the photolytic process. Following the studies the authors established that 3,5-dimethoxybenzoin (DMB, **68**, X = OAc) derivative was the most appropriate as PPG of acetate. The expected byproduct 2-phenyl-5,7-dimethoxybenzofuran **69**, was formed in quantitative yield, with a photochemical quantum yield (Φ_{Phot}) of 0.64 ± 0.03 (Scheme 18).



68 X = OAc **69**

Scheme 18. Photocyclization of DMB acetate.

Based on experimental evidences, the authors concluded that the reaction proceeds from the excited singlet state or an extremely short-lived triplet state. Nevertheless, the detailed mechanism of this reaction has only recently arisen and results from the contribution of several researchers (Scheme 19).[109,110]



Scheme 19. Mechanism of the photocyclization of 3,5-dimethoxybenzoin derivatives.

The applications of 3,5-dimethoxybenzoin as photolabile group include drug delivery,[111] protein folding and unfolding,[112] neurotransmitter amino acids (glutamate and GABA), and also amines.[113]

The existence of a stereogenic center on the phototrigger can be a limitation in the use of DMB as a protecting group of chiral molecules (such as nucleotides or amino

acids/ peptides), since the use of an enantiopure derivative of benzoin or a stereospecific synthesis is required to obtain a diastereomerically clean phototrigger.

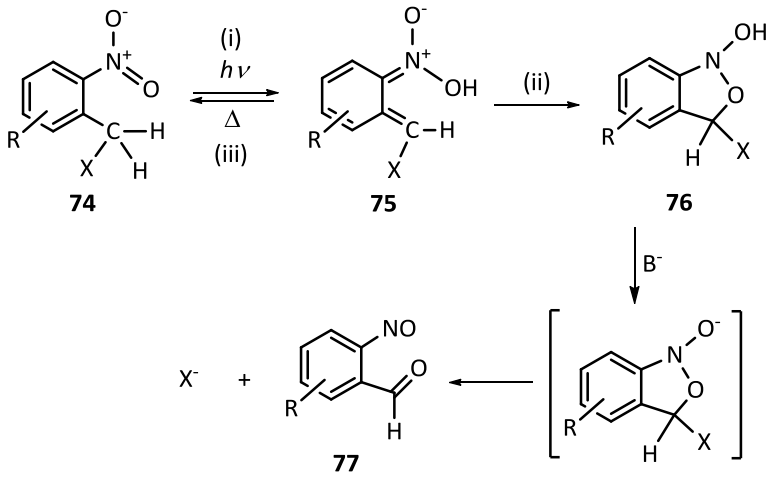
2.3. *o*-Nitrobenzyl groups

The *o*-nitrobenzyl group (NB) was reported for the first time to release benzoic acid from the corresponding ester by Barltrop *et al.* in 1966.[114] Since this initial work, numerous NB derivatives have been developed for the protection of molecules possessing various functional groups, namely acids, alcohols, amines, phosphates and ketones in a wide range of applications.[93,115,116] Among them, the caging of biologically relevant moieties, including neurotransmitters, steroid hormones, lipids, messengers and cellular signaling molecules, proteins and nucleic acids are of particular interest. Also, the application in polymer science has attracted increasing attention.[117]

o-Nitrobenzyl derivatives are undoubtedly the most used PPGs, although their known disadvantages: (i) short-wavelength UV light is required for deprotection (uncaging); (ii) following electronic excitation, the molecule goes through a series of "dark" steps before the bioactive molecule is released, being estimated that there is a lag of a few milliseconds after photolysis before the bioactive molecule is released. This makes the caging group unsuitable for studying fast reactions, which frequently occur on the sub-millisecond time scale; (iii) a reactive *o*-nitrosobenzaldehyde is formed as a byproduct of the photolysis reaction, which can damage cells and absorbe the incident light, thus creating an internal filter.[118] A further complication of *o*-nitrobenzyl photochemistry arises from the formation of radical species. No further reports of long-lived radical species from other caged compounds have appeared, but probably all such *o*-nitrobenzyl systems generate a proportion of a radical species upon photolysis. Evidently the formation of low amounts of such radicals does not usually have deleterious effects.

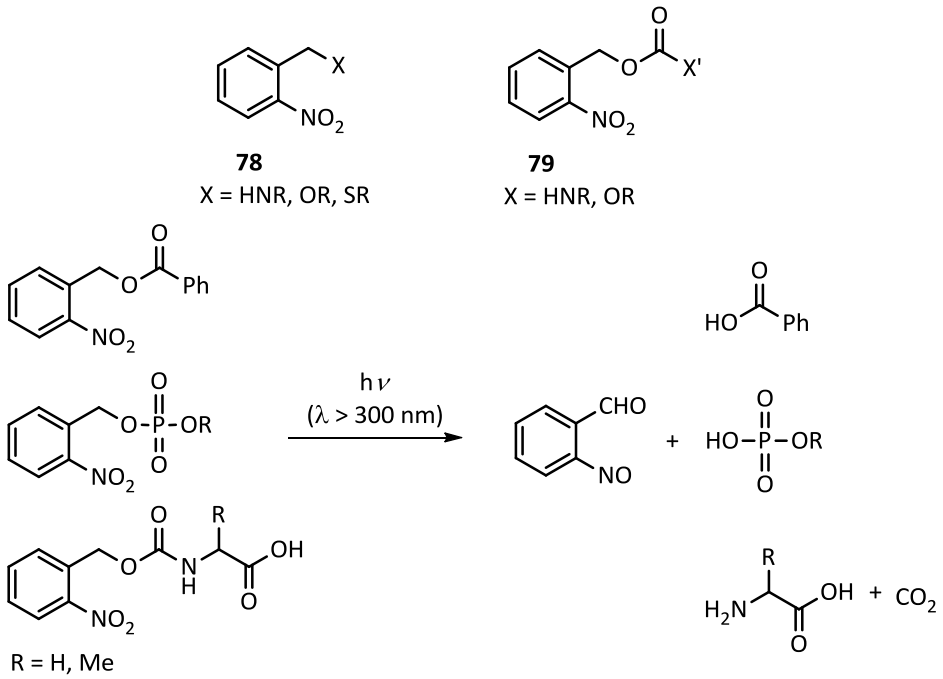
Regarding the release mechanism of *o*-nitrobenzyl derived PPGs, three critical steps are included (Scheme 20): (i) the photoinduced H-atom transfer (HAT*) leading to the primary *aci*-nitro intemediates **75**, (ii) ground state cyclization of **75** to benzo[*c*]isoxazol-1(3*H*)-ols **76**, which competes with (iii) reverse HAT to re-form **74**. In basic media, **76** is deprotonated and cleaves irreversibly, by concomitant release of X^-,

to *o*-nitrosobenzaldehydes **77**.[119]



Scheme 20. Simplified mechanism of the photofragmentation of *o*-nitrobenzyl compounds.

The parent *o*-NB compound **78**, although not the most extensively used, represents the generic form of this family of PPGs. The leaving group is directly attached to the benzylic site, which is the typical way for the release of carboxylic acids, thiols, histidine, and phosphates (Scheme 21; see some examples).[3,120]

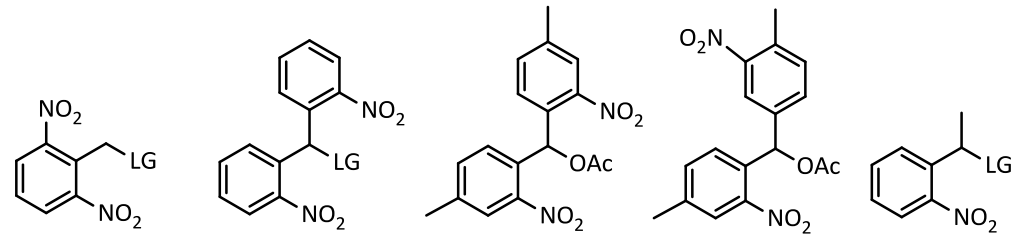


Scheme 21. Photolysis of some *o*-nitrobenzyl derivatives.

It is possible to directly link alcohols and amines, however they are most frequently attached as carbonic acid derivatives **79** (X = OCO-X´), which are better leaving groups (LGs). Nevertheless these strategies are unsuitable when rapid release is required as the postphotolytic fragmentation is the rate-limiting step for release (Scheme 21).[113,121] Another disadvantage of theses LGs is the slow rate of release of alcohols, and the formation of a stable imine in the release of amines, due to a condensation reaction with the nitrosoaldehyde side-product **77**. Although, this effect can be overcome by the addition of semicarbazide hydrochloride as carbonyl scavenger of **77**.

To improve the photochemical properties of NB as a PPG, various structural modifications have been conducted over the years, not only to increase the photo-deprotection quantum yield, but also to create a bathocromic shift in the absorbance.[122]

Substitution at the benzylic site mainly affects quantum yields, whereas modification on the aromatic moiety affects the absorbance. Introducing another *ortho*-nitro group into the chromophore statistically increases the probability of producing the *aci*-nitro moiety through hydrogen abstraction by the excited nitro groups. To this end, the 2,6-dinitrobenzyl PPG (DNB) in **80** was investigated (Figure 15). This group gives an improved quantum yield when compared with the NB chromophore **78**.[123,124] Likewise, a comparison between the 2-nitro-1,3-benzenedimethyl group (with two benzylic sites available for hydrogen abstraction and consequent leaving group release) and the 3-methyl-2-nitrobenzyl group further confirmed that the quantum yield can be increased by statistically increasing the probability of forming the *aci*-nitro compound. This was also observed by introducing a 2-nitrobenzyl group at the benzylic site by using 2,2'-dinitrophenylmethyl group (DNPM) **81**.



2,6-dinitrobenzyl (DNB) **80** | 2,2'-dinitrophenyl-methyl (DNPM) **81** | **82** | **83** | **84**

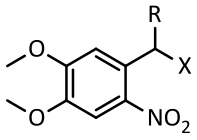
LG - leaving group

Figure 15. NB analogues **80**-**84**.

Cameron and Frechet obtained an increase in photolysis quantum yields from NB (Φ_{Phot} = 0.13) to **81**, DNPM (Φ_{Phot} = 0.26) in releasing a carbamate at 254 nm.[124] Hasan *et al.* also obtained the same effect in releasing a carbonate at 365 nm, NB (Φ_{Phot} = 0.033) to DNPM **81** (Φ_{Phot} = 0.12).[125] However, the quantum yields of releasing an acetate from **82** and **83** are identical (Φ_{Phot} = 0.2) under the same conditions, which suggests that the improved quantum yields of **81** and **82**, **83** relative to their NB counterparts can probably be attributed to the known α-substitution effect.[126]

Reichmanis *et al.* reported a fivefold increase in quantum yield in acetonitrile and a twofold increase in a polymeric matrix for releasing methyl-substituted NB, that is, 1-(2-nitrophenyl)ethyl (1-NPE), **84**, compared with its NB counterparts under the same conditions.[123]

Aujard and co-workers prepared and evaluated the 6-nitroveratryl (NV, or 4,5-dimethoxy-2-nitrobenzyl, DMNB) derivatives **85**-**88**, with electron-withdrawing substituents at the benzylic position (Figure 16).[127] The authors found that the substituents at the benzylic site despite their significant effect (up to a factor of 3 between the most and the least efficient), were not able to induce a significative increment in the quantum yield (Φ = 0.013 for -Br and Φ = 0.003 for -CN).



85 R = CF_3
86 R = CBr_3
87 R = CCl_3
88 R = CN

Figure 16. 6-Nitroveratryl derivatives **85**-**88**.

Concerning the effect of substituents on the aromatic ring of *o*-nitrobenzyl chromophore, the introduction of two methoxy groups, leading to the 6-nitroveratryl (NV, **89**) and 6-nitroveratryloxycarbonyl (NVOC, **90**) moieties was advantageous, since it shifted the absorbance to longer wavelengths (λ > 350 nm, and Rayonet lamps at 420 nm still provide photolysis at reasonable rates).[3] These are definitely the most frequently used groups to date, both in solution and in the solid phase (Figure 17).[128]

Other possibilities have been studied, like the introduction of an α-methyl group at the benzylic site: the methyl-6-nitroveratryl (MeNV, **91**) and methyl-6-nitroveratryloxycarbonyl (MeNVOC, **92**) groups. Related analogues, such as the methylenedioxy derivatives **93** and **94** showed little difference, possibly related to the impeditive alignment of the nonbonding orbitals of the oxygen with the aromatic π system. Addition of an extra α-methyl group results in α-methyl-(6-nitropiperonyloxymethyl) (MeNPOM, **95**) and 3,4-(methylenedioxy)-6-nitrophenylethoxycarbonyl (MeNPOC **96**) groups.[129] Berroy *et al.* reported a solution-phase comparison on the quantum yield of release of thymidine derivatives with the NVOC (**90**) groups (Φ = 0.0013) and the MeNPOC (**96**) groups(Φ = 0.0075).[130]

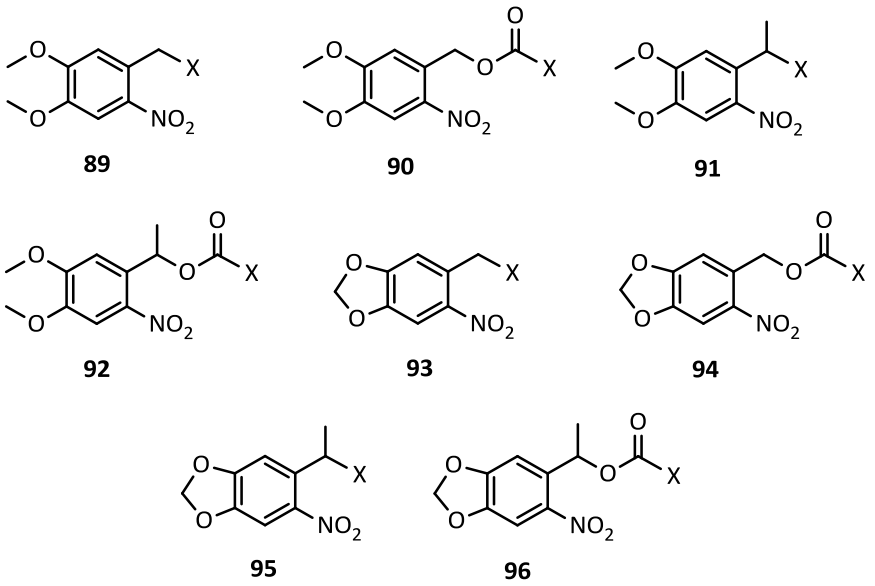
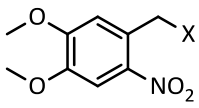


Figure 17: Analogues of *o*-nitrobenzyl group **89**-**96**.

Owing to the interest in nitrobenzyl PPGs, namely the nitroveratryl derivatives (NV), recently Šolomek *et al.*[119] measured quantum yields for the photolytic release (Φ_{Phot}) of seven different common LGs, linked through ether, amine, urethane, amide and carbonate, from the corresponding *o*-nitroveratryl conjugates **97** (Figure 18). The authors verified that these quantum yields depend strongly on the nature of the LGs (Table 2). The quantum efficiency with which the LGs are released, correlates with the stabilization that these LGs provide to the *o*-nitrobenzyl-type radicals, once radical stabilizing groups weaken the C-H bond that is cleaved in the HAT step and hence, lower

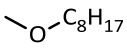
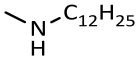
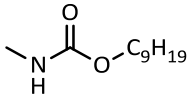
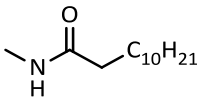
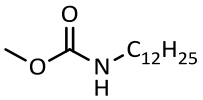
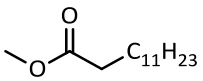
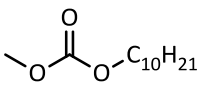
the barrier for this process. Simultaneously these substituents lower the endothermicity of the thermal HAT and thus, increase the barrier for the reverse process, enhancing the part of the initially formed *aci*-nitro intermediate that undergoes cyclization (which ultimately leads to LG release).



97

Figure 18. *o*-Nitroveratryl conjugates **97**.

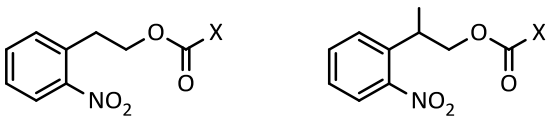
Table 2. Compounds **97a**-**g** investigated and quantum yields for their disappearance on photolysis at 360 nm.

Compound	X	Φ_{Phot}[a]
a		0.22 ± 0.04
b		0.14 ± 0.01
c		0.080 ± 0.003
d		0.072 ± 0.013
e		0.019 ± 0.005
f		0.0081 ± 0.0005
g		0.0091 ± 0.0013

[a]Acetonitrile solutions (~1 x 10^{-3} M) were irradiated at λ = 360 ± 5 nm (LED-based reactor). Φ_{Phot} was determined by actinometry using phenylglyoxylic acid. Standard deviations from two independent measurements are shown.

2.4. *o*-Nitro-2-phenethyloxycarbonyl groups

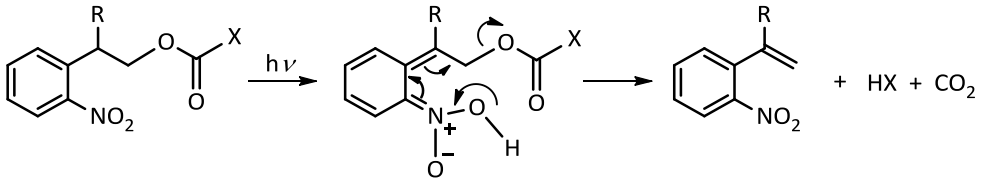
Hasan and co-workers designed the *o*-NB homologue **98**, 1-(2-nitrophenyl)ethyloxycarbonyl (NPEOC) and its α-methylated analogue **99** (NPPOC) and evaluated them in the photorelease of thymidine derivatives protected at the 5´-hydroxyl function (Figure 19).[125]



98 **99**

Figure 19. *o*-Nitrobenzyl homologues **98** and **99**.

Despite the structural similarity between **98**, **99** and **78**, the release mechanism proposed differs markedly, being in this case through the formation of an *aci*-nitro intermediate **100**, which can undergo rearrangement to photolabile *o*-nitrostyrene **101** with β-elimination of the protected group (Scheme 22).[125] In fact, **98** eliminated thymidine with a higher quantum yield (Φ = 0.042) than that of its *o*-NB analogue **78** (Φ = 0.033). The substitution of the benzylic center with a methyl considerably added to the efficiency (Φ = 0.35), turning **99** into a candidate of choice for oligonucleotide synthesis.[131]



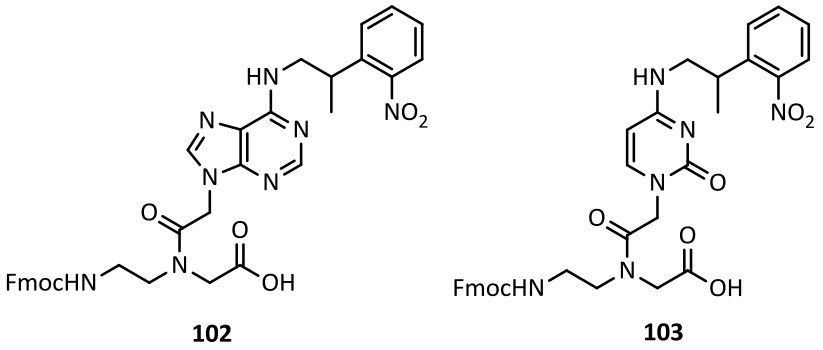
98: R = H
99: R = Me

100 **101**

Scheme 22. Photolysis of 2-nitro-2-phenethyl derivatives **98** and **99**.

Recently, the 2-(*o*-nitrophenyl)-propyl (NPP) group was used in a solid phase peptide synthesis, as caging group to mask the nucleobases adenine and cytosine, in *N*-(2-aminoethyl)glycine peptide nucleic acids (aeg-PNA), since it is compatible with the cleavage conditions required. Removal of the nucleobase caging group in compounds

102 and **103** was achieved by UV/LED irradiation at 365 nm (Figure 20).[132]



102 **103**

Fmoc = 9-fluorenylmethyloxycarbonyl

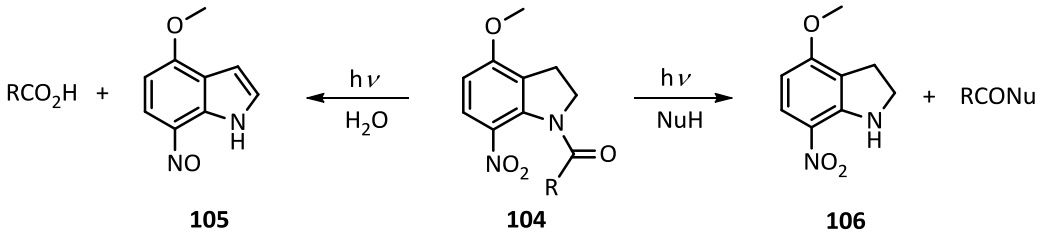
Figure 20. Caged aeg-PNA building block Fmoc-a^{NPP}-aeg-OH **102** and aeg-PNA building block Fmoc-c^{NPP}-aeg-OH **103**.

2.5. *o*-Nitroindoline derivatives

7-Nitroindoline (NI) derivatives were introduced by Amit *et al*. in the mid-1970s as PPGs for carboxylic acids.[133]

The NI caged amino acids, unlike the nitrobenzyl analogues showed negligible hydrolysis at physiological pH. Mechanistic investigations have shown that the product of the photolysis of 7-nitroindolines depends on the medium, being different when the irradiation is performed in water or in moist organic solvents. In water, deprotonation releases a carboxylic acid, with the concomitant formation of a nitrosoindole **105**, stable in physiological solutions, whereas in organic solvents, the acyl moiety undergoes a nucleophilic attack, either by the solvent (water or alcohol), or by an external nucleophile, to form the product 7-nitroindoline **106** (Scheme 23).[133]

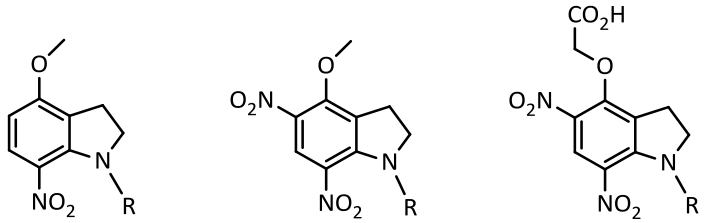
Variations in the substitution pattern on the indoline aromatic ring can, to a certain extent, influence the ratio of the side-products.[134] It was found that the maximum on photolysis efficiency occurred with 4-methoxy-7-nitroindolinyl (MNI) derivatives **104**.



105 **104** **106**

Scheme 23. Differences in the photolysis of the MNI-caged carboxylates in an aprotic solvent or in water.

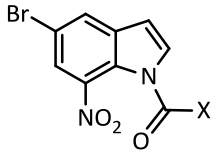
The 4-methoxy-7-nitroindolinyl glutamate (MNI-R, R = Glu **107**, Figure 21) is widely used in neuroscience for one- and two-photon excitation and is sufficiently inert at glutamate receptors to be tolerated at high concentration, thus compensating for the low efficiency with two-photon photolysis. Other 7-nitroindoline derivatives have also been used in many studies of glutamate signaling in neurons (see as examples compounds **108** and **109**, Figure 21).[135,136]



107 **108** **109**

Figure 21. 7-Nitroindoline-derived caged compounds **107**-**109**.

In addition to the carboxylic acid, other functional groups were released from nitroindoline derivatives. Hassner *et al.* published the release of alcohols from carbamates (X = OR), or amines from ureas (X = NR_1R_2) of **110** (Figure 23).[137]

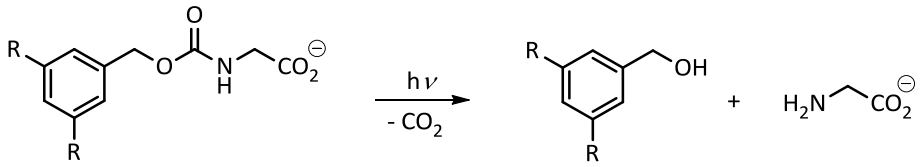


110

Figure 23. Nitroindoline carbamate derivatives **110**.

2.6. Benzyl and polyaromatic benzyl-based protecting groups

The first application of a photoremovable protecting group in organic synthesis was demonstrated by Barltrop and Schofield in 1962, and consisted in the photolytic release of glycine **114** from a benzyl protection, *N*-benzyloxycarbonylglycine **111,** by irradiation at 254 nm (Scheme 24). The following research focused in structural alterations on the benzyl group and resulted in a large family of PPGs, namely substitution and expansion of the benzene ring, as well as modification at the benzylic carbon center.[3]



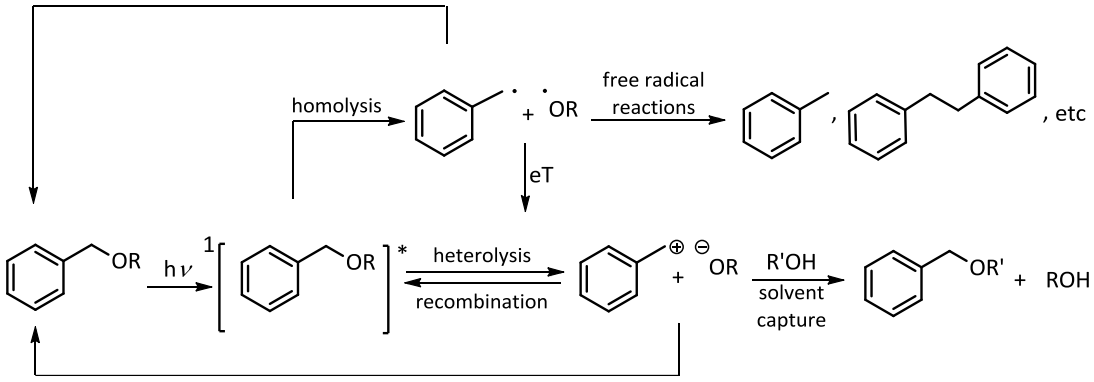
111: R = H
112: R = OCH_3

113 **114**

Scheme 24. Photorelease of glycine from the *N*-benzyloxycarbonyl glycine **111**.

The proposed mechanism for the photosolvolysis admitted that it could proceed *via* initial light-induced homolysis of the C-O bond, followed by rapid electron transfer (eT) to give an ion pair, which is then trapped by solvent to provide the final products (Scheme 25). In the photolysis of simple benzyl derivatives this mechanism is shown to be predominant.[138] Actually, the formation of radical products often arises in photosolvolysis of benzyl esters.

Zimmerman and collaborators[139] suggested that heterolysis of the C–O bond is energetically favoured over the homolysis, especially in the presence of *ortho* and *meta* substituents stabilizing the cation. In solvolysis studies of benzyl acetates, the authors observed the *meta* effect through a higher efficiency of photorelease in benzyl esters possessing methoxy substituents in *meta* positions.



Scheme 25. Photochemistry of arylmethyl derivatives.

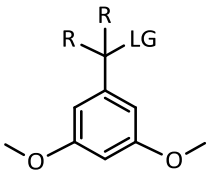
The direct photoheterolysis of the C–O bond can be stimulated by structural alterations that increase the delocalization of positive charge in the ground state and improve the efficiency of the eT step, or through moieties that stabilize the cation in the excited-state. The efficiency of the photorelease process is dependent on the competition between solvent capture of the ion pair to give the free hydroxyl compound ROH and recombination resulting in the starting material, as can be seen in Scheme 25.[3,139] The use of polar protic solvents favors both heterolytic cleavage and eT processes.

Carboxylates, carbonates, carbamates and phosphates are photolysed with relative efficiency, while the uncaging of alcohols is more challenging. Taking into account that the ion recombination can be reduced by increasing the benzyl cation stability and steric hindrance around the cationic center, the search for new PPGs on this family has to include these alterations, in order to help the positive charge to delocalise.

2.6.1. Aromatic Substitution

As mentioned before, the presence of two methoxy groups at the *meta* positions of the benzyl group in **115** (Figure 23), the *meta* effect, has significantly increased the yields of the released molecules.[139] This was observed in the photorelease of glycine from 3,5-dimethyoxybenzyloxycarbonylglycine **112**, which occurred in 85% yield, while from *N*-benzyloxycarbonylglycine resulted only in 10%, in the same irradiation conditions.

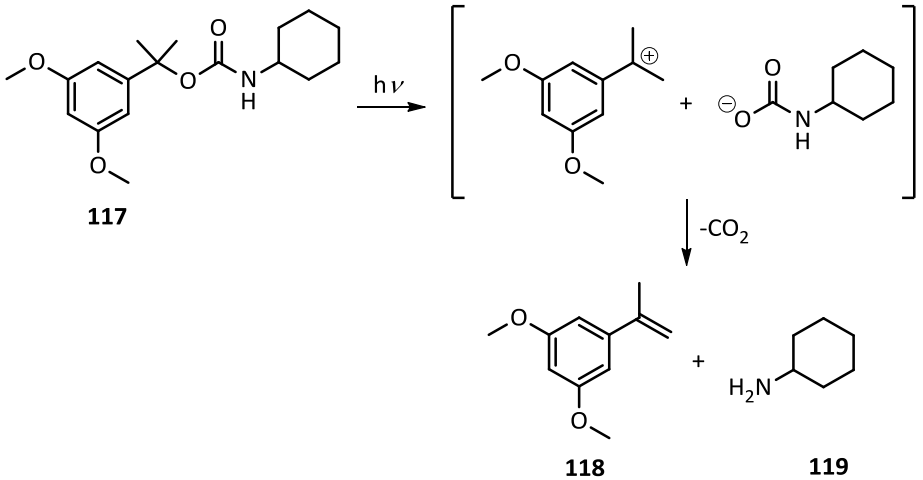
With the aim to stabilize the positive charge at the benzylic position in the transition state and consequently to facilitate the cleavage of C-O linkage, two methyl groups were introduced at the benzylic position in **116** (Figure 23).[122] Although having decreased in stability under acidic conditions, the resulting α,α-dimethyl-3,5-dimethoxybenzyl group displayed better photolysis reactivity.



115: R = H
116: R = Me

Figure 23. Conjugates based on PPGs of the benzyl-type **115** and **116**.

Cameron and Frechet used α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl Ddz PPG **117**, in the caging of amines,[140] which were released by irradiation of the corresponding carbamates with a medium-pressure mercury lamp in a THF solution and also in the solid state (Scheme 26). These PPGs are only suitable for the release of good leaving groups, such as carbamates or carboxylates, but not for alkoxides.

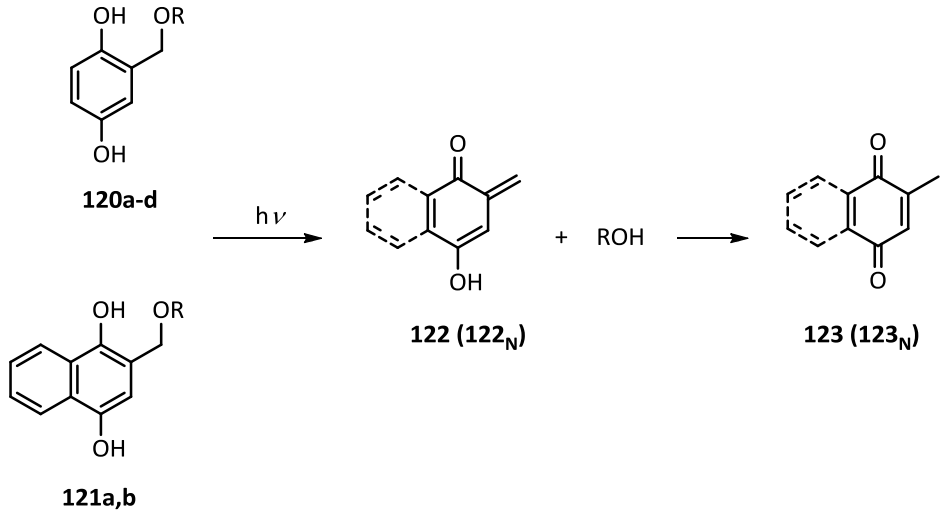


117 **118** **119**

Scheme 26. Photorelease of amines from benzyl-type conjugates **117**.

Popik and collaborators have demonstrated the interest in introducing hydroxy substituents on the benzyl group.[141] Recently, the authors reported the use of 1,4-

dihydroxybenzyl or its naphthalene analogue, (1,4-dihydroxynaphthyl)methyl group in the caging of alcohols, phenols, and carboxylic acids.[143] The substrates were efficiently released upon irradiation at 300 or 350 nm in a good to excellent yield. It was possible to directly link alcohols to these PPGs without the need of a carbonate linker, which enhances the hydrolytic stability of the caged alcohols and makes faster the release of substrates (Scheme 27). The authors observed that the initial byproduct of the photoreaction, 4-hydroxyquinone-2-methide **122**, undergoes rapid tautomerization into methyl *p*-quinone **123**. The UV spectrum of the latter is different from that of the caging chromophore, thus allowing selective irradiation of the starting material in the presence of photochemical products.



a R = $PhCH_2CH_2$; **b** R = *p*-Cl-C_6H_4; **c** R = PhCO; **d** R = *t*-BuCO

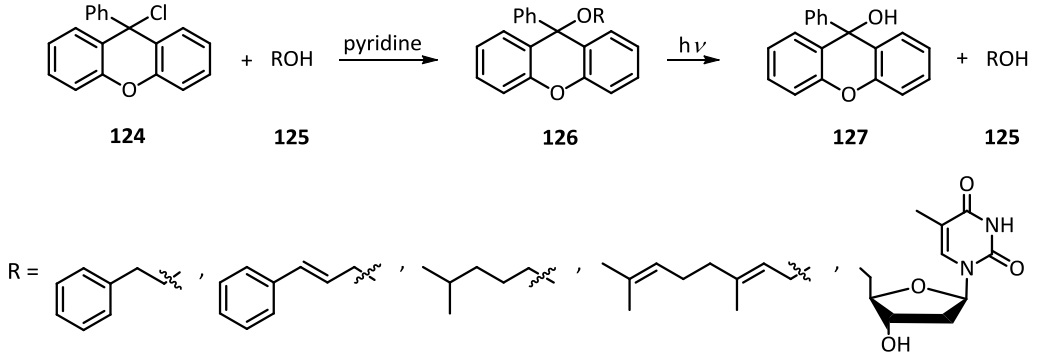
N = naphthyl derivative

Scheme 27. Photocleavage of 1,4-dihydroxybenzyl and 1,4-dihydroxynaphthyl cages **120** and **121**.

2.6.2. α-Substitution

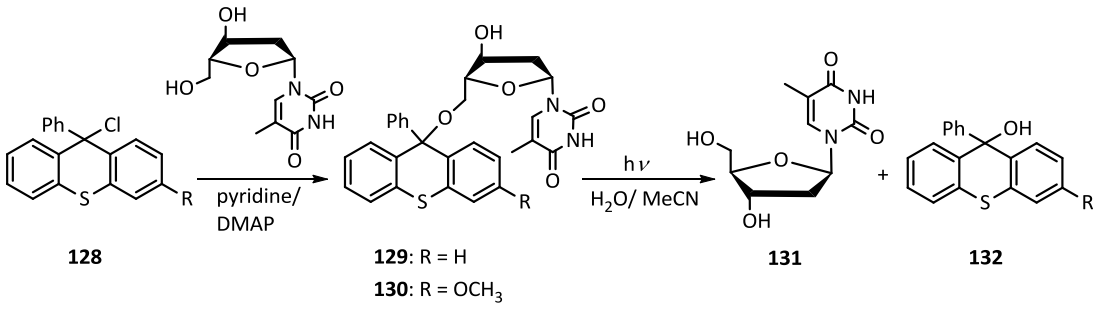
The development of 9-phenylxanthyl (pixyl, Px) as PPG for application in primary alcohols was accounted in the stability of 9-phenylxanthylium cation, which possesses a highly rigid and planar backbone (Scheme 28).[144] The pixyl conjugates **126** were prepared by reaction of various alcohols with commercially available pixyl chloride **124** in the presence of pyridine. In order to extend the utility of the pixyl group to nucleoside chemistry, the thymidine nucleoside was among the substrates used. Photolysis studies

of the pixyl ethers **126** under irradiation at 254 or 300 nm resulted in the release of the alcohols **125** in 78-97% yield. The authors referred that Px could be an effective PPG for the combinatorial synthesis of oligoribonucleotides.



Scheme 28. Protection and photorelease of primary alcohols.

The 9-phenylthioxanthyl group (S-pixyl, S-Px) was found to release primary alcohols at longer wavelengths and within higher quantum yield of deprotection, having the chemical yield of deprotection varied from 75% for cytidine to 97% for thymidine (Scheme 29).[145] Later studies by the same authors revealed that the efficiency of releasing thymidine from the S-Px cage **129** was enhanced by a factor of three in the presence of an electrondonating group at the 3-position **130**.[146] Furthermore, some of these compounds underwent photorelease when irradiated at 350 nm, beeing 5-*o*-(3-methoxy-9-phenylthioxanthyl)-2-deoxythymidine **131**, an example of quantitative release of thymidine upon irradiation at 300 or 350 nm.



Scheme 29. Use of the S-pixyl PPG for caging and release of nucleosides.

2.6.3. Expansion of the aromatic system

The largest part (or almost all) of the above mentioned benzyl-based photoremovable protecting groups requires irradiation with quite short wavelengths to accomplish the efficient release of substrates. In order to bathochromically shift the wavelengths of absorbance of the chromophore's protecting group, and consequently the wavelengths of photolysis, polyaromatic analogues of the benzyl protecting group, namely (2-naphthyl)methyl **133**,[147] (anthracen-9-yl)methyl **134**,[148] (phenanthren-9-yl)methyl **135**,[149] (pyren-1-yl)methyl **136**,[150,11,151] and (perylen-3-yl)methyl **137** groups,[92] have been introduced (Figure 24).

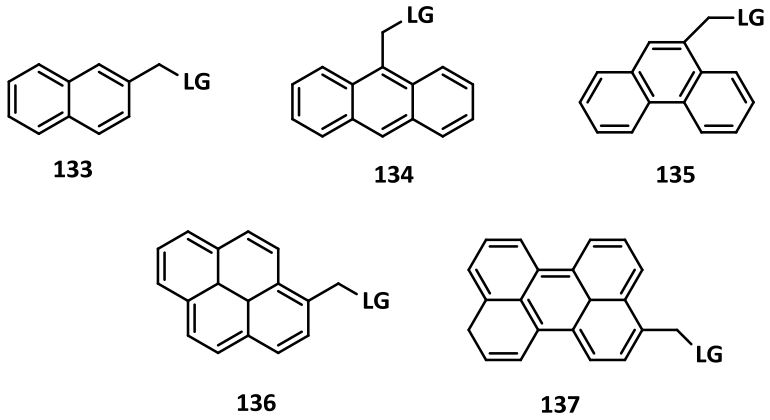


Figure 24. Polyaromatic analogues of the benzyl protecting group.

(2-Naphthyl)methyl group **133** was applied in the release of diethyl phosphate with only traces of side products, and the reactive excited state appeared to be the singlet state. The molar extinction coefficient at 340 nm and the photolytic sensitivity under irradiation at this wavelenght in 1,4-dioxane/water (1:1), were much lower than with the 2-nitrobenzyl and benzoin groups. In the protection of cAMP, (2-naphthyl)methyl group has comparable dark stability to the benzoin group, nevertheless much lower stability than the α-methyl-nitrobenzyl.[147]

The use of (anthracen-9-yl)methyl **134** in the protection of carboxylic acids was reported by Singh and Khade.[148] The reaction between anthracene-9-methanol and several carboxylic acids, namely *o*-chlorobenzoic acid, hippuric acid, *m*-nitrobenzoic acid, phenylacetic acid and acetic acid resulted in the corresponding esters **138** (Figure 25).

Photolysis at 386 nm of anthracen-9-methanol esters **138a**-**e** in acetonitrile/water (3:2) resulted in the release of carboxylic acids in good chemical yields (43-100%), and quantum yields of photoreaction in the range 0.07-0.43. The fluorescence emission of the conjugates **138a**-**e** occurred in the range 380-480 nm, with quantum yields of fluorescence of 0.01-0.09.

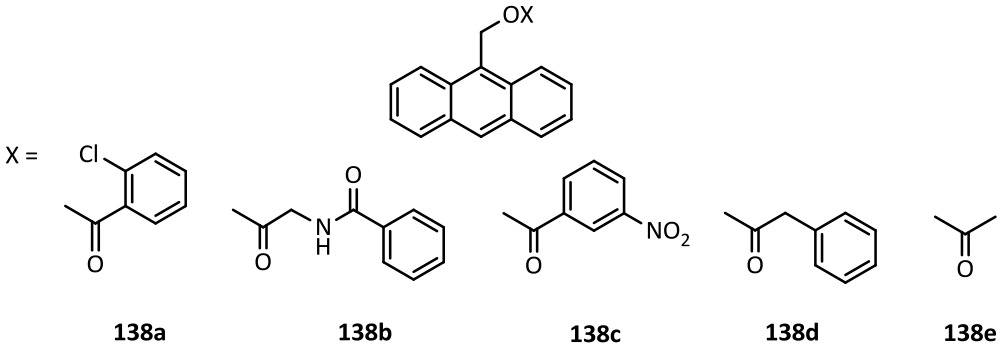
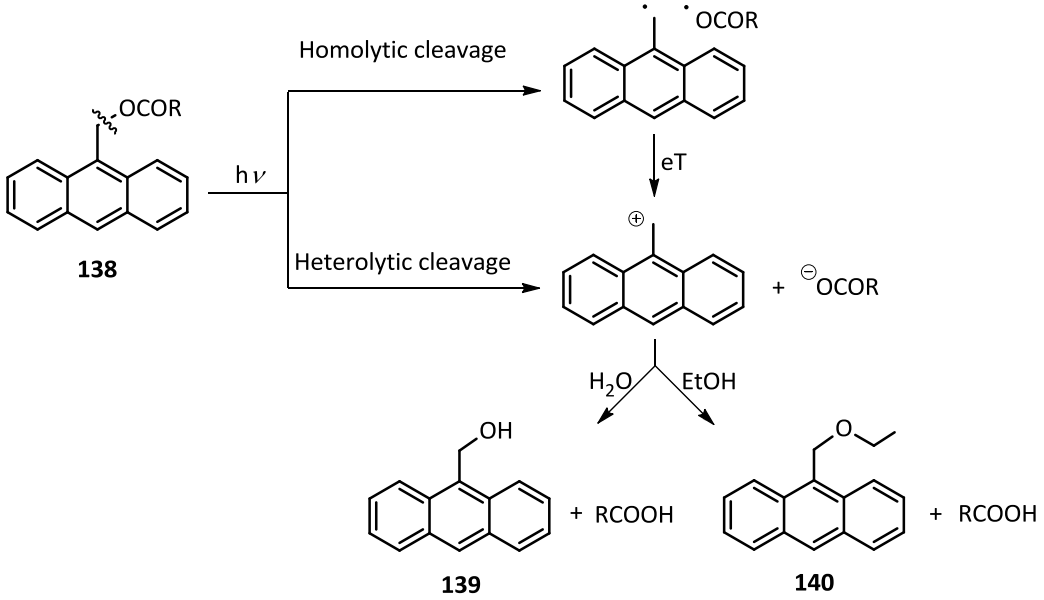


Figure 25. (Anthracen-9-yl)methyl ester derivatives **138a**-**e**.

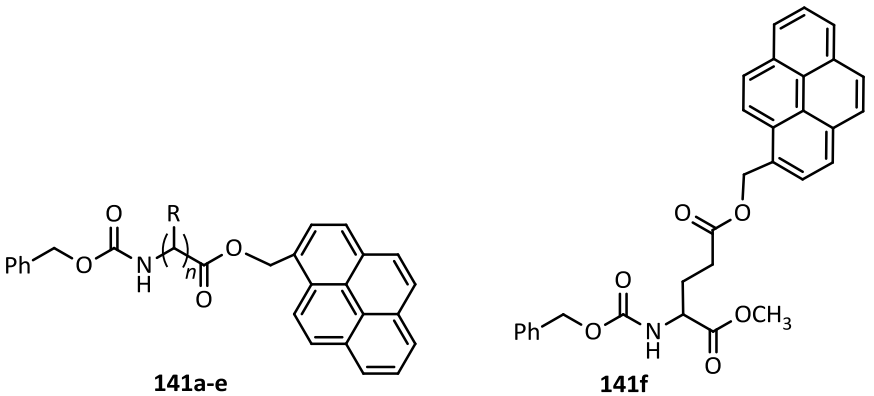
The photochemical mechanism proposed by the authors is based in the homolytic cleavage of the C-O bond followed by eT to give an anthryl-9-methylene carbocation, which undergoes nucleophilic attack by the solvent molecule yielding the respective products. Furthermore, the mechanism based on heterolytic cleavage of the C-O bond, which can directly yield the 9-anthryl-methylene carbocation is also another possibility (Scheme 30).



Scheme 30. Proposed mechanism for the photolysis of (anthracen-9-yl)methyl ester derivatives **138a**-**e**.

Studies of photolytic efficiency of the (phenanthren-9-yl)methyl group **135** (ε_{297nm} = 9530 $M^{-1}cm^{-1}$) in comparison with (pyren-1-yl)methyl group **136** (ε_{323nm} = 13100 $M^{-1}cm^{-1}$) revealed that under irradiation in THF/water (1:1) at 350 nm, the quantum yield of releasing a carbonate from (phenanthren-9-yl)methyl group **135** was 8.9×10^{-4} (ε_{350nm} = 190 $M^{-1}cm^{-1}$), while from (pyren-1-yl)methyl group **136** was 2.9×10^{-3} (ε_{350nm} = 3400 $M^{-1}cm^{-1}$).[150]

The (pyren-1-yl)methyl **136** was used as fluorescent photolabile protecting group for alcohols,[150] carboxylic acids,[151] phosphates,[149] and amines.[153] Fernandes and co-workers[11] described the caging of several neurotransmitter amino acids, such as glycine, alanine, β-alanine, glutamic acid and γ-aminobutyric acid through the ester linkage between the carboxylic acid funtion and the (pyren-1-yl)methyl unity (Figure 26). The photolytic studies of the fluorescent cages **141a**-**f** carried out in acetonitrile and mixtures of acetonitrile, methanol and tetrahydrofuran with aqueous HEPES buffer or water, in different proportions, at different wavelengths of irradiation (254, 300, 350 and 419 nm), showed that the use of a polar protic solvent like methanol and a higher percentage of water resulted in a significant increase in the photocleavage rate.



141a-e **141f**

a *n* = 1, R = H
b *n* = 1, R = CH_3
c *n* = 2, R = H
d *n* = 3, R = H
e *n* = 1, R = $(CH_2)_2CO_2CH_3$

Figure 26. (Pyrenyl-1-yl)methyl ester conjugates of neurotransmitter amino acids **141a**-**f**.

More recently, Fernandes and co-workers also reported the use of (pyren-1-yl)methyl in the caging of the amino function of several amines, catecholamines (dopamine, norepinephrine, tyramine and octopamine), and the related amino acids L-

phenylalanine, L-tyrosine and L-3,4-dihydroxyphenylethylamine (DOPA) (Figure 27, see catecholamine conjugates **142a**-**d**).[151] The caged compounds displayed maxima absorption (λ_{max}) at about 340 nm and the carbamate linkage between the active amine and the pyrenylmethyl moiety cleaved readily with the most promising results obtained by irradiation at 300 and 350 nm, in a methanol/HEPES buffer (80:20) solution. The emission of these compounds occurred at about 375 nm with Φ_F on the range of 0.06-0.17.

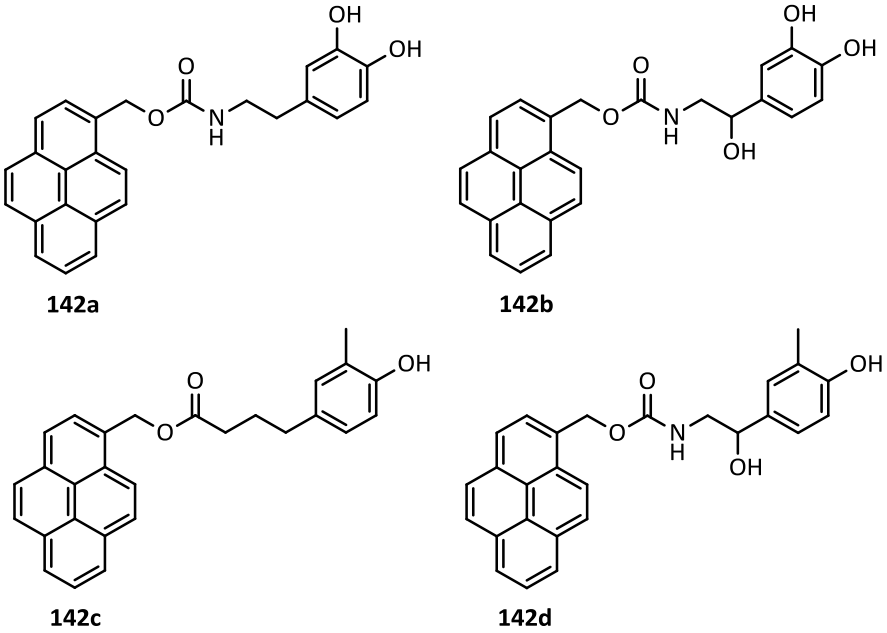
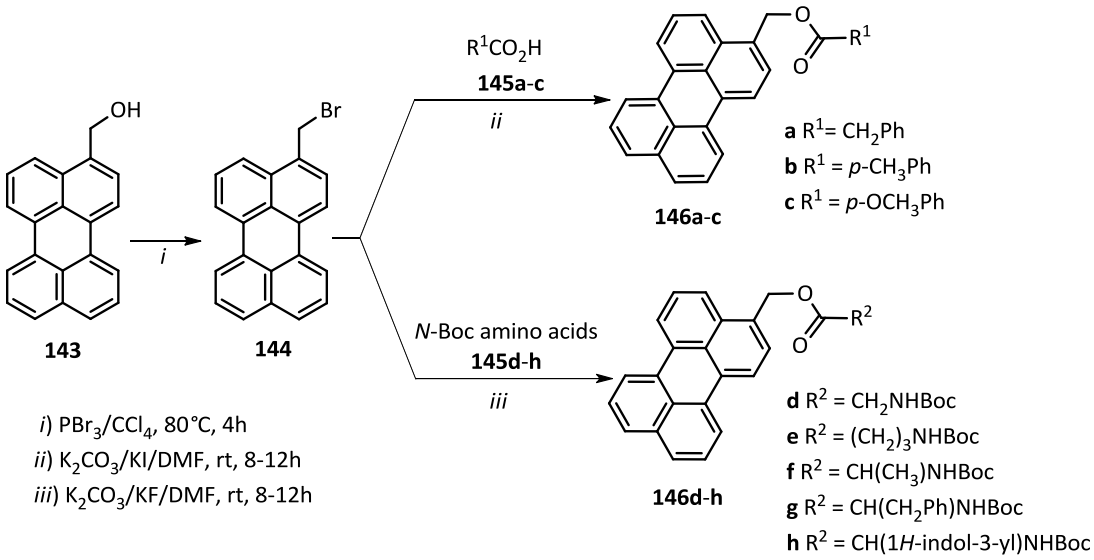


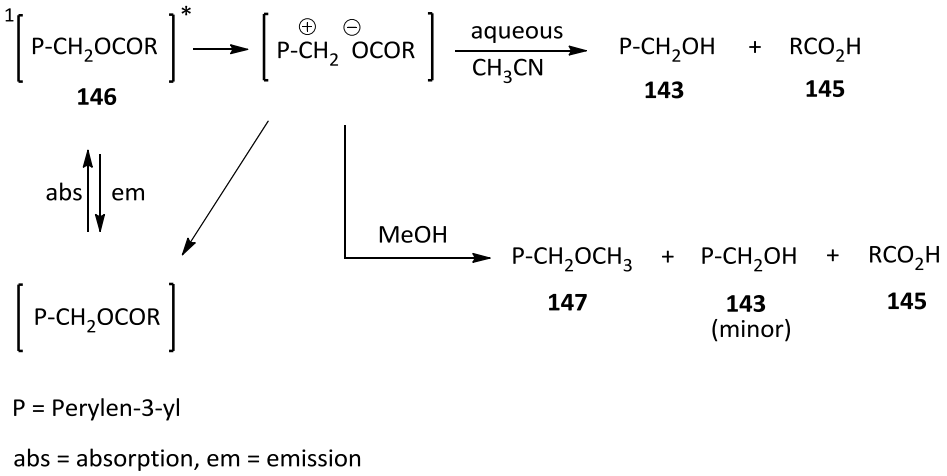
Figure 27. (Pyren-1-yl)methyl carbamate cages of catecholamines **142a**-**d**.

Recently, Jana and collaborators demonstrated the capability of (perylen-3-yl)methyl **137** to act as a fluorescent photoremovable protecting group for carboxylic acids and alcohols.[92] Carboxylic acids including amino acids **145a**-**h** protected as their corresponding esters (compounds **146a**-**h**), displayed strong fluorescence with Φ_F in the range 0.85-0.95 (Scheme 31). Photolysis of the caged esters **146** under visible light (λ = 410 nm) in aqueous acetonitrile released the expected carboxylic acids **145** in high chemical (94-97%) and quantum (0.072-0.093) yields. The authors observed that the rate of photorelease was influenced by the solvent.



Scheme31. Synthesis of (perylen-3-yl)methyl caged esters **146a**-**h**.

An ionic mechanism for the photorelease of carboxylic compounds **145** was proposed, with the initial step involving excitation of the (perylen-3-yl)methyl cage **146** to its singlet excited state, which then undergoes heterolysis of the C-O ester bond through an ion pair of (perylen-3-yl)methyl carbocation and carboxylate anion. The ion-pair separation in a polar solvent, followed by the methylenic carbocation and carboxylate anion reaction with solvent molecules resulted in 3-(hydroxymethyl) perylene **143** and carboxylic acid **145**, respectively (Scheme 32).

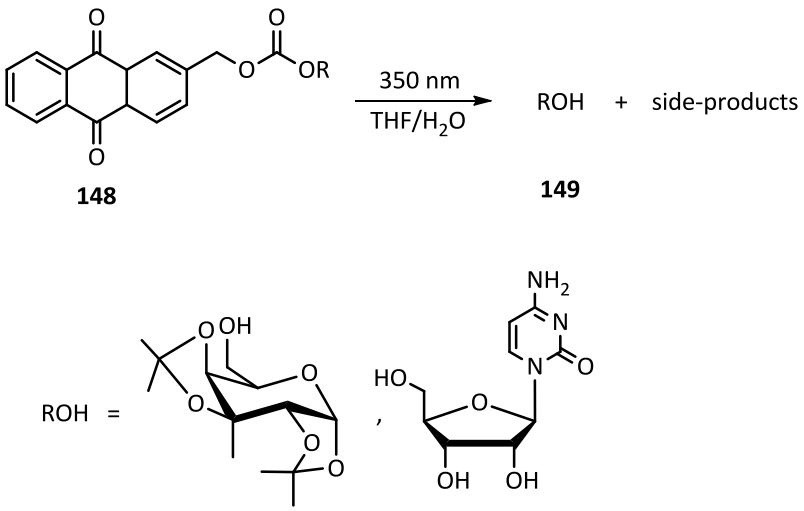


Scheme 32. Possible mechanism for the photolysis of (perylen-3-yl)methyl caged esters **146**.

The capability of (perylen-3-yl)methyl group to act as PPG for alcohols and phenols was also tested through photolysis studies of the corresponding caged carbonates in the same conditions as described before.

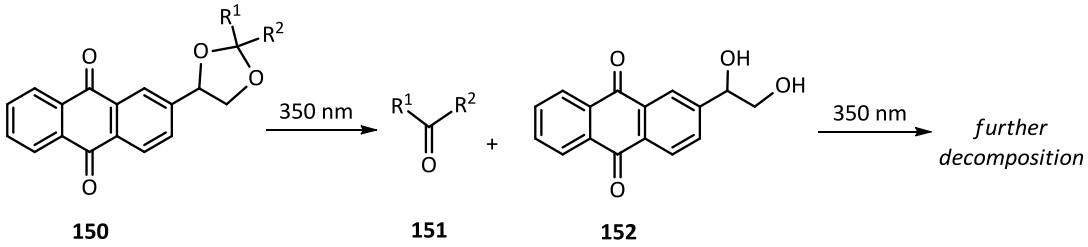
The release of alcohols occurred in quantitative yield (95-96%). As in the case of caged esters, the authors suggested that the mechanism of the photocleavage of carbonates involves heterolysis of the C-O ester bond to produce an ion pair of (perylen-3-yl)methyl carbocation and carbonate anion. The carbonate anion undergoes decarboxylation followed by proton abstraction from the solvent to produce uncaged alcohol.

Overall, the above mentioned polyaromatic benzyl-type cages displaying significant absorbance at 350 nm with weak to excellent fluorescence (Φ_F = 0.01-0.95) and are suitable for the release of various functional groups, with fair to low quantum efficiency of release. A common drawback of these PPGs is their poor aqueous solubility. Considering it, Furuta and co-workers have described the (anthraquinon-2-yl)methoxycarbonyl cage (Aqmoc) **148** which undergoes fairly efficient photocleavage at 350 nm ($\Phi_{350\ nm}$ = 0.10) and has good aqueous solubility (Scheme 33).[147,150] The suitability of Aqmoc group in the protection of the alcohol function of carbohydrates and nucleosides *via* carbonate bond has been demonstrated with 68% and 91% chemical yields, respectively.



Scheme 33. Aqmoc caging of primary alcohols.

In addition, (anthraquinon-2-yl)methyl was also applied in the protection of carbonyl groups of ketones and aldehydes *via* photolabile acetals **150**. By irradiation at 350 nm in acetonitrile/aqueous buffer (1:1) carbonyl compounds **151** were released with 0.03−0.09 quantum efficiency in 60-90% chemical yield (scheme 34).[154]



Scheme 34. (Anthraquinon-2-yl)methyl-based photolabile acetals **150**.

A number of the aforementioned groups have been demonstrated as fluorescent phototriggers, possessing interesting advantages over non-fluorescent protecting groups, since, in addition to release the desired compound at a particular location for a specific period of time, they enable the visualization, quantification and pursuit of the spatial distribution, localization, and depletion of the uncaged compound.[155,156]

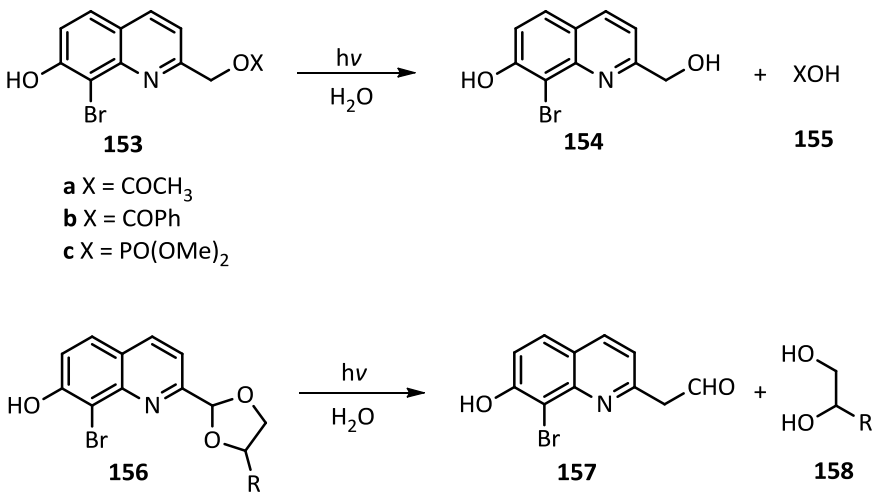
Fluorescent PPGs have been successfully applied for temporal and spatially controlled release of bioactive molecules in studies of various processes in biological[93] and medical areas.[157] Of particular interest, for example, is the labelling of amino acids by fluorescent PPGs, since it allows detection of small aliphatic amino acids with neither fluorescence nor strong absorption in the UV/vis region by a far more sensitive technique than common UV absorption.[11,151] Also, it allows the visualization of the amino acids involved in biological processes and simultaneously their release over a specific period of time at a particular location.

2.7. Polyheteroaromatic analogues of the benzyl group

Owing to the interest of fluorescent PPGs, together with the development of polyaromatic groups, polyheteroaromatic analogues of the benzyl protecting group have also emerged, and some representative examples will be indicated below.

The use of oxazole heterocycle as the basis of protecting groups for carboxylic acids was recently demonstrated, for the first time, by our research group.[54] The oxazole conjugates required short irradiation times for the quantitative release of the caged amino acids at 254 and 300 nm, the best result was obtained for naphtho[2,3-*d*]oxazole (1-5 min) in methanol/HEPES buffer (80:20) solution.

(8-Bromo-7-hydroxyquinoline-2-yl)methyl (BHQ), which displays a strong absorption at 370 nm was suggested as photolabile protecting group for carboxylic acids through the corresponding ester cage (Scheme 35).[158-160] The authors observed that photorelease of acetic acid was more efficient from the hydroxyquinoline **153a** (Scheme 35) than from 4,5-dimethoxy-2-nitrobenzyl and (6-bromo-7-hydroxycoumarin-4-yl)methyl acetates.[161] This group can also be cleaved by two-photon excitation.[162]



Scheme 35. (8-Bromo-7-hydroxyquinoline-2-yl)methyl (BHQ)-based cages **153** and **156**.

Carboxylate (**153b**), phosphate (**153c**), and diol (**156**) BHQ-based cages were also efficiently photolysed under simulated physiological conditions by one-photon and two-photon excitation (Scheme 35).[161] Moreover, a (2-phenylquinolin-4-yl)methyl group[163] and the corresponding arylmethylsulfonyl analogue[164] have been suggested as PPGs for alcohols and amines, respectively.

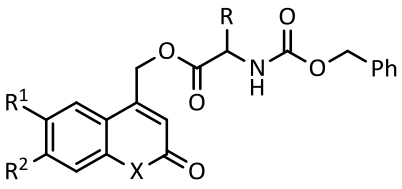
The quinoline skeleton could be tailored by the introduction of appropriated substituents in the aromatic ring to tune the photophysic characteristics of this PPG.[165,166] A significant bathochromic shift occurred by replacing the bromine substituent in BHQ with a cyano group to obtain ((8-cyano-7-hydroxyquinolin-2-

yl)methyl, CyHQ). However, from the (quinoline-2-yl)methyl derivatives, BHQ group showed to have the highest sensitivity towards two-photon absorption.

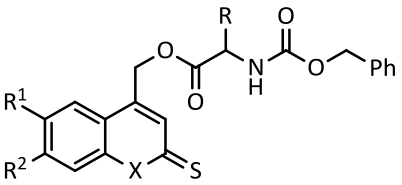
Even though quinolines have been considered for application as PPGs with promising results as it was mentioned above, the parent nitrogen heterocyclic quinolones, with structural resemblance to coumarins, contrary to the latter, have not been reported until recently by our research group. Evaluation of the photosensitivity of quinolones in methanol/HEPES buffer (80:20) solution was performed in comparison to coumarins by using model phenylalanine conjugates of both heterocycles. It was found that the quinolone conjugates were readily photolysed, with complete release of the amino acid within shorter irradiation times than the corresponding coumarin conjugates, at various wavelengths of irradiation, including at 419 nm.[167]

As an attempt to improve the previously reported promising outcomes, more recently, our research group reported the use of thionated analogues of quinolone **160a**-**f** and coumarin **160g**,**h** as PPGs for carcboxylic acids (Figure 28).[168] The introduction of the sulfur atom resulted in a considerable bathochromic shift in the maxima wavelength of absorption (λ_{max} between 47 and 73 nm, being larger for coumarins) and emission (λ_{max} between 10 and 60 nm) in comparison to the carbonyl related compounds **159**. All thionated conjugates **160** displayed λ_{abs} and λ_{em} located in the range 377-401 nm and 425-469 nm, respectively, with lower fluorescence quantum yields than the corresponding quinolones **159a**-**f** and coumarins **159g**,**h**.

	X	R^1	R^2	R
a	NCH_3	H	OCH_3	CH_2Ph
b	NCH_3	H	H	CH_2Ph
c	NCH_3	CH_3	H	CH_2Ph
d	NCH_3	OCH_3	H	CH_2Ph
e	NCH_3	OCH_3	H	CH_3
f	NCH_3	OCH_3	H	$(CH_2)_2CO_2CH_3$
g	O	H	CH_3	CH_2Ph
h	O	H	OCH_3	CH_2Ph



159



160

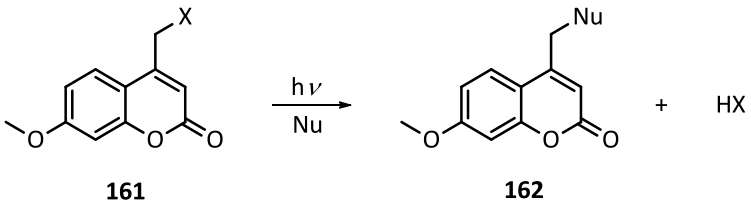
Figure 28. Structures of thionated amino acid ester conjugates **159** and **160**.

Upon photolysis, it was found that the thionated conjugates **160** cleaved significantly faster than the parent conjugates **159** at 419 nm, being thioquinolone conjugates **160a**-**d** photolysed in shorter irritation times in the tested conditions. These results are particularly interesting for caging strategies as it avoids cell damage due to short wavelengths. Moreover, the fact that some of the proposed heterocycles display very different photocleavage rates at the same wavelength may provide the possibility of selective removal of one group in the presence of the other group.

Although the importance of the abovementioned examples of heteroaromatics analogues of the benzyl protecting group, they have only recently emerged. However, during the last three decades a special attention has been given to the development of coumarin photolabile protecting groups which will be mentioned with more detail in the next section.

2.7.1. Coumarinylmethyl group

In 1984, Givens and Matuszewski noticed the photosensitivity of (coumarin-4-yl)methyl group in the release of phosphate esters.[96] Photolysis of a benzene solution of (7-methoxycoumarin-4-yl)methyl ester of diethylphosphate **161** occurred with a quantum yield of 0.038. The authors also demonstrated its use as fluorescent tag for nucleophiles (Nu) including proteins, proposing the generation of an electrophilic (coumarin-4-yl)methyl cation upon photolysis (Scheme 36).[3] This work stated the beginning of the development of a new family of PPGs based on (coumarin-4-yl)methyl derivatives, that have been used in the caging of phosphates, carboxylates, amines, alcohols, phenols and carbonyl compounds.



161 **162**

X = $OP(O)(OEt)_2$
Nu = MeOH, piperidine, cysteine, tyrosine, α-chymotrypsin

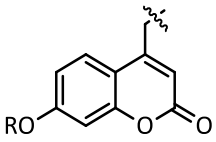
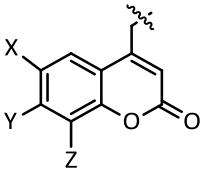
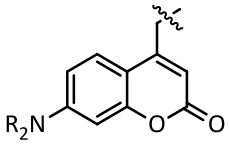
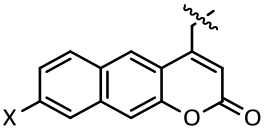
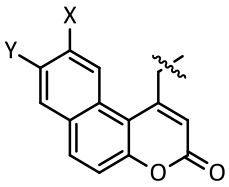
Scheme 36. Release of phosphates from 7-methoxycoumarin derivatives **161**.

The attractive properties of coumarin-based PPGs, namely large molar absorption coefficients (ε) at longer wavelengths, extending into the visible region (400-500 nm) and the fast release rates from its excited singlet state, have motivated their interest specially for bioapplications.[169] These advantages may compensate the low photochemical quantum yields, reduced aqueous solubility and the inconvenience of strong fluorescence and competitive absorptivity from the coumarin photolysis byproducts. Structural modifications performed by several researchers allowed for higher quantum yields, increased aqueous solubility, and the resulting desired features enabled the expansion of the leaving group range (including alcohols and amines).

Concerning the substituents at C-6 and C-7, it is possible to state that the first generation of coumarylmethyl-based phototriggers possessing hydroxyl or methoxyl substituents at C-7, displayed high release rates and hydrolytic stability. Nevertheless, they showed poor solubility and relatively low photochemical quantum yields for all leaving groups except for phosphates. 6-Bromo-derivatives lowered the pK_a of the 7-hydroxy group by two units, resulting in complete deprotonation at physiological pH, improved water solubility and a bathochromic shift of 60 nm (Table 3). However, some of these caged compounds presented increased susceptibility to hydrolysis in the dark.

The introduction of an amino group at C-7 can be considered as a second-generation of coumarinylmethyl-based PPGs, resulting in an improvement in photophysical and photochemical properties of the cage, moving the absorption maxima to 350-400 nm and recording the highest photochemical quantum yields among the analogues. In order to circumvent the problem of water solubility, polar groups such as carboxylates have been added as substituents of the aniline moiety. Extension of the aromatic ring of coumarins, resulting in polyaromatic analogues have led to the enhancement of the fluorophoric properties.[12,13,170,171]

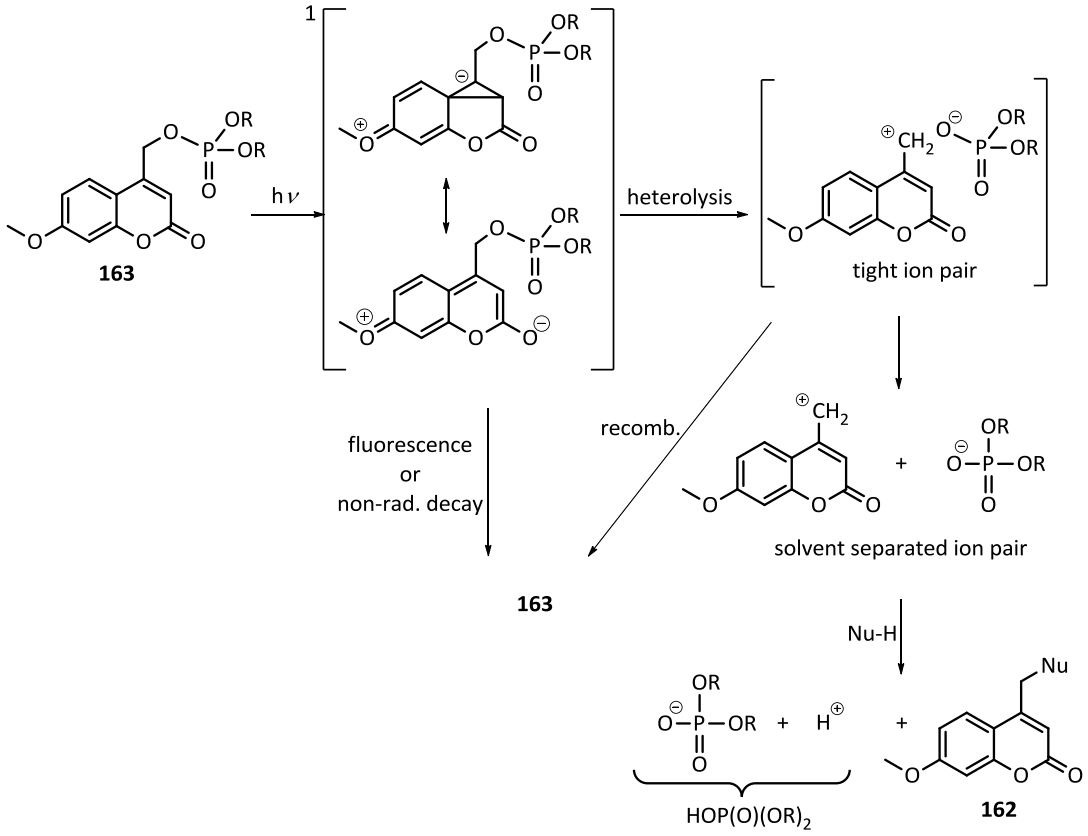
Table 3. Typical coumarinyl chromophores.

λ_{max} (nm)	Structure	Abbreviation
320-330		HCM (R = H) 7-MCM, Bpm (R = Me) CMCM (R = CH_2CO_2H)
330-380		DMCM (X = Y = OMe, Z = H) BCMCM (X = Y = OCH_2CO_2H, Z = H) 7,8-BCMCM (X = H, Y = Z = OCH_2CO_2H) BHCM, Bhc (X = Br, Y = OH, Z = H) BMCM (X = Br, Y = OMe, Z = H) BBHCM (X = Br, Y = OH, Z = $N(CH_2CO_2tBu)_2$) (X = Br, Y = *n*-$C_{17}H_{35}CO_2$, Z = H)
350-400		ACM (R = H) DMACM (R = Me) DEACM (R = Et) BCMACM (R = CH_2CO_2H) BBCMACM (R = CH_2CO_2tBu)
340-360		Obb (X = H) Bbl (X = OMe)
		Obc (X = OH, Y = H) Obm (X = OMe, Y = H) Bba (X = H, Y = OMe)

Schmidt and colaborators have proposed a general mechanism of photocleavage for the most efficiently applied leaving groups, i.e. phosphates, sulfonates, and carboxylic acids (Scheme 37).[172,173] After light absorption by the ground-state caged compound, relaxation occurs to the lowest $^1(\pi,\pi^*)$ excited singlet state, with competition between unproductive non-radiative decay and fluorescence, and

heterolytic C–X bond cleavage. The main intermediate of this process is the initially formed tight ion pair (coumarinylmethyl cation and leaving group conjugate base). Regarding the coumarinylmethyl cation, it can either react directly with accessible nucleophiles or solvent, yielding a new stable coumarylmethyl product, or instead escape from the solvent cage and react with available nucleophiles. Even though there is evidence of intersystem crossing occurring with 7-aminocoumarins,[3] there are no indications of triplet reactivity. An additional unproductive pathway can occur through recombination of the tight ion pair to regenerate the ground-state caged compound.

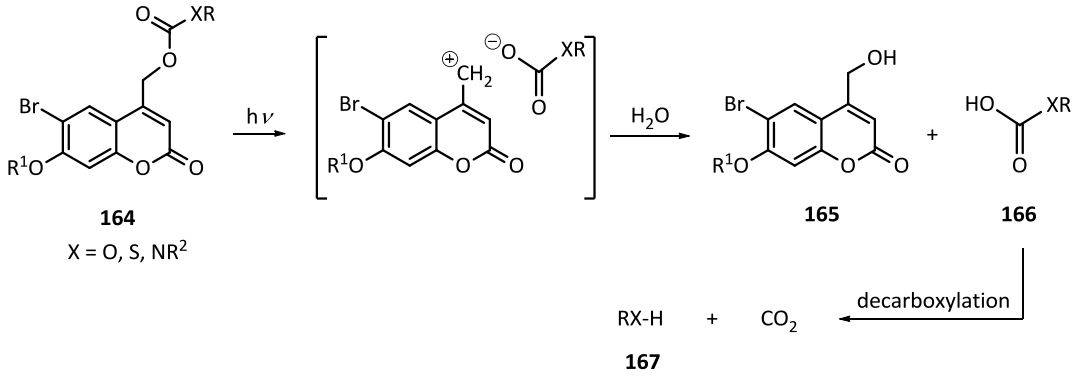
Based on time-resolved absorption studies it was demonstrated that the heterolytic bond cleavage is among the fastest photorelease for any caged compounds, with rate constants up to 2×10^{10} s^{-1} (determined for phosphate esters).[174] Nevertheless, ion pair recombination dominates the subsequent reactions and is about 10 times faster than the nucleophilic trapping of coumarinylmethyl cation by the solvent.[172,173] In an attempt to facilitate the reaction and simultaneously ion pair recombination, modifications on the coumaryl moiety by inserting electron-donating substituents or selecting leaving groups with low pK_a values can be performed.[172]



Scheme 37. General mechanism of photorelease of coumarin-caged compounds **163**.

(Coumarin-4-yl)methyl cages of poor leaving groups such as alcohols, phenols, and thiols revealed to be resistant to photoheterolysis. However, when caged through the carbonate linkage with the (6-bromo-7-hydroxy/alkoxycoumarin-4-yl)methyl group **164** (X = O, S),[3] they can be photoreleased more efficiently (Scheme 38). The free alcohol or thiol is obtained after decarboxylation of the firstly unstable carbonic or thiocarbonic acid formed,[91,175,176] being this reaction frequently rather slow, and subjected to both acid and base catalysis.[177]

Concerning the photorelease of amines from the corresponding coumarin-4-ylmethyl carbamates **164** (X = NR^2), it is similar to alcohols, but with a slower rate (Scheme 38).[178-181] The rate-limiting step is decarboxylation of the released carbamate anion, which is more strongly dependent on the pH and the nature of the released amine or amino acid, than the carbonates in the release of alcohols.[182]



164
X = O, S, NR^2

165 **166**

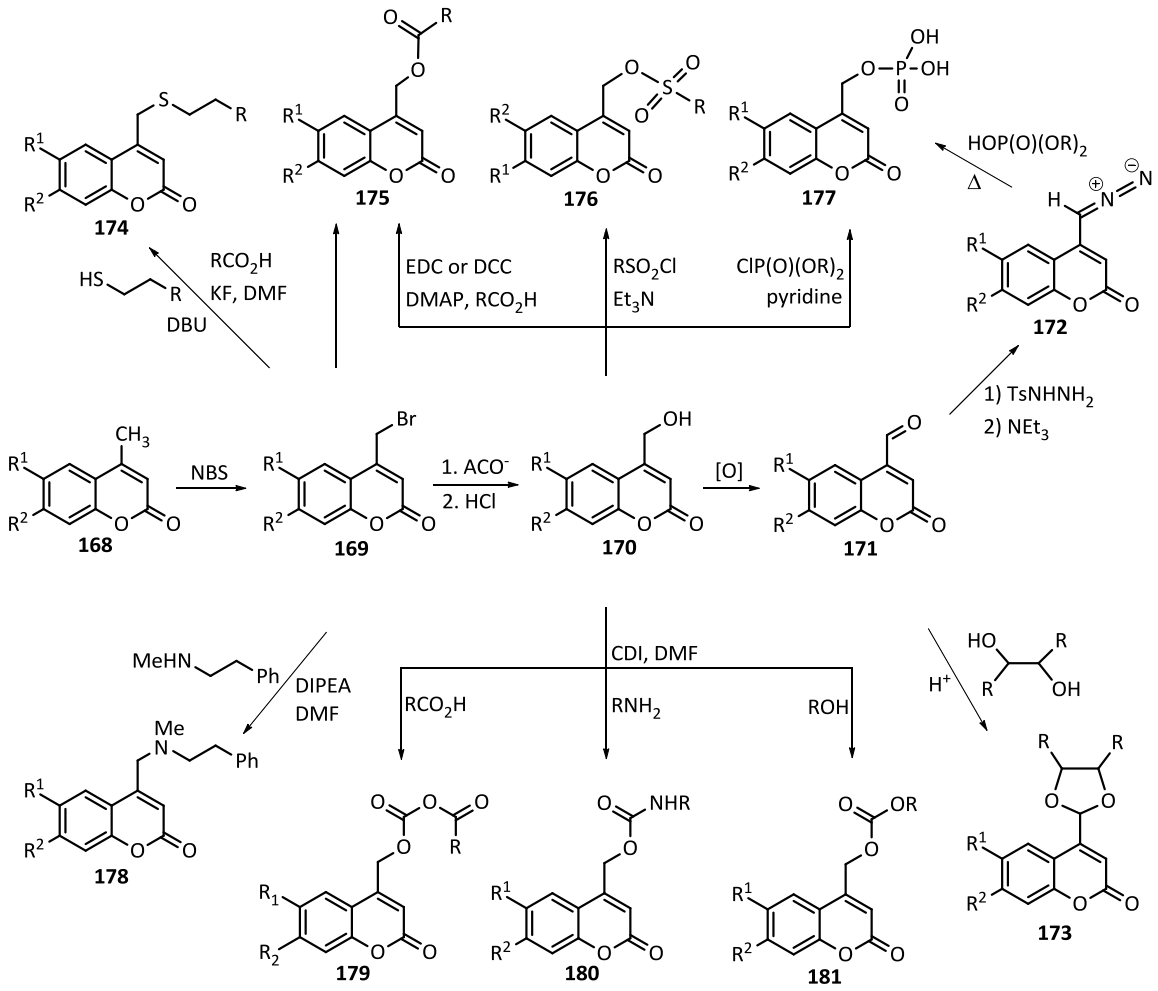
167

Scheme 38. Photorelease of alcohols, thiols and amines from coumarinylmethyl conjugates **164**.

The photophysical properties of the coumarin system accomplished with clever modifications on the substituents present in certain positions, such as C-6 and C-7, as above mentioned, resulted in interesting performances of its derivatives in the field of PPGs. In addition, the versatility to achieve derivatives with reactive groups, capable of establishing covalent linkages with the functional groups of a broad range of target molecules, to attain the corresponding caged compounds is significant, in the widespread applications of these compounds.

The usual precursor of coumarin-caged esters, including phosphates, carboxylates, and sulfonates as well as carbonate, carbamate, and anhydride derivatives

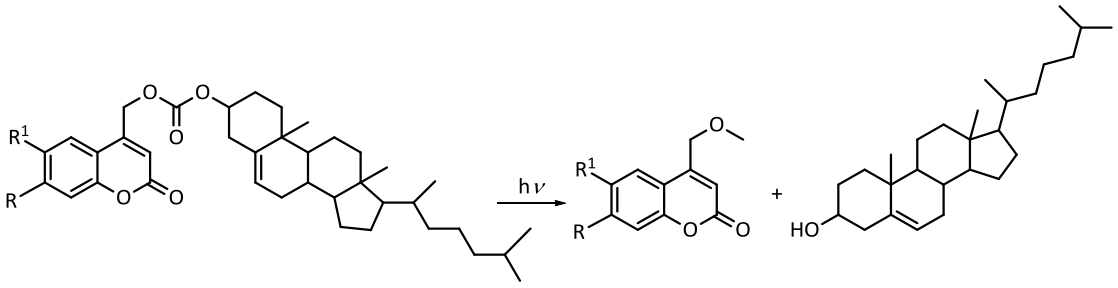
is the coumarin-4-ylmethanol **170**, obtained from the corresponding halides **169**. Coumarinylmethyl bromide **169** is suitable for caging carboxylic acids, including amino acids, through S_N2 substitution. It can also be used in the preparation of coumarin-4-methylamines **178** and -thiols **174**, by standard alkylation. The aldehyde **171** obtained by manganese dioxide oxidation from alcohol **170**, showed to be appropriate to cage diols, e.g., acetals **173**.[183] This aldehyde is also an adequate precursor to 4-diazomethylcoumarin **172**, which was reported to be effective in caging complex phosphates **177**, mainly cyclic adenosine nucleotides, whene coumarin-4-ylmethanol **170** or coumarin-4-yl bromide **169** didn´t succeed by standard methods (Scheme 39).[184,185]



Scheme 39. Strategies for the synthesis of coumarin-caged compounds **173**-**181**.

The importance of the (coumarin-4-yl)methyl derivatives as protecting groups, especially in biological applications is notable. In order to illustrate some of them, several recently reported examples have been selected and will be mentioned below.

In the present year, Bourbon *et al.* reported the release of cholesterol **187** from the corresponding coumarin-cages **182**-**184**, possessing the 7-diethylaminocoumarin (DEACM) **182**, 6-bromo-7-hydroxycoumarin (BHC) **183** and 6-bromo-7-methoxycoumarin (BMCM) **184** photocleavable groups.[186] Owing to their absorption properties, solutions of compounds **182**-**184** in methanol, 20% tetrahydrofuran in methanol and 1% chloroform in methanol, were photolysed at 380 nm (compound **184**) and 350 nm (compounds **183** and **184**) (scheme 40).



182 R = NEt_2, R^1 = H (DEACM-cholesterol)
183 R = OH, R^1 = Br (BHC-cholesterol)
184 R = OMe, R^1 = Br (BMCM-cholesterol)

185 R = NEt_2, R^1 = H
186 R = OMe, R^1 = Br

187

Scheme 40. Photolysis reaction of caged cholesterol **182**-**184**.

The authors showed that BHC-cholesterol **183** was the most efficient of the three caged compounds in the release of cholesterol, with a photochemical quantum yield of 0.032. Moreover, it is superior to the previously published cholesterol caging group, the perylen-3-ylmethyl group (with a photochemical quantum yield of 0.0085) and also at a more appropriate wavelength (Table 4).

(6-Bromo-7-hydroxycoumarin-4-yl)methyl (BHC)[176,183,187-190] and [7-[bis(carboxymethyl)amino]coumarin-4-yl]methyl (BCMACM)[191,192] have been applied to protect carboxylates, phosphates, amines, alcohols, and also carbonyl compounds. Although the two (coumarin-4-yl)methyl phototriggers displayed favourable photophysical and photochemical properties, the application of BHC cages in hydrophobic biomolecules is limited by their low solubility in aqueous buffer and BCMACM derivatives by their low quantum yields in the case of poor leaving groups.

Table 4. Properties of caged cholesterol **182**-**184** in methanol.

Compound	λ_{max} (nm)	ε ($M^{-1}cm^{-1}$)	Φ_{Phot}[a]
182	380	13000	0.010[b]
183	318	6700	0.032[c]
184	327	5000	0.022[c]

[a]With methanol as explicit solvent model; [b]at 380 nm; [c]at 350 nm.

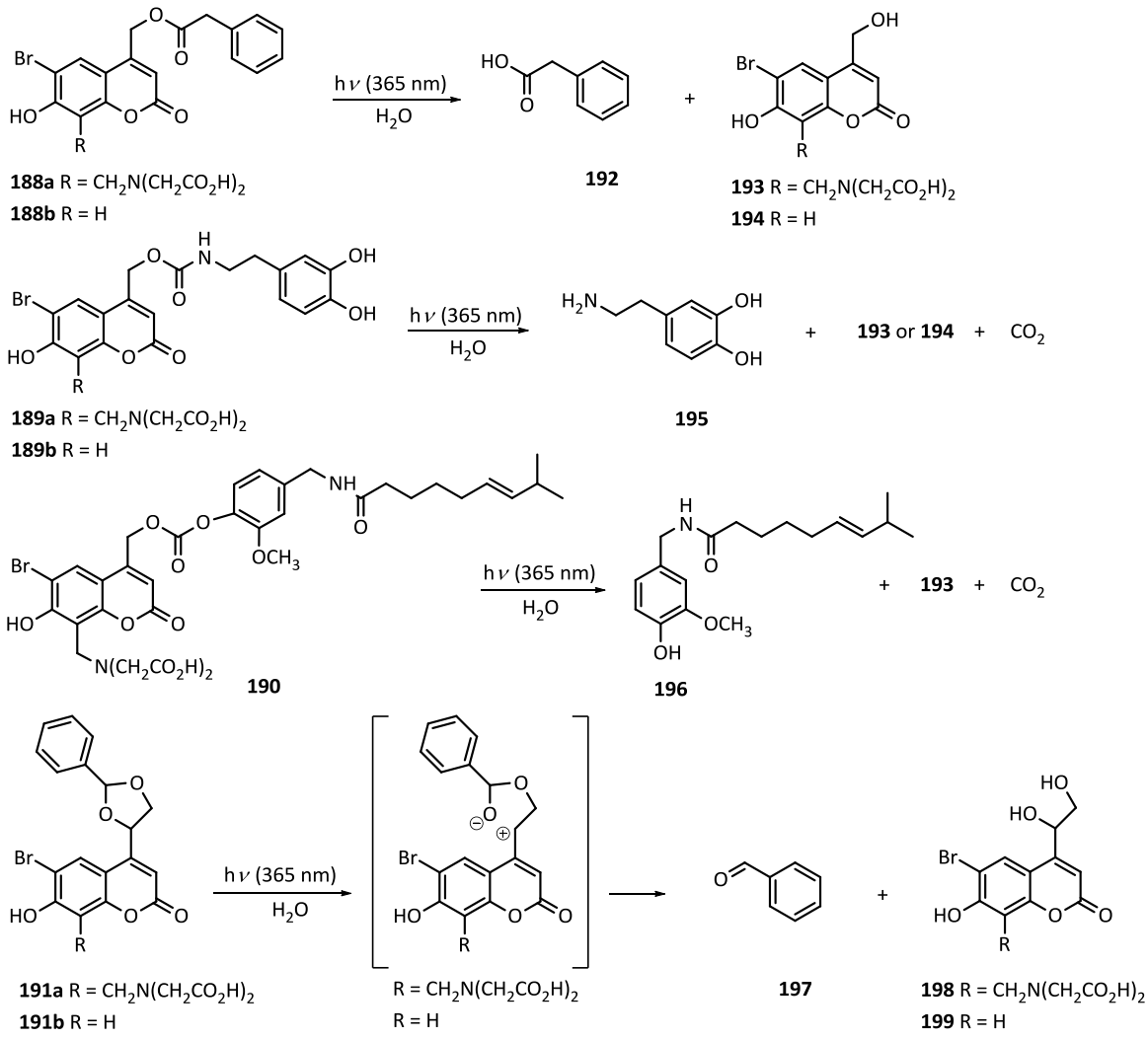
In order to circumvent these limitations, Hagen and co-workers evaluated the sensitivity to photolysis of [8-[bis(carboxymethyl)aminomethyl]-6-bromo-7-hydroxycoumarin-4-yl]methyl (BBHCM) ester **188a**, BBHCM carbamate **189a**, BBHCM carbonate **190**, and the [8-[bis(carboxymethyl)aminomethyl]-6-bromo-7-hydroxycoumarin-4-yl] (BBHC)-substituted cyclic acetal **191a** in the release of a carboxylic acid, an amine, a phenol, and a carbonyl compound, respectively (Scheme 41).[91] The photophysical properties and the photochemical behaviour of these compounds was compared with those of the corresponding BHC cages **188b**, **189b** and **191b** and are summarised in Table 5.[188]

Table 5. Photophysical and chemical properties of the caged compounds **188**-**191**, and of **193** and **194** in acetonitrile/HEPES-potassium chloride buffer (5:95), pH 7.2.

Compound	λ_{abs} (nm)	ε ($M^{-1}cm^{-1}$)	Φ_{Phot}[a]	λ_{em} (nm)	Φ_F	τ_F[b] (ns)
188a	375	17600	0.10	472	0.08	0.66
188b	375	17200	0.10	476	0.04	0.44
189a[c]	372	18000	0.12	470	0.12	1.37
189b[c]	372	17400	0.10	471	0.11	1.04
190	376	17000	0.06	468	0.03	ND
191a	372	18500	0.14	470	0.36	ND
191b	371	18300	0.06[d]	476	0.58	4.37
194	367	17600	ND	471	0.60	4.21
193	369	18200	ND	465	0.57	4.72

[a]At 365 nm irradiation; [b]Fluorescence lifetimes of the lowest excited singlet state; [c]Measured in CH_3CN/phosphate buffer (5:95), pH 7.2; [d]Literature value 0.06; ND not determined.

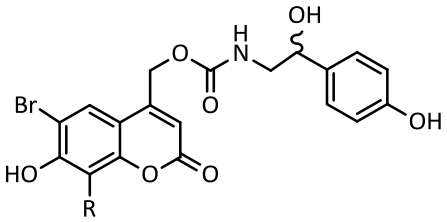
Photolysis of caged derivatives **188**-**191** by irradiation in the range 333-420 nm led to the efficient release of the expected active compounds. The results showed that in comparison to the corresponding BHC derivatives, the new coumarin-based caged compounds displays dramatically increased solubilities in aqueous buffers; lower pK_a values of the C-7 hydroxyl of the coumarin chromophore, thus allowing efficient photorelease at lower pH; and higher photolysis quantum yields in the case of carbonyl compounds. The primary step of the photocleavage is very fast, with rate constant of about 10^9 s^{-1}.



Scheme 41. Photolysis of conjugates **188**-**191**.

As a continuation of the work performed by Hagen's research group regarding the evaluation of coumarin derivatives as PPGs, the authors recently reported the

efficient caging of the neurotransmitter octopamine with (6-bromo-7-hydroxycoumarin-4-yl)methoxycarbonyl (BHCMOC) **200**, [8-[bis(carboxymethyl)aminomethyl]-6-bromo-7-hydroxycoumarin-4-yl]methoxycarbonyl (BBHCMOC) **201** moieties,[91] as well as the [6-bromo-8-(diethylaminomethyl)-7-hydroxycoumarin-4-yl]methoxy-carbonyl (DBHCMOC) **202** and {6-bromo-7-hydroxy-8-[(piperazin-1-yl)methyl]coumarin-4-yl}methoxycarbonyl (PBHCMOC) **203** moieties, the two latter being described for the first time (Figure 29). The resulting cages **200**-**203** were functionally inactive when applied to heterologous expressed octopamine receptors (AmOcta1R). From the photophysical data of caged compounds **200**-**203** in aqueous buffer (pH 7.2), it was possible to see that absorption occurred in the range 371-375 nm with high molar extinction coefficients (Table 6).



200 R = H
201 R = $CH_2N(CH_2CO_2H)_2$
202 R = $CH_2N(CH_2CO_2H)_2$
203 R = 

Figure 29. Caging BHCMOC **200**, BBHCMOC **201**, DBHCMOC **202** and PBHCMOC **203** moieties.

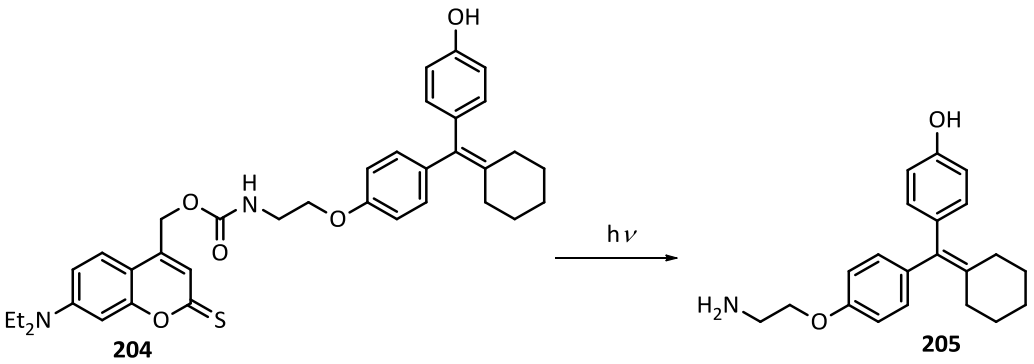
Photolysis at 360 nm or IR light (two-photon approach) in aqueous buffer solutions led to the release of octopamine and evoked Ca^{2+} signals in AmOcta1R-expressing cells. The photoreaction was clean, the recovery rate of octopamine was very high (88-100%) and the single-photon photochemical quantum yields for the disappearance of **200**-**203** were also rather high. The marked water solubility of compounds **201**-**203** in particular and the fact that they did not measurably interact with phospholipid membranes, made them useful phototriggers for this important neurotransmitter.

Table 6. Long-wavelenght absorption maxima (λ_{abs}), molar extinction coefficients at the absorption maxima (ε), photochemical quantum yields (Φ_{phot}), fluorescent quantum yields (Φ_F) and recovery rates of caged compounds **200**-**203** measured in acetonitrile/phosphate (5:95) buffer, pH 7.2.

Compound	λ_{abs} (nm)	ε ($M^{-1}cm^{-1}$)	Φ_{phot}	λ_{em} (nm)	Φ_F	Recovery (%)
200	373	17150	0.16	476	0.29	100
201	372	15000	0.11	470	0.23	100
202	371	14500	0.13	470	0.26	100
203	375	12150	0.13	471	0.15	88

As an attempt to contribute for developing PPGs with maximum wavelength of absorption in the visible region, which is desirable for caging applications to minimise interferences from biological media absorption, Fournier *et al.* recently evaluated the behaviour of 7-diethylamino-4-methanol-thiocoumarin as a blue-absorbing caging group.[193]This group takes advantage from its strongly donating substituent conjugated to the thiocarbonyl group, to shift the wavelength of maximum absorption, as previously reported by our research group with methoxylated coumarins.[168] It displays a significant cross-section for uncaging in the 470-500 nm wavelength range and a low light absorption between 350 and 400 nm.

To access the suitability of the present moiety as caging group, the authors chose an analogue of the previously investigated cyclofen-OH **205** as active target molecule (Scheme 42).[194] Compound **205** possess a terminal primary amino function, which was masked through a carbamate linkage, as in previous caging studies of coumarin series for biological purposes.[187] After evidencing **204** uncaging *in vitro*, and taking advantage of the low absorption of 7-diethylamino-4-methanol-thiocoumarin in the 350-400 nm wavelength range, the authors evaluated the relevance of this system *in vivo*, using zebra fish embryos. Two different wavelengths (365 and 488 nm) were used to orthogonally photoinduce two distinct phenotypes upon independently having photogenerated two biologically active substrates, the analogue of cyclofen-OH **205** and 13-*cis*-retinoic acid.



Scheme 42. Photorealease of cyclofen-OH analogue **205** from the corresponding cage **204**.

The use of diethylaminocoumarin (DEACM) as appropriate caging group for nucleobases in oligonucleotides was demonstrated by Menge and Heckel.[195] An O^6-DEAC-caged deoxyguanosine phosphoramidite (dG^{DEACM}) **206** was synthesized and photolysed at 365 and 405 nm (Figure 30, Table 7). Compared to 2-(*o*-nitrophenyl)-propyl (NPP)-caged analogues **207** under irradiation at 405 nm, DEACM group showed superior photochemical properties with an uncaging efficiency ($\varepsilon.\delta_{unc}$) 17 times higher and time of release 80 times faster.

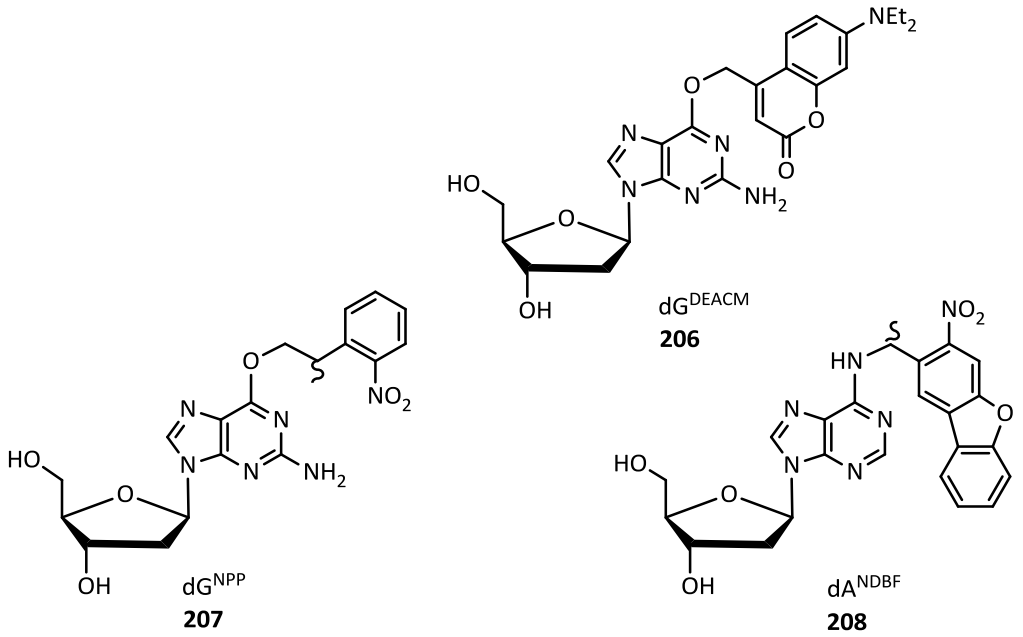


Figure 30. Residue dG^{DEACM} **206**, dG^{NPP} **207** and dA^{NDBF} **208** for comparison.

Regarding the *o*-nitrobenzyl-type caging group, the nitrodibenzofuran (NDBF) chromophore previously reported by the authors for protection of deoxycytidine (dC)

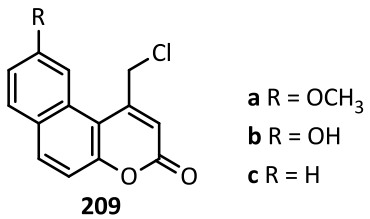
and deoxyadenosine (dA) residues into DNA (see for example dA^{NDBF} **208** in Figure 30),[196] dG^{DEACM} **206** behaves around 7 times better at this wavelength. Moreover, deprotection can still be performed at 470 nm to reduce near UV irradiation damage on biological probes. The dG^{DEACM} **206** represents an interesting improvement in the photocleavable caged nucleotide building blocks that permits efficient wavelength-selective uncaging for more sophisticated light regulation strategies in oligonucleotide applications.

Table 7. Photochemical properties uncaging quantum yield (δ_{unc}) and molar extinction coefficient (ε) of investigated compounds **206**-**208** in PBS buffer, pH 7.4 at different wavelengths.

Compound	λ_{abs} (nm)	δ_{unc}	ε ($M^{-1}cm^{-1}$)	$\varepsilon.\delta_{unc}$ ($M^{-1}cm^{-1}$)
206	365	0.01	15318	178
206	405	0.06	27321	1672
207	365	0.16	625	97
208	365	0.13	9801	1171

As mentioned before, an improvement in photophysical and photochemical properties of the cage, moving the absorption maxima to 350-400 nm, can be achieved by tailoring the coumarin skeleton, through the introduction of substituents, namely the amino group at C-7. Furthermore, the addition of a second benzene ring in the aromatic system leading to benzocoumarins can also result in enhanced properties, especially interesting for fluorescent detection of uncaging processes.

Benzocoumarin chromophores were introduced for the first time by our research group in 2006, being (1-chloromethyl-3-oxo-3*H*-naphto[2,1-*b*]pyrans) **209** used in the caging of *N*-benzyloxycarbonyl and toluenesulfonyl phenylalanine, as model carboxylic compounds (Figure 31).[170]

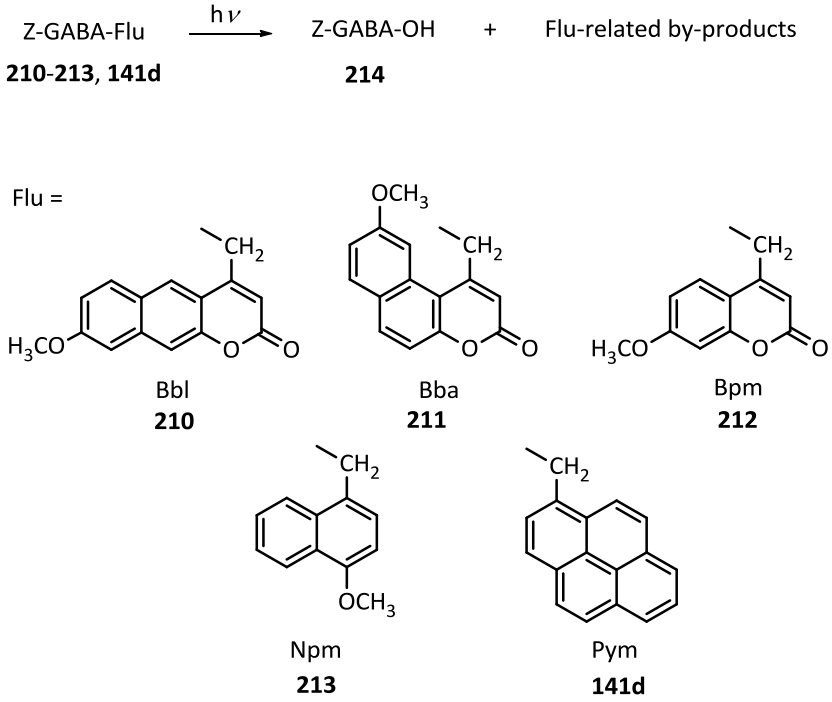


209

a R = OCH_3
b R = OH
c R = H

Figure 31. Structures of benzocoumarins **209a**-**c**.

Later, we reported a comparative study using various fluorophores of aromatic, namely naphthalene and pyrene, and heteroaromatic nature, such as oxobenzopyran derivatives, undertaken to evaluate their performance as photolabile groups in the release of *N*-benzyloxycarbonyl-γ-aminobutyric acid (Z-GABA-OH) **214** (Scheme 43).[12]



Scheme 43. Photorelease of Z-GABA-OH from cages **210**-**213** and **141d**.

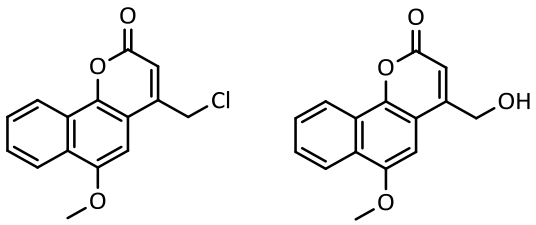
Table 8 summarises the photophysical data of the corresponding fluorescent conjugates **210**-**213** and **141d**. Concerning the photocleavage studies, in methanol/HEPES buffer solution (80:20) at different wavelengths (254, 300 and 350 nm), it was possible to conclude that the irradiation time depended on the structure of the label. The benzocoumarin (Bba) was the most promising at 350 nm, naphthalene (Npm) at 300 nm and pyrene (Pym) at 254 nm.

Relying on the preliminary encouraging results obtained, we applied the Bba group in the caging of the carboxylic acid of other neurotransmitter amino acids in the same conditions as previously mentioned.[12,13]

Table 8. UV/vis and fluorescence data for GABA ester conjugates **210**-**214** and **141d** in absolute etanol.

Compound	λ_{abs} (nm)	log ε	λ_{em} (nm)	Stokes´s shift (nm)	Φ_F
210 Z-GABA-OBbl	345	3.91	503	158	0.21 ± 0.03
211 Z-GABA-OBba	345	3.95	472	127	0.76 ± 0.02
212 Z-GABA-OBpm	320	4.22	393	73	0.27 ± 0.03
213 Z-GABA-ONpm	295	3.80	339	44	0.20 ± 0.02
141d Z-GABA-Opym[11]	342	4.61	375	33	0.15 ± 0.01

A new benzocoumarin-4-yl-methyl moiety, (4-(chloromethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran **215** or 4-(hydroxymethyl)-6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran **216**), with a different benzene ring fusion was designed and evaluated as phototrigger using the same class of compounds as targets, neurotransmitter amino acids, being the caging through ester or carbamate linkages to the carboxylic acid or the amine functions, respectively (Figure 32).[171] Furthermore, the cages obtained from this benzo[*h*]benzopyran showed absorption in ethanol at about 470 nm whereas the Bba absorbed in the range 446-449 nm. This resulted in a considered enhancement in relation to our previously fluorescent fused heterocycles studied as PPGs.

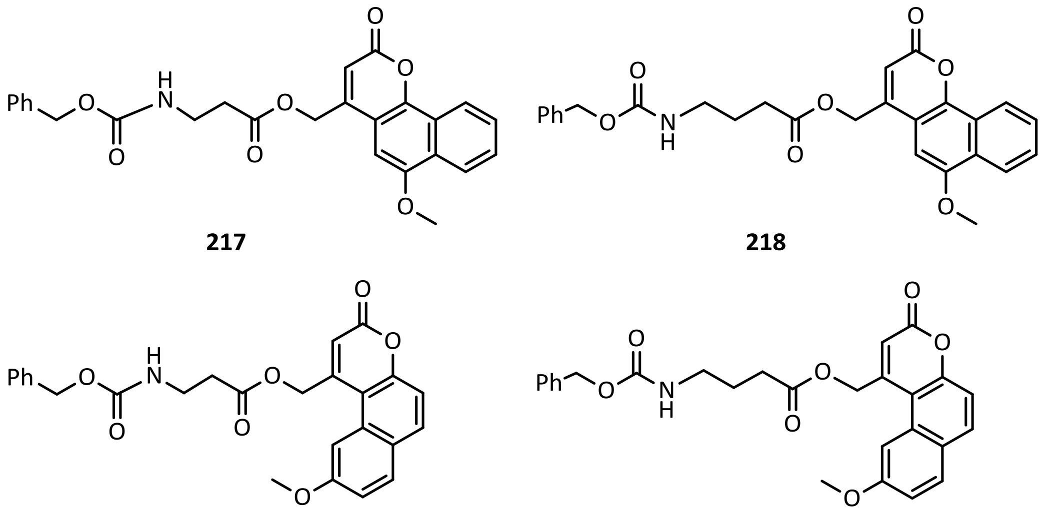


215 **216**

Figure 32. Structures of benzocoumarinyl-methyl moiety **215** and **216**.

A comparison between data of β-alanine and GABA conjugates of benzocoumarins **217**, **218**[171] and **219**, **220** (Figure 33)[12,13] revealed that photolysis of **217**

and **218** occurred with shorter irradiation times at 300 and 350 nm (up to three times), as well as at 254 nm (up to eight times).



217 **218** **219** **220**

Figure 33. Structures of benzocoumarin neurotransmitter conjugates **217**-**220**.

Owing to the convenience of these two benzocoumarins as PPGs and the interest in exploring and extending the applicability of these moieties, and also to make structural and photolytic alterations in order to improve their performance, several studies were carried out under the present dissertation and will be discussed in Chapter 2.

3. Two-photon excitation photorelease

3.1. One-photon *versus* two-photon excitation

The PPGs that have been discussed in the present dissertation are mostly cleaved by UV and in some cases by visible wavelengths through one photon excitation (OPE). However, these conditions limit their applications in the biomedical field, since UV/vis light is efficiently absorbed by the tissue.[3]

Oxyhemoglobin is the responsible species in vertebrate tissues for filtering most of the radiation below 650 nm. On the contrary, water becomes progressively absorbent at wavelengths above 950 nm. In order to minimize background absorbance by biological tissues, the studies have to be conducted within harmless radiation, that is

between 650 and 950 nm.[197] Some of the beneficts taken from working within this interval include reduced phototoxic effects and the possibility to reach deeper tissues, minimizing the scattering of the radiation. However, given the low energy of red and NIR photons, the number of processes in which they can be used is limited. In addition, most of the chromophores that are suitable for single-photon absorbance within this spectral region, are also sensitive to visible light, difficulting the work with caged substrates.

To overcome these problems two-photon excitation (TPE) has been applied, where at high light intensity, chromophores may simultaneously absorb two red/NIR photons, to produce the same or similar higher-energy excited states as those accessible by direct excitation with UV photons at about twice the frequency (Figure 34).[198,199]

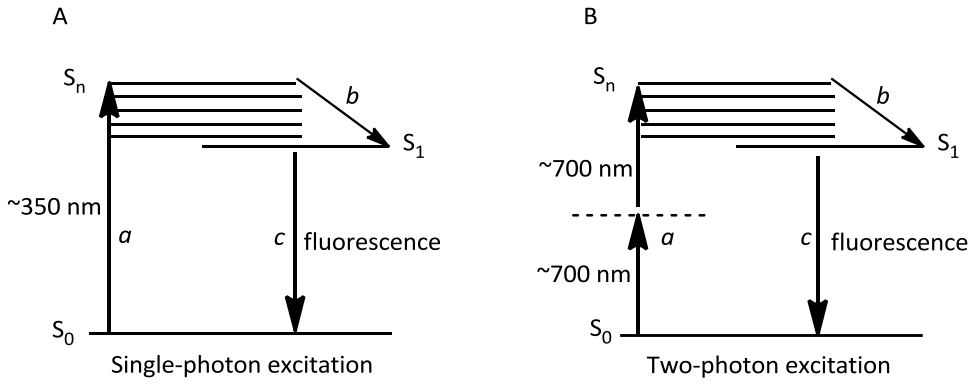


Figure 34. Jablonski diagrams showing the three-stages of excitation *a*, internal conversion *b*, and emission *c*, involved in the process of fluorescence induced by the absorption of a single photon (A) or two photons (B).

Additionally, focusing the irradiation of TPE on UV chromophores within UV-absorbing materials provides an opportunity to control substrate release in three dimensions.[200]

The overall efficiency of the TPE-induced substrate release (δ_{unc}) can be defined by the product $\delta_a \Phi_{Phot}$, whereas δ_a also called σ_2, is the two-photon absorption cross-section (expressed in Göppert-Mayer (GM) (1 GM = 10^{-50} cm^4 s^{-1} $photons^{-1}$) and Φ_{Phot} is the photorelease quantum yield. As a result of the difficulty of measuring photorelease quantum yields under two-photon conditions, the value derived from one-photon

photolysis is used with the assumption that one- and two-photon absorption populate the same excited states and therefore induce the same uncaging quantum yield.

The value of δ_{unc} is strongly dependent on the wavelength of irradiation. Many cages that show reasonable efficiency of substrate release at 740-750 nm have very low δ_{unc} at 800nm.[3]

TPE requires very high light intensities at minimum absorbance by vertebrate tissues, which can be achieved by using ultrafast-pulsed laser, such as Ti: sapphire. This type of laser can provide a photon irradiance of 10^{25} photons cm^{-2} s^{-1} (3 mm beam at 100 fs pulse duration, and repetition frequency ν = 90 MHz) with principal emission at 800 nm. Phototoxicity limits two-photon irradiation with ultrafast-pulsed lasers up to 5 mW average power.[201] It is possible to predict that two-photon photolysis cross-sections with large GM values are required, together with fragmentation rates superior to 10 000 s^{-1}. Cages currently available have low cross-sections, in the range 0.01-0.1 GM at 720 nm, with fragmentation rates typically less than 10 000 s^{-1}.

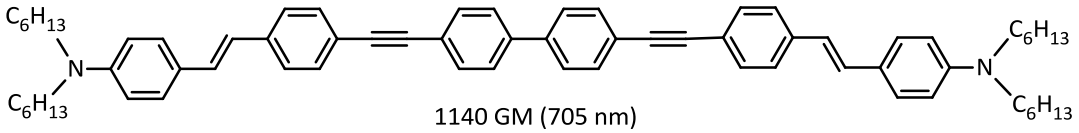
Increasing the cross-sections will likely increase the size and lipophilicity of the cages, thus requiring strategies to increase the aqueous solubility, while retaining high fragmentation rates.[133]

3.2. Two-photon activable caged compounds

The main structural parameters that increase TPE efficiency are extended conjugation and the presence of strong donor and/or acceptor couples that promote strong internal charge transfer. The efficient conjugation requires planarity, which can be achieved by introducing rings or by complexation of a flexible chromophore to constrain a conformation (as examples see compounds **221** and **222** in Figure 35).[202]

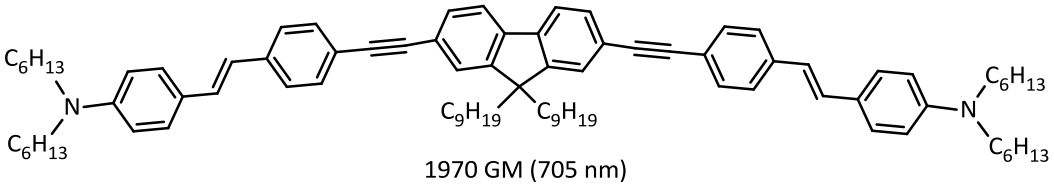
The suitability of several conventional (single-photon) PPGs for TPE-induced substrate release has been explored instead of the development of specific groups for TPE and some examples are discussed below. Caged compounds currently in use for TPE photolysis are derived from photochemical systems developed for near UV-absorbing protecting groups. Although several PPGs have been adapted for cell physiology, none of them adequately satisfies the criteria needed for application in biological conditions and for TPE photolysis. Fluorophores containing D–π–A–π–D (D = donor, A = acceptor) or a

similar structural motif, with δ_a over 1000 GM, unfortunately have not been applied so far in the design of two-photon chromophores for photorelease purposes.



1140 GM (705 nm)

221



1970 GM (705 nm)

222

Figure 35. TPA compounds with high molecular planarity, efficient conjugation and π-electron delocalization.

3.2.1. Nitrobenzyl derivatives

The most widely used *o*-nitrobenzyl PPGs have a rather low two-photon uncaging cross-section δ_{unc}, which varies from 0.01 to 0.035 GM at 730 nm depending on the substitution in the ring and the wavelength of excitation. The 6-nitroveratryl cage **97** shows the highest sensitivity to TPE in this family, with δ_{unc} = 0.035 GM at 730 nm.[203]

The 1-(2-nitrophenyl)ethyloxycarbonyl, NPEOC **98**, and 2-(2-nitrophenyl)prop-1-oxycarbonyl, NPPOC **99**, or (3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl, DMNPB **223**, possess the *o*-nitrophenyl chromophore, but all of them have 5−12 times higher δ_{unc} due to more efficient photochemistry (Figure 36).[204] Also in the presence of a triplet sensitizer with a large TPE cross-section (i.e. thioxanthone), the two-photon uncaging cross-section δ_{unc} of NPPOC **99** is enhanced from 0.12 to 0.86 GM.[204]

Extension of the conjugated π-system of the chromophores, such as in (4'-methoxy-4-nitrobiphenyl-3-yleth-2-yl)methyl, PMNB, **224**, (4'-tris-ethoxymethoxy-4-nitrobiphenyl-3-yleth-2-yl)methyl, PENB **225,**[206,207] and especially when combined with a symmetrical structure **226** (Figure 36),[208] enhances dramatically the two-photon uncaging cross-sections δ_{unc} into 3-5 GM range. However, these groups are rather bulky and suffer from poor solubility.

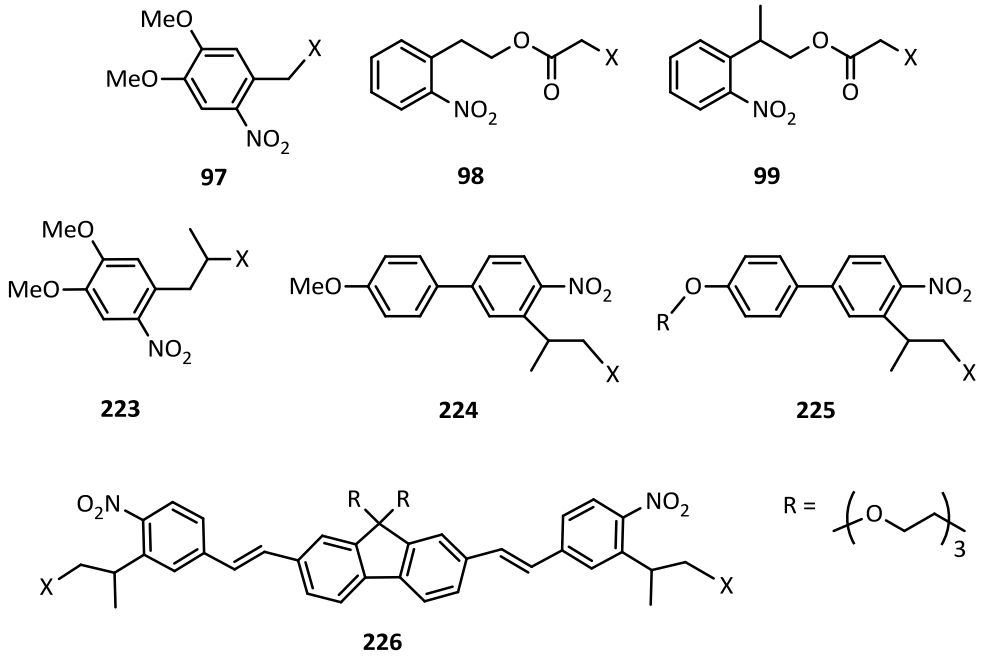


Figure 36. Structures of compounds **97**-**99**, **223**-**226** useful for TPE.

3.2.2. *o*-Nitroindolines

As previously mentioned, within the 7-nitroindoline-based PPGs maximum photolysis efficiency was found with 4-methoxy-7-nitroindolinyl (MNI) derivatives **107**, being MNI-glutamate used in neuroscience for one and two photon excitation.

5,7-Dinitroindolinyl-L-glutamates, exemplified by 4-methoxy-5,7-dinitroindolinylglutamate (MDNI-Glu) **108** and 4-carboxymethyl-5,7-dinitroindolinylglutamate **109**[134,208] were found to be about twice as sensitive to near-UV excitation as MNI-Glu. Even though the photolytic efficiency of the dinitroindolines is 3.5 fold greater than the mono-nitro derivatives (δ_a = 0.06 GM), the reduced stoichiometry results in doubts regarding their use.[134,209]

3.2.3. Hydroxyquinolines

As mentioned before, quinoline-based phototriggers such as (8-chloro-7-hydroxyquinolin-2-yl)methyl acetate (CHQ-OAc) and also (8-bromo-7-hydroxyquinolin-2-yl)methyl acetate (BHQ-OAc) photolyze in response to irradiation with ultraviolet (using OPE) or near-visible light (using TPE), releasing the correspondent acetate. In particular

BHQ, the most efficient two-photon-indutive substrate releaser (δ_{unc} = 0.6-0.9 GM at 740 nm) in this family[158] has been reported to be a good phototrigger for mediating the release of biologically relevant effectors *in vivo* through TPE. It has been used to pinpoint the timing and location of morpholino activation in zebrafish with TPE,[210] to regulate the action of a thrombin aptamer[211] and to initiate the expression of green fluorescent proteins.[83] Owing to its interest in TPE-uncaging, mechanistic studies of the photodeprotection of BHQ and also CHQ caged acetates have recently been reported.[162]

3.2.4. Hydroxycoumarin

(Coumarin-4-yl)methyl-based PPGs are usually more efficient two-photon cages than simple 1-(2-nitrophenyl)ethyl (1-NPE) and 2-(2-nitrophenyl)prop-1-yl (NPP) analogues. The uncaging cross-sections of 6-bromo-7-hydroxycoumarin-4-ylmethyl (BHCM) group **227** range from 0.35 to 2 GM at 740 nm depending on the caged substrate.[179,188] Introduction of two additional bromine atoms in the benzene ring of BHCM **228** results in a red-shift of the TPE maximum and also in the increase of the two-photon uncaging efficiency (δ_{unc} 0.96 GM, **228**, *versus* 0.35 GM, **227**) (Figure 37).

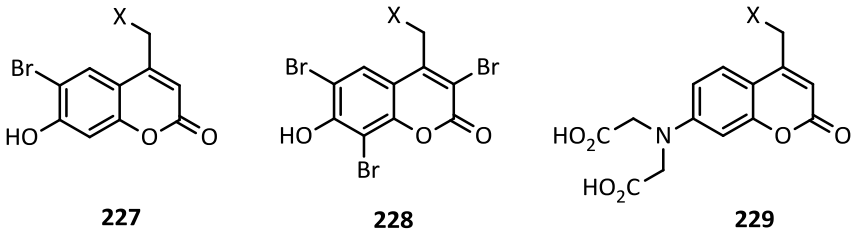


Figure 37. Structure of (coumarin-4-yl)methyl-based PPGs **227**-**229** useful for TPE.

The presence of the substituted amino group in [7-[bis(carboxymethyl)amino]coumarin-4-yl]methyl, BCMACM, **229,** red shifted the chromophore absorption (λ_{max} 330-430 nm), being the two-photon cross-section of uncaging δ_{unc} at 800 nm (1 GM) one of the most interesting of this family of PPGs.[191]

Recently, Bao and co-workers reported the synthesis of a new photottigger based on {7-[4-(dimethylamino)styryl]coumarin-4-yl}methoxycarbonyl for alcohols (Figure 38).[212] Thus, the caged model compound was prepared by the protection of 4-

methoxy-benzyl alcohol **230**. The photolytic properties were evaluated and compared to those of carbonate **231** from the known [7-(diethylamino)coumarin-4-yl]methoxycarbonyl (DEACM) phototrigger.[213]

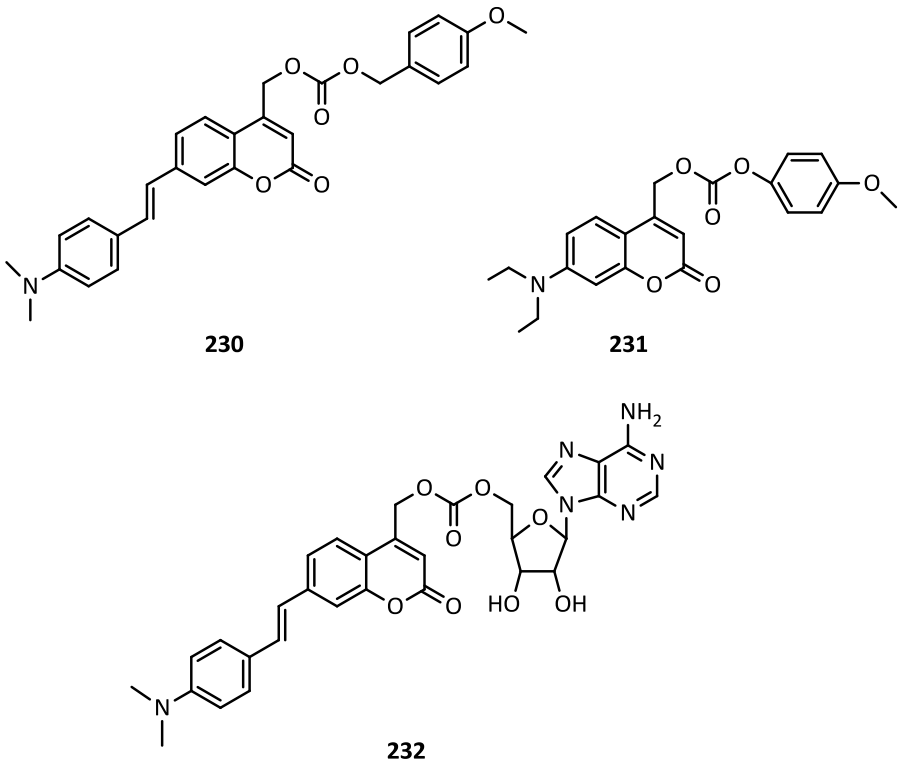
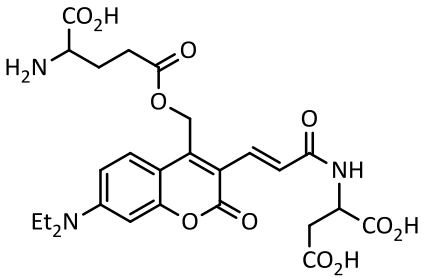


Figure 38. Structures of compounds **230**-**232**.

The authors found that conjugate **230** displayed long wavelength absorption with a maximum at 407 nm (*ca*. 30 nm red shifted), an expedite one-photon photolysis efficiency upon irradiation at 475 nm and a strong two-photon photolysis sensitivity with 800 nm laser light (TPA cross-section with 309.5 GM, which is 135 times higher than **231**, 2.3 GM). A caged purine nucleoside **232** using {7-[4-(dimethylamino)styryl]coumarin-4-yl}methoxycarbonyl as a phototrigger was also prepared and the photolytic properties revealed a similar behaviour to those of compound **230** in the release of free adenosine. The results obtained with the reported caging group seemed to be promising for biological applications.

In the present year, Olson *et al.* have synthesized a 7-diethylaminocoumarin (DEAC) derivative that enables for the first time wavelenght-selective two-photon uncaging of a biomolecule at long wavelenghts, 900 nm (δ_{unc} 0.5 GM) *versus* 720 nm

(Figure 39).[214] This new phototrigger chromophore, designated DEAC450, possesses an extended π-electron moiety at the 3-position that shifts the DEAC absorption spectrum maximum from 375 to 450 nm. Two-photon excitation at 900 nm was more than 60 times greater than at 720 nm. At 900 nm, two photon uncaging of the new caged glutamate compound, DEAC450-Glu **233**, at spine heads on pyramidal neurons in highly isolated mouse brain slices, generated postsynaptic responses that were similar to spontaneous postsynaptic excitatory miniature currents, whereas significantly higher energies at 720 nm induced no currents.[214] The two-photon sensitive caged compounds are almost active at 720 nm, optically selective uncaging of DEAC450-caged biomolecules at 900 nm may pursuit for two-color optical approaches of bimodal signaling pathways in living tissue with high resolution for the first time.[96]

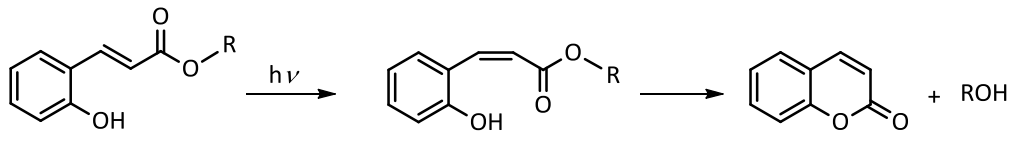


DEAC450-Glu
233

Figure 39. Structure of DEAC450-Glu **233**.

3.2.5. *o*-Hydroxycinnamyl derivatives

o-Hydroxycinnamate (*o*-HC) derivatives **234** introduced by Porter and co-workers in 1987, were further developed as PPG for alcohols. In general, these systems undergo *E* to *Z* photoisomerization followed by lactonization, to form a coumarin and release of caged substrates (ROH) (Scheme 44).



234

Scheme 44. Photochemistry of *o*-hydroxycinnamic derivatives **234**.

These compounds displayed high uncaging cross-sections and water solubility, which allowed their use in physiological experiments, like the case of 3,5-dibromo-2,4-dihydroxycinnamate (δ_{unc} = 1.6 GM at 750 nm), that was validated on an embryo zebrafish model (60 min irradiation at 750 nm, using < 5 mW maximum power in 10 mM aqueous solution) (Figure 40).[215]

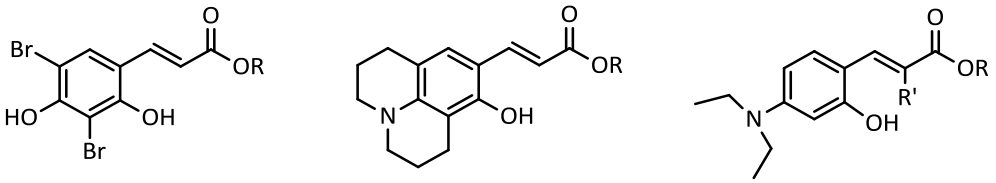


Figure 40. Structures of compounds **235**-**237**.

Even though the rate into coumarin conversion by TPE in *o*-HC cages **235**-**237** is considerably higher than the photo-isomerization step, the substrate release *in vivo* conditions was slow (10^{-2} - 10^{-3} s^{-1} at 750 nm), thereby limiting their application as PPGs to biological processes whose slow substrate release is not detrimental. Notably, many *o*-hydroxycinnamate derivatives have high cell membrane permeability, which allows the use of the spatial localization of TPE, to restrict activation to one cell.

4. Photoactivation with chromatic selectivity

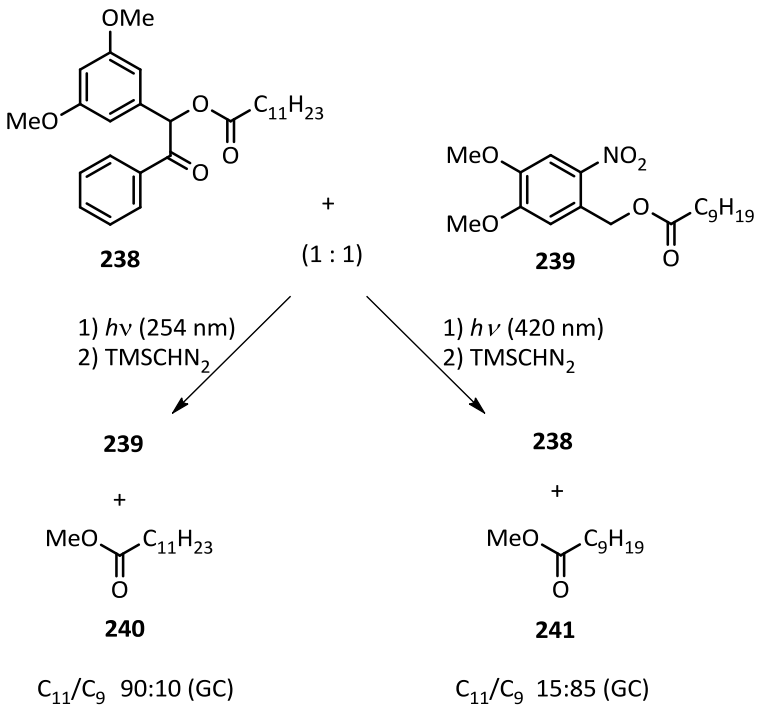
Orthogonality is the possibility of causing certain functional groups to react under a defined set of conditions without affecting others.[216] In the specific case of photochemical reactions, *chromatic orthogonality* is defined as the possibility of transforming photochemically a specific chromophore at a specific wavelength, without disturbing other photosensitive moieties.[217]

The diversity of PPGs operating by different mechanisms and bearing different types of chromophores, opens the door for wavelength-selective deprotection. The possibility of releasing on demand various types of molecules (e.g., in a cell, or wherever access by conventional injection techniques is not possible) is a very appealing prospect.

A first-order approximation would suggest that absorption maxima sufficiently distant could work; however, fast energy transfer may destroy such a strategy, in particular for cases where both chromophores are part of the same molecular entity.[3]

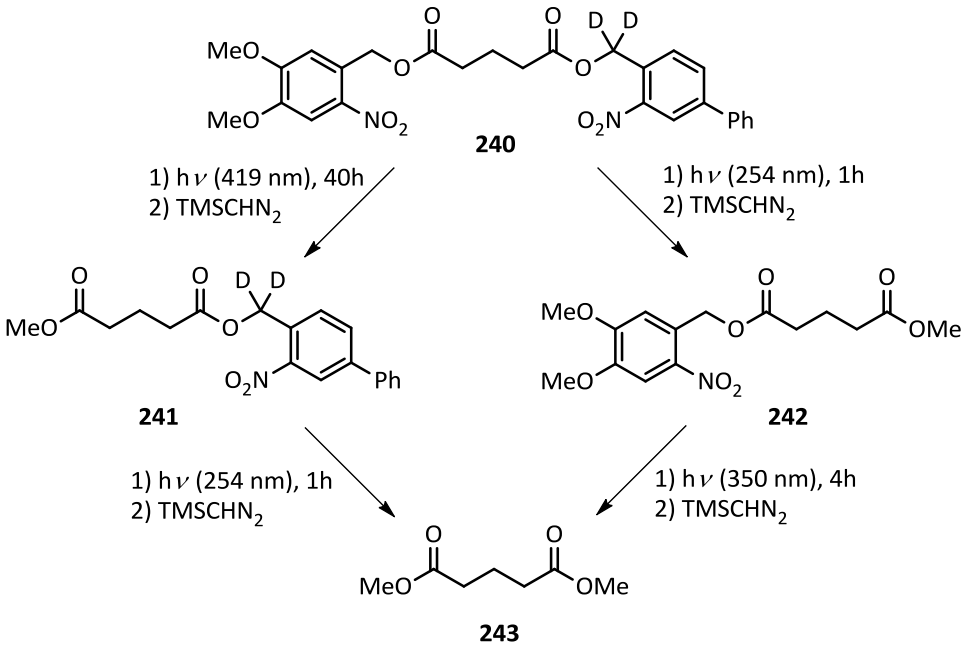
By the year 2000, the idea of achieving multifunctionality by combining PPGs that could be independently photocleaved at selective wavelengths and intensities was proposed and demonstrated for solution experiments[217,218] and solid phase peptide synthesis[219] using derivatives of 3,5-dimethoxybenzoin (DMB) and 4,5-dimethoxy-2-nitrobenzyl (DMNB). The principle is based on the different photosensitivities of the PPGs at different wavelengths, as given by the product of the absorbance and the photochemical quantum yield.

Blanc and Bochet evaluated the selectivity of release of a 1:1 solution mixture of esters **238** and **239** by the choice of wavelength.[218] The crude mixture resulting from the photolysis at 254 nm for 5 min showed a 90:10 ratio of C_{11}/C_9 esters **240** and **241**, confirming the much higher reactivity of the benzoin derivative **238** at this wavelength. On the other hand, the 420 nm irradiation for 16 h yielded a 15:85 ratio, favoring the C_9 ester **241**, again confirming the higher reactivity of the 2-nitroveratryl ester **239** at long wavelengths (Scheme 45).[218]



Scheme 45. Example of intermolecular chromatic orthogonally.

Bochet and co-workers were interested in the possibility of a real chromatic orthogonality, i.e., the deprotection of either terminus by choosing the appropriate wavelength. To check this, they exposed several diesters (e.g. given by **240**) to monochromatic light at 254 and 419 nm. By carefully choosing a protecting group pair, such as in **240**, chromatic orthogonality was found. Irradiation at 419 nm cleanly converted **240** into **241** after treatement with trimethylsilyldiazomethane ($TMSCHN_2$), with a yield of 69%, whereas irradiation at 254 nm gave **242** with 61%. Further photolysis at the complementary wavelengths converted both **241** and **242** into glutaric diester **243** in 71% and 85%, respectively (Scheme 46).



Scheme 46. Example of intramolecular chromatic orthogonally.

This selectivity can be explained as follows: the 4,5-dimethoxy-2-nitrobenzyl group is known to yield products quite efficiently despite its very low photochemical quantum yield (Φ_{Phot} = 0.0013),[123] because of its significant absorbance above 350 nm, whereas the 4-phenyl-2-nitrobenzyl group is highly reactive at 254 nm by both high photochemical quantum yield and absorbance.[220] Reactivity tuning by way of a kinetic isotope effect was also exploited to make two derivatives of *o*-nitrobenzylic esters chromatically orthogonal.[221]

Examples of chromatic orthogonality were soon shown in the solid-phase

synthesis of peptides. Kessler *et al.* developed a sequential solid-phase peptide synthesis using both photolabile linker and protecting groups.[222] The chromatic sequential lability between a *tert*-butyl ketone-derived linker (sensitive to irradiation at 305 nm) and a nitroveratryloxycarbonyl (NVOC) group (sensitive at 360 nm) was exploited to prepare Leu-Enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) in a 55% overall yield.[223] This strategy allows the preparation of peptides in essentially neutral medium, avoiding the use of common deprotection reagents such as trifluoroacetic acid or piperidine.

Analysis on the photolytic characteristics of the PPGs helped to establish several protecting strategies that enable the simultaneous use of up to four orthogonal photoactivated moieties, such as benzoin (BNZ, λ_{ex} = 255 nm), *p*-hydroxyphenacyl (*p*-HP, λ_{ex} = 275 nm), 5,7-dinitroindolinyl (DNI, λ_{ex} = 360 nm), and [7-(diethylamino)coumarin-4-yl]methyl (DEAC, λ_{ex} = 435 nm) which can be cleaved sequentially from the same solid support under different irradiation conditions (Scheme 46).[224]

Recently, del Campo *et al.* studied the possibility of wavelength-selective photolysis of seven different families of PPGs, on various functionalities immobilized through an organosilane tether on a glass surface. Amine, thiol-, and carboxylic-terminated organosilanes were caged with *o*-nitrobenzyl (NVOC, NPPOC), benzoin (DMB), (coumarin-4-yl) methyl (DEAC), 7-nitroindoline (DNI, BNI), and *p*-hydroxypehancyl (*p*-HP) derivatives, and the corresponding silanes **244**-**250** were irradiated at different wavelengths (Figure 41).[224,225]

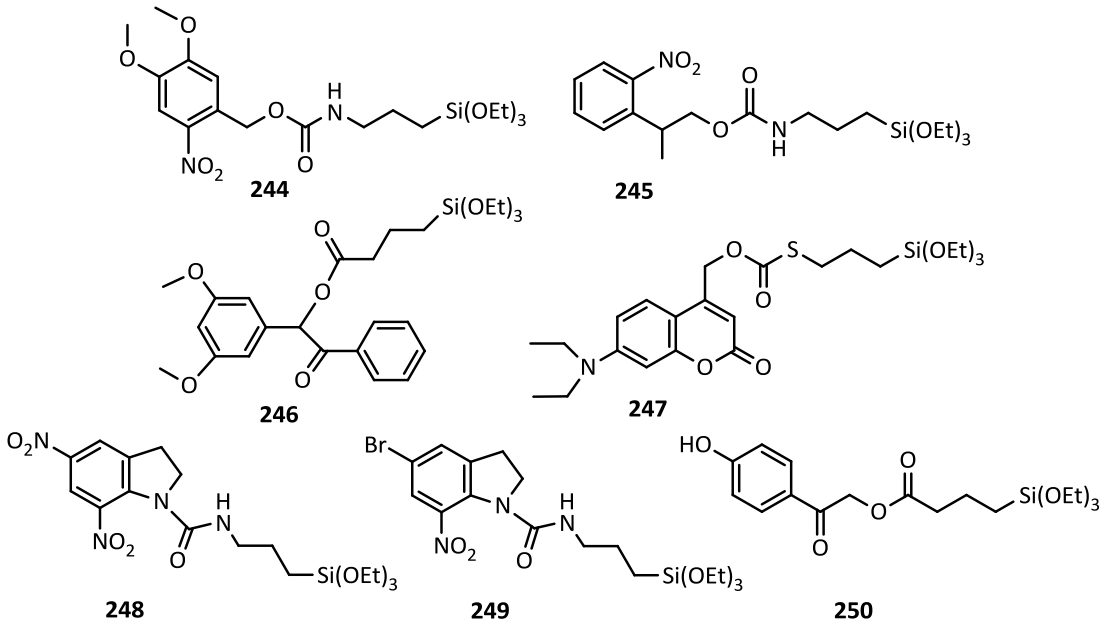
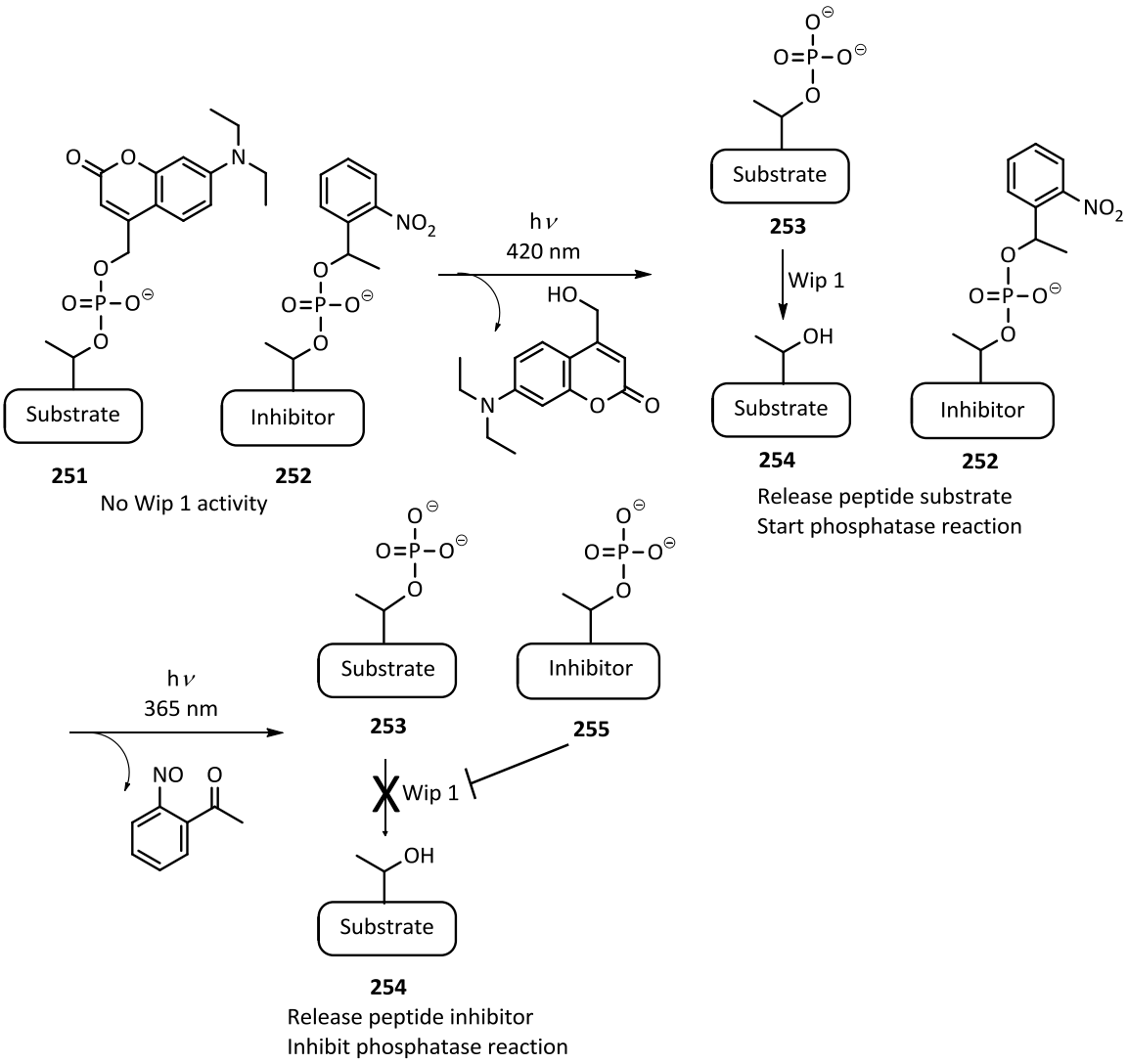


Figure 41. Structures of organosilane caged derivatives **244**-**250**.

Goguen *et al.* developed and approach for sequentially uncage two different phosphopeptides in the same solution, by investigating the photolysis of [7-(diethylamino)coumarin-4-yl]methyl (DEAC) and 1-(2-nitrophenyl)ethyl (1-NPE) groups. Exposure of DEAC- and NPE-caged phosphopeptides **251** and **252** simultaneously to 420 nm light, selectively releases the DEAC group without affecting the NPE-caged peptide **252**. As a model system, they chose a serine/threonine protein phosphatase, Wip1 phosphatase. Subsequent irradiation at 365 nm removed the NPE-group to attenuate the Wip1 activity (Scheme 47).[226]



251 **252**
No Wip 1 activity

253 **254** **252**
Release peptide substrate
Start phosphatase reaction

253 **255** **254**
Release peptide inhibitor
Inhibit phosphatase reaction

Scheme 47. Sequential uncaging to control the activity of Wip1 phosphatase.

The discussed studies involved photolysis of mixtures of the caged molecules bearing in each case only one photolabile protecting group, whereas a single report[222] refers to the use of two different groups in the same molecule.

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Chapter 2

Results and discussion as a compilation of articles

2.1

Photorelease of amino acids from novel thioxobenzo[*f*]benzopyran ester conjugates

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Amino Acids, **2012**, *42*, 2275-2282

INDEX

1. Abstract

Aiming at the enhancement of the performance of (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester as photocleavable protecting group for the carboxylic acid function at long-wavelengths, 9-methoxy-3-thioxo-3*H*-benzo[*f*]benzopyran-L-valine and L-phenylalanine model conjugates were prepared through a thionation reaction of the corresponding oxo-benzobenzopyrans. These thioxobenzobenzopyran derivatives were subjected to photocleavage reactions in the same conditions as the parent oxo-benzobenzopyrans at different wavelengths of irradiation, and photocleavage data were obtained. It was found that the exchange of the carbonyl by a thiocarbonyl group enhanced the performance of the heterocyclic protecting group at 419 nm by improving the photolysis rates, making it an appropriate group for practical applications at long wavelengths.

2. Introduction

The choice of specific protecting groups remains of crucial importance in the success of many steps of organic synthesis and manipulation of polyfunctional molecules, since they prevent the formation of undesired side products and reactions.[1]

Photoremovable protecting groups (PPGs) exhibit numerous advantages, such as the relatively soft conditions required for their deprotection and orthogonality with respect to acid- or base-sensitive groups.[2-6] PPGs have become broadly reported for various functional groups, including carboxylic acids,[7-9] amines,[10,11] alcohols,[12] phosphates,[13,14] aldehydes and ketones[15,16] for convenient and controlled release of molecules in a variety of environments, including in materials science,[17,4] the caging and release of biologically significant compounds,[3,4,18-23] and in synthetic organic chemistry.[24,25]

The design and application of polyaromatic compounds, as well as oxygen and nitrogen polycyclic heterocycles as novel photocleavable protecting groups for the carboxylic acid and amine functions of amino acids is part of our current research interests. Carboxylic acid containing molecules (amino acids, including neurotransmitters) have been effectively protected by a variety of PPGs, such as 1-

pyrenylmethyl,[26,27] quinolones,[28] benzoxazoles,[29] as well as 2-oxo-benzopyrans (trivially known as coumarins)[11,28] and 2-oxo-benzobenzopyrans (benzocoumarins).[27,30-32] Their behaviour towards photocleavage under UV irradiation was studied at different wavelengths, in simulated physiological environment in mixtures of aqueous buffer and different organic solvents. In studies involving biomolecules, photocleavage at 350 nm or longer wavelengths is preferable, as it avoids cell damage due to short-wavelength light. According to recent results reported by us, 1-chloromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran is a suitable photocleavable protecting group for carboxylic acids, with release at 350 nm occurring efficiently in short times.[27,31] However, photocleavage at longer wavelengths, namely at 419 nm usually resulted in a large increase of the irradiation time, with a reduction of the scope of its applications at this wavelength.

Considering these facts, we now report the synthesis of novel ester conjugates of 9-methoxy-3-thioxo-3*H*-benzo[*f*]benzopyran-L-valine and L-phenylalanine obtained by a thionation reaction of the 3-oxo-3*H*-benzo[*f*]benzopyran bioconjugates, and compare their behaviour towards irradiation with the parent compounds. To the best of our knowledge, the use of thioxo derivatives as photocleavable protecting groups has not been previously reported. Photocleavage studies were carried out in a photochemical reactor under irradiation at 350 and 419 nm, and kinetic data was obtained.

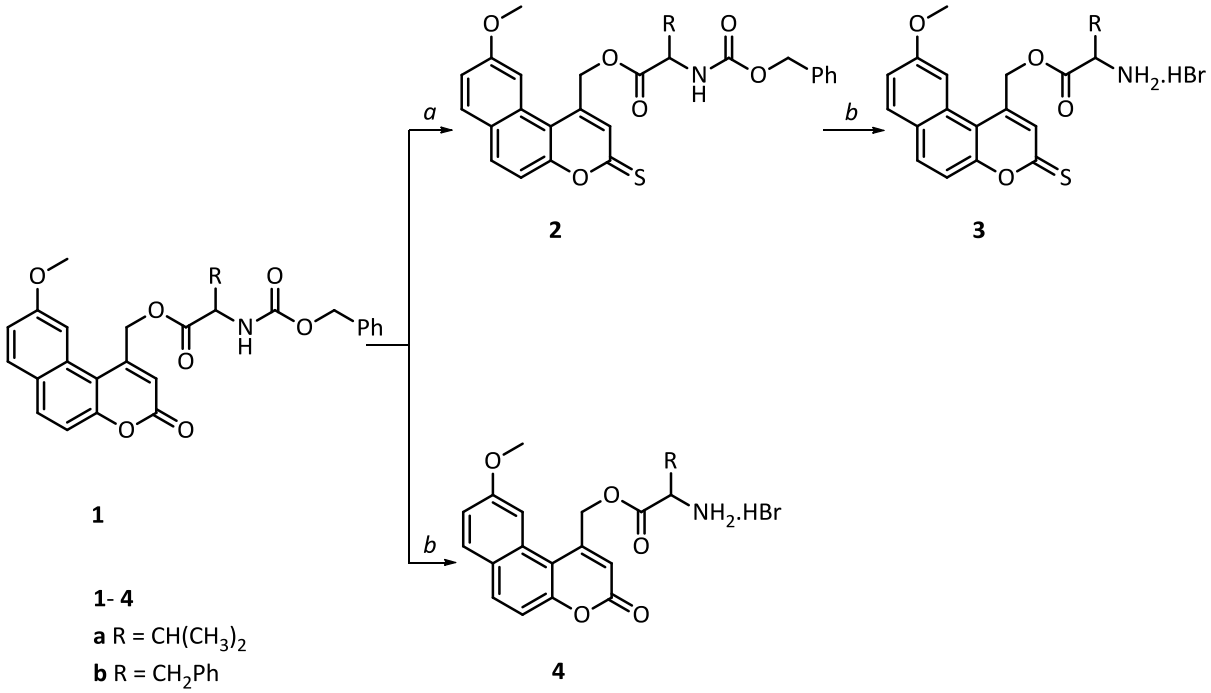
3. Results and discussion

3.1. Synthesis of amino acid conjugates 2, 3 and 4

N-(Benzyloxycarbonyl)-L-valine (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester, Z-Val-OBba **1a** and *N*-(benzyloxycarbonyl)-L-phenylanaline (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester, Z-Phe-OBba **1b** were prepared by reaction of 1-chloromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran with *N*-(benzyloxycarbonyl)-L-valine and *N*-(benzyloxycarbonyl)-L-phenylanaline in the presence of potassium fluoride in DMF, at room temperature, as previously described.[30]

Conjugates **1a** and **1b** were reacted with Lawesson's reagent in toluene,[33] under reflux conditions, followed by column chromatography purification affording corresponding thioxobenzopyran conjugates **2a** (55%) and **2b** (42%). In addition to the

use of this heterocycle for molecules that require multiple protecting groups, it was intended to evaluate its usefulness in the release of unprotected moieties in a biological perspective. Therefore, the *N*-benzyloxycarbonyl-protecting group was removed by acidolysis with hydrobromic acid in acetic acid yielding conjugates **3a** and **3b** bearing the amino acid and the photosensitive tag.



a) Lawesson´s reagent/toluene; *b)* HBr/CH_3CO_2H

Scheme 1. Synthesis of thioxobenzo[*f*]benzopyran and oxobenzo[*f*]benzopyran conjugates **2**-**4**.

In order to compare the behaviour towards irradiation of the thioxobenzopyran conjugates **3a** and **3b** with the related oxobenzopyran derivatives, cleavage of the *N*-benzyloxycarbonyl-protecting group was also carried out in compounds **1a** and **1b**, following the same conditions as mentioned above, to provide compounds **4a** and **4b** (Scheme 1, Table 1). The oxo-benzobenzopyran (Bba) and the thioxo-benzobenzopyran (Tba) moieties will be designated in this report by a three-letter code for simplicity of naming the various fluorescent conjugates, as indicated in Tables 1 and 2.

Table 1. Yields, UV/vis and fluorescence data for conjugates **1**-**4** in absolute ethanol and methanol/HEPES buffer (80:20) solution.

Compound		Yield (%)	EtOH					MeOH/HEPES (80:20)				
			λ_{abs} [a]	log ε	λ_{em} [a]	Φ_F	$\Delta\lambda$ [a]	λ_{abs} [a]	log ε	λ_{em} [a]	Φ_F	$\Delta\lambda$ [a]
1a	Z-Val-OBba[b]	75	348	4.07	478	0.58	130	351	4.19	490	0.23	139
1b	Z-Phe-OBba[b]	94	347	4.08	478	0.59	131	349	4.09	490	0.25	141
2a	Z-Val-OTba	55	407	3.70	458	0.01	51	417	4.14	476	0.002	59
2b	Z-Phe-OTba	42	408	3.83	464	0.02	56	417	4.19	477	0.004	60
3a	H-Val-OTba	64	407	3.74	461	0.02	54	417	4.44	477	0.004	60
3b	H-Phe-OTba	93	407	3.75	461	0.04	54	416	4.13	476	0.004	60
4a	H-Val-OBba	72	344	3.76	468	0.71	124	348	4.35	478	0.36	130
4b	H-Phe-OBba	61	345	3.79	468	0.55	123	345	3.94	473	0.29	128

[a] in nm. [b] Data in ethanol was previously reported.[30]

All new compounds were fully characterised by high resolution mass spectrometry, IR, ^{1}H and ^{13}C NMR spectroscopy. ^{1}H NMR spectra showed signals of the amino acid residues, such as the α-CH (δ 4.19-4.85 ppm), β-CH (δ 2.10-2.30 ppm), β-CH_2 (δ 3.0-3.30 ppm), as well as γ-CH_3 (δ 0.91-1.04 ppm). The heterocycle methylene group was also visible for all conjugates (δ 5.56-6.01 ppm). The effect of the thiocarbonyl group in compounds **2a**, **2b**, **3a** and **3b** was notorious in the chemical shift of the pyran proton H-2, which appeared downfield in the range δ 7.08-7.60 ppm, whereas in compounds **4a** and **4b**, having a carbonyl group, it occurred at δ 6.49 or 6.73 ppm.

The confirmation of the presence of the new C=S bond (C-3) at the heterocyclic ring (**2a**, **2b**, **3a** and **3b**) was also supported by ^{13}C NMR spectra signals at δ 194.23-195.13 ppm, instead of the carbonyl group, which occurred at δ 159.14 or 159.48 ppm, in conjugates **4a** and **4b**. The chemical shift of the pyran carbon C-2 was also influenced by the proximity of the carbon-sulphur double bond, being in the range δ 126.58-127.98 ppm for compounds **2a**, **2b**, **3a** and **3b**, and δ 112.17 or 112.20 ppm for compounds **4a** and **4b**.

3.2. Evaluation of the photophysical properties of amino acid conjugates 1, 2, 3 and 4

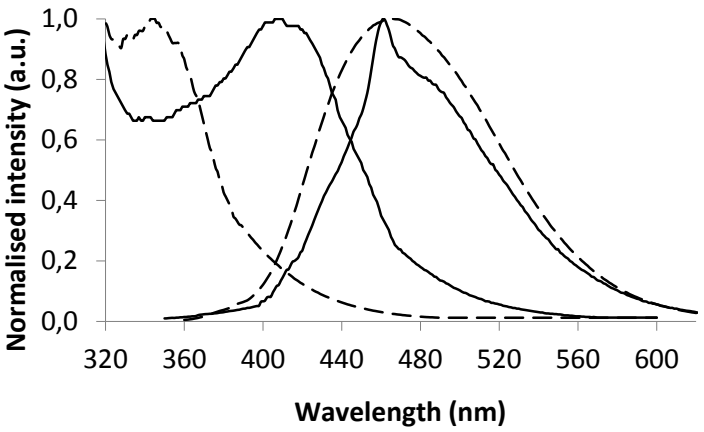


Figure 1. Normalised UV/vis absorption and fluorescence spectra of conjugates **3a** (full line) and **4a** (spaced line) in methanol/HEPES (80:20) solution (**3a**, λ_{exc} = 417 nm; **4a**, λ_{exc} = 348 nm).

The UV/vis absorption and emission spectra of degassed 10^{-5} M solutions in absolute ethanol and in a methanol/HEPES buffer (80:20) solution of thioxobenzopyran conjugates **2a**, **2b**, **3a**, **3b**, in comparison with the corresponding oxobenzopyran conjugates **1a**, **1b**, **4a**, **4b** were measured; absorption and emission maxima, molar extinction coefficients and relative fluorescence quantum yields are also reported (Table 1). Relative fluorescence quantum yields were calculated using 9,10-diphenylanthracene as standard (Φ_F = 0.95 in ethanol).[34] For the Φ_F determination, the fluorescence standard was excited at the wavelengths of maximum absorption found for each one of the compounds to be tested and in all fluorimetric measurements the absorbance of the solution did not exceed 0.1.

By comparison of absorption maxima for all compounds in both solvents, a slight bathochromic shift (2 to 10 nm) was observed in methanol/HEPES buffer (80:20) solution (except for compound **4b**, no variation) (λ_{abs} 344-408 nm, in ethanol, and 345-417 nm, in methanol/HEPES buffer). Furthermore, upon exchange of the carbonyl group at the heterocycle in compounds **1a** and **1b** for the thiocarbonyl group in **2a** and **2b**, a considerable bathochromic shift (59 to 68 nm) was observed. The same trend occurred for Z-deprotected conjugates **4a**, **4b** in comparison with **3a**, **3b** (62-71 nm), being the

highest λ_{abs} values in methanol/HEPES buffer, as in the latter conjugates. Absorption maxima for thioxo- and oxo-conjugates were independent of the amino acid residue.

Bioconjugates **1a**, **1b**, **4a** and **4b** exhibited high fluorescence quantum yields in ethanol ($0.55 < \Phi_F < 0.71$) and in methanol/HEPES buffer ($0.23 < \Phi_F < 0.36$), contrarily to the minor fluorescent thioxo analogues **2a**, **2b**, **3a** and **3b**.

Compounds **2a**, **2b**, **3a** and **3b** displayed emission maxima between 458-464 nm (in ethanol) and at about 477 nm (in methanol/HEPES buffer), with moderate Stokes's shifts ($\Delta\lambda$, 51-60 nm), but inferior to those of the corresponding oxobenzopyran conjugates **1a**, **1b**, **4a** and **4b** (123-141 nm) (Table 1, Figure 1).

3.3. Photolysis studies of amino acid conjugates 1-4

The sensitivity of thioxo-conjugates **2a**, **2b**, **3a** and **3b** in comparison with oxo-conjugates **1a**, **1b**, **4a** and **4b** towards UV/vis irradiation was evaluated by exposing solutions of the mentioned compounds in methanol/HEPES buffer (80:20) solution in a Rayonet RPR-100 reactor at 350 and 419 nm (Scheme 2).

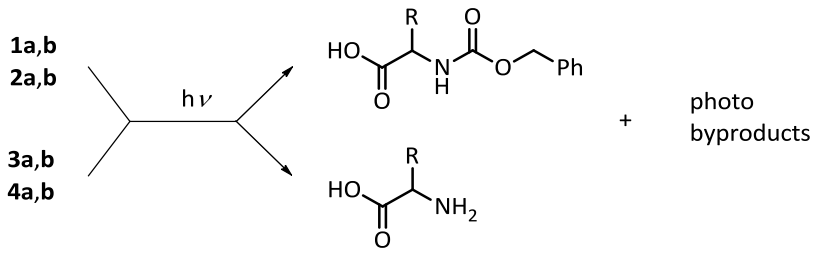
The course of the photocleavage reaction was followed by reverse phase HPLC with UV detection. The plots of peak area (*A*) of the starting material *versus* irradiation time were obtained for each compound, at the considered wavelengths. Peak areas were determined by HPLC, which revealed a gradual decrease with time, and were the average of three runs. The determined irradiation time represents the time necessary for the consumption of the starting materials until less than 5% of the initial area was detected (Table 2). For each compound and based on HPLC data, the plot of ln *A versus* irradiation time showed a linear correlation for the disappearance of the starting material, which suggested a first order reaction, obtained by the linear least squares methodology for a straight line (Figure 2). The corresponding rate constants were calculated and are presented in Table 2. The photochemical quantum yields (Φ_{Phot}) were calculated based on half-lives ($t_{1/2}$), molar extinction coefficient (ε) and the incident photon flux (I_0), which was determined by potassium ferrioxalate actinometry.[33] The calculated photochemical quantum yields indicated that the photocleavage process was not as efficient as desirable, probably due to the dissipation of part of the absorbed

energy *via* fluorescence pathways that compete with the photochemical reaction, as well as the type of reactor used (open chamber reactor).

Table 2. Irradiation times (t_{irr} in min), rate constants (k, $\times 10^{-2}$ min^{-1}) and photochemical quantum yields (Φ_{Phot}, $\times 10^{-3}$) for the photolysis of conjugates **1**-**4** at 350 and 419 nm in methanol/HEPES buffer (80:20) solution.

Compound		350 nm			419 nm		
		t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}
1a	Z-Val-OBba	33	9.08	0.098	462	0.66	0.079
1b	Z-Phe-OBba	38	8.28	0.093	301	1.0	0.144
2a	Z-Val-OTba	66	4.76	0.130	30	9.68	0.119
2b	Z-Phe-OTba	354	0.84	0.020	52	5.80	0.062
3a	H-Val-OTba	162	1.97	0.015	48	6.07	0.041
3b	H-Phe-OTba	146	2.12	0.043	49	6.49	0.078
4a	H-Val-OBba	607	0.49	0.004	3304	0.09	0.006
4b	H-Phe-OBba	520	0.58	0.011	7478	0.04	0.004

The release of *N*-benzyloxycarbonyl valine and phenylalanine required shorter irradiation times than the corresponding free amino acids at 350 nm (except for **3b**). The presence of the thiocarbonyl group at the heterocycle clearly affected the photolysis rate of the amino acid-heterocycle ester bond. Comparison of thioxo-conjugates **3** with the corresponding oxo-conjugate **4**, showed that irradiation times were shorter for **3** revealing the significance of the thioxo-group in the release of the free amino acids. However, this trend was reversed when the *N*-protecting group was present (compare data for conjugates **2** and **1**).



Scheme 2. Photocleavage reactions of conjugates **1**-**4**.

It was expected that the bathochromic shift in the maximum absorption wavelengths related to the presence of the C=S bond would result in an increase in the efficiency of the photolysis at longer wavelengths, leading to shorter irradiation times. The obtained results confirmed this expectation, as the photocleavage at 419 nm of thioxo-conjugates **2a**, **2b**, **3a** and **3b** occurred rapidly (30 to 52 min), in comparison with the corresponding oxo-conjugates **1a**, **1b**, **4a** and **4b** (301-7478 min). For these compounds and at this wavelength, the release of *N*-protected or free valine and phenylalanine occurred in similar irradiation times. The results indicated that the release of the amino acids at 419 nm from thioxobenzobenzopyrans is practicable for organic synthesis and also for caging applications, considering the short irradiation times and the fact that it minimises the side reactions for the remaining functionalities of the molecule.

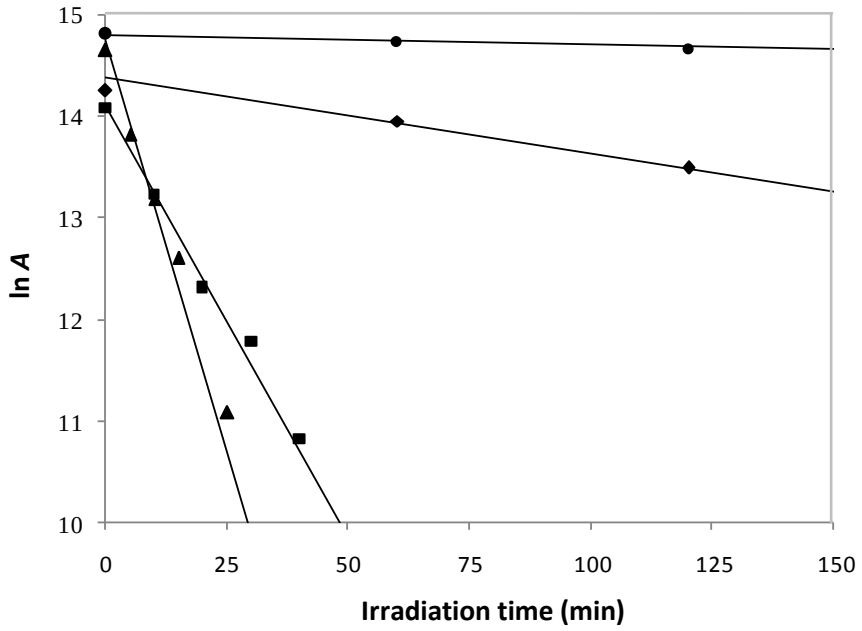


Figure 2. Plot of ln *A versus* irradiation time for the photolysis at 419 nm of conjugates **1a** (*diamond*), **2a** (*triangle*), **3a** (*square*) and **4a** (*circle*) in methanol/HEPES buffer (80:20) solution (for better visualisation, time scale is shown only up to 150 min, although photolysis for **4** proceeded until *ca*. 7500 min).

Given the interest in the development of novel protecting groups cleavable with UV A or even visible radiation, it was found that a simple modification involving the exchange of the carbonyl group of the light sensitive 9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester reported before by us, for a thiocarbonyl group by a thionation reaction, lead to a enhancement of the photolysis performance at 419 nm. This newly reported thioxobenzo[*f*]benzopyran can be considered an addition to the collection of photolabile protecting groups for application at long wavelengths.

4. Conclusions

The synthesis of novel thioxobenzo[*f*]benzopyran ester conjugates was achieved in good yields through a simple thionation reaction with Lawesson's reagent from the corresponding oxobenzo[*f*]benzopyrans. In order to obtain the parameters necessary for monitoring the photolysis reaction, as well as the fluorescence properties, the UV/vis characterization was carried out in absolute ethanol and methanol/HEPES buffer (80:20) solution.

Photocleavage studies of the oxo- and thioxo-conjugates, in methanol/HEPES buffer (80:20) solution at 350 nm revealed that the amino acid-heterocycle ester bond was efficiently photolised, releasing the *N*-protected and free amino acids from both types of precursors. However, the most interesting results were obtained at 419 nm for the thioxoconjugates, revealing that the presence of the thiocarbonyl clearly improved the photolysis rates, giving practicable irradiations times (30-52 min).

Although the parent oxo-protecting group still represents an interesting alternative as photocleavable group for the carboxylic acid, the newly reported thioxo-protecting group, (9-methoxy-3-thioxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester, emerges as an innovation for the release at longer wavelengths, since it photolyses with short irradiation times at wavelengths that are not detrimental to a variety of applications.

5. Experimental section

5.1. General

All melting points were measured on a Stuart SMP3 melting point apparatus and are uncorrected. TLC analyses were carried out on 0.25-mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230-240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer using KBr discs. UV/vis absorption spectra (200-700 nm) were obtained using a Shimadzu UV/2501PC spectrophotometer. NMR spectra were obtained on a Varian Unity Plus Spectrometer at an operating frequency of 300 MHz for ^{1}H NMR and 75.4 MHz for ^{13}C NMR or a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H NMR and 100.6 MHz for ^{13}C NMR using the solvent peak as internal reference at 25 °C. All chemical shifts are given in ppm using δ_H Me_4Si = 0 ppm as reference and *J* values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. Mass spectrometry analyses were performed at the "C.A.C.T.I. - Unidad de Espectrometria de Masas", at University of Vigo, Spain. Fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer. Photolyses were carried out using a Rayonet RPR-100 chamber reactor equipped with 10 lamps of 350 and 419 ± 10 nm. HPLC analyses were performed using a Licrospher 100 RP18 (5 µm) column in a JASCO HPLC system composed by a PU-2080 pump and a UV-2070 detector with ChromNav software. All reagents were used as received.

5.1.1. Synthesis of *N*-(benzyloxycarbonyl)-L-valine (9-methoxy-3-thioxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester, Z-Val-OTba (2a). Lawesson's reagent (0.231 g, 5.71×10^{-4} mol) was added to a solution of *N*-(benzyloxycarbonyl)-L-valine (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester, Z-Val-OBba **1a** in toluene (5 mL), with stirring, at room temperature. The reaction mixture was refluxed for 48 h and the process was followed by TLC (ethyl acetate/*n*-hexane, 2:8). The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by

column chromatography using dichloromethane/*n*-hexane, with mixtures of increasing polarity as eluent. Compound **2a** was obtained as an orange solid (0.081 g, 55%). Mp = 131.7-132.0 °C. TLC (ethyl acetate/*n*-hexane, 2:8): R_f = 0.85. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 0.91 (d, *J* = 6.8 Hz, 3 H, γ-CH_3 Val), 1.04 (d, *J* = 6.8 Hz, 3 H, γ-CH_3 Val), 2.20-2.30 (m, 1 H, β-CH Val), 3.96 (s, 3 H, OCH_3), 4.40-4.50 (m, 1 H, α-CH Val), 5.13 (s, 2 H, CH_2 Z), 5.27 (d, *J* = 8.8 Hz, 1 H, α-NH Val), 5.60-5.74 (m, 2 H, CH_2), 7.26 (dd, *J* = 8.8 and 2.4 Hz, 1 H, H-8), 7.30-7.40 (m, 5 H, 5×Ar-H Z), 7.45 (s, 1 H, H-2), 7.48 (d, *J* = 7.2 Hz, 1 H, H-5), 7.49 (s, 1 H, H-10), 7.85 (d, *J* = 8.8 Hz, 1 H, H-7), 7.96 (d, *J* = 8.8 Hz, 1 H, H-6) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 17.44 (γ-CH_3 Val), 19.21 (γ-CH_3 Val), 30.96 (β-CH Val), 55.51 (OCH_3), 59.26 (α-CH Val), 64.62 (CH_2), 67.28 (CH_2 Z), 106.25 (C-10), 114.21 (C-4b), 114.93 (C-5), 117.21 (C-8), 126.60 (C-6a), 127.50 (C-2), 128.19 (1×Ar-C Z), 128.24 (2×Ar-C Z), 128.52 (2×Ar-C Z), 130.24 (C-6b), 131.46 (C-7), 134.36 (C-6), 136.02 (C-1 Z), 141.32 (C-1), 156.24 (CONH), 158.79 (C-4a), 159.96 (C-9), 171.55 (CO_2CH_2), 195.13 (C-3) ppm. IR (KBr 1%, cm^{-1}): υ = 3339, 2936, 2964, 1720, 1623, 1606, 1591, 1538, 1455, 1444, 1432, 1363, 1346, 1296, 1230, 1217, 1181, 1139, 1096, 1052, 1026, 1009, 985, 838. HRMS (ESI): calcd for $C_{28}H_{28}NO_6S$ [M^++H]: 506.16318; found 506.16317.

5.1.2. Synthesis of *N*-(benzyloxycarbonyl)-L-phenylalanine (9-methoxy-3-thioxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester, Z-Phe-OTba (2b). Starting from Lawesson's reagent (0.120 g, 2.97×10^{-4} mol), *N*-(benzyloxycarbonyl)-L-phenylalanine (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester, Z-Phe-OBba **1b** and toluene (5 mL), following the same procedure as described for **2a**, compound **2b** was obtained as a yellow solid (0.035 g, 42%). Mp = 155.8-156.9 °C. TLC (ethyl acetate/*n*-hexane, 2:8): R_f = 0.87. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 3.16 (d, *J* = 6.4 Hz, 2 H, β-CH_2 Phe), 3.94 (s, 3 H, OCH_3), 4.70-4.85 (m, 1 H, α-CH Phe), 5.05-5.20 (m, 2 H, CH_2 Z), 5.30 (d, *J* = 5.6 Hz, 1 H, α-NH Phe), 5.56 (s, 2 H, CH_2), 7.08-7.14 (m, 2 H, 1×Ar-H Phe and H-2), 7.15-7.23 (m, 4 H, 4×Ar-H Phe), 7.27 (dd, *J* = 9.2 and 2.4 Hz, 1 H, H-8), 7.30-7.38 (m, 5 H, 5×Ar-H Z), 7.42-7.47 (m, 2 H, H-5 and H-10), 7.84 (d, *J* = 8.8 Hz, 1 H, H-7), 7.95 (d, *J* = 8.8 Hz, 1 H, H-6) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 38.18 (β-CH_2 Phe), 55.11 (α-CH Phe), 55.47 (OCH_3), 64.71 (CH_2), 67.22 (CH_2 Z), 106.07 (C-10), 114.20 (C-4b), 114.85 (C-5), 117.26 (C-8), 126.53 (C-6a), 127.39 (1×Ar-C Phe), 127.98 (C-2), 128.13 (1×Ar-C Z), 128.20 (2×Ar-C Phe), 128.47 (2×Ar-C Z), 128.76 (2×Ar-C Phe), 128.98 (2×Ar-C Phe), 130.17 (C-6b), 131.38

(C-7), 134.27 (C-6), 135.02 (C-1 Phe), 135.95 (C-1 Z), 140.66 (C-1), 155.64 (CONH), 158.73 (C-4a), 159.90 (C-9), 171.16 (CO_2CH_2), 195.03 (C-3) ppm. IR (KBr 1%, cm^{-1}): υ = 3330, 3063, 3030, 2934, 1722, 1623, 1606, 1592, 1538, 1520, 1498, 1455, 1444, 1432, 1362, 1343, 1295, 1230, 1216, 1178, 1139, 1096, 1056, 1027, 1009, 838. HRMS (ESI): calcd for $C_{32}H_{28}NO_6S$ [M^++H]: 554.16318; found 554.16358.

5.1.3. Synthesis of L-valine (9-methoxy-3-thioxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester hydrobromide, HBr.H-Val-OTba (3a). A 45% solution of hydrobromic acid in acetic acid (60 μL), and acetic acid (1 mL) were added to Z-Val-OTba **2a** (0.017 g, 3.36×10^{-5} mol) with stirring, at room temperature. The reaction mixture was maintained in these conditions for four days, and the process was followed by TLC (ethyl acetate/methanol, 1:1). During this time, additional amounts of 45% solution of hydrobromic acid in acetic acid were added until the total volume of 1.76 mL. When the reaction was completed, cold diethyl ether was added (2 mL), and the precipitate filtered off and washed with the same solvent to give compound **3a** as a brown solid (0.010 g, 64%). Mp = 181.7-183.4 °C. TLC (ethyl acetate/methanol, 1:1): R_f = 0.61. ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H = 0.94 (d, *J* = 2.1 Hz, 3 H, γ-CH_3 Val), 0.97 (d, *J* = 2.1 Hz, 3 H, γ-CH_3 Val), 2.10-2.30 (m, 1 H, β-CH Val), 3.98 (s, 3 H, OCH_3), 4.23 (br s, 1 H, α-CH Val), 6.01 (s, 2 H, CH_2), 7.40 (dd, *J* = 9.3 and 1.8 Hz, 1 H, H-8), 7.49-7.60 (m, 3 H, H-10, H-5 and H-2), 8.09 (d, *J* = 9.0 Hz, 1 H, H-7), 8.27 (d, *J* = 9.0 Hz, 1 H, H-6) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C = 17.49 (γ-CH_3 Val), 19.31 (γ-CH_3 Val), 31.01 (β-CH Val), 55.60 (OCH_3), 59.28 (α-CH Val), 64.24 (CH_2), 106.86 (C-10), 114.45 (C-5), 117.50 (C-8), 126.40 (C-4b), 126.58 (C-2), 127.31 (1×Ar-C Phe), 128.56 (2×Ar-C Phe), 129.24 (2×Ar-C Phe), 129.32 (C-6a), 129.73 (C-6b), 131.50 (C-7), 134.25 (C-1 Phe), 135.01 (C-6), 142.62 (C-1), 158.25 (C-4a), 159.44 (C-9), 168.75 (CO_2CH_2), 194.23 (C-3) ppm. IR (KBr 1%, cm^{-1}): υ = 3427, 2968, 1750, 1739, 1623, 1591, 1537, 1501, 1465, 1444, 1430, 1372, 1296, 1231, 1218, 1198, 1139, 1096, 1047, 1028, 839. HRMS (ESI): calcd for $C_{20}H_{22}NO_4S$ [M^++H]: 372.12641; found 372.12595.

5.1.4. Synthesis of L-phenylalanine (9-methoxy-3-thioxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester hydrobromide, HBr.H-Phe-OTba (3b). Starting from Z-Phe-OTba **2b** (0.015 g, 2.71×10^{-5} mol), 45% solution of hydrobromic acid in acetic acid (65 μL), and acetic acid (1 mL), following the same procedure as described for **3a** (total volume of hydrobromic

acid in acetic acid 2.2 mL; reaction time: two days), compound **3b** was obtained as a yellow solid (0.013 g, 93%). Mp = 188.3-189.9 °C. TLC (ethyl acetate/methanol 1:1): R_f = 0.61. ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H = 3.0-3.20 (m, 2 H, β-CH_2 Phe), 3.95 (s, 3 H, OCH_3), 4.60 (br s, 1 H, α-CH Phe), 5.90-6.0 (m, 2 H, CH_2), 7.10-7.30 (m, 5 H, 5×Ar-Phe), 7.33 (s, 1 H, H-2), 7.38 (dd, *J* = 8.9 and 2.1 Hz, 1 H, H-8), 7.53 (d, *J* = 1.2 Hz, 1 H, H-10), 7.60 (d, *J* = 8.7 Hz, 1 H, H-5), 8.08 (d, *J* = 9.0 Hz, 1 H, H-7), 8.26 (d, *J* = 9.0 Hz, 1 H, H-6), 8.53 (br s, 3 H, NH_3^+) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C = 36.13 (β-CH_2 Phe), 53.21 (α-CH Phe), 55.42 (OCH_3), 65.16 (CH_2), 106.84 (C-10), 114.41 (C-5), 117.46 (C-8), 126.36 (C-4b), 126.62 (C-2), 127.33 (1×Ar-C Phe), 128.58 (2×Ar-C Phe), 129.26 (2×Ar-C Phe), 129.34 (C-6a), 129.75 (C-6b), 131.52 (C-7), 134.27 (C-1 Phe), 135.01 (C-6), 142.61 (C-1), 158.24 (C-4a), 159.43 (C-9), 168.73 (CO_2CH_2), 194.42 (C-3) ppm. IR (KBr 1%, cm^{-1}): υ = 3440, 2924, 1753, 1623, 1538, 1455, 1364, 1296, 1231, 1139, 1098, 840, 751, 701. HRMS (ESI): calcd for $C_{24}H_{22}NO_4S$ [M^++1]: 420.12641; found 420.12568.

5.1.5. Synthesis of L-valine (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester hydrobromide, HBr.H-Val-OBba (4a). Starting from Z-Val-OBba **1a** (0.030 g, 6.13×10^{-5} mol), 45% solution of hydrobromic acid in acetic acid (32 μL), and acetic acid (1.5 mL), following the same procedure as described for **3a** (total volume of hydrobromic acid in acetic acid 1.23 mL; reaction time: three days), compound **4a** was obtained as a yellow solid (0.019 g, 73%). Mp = 218.9-220.1 °C. TLC (ethyl acetate/methanol, 1:1): R_f = 0.56. ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H = 0.95 (d, *J* = 0.9 Hz, 3 H, γ-CH_3 Val), 0.98 (d, *J* = 0.9 Hz, 3 H, γ-CH_3 Val), 2.19-2.26 (m, 1 H, β-CH Val), 3.95 (s, 3 H, OCH_3), 4.19 (br s, 1 H, α-CH Val), 5.90-6.10 (m, 2 H, CH_2), 6.73 (s, 1 H, H-2), 7.31 (dd, *J* = 8.7 and 2.1 Hz, 1 H, H-8), 7.39 (d, *J* = 9.0 Hz, 1 H, H-5), 7.49 (s, 1 H, H-10), 8.02 (d, *J* = 9.0 Hz, 1 H, H-7), 8.15 (d, *J* = 9.0 Hz, 1 H, H-6), 8.48 (br s, 3 H, NH_3^+) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C = 17.84 (γ-CH_3 Val), 18.15 (γ-CH_3 Val), 29.60 (β-CH Val), 55.54 (OCH_3), 57.49 (α-CH Val), 65.46 (CH_2), 106.51 (C-10), 111.53 (C-4b), 112.20 (C-2), 114.95 (C-5), 116.74 (C-8), 126.13 (C-6a), 130.18 (C-6b), 131.52 (C-7), 134.29 (C-6), 151.20 (C-1), 155.02 (C-4a), 159.31 (C-9), 159.48 (C-3), 168.53 (CO_2CH_2) ppm. IR (KBr 1%, cm^{-1}): υ = 3407, 2969, 1757, 1694, 1624, 1550, 1519, 1495, 1470, 1447, 1428, 1378, 1366, 1340, 1282, 1251, 1235, 1214, 1173, 1140, 1100, 1062, 1026, 973, 962, 844. HRMS (ESI): calcd for $C_{20}H_{22}NO_5$ [M^++1]: 356.14925; found 356.14894.

5.1.6. Synthesis of L-phenylalanine (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl) methyl ester hydrobromide, HBr.H-Phe-OBba (4b). Starting from Z-Phe-OBba **1b** (0.058 g, 1.08×10^{-3} mol), 45% solution of hydrobromic acid in acetic acid (72 µL), and acetic acid (1.5 mL), following the same procedure as described for **3a** (total volume of hydrobromic acid in acetic acid 2.9 mL; reaction time: three days), compound **4b** was obtained as a yellow solid (0.032 g, 61%). Mp = 231.1-232.9 °C. TLC (ethyl acetate/methanol, 1:1): R_f = 0.58. ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H = 3.0-3.26 (m, 2 H, β-CH_2 Phe), 3.94 (s, 3 H, OCH_3), 4.57 (t, *J* = 6.9 Hz, 1 H, α-CH Phe), 5.80-6.0 (2 H, m, CH_2), 6.49 (s, 1 H, H-2), 7.15-7.28 (m, 5 H, 5×Ar-H Phe), 7.31 (dd, *J* = 8.7 and 2.1 Hz, 1 H, H-8), 7.41 (d, *J* = 8.7 Hz, 1 H, H-5), 7.44 (s, 1 H, H-10), 8.02 (d, *J* = 9.0 Hz, 1 H, H-7), 8.16 (d, *J* = 9.3 Hz, 1 H, H-6), 8.60 (br s, 3 H, NH_3^+) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C = 36.14 (β-CH_2 Phe), 53.28 (α-CH Phe), 55.31 (OCH_3), 65.21 (CH_2), 106.13 (C-10), 111.41 (C-4b), 112.17 (C-2), 114.82 (C-5), 116.75 (C-8), 126.00 (C-6a), 127.31 (1×Ar-C Phe), 128.56 (2×Ar-C Phe), 129.31 (2×Ar-C Phe), 130.04 (C-6b), 131.32 (C-7), 134.06 (C-1 Phe), 134.48 (C-6), 150.56 (C-1), 154.86 (C-4a), 159.14 (C-3 and C-9), 168.67 (CO_2CH_2) ppm. IR (KBr 1%, cm^{-1}): υ = 3419, 2914, 1749, 1715, 1626, 1594, 1552, 1519, 1498, 1456, 1445, 1428, 1377, 1363, 1337, 1280, 1234, 1216, 1204, 1141, 1121, 1082, 1063, 1021, 855. HRMS (ESI): calcd for $C_{24}H_{22}NO_5$ [M^++H]: 404.14925; found 404.14858.

5.2. General photolysis procedure

A 1×10^{-4} M methanol/HEPES (80:20) solution of compounds **1**-**4** (5 mL) were placed in a quartz tube and irradiated in a Rayonet RPR-100 reactor at the desired wavelength. The lamps used for irradiation were of 350 and 419 ± 10 nm.

Aliquots of 100 µL were taken at regular intervals and analysed by RP-HPLC. The eluent was acetonitrile/water, 75:25 or methanol/water, 64:36 (**4a** and **4b**), at a flow rate of 0.8 mL/min, previously filtered through a Millipore, type HN 0.45 µm filter and degassed by ultra-sound for 30 min. The chromatograms were traced by detecting UV absorption at the wavelength of maximum absorption for each compound (retention time: **1a**, 7.1; **1b**, 7.4; **2a**, 9.7; **2b**, 10.6; **3a** and **3b**, 4.2; **4a** and **4b**, 8.0 min).

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2.2

Long-wavelenght photolysis of amino acid 6-(methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl)methyl esters

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INDEX

1. Abstract

(6-Methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl)methyl esters of valine or phenylalanine as model bifunctional molecules were synthesized to assess its applicability as a photocleavable protecting group for solution phase organic synthesis and in caging applications at longer wavelengths. The behaviour of the corresponding derivatives towards photolysis was evaluated by irradiation at 350 and 419 nm in a mixture of HEPES buffer and acetonitrile or methanol in a photochemical reactor, followed by HPLC/UV monitoring. Time-resolved fluorescence measurements were used to elucidate the dynamics.

2. Introduction

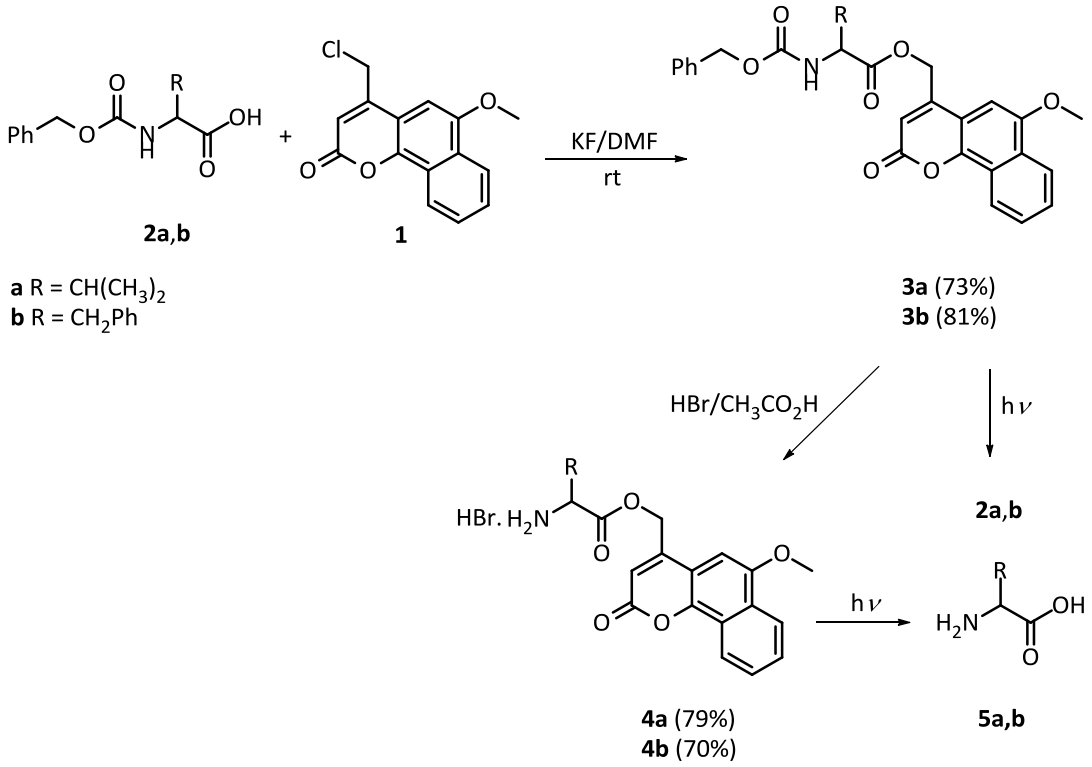
The use of light of appropriate wavelength can liberate synthetic and biochemically relevant molecules from their corresponding nonreactive/inactive conjugate/caged precursors possessing suitable light-sensitive moieties covalently bonded to the functional groups of the molecule of interest. In recent years this approach has been widely applied in organic synthesis as an alternative to classical acid- and base-labile protecting groups, as well as, in life sciences, to investigate signal transduction mechanisms at the cellular level and in drug delivery.[1] This broad range of applications supports the development of new photolabile groups with enhanced properties that will allow fast cleavage and release of various functionalities (such as alcohols, amines, phosphates, aldehydes, ketones and carboxylic acids).[2-6] Additionally, the use of longer wavelengths can minimise side reactions and/or cell damage. Nevertheless, among the considerable number of light-sensitive groups that have been reported, only a few cleave in a practical time by irradiation at wavelengths longer than 400 nm.

In addition to the interest in natural or synthetic derivatives of 2*H*-benzopyran-2-ones (coumarins) as antioxidants, food components, stabilisers, immunomodulatory substances, fluorescent markers in analysis, laser dyes and in clinical use,[7-12] they have also been recognised for their application as photolabile protecting groups for the caging of biomolecules. Oxobenzopyrans have been reported in cell biology and biophysical

studies with a range of chemical functionalities.[3-6,13] As part of our research on the design and evaluation of new polyaromatic and polyheteroaromatic systems, which include oxobenzopyrans and their fused derivatives as photolabile protecting groups for biologically relevant molecules,[14-19] we report the synthesis of new valine and phenylalanine derivatives bearing a 2*H*-naphtho[1,2-*b*]pyran-2-one moiety as a light-sensitive group. In order to evaluate the photorelease of the amino acids in *N*-protected and free forms in practical times at longer wavelengths, photolysis studies were carried out in mixtures of aqueous 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and an organic solvent at 350 and 419 nm. Cleavage kinetic data obtained by HPLC/UV monitoring was also collected and time-resolved fluorescence measurements were used to elucidate the dynamics.

3. Results and discussion

4-(Chloromethyl)-6-methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran **1**,[18] was treated with *N*-(benzyloxycarbonyl)-L-valine **2a** or *N*-(benzyloxycarbonyl)-L-phenylalanine **2b** in the presence of potassium fluoride in DMF, at room temperature,[20] to give *N*-(benzyloxycarbonyl)-L-valine (6-methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl) methyl ester **3a** and *N*-(benzyloxycarbonyl)-L-phenylalanine (6-methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl) methyl ester **3b** in good yields. In order to compare the release of the model amino acids in their *N*-protected **2a** and **2b** and free forms **5a** and **5b**, under irradiation, the *N*-benzyloxycarbonyl protecting group was removed by acidolysis with hydrobromic acid in acetic acid giving compounds **4a** and **4b** bearing the amino acid and the photosensitive tag (Scheme 1).



Scheme 1. Synthesis of **3** and **4** and photorelease of **2** and **5**.

The 2-oxonaphtho[1,2-*b*]pyran (Bbp) moiety will be designated in this report by a three-letter code for simplicity of naming the various fluorescent derivatives, as indicated in Tables 1-4.

All new compounds were fully characterised by high resolution mass spectrometry, IR, ^{1}H and ^{13}C NMR spectroscopy. ^{1}H NMR spectra showed signals of the amino acid residues, such as α-CH (δ 4.24-4.80 ppm), β-CH (δ 2.00-2.40 ppm), β-CH_2 (δ 3.10-3.30 ppm), and γ-CH_3 (δ 0.95-1.05 ppm). The methylene group bound to the heterocycle was also visible for all derivatives (δ 5.30-5.90 ppm). The confirmation of the presence of the ester bond was also supported by ^{13}C NMR spectra signals at δ 168.43 to 171.60 ppm.

In order to obtain the parameters necessary for the monitoring of the photolysis reaction, as well as the fluorescence properties, the UV/vis characterization was carried out in mixtures of different organic solvents (acetonitrile or methanol) with HEPES buffer.

Table 1. UV/vis absorption and emission data (λ, in nm) for amino acid derivatives **3**, **4**, **6** and **7** in mixtures of acetonitrile/HEPES buffer (80:20).

	Compound	λ_{abs}	log ε	λ_{em}	Φ_F	$\Delta\lambda$
3a	Z-Val-OBbp	373	3.82	479	0.80	106
3b	Z-Phe-OBbp	373	3.92	476	0.50	103
4a	H-Val-OBbp	371	3.68	475	0.84	104
4b	H-Phe-OBbp	373	3.78	478	0.58	105
6a	Z-Val-OBba	346	4.07	481	0.54	135
6b	Z-Phe-OBba	346	4.06	478	0.59	132
7a	H-Val-OBba	345	4.02	476	0.63	129
7b	H-Phe-OBba	347	4.00	480	0.77	135

The absorption and emission spectra of **3** and **4** in degassed mixtures of acetonitrile and HEPES buffer (80:20), methanol and HEPES buffer (80:20), and absolute ethanol were measured; absorption (λ_{abs}) and emission maxima (λ_{em}), molar extinction coefficients (ε) and relative fluorescence quantum yields (Φ_F) are reported (see Tables 1 and 2). Values of Φ_F were calculated using 9,10-diphenylanthracene as standard (Φ_F = 0.95 in ethanol).[21] For Φ_F determination, the fluorescence standard was excited at the wavelengths of maximum absorption found for each of the compounds to be tested, and in all fluorimetric measurements the absorbance of the solution did not exceed 0.1.

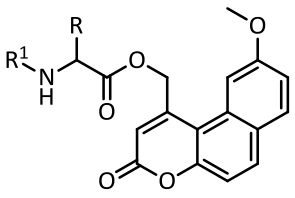
Table 2. UV/vis absorption and emission data (λ, in nm) for amino acid derivatives **3** and **4** in mixtures of methanol and HEPES buffer (80:20) and absolute ethanol.

	Methanol/HEPES (80:20)					Ethanol				
	λ_{abs}	log ε	λ_{em}	Φ_F	$\Delta\lambda$	λ_{abs}	log ε	λ_{em}	Φ_F	$\Delta\lambda$
3a	376	3.70	481	0.51	105	376	3.66	474	0.65	98
3b	375	3.91	481	0.72	106	374	3.63	474	0.49	100
4a	375	3.75	474	0.70	99	375	3.65	476	0.51	101
4b	374	3.71	471	0.94	97	375	3.69	472	0.58	97

By comparison of the absorption and emission maxima, no significant differences

were observed in these solvents, with values in the range 371-376 and 471-481 nm, respectively. The Stokes's shift ($\Delta\lambda$) was between 97 and 106 nm, which is an advantageous property in fluorescence techniques as it minimizes self-quenching phenomena. It was found that all compounds were highly emissive in mixtures of HEPES buffer and acetonitrile or methanol (Φ_F from 0.50 to 0.94) as well as in ethanol (Φ_F from 0.49 to 0.65).

Absorption maxima at longer wavelengths indicates that photolysis could be possible with shorter irradiation times. Therefore considering that the present heterocycle is associated with a λ_{abs} at about 370 nm, which is 25 nm bathochromically shifted from the previously reported derivatives **6** and **7**, possessing the same heterocycle with a different ring fusion, namely 9-methoxy-3*H*-naphtho[2,1-*b*]pyran-3-one (Bba) (Figure 1, Table 1), it was expected that the behaviour of **3** and **4** towards irradiation in mixtures of acetonitrile/HEPES buffer (80:20) would be improved in the visible region compared with results obtained with **6** and **7**.[19]



6a R = $CH(CH_3)_2$, R^1 = CO_2CH_2Ph

6b R = CH_2Ph, R^1 = CO_2CH_2Ph

7a R = $CH(CH_3)_2$, R^1 = H.HBr

7b R = CH_2Ph, R^1 = H.HBr

Figure 1. Structure of **6** and **7**.[19]

The evaluation of **3**, **4**, **6** and **7** towards UV/vis irradiation was carried out by exposing solutions of the compounds in mixtures of acetonitrile/HEPES buffer (80:20) and methanol/HEPES buffer (80:20) with a Rayonet RPR-100 reactor at 350 and 419 nm (Scheme 1). The course of the photocleavage reaction was followed by reverse phase HPLC with UV detection. The plots of peak area (*A*) of the starting material *versus* irradiation time (t_{irr}) were obtained for each compound at the considered wavelengths. Peak areas were determined by HPLC, which revealed a gradual decrease with time and were the average of three runs.

The determined t_{irr} represents the time necessary for the consumption of the

starting materials until less than 5% of the initial area was detected (Table 3). The photochemical quantum yields (Φ_{Phot}) were calculated based on half-lives ($t_{1/2}$), molar extinction coefficients (ε) and the incident photon flux (I_0), which was determined by potassium ferrioxalate actinometry.[22]

Table 3. Irradiation times (t_{irr}, in min) and photochemical quantum yields (Φ_{Phot}, x 10^{-3}) for the photolysis of **3** and **4** at 350 and 419 nm in mixtures of acetonitrile/HEPES buffer (80:20) and methanol/HEPES buffer (80:20).

Cpd	Acetonitrile/HEPES (80:20)				Methanol/HEPES (80:20)			
	350 nm		419 nm		350 nm		419 nm	
	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}
3a	86	0.121	77	0.645	59	0.243	270	0.164
3b	35	0.210	53	0.512	47	0.176	148	0.158
4a	35	0.338	23	2.439	231	0.055	1253	0.047
4b	33	0.304	45	0.802	632	0.023	1153	0.042

The photolysis irradiations times at 419 nm in a mixture of acetonitrile/HEPES buffer (80:20) obtained for **3** and **4** in comparison with those of **6** and **7**, between 598 and 1155 min, confirmed the expectations related to the difference in the absorption maxima. Quantitative release of **2a** and **2b** and the corresponding free amino acids **5a** and **5b** from **3** and **4**, bearing a naphtho[1,2-*b*]pyran group, was achieved in less than 1.3 h, whereas cleavage from **6** and **7** required from 10 to 20 h. At 350 nm, differences in the photolysis times were not significant enough to make a clear distinction in the behaviour of both systems. Regarding irradiation of **3** and **4** at 350 nm, it was found that irradiation times were short, 33-86 min, and comparable to those obtained at 419 nm (23-77 min). However, considering the benefits of the use of longer wavelengths for irradiation in photolytic processes, the selected irradiation wavelength for these conjugate systems would be 419 nm.

For both irradiation wavelengths, it was found that the presence of the *N*-benzyloxycarbonyl protecting group influenced the cleavage times, as the release of **5a** was faster than the corresponding *N*-blocked form **2a**. In the case of phenylalanine, the cleavage times were similar. As reported previously,[14] the *N*-benzyloxycarbonyl group

was photostable under the test conditions.

Photolysis of **3** and **4** was also carried out in a mixture of HEPES buffer with a protic solvent, methanol, in the same proportion as before, in order to compare the performance of the (6-methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl)methyl ester in different solvent systems. Overall, the sensitivity of the ester linkage between the protecting group and the amino acid was inferior in this solvent system at both wavelengths tested. In addition, the release of the free form of both amino acids required much longer irradiation periods.

Very low photochemical quantum yields were obtained in mixtures of acetonitrile/HEPES buffer (80:20) and methanol/HEPES buffer (80:20), indicating strong competition between the bond scission and radiative and nonradiative relaxation. To help elucidate the photophysics of the processes involved a preliminary time-resolved fluorescence study was performed using both acetonitrile/HEPES and methanol/HEPES mixtures. The excitation wavelength of 375 nm (with λ_{em} = 474 nm) was chosen to monitor the coumarin species present. The expected cleavage alcohol product, Bbp-OH, was found to decay monoexponentially [Φ_F determined as 0.45 in both ethanol and methanol/HEPES (80:20)].[18] This allowed the estimation of the radiative and nonradiative rate constants (k_r and k_{nr}) of 5.2 x 10^7 and 6.3 x 10^7 s^{-1}, respectively, in methanol/ HEPES and 4.6 x 10^7 and 9.4 x 10^7 s^{-1} in acetonitrile/HEPES (Φ_F = 0.45 and 0.33 for the two solvent systems, respectively).[23]

With the exception of **3a**, which decayed biexponentially, the sum of three exponential components was required to fit the decay data (see Supporting information), indicating the presence of different coumarin species. From the lifetimes alone, it was not possible to clearly ascribe decay rates to individual species, which necessitated the measurement of time-resolved emission spectra to obtain the decay associated spectra (see Supporting information). Comparison with the steady state spectrum (measured under the same experimental conditions) was in accordance in terms of both spectrum and lifetime, and the presence of the alcohol product in the methanol/HEPES mixture was seen for **4a** and **4b**. The other assignments are an area of continued investigation, but assuming that the spectrum for the ester is likely to be at longer wavelengths and its contribution to the fluorescence significant (shown by pre-exponential value), it is possible to estimate the rate constants for the initial bond

cleavage (k_c), shown in Table 4. This generally shows faster rates for **4**, although these results are still preliminary, there appears to be a correlation with the photochemical yield data presented in Table 3, and a full photophysical investigation is underway.

Table 4. Cleavage rate constants (k_c in s^{-1}) calculated from the fluorescence lifetime data in mixtures of acetonitrile/HEPES buffer (80:20) and methanol/HEPES buffer (80:20).

Cpd	Acetonitrile/HEPES (80:20)	Methanol/HEPES (80:20)
3a	0.25×10^8	0.53×10^8
3b	0.58×10^8	1.11×10^8
4a	5.69×10^8	4.70×10^8
4b	9.35×10^8	6.60×10^8

The disappearance of the starting material can also be illustrated for each compound, and the plot of ln *A versus* irradiation time based on HPLC data showed a linear correlation for the disappearance of the starting material, which suggests a first-order reaction, obtained by the linear least squares methodology for a straight line (Figure 2).

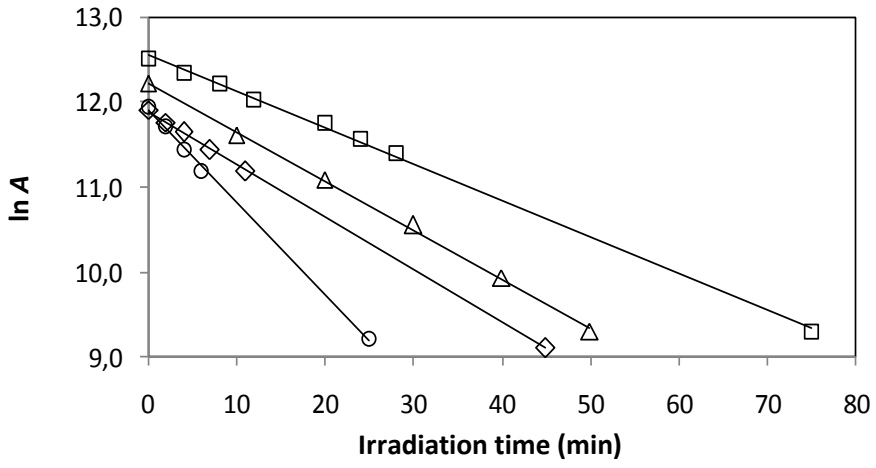


Figure 2. Plot of ln *A versus* irradiation time for the photolysis of compounds **3a** (□), **3b** (Δ), **4a** (○) and **4b** (◊) at 419 nm in mixtures of acetonitrile/HEPES buffer (80:20).

4. Conclusions

The synthesis of new valine and phenylalanine oxonaphtho[1,2-*b*]pyran esters was achieved in good yields through a standard procedure from the corresponding chloromethylated oxonaphtho[1,2-*b*]pyran. In order to obtain the parameters necessary for monitoring the photolysis reaction, the UV/vis characterization was carried out in mixtures of acetonitrile/HEPES buffer (80:20), methanol/HEPES buffer (80:20) and absolute ethanol. Photocleavage studies of the ester compounds, in a mixture of acetonitrile and HEPES buffer (80:20), at 350 and 419 nm, revealed that the amino acid-heterocycle ester bond was readily photolysed, releasing the *N*-protected and free amino acids. The most interesting results were obtained at 419 nm for all compounds in practical irradiations times (23-77 min), confirming the suitability of oxonaphtho[1,2-*b*]pyran as a photocleavable protecting group for release at longer wavelengths, which is an interesting improvement compared to the previously reported 3-oxo-3*H*-naphtho[2,1-*b*]pyran with a different ring fusion. The use of time-resolved fluorescence techniques can further elucidate the dynamics of the photocleavage processes of these compounds.

5. Experimental section

5.1. General

All melting points were measured with a Stuart SMP3 melting point apparatus. TLC analyses were carried out with 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230-240 mesh). IR spectra were determined with a BOMEM MB 104 spectrophotometer. UV/vis absorption spectra (200-700 nm) were obtained using a Shimadzu UV/2501PC spectrophotometer. NMR spectra were obtained with a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H NMR and 100.6 MHz for ^{13}C NMR using the solvent peak as internal reference at 25 °C. All chemical shifts are given in ppm using $\delta(Me_4Si) = 0$ ppm as reference and *J* values are given in Hz. Assignments were made by comparison of

chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. HRMS analyses were performed at the "C.A.C.T.I. – Unidad de Espectrometria de Masas", at University of Vigo, Spain. Time-resolved fluorescence measurements were performed using a HORIBA Scientific UltraFast-01 system equipped with a DeltaDiode (DD-375L) excitation source running at 8 MHz, emitting at 374 nm and a microchannel plate detector. Data were analysed using DAS6 software, with the TRES (time-resolved emission spectra) fitting using a global analysis module linking common lifetimes, generally fixed from the simple decay analysis, from which decay associated spectra were then obtained.

5.2. Synthetic procedures for 3 and 4

5.2.1. *N*-(Benzyloxycarbonyl)-L-valine (6-methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl)methyl ester, Z-Val-OBbp (3a). 4-(Chloromethyl)-6-methoxy-2*H*-naphtho[1,2-*b*]pyran-2-one (Bbp-Cl, **1**) (0.047 g, 1.7 x 10^{-4} mol) was dissolved in dry DMF (2 mL) and potassium fluoride (0.030 g, 5.1 x 10^{-4} mol) and Z-Val-OH (**2a**) (0.047 g, 1.9 x 10^{-4} mol) were added. The reaction mixture was stirred at room temperature for 32 h and followed by TLC (ethyl acetate/*n*-hexane, 3:7). The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography with silica gel using dichloromethane/methanol mixtures with increasing polarity as eluent, to give **3a** as a yellow solid (0.061g, 73%). Mp = 142.2-143.6 °C. TLC (ethyl acetate/*n*-hexane, 3:7): R_f = 0.53. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 0.95 (d, *J* = 6.8 Hz, 3 H, γ-CH_3 Val), 1.04 (d, *J* = 6.8 Hz, 3 H, γ-CH_3 Val), 2.22–2.36 (m, 1 H, β-CH Val), 4.04 (s, 3 H, OCH_3), 4.40-4.50 (m, 1H, α-CH Val), 5.16 (s, 2H, CH_2 Z), 5.26 (d, *J* = 8.8 Hz, 1 H, α-NH Val), 5.35-5.50 (m, 2 H, CH_2), 6.60 (s, 1 H, H-3), 6.65 (s, 1 H, H-5), 7.25-7.40 (m, 5 H, 5 x Ar-H Z), 7.60-7.74 (m, 2 H, H-8 and H-9), 8.25-8.34 (m, 1 H, H-7), 8.50-8.59 (m, 1 H, H-10) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 17.58 (γ-CH_3 Val), 19.15 (γ-CH_3 Val), 31.04 (β-CH Val), 55.89 (OCH_3), 59.33 (α-CH Val), 62.38 (CH_2), 67.29 (CH_2 Z), 95.27 (C-5), 112.14 (C-4a), 113.20 (C-3), 122.29 (C-7), 122.44 (C-10), 124.01 (C-10a), 127.43 (C-6a), 128.00 (C-9), 128.21 (2 x Ar-C), 128.28 (2 x Ar-C), 128.53 (Ar-C), 128.55 (C-8), 136.00 (C-1 Z), 145.82 (C-

10b), 148.94 (C-4), 152.38 (C-6), 156.25 (CONH), 160.59 (C-2), 171.60 (CO_2CH_2) ppm. IR (KBr 1%, cm^{-1}): υ = 3758, 3691, 3054, 2987, 2686, 2522, 2411, 2306, 1726, 1603, 1551, 1508, 1422, 1386, 1265, 1160, 1114, 1029, 896, 738, 704 cm^{-1}. HRMS (ESI): calcd for $C_{28}H_{28}NO_7$ [M^+ + H]: 490.18603; found 490.18729.

5.2.2. *N*-(Benzyloxycarbonyl)-L-phenylalanine (6-methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl)methyl ester, Z-Phe-OBbp (3b). Starting from Bbp-Cl (**1**) (0.060 g, 2.2 x 10^{-4} mol), DMF (2 mL), potassium fluoride (0.040 g, 6.9 x 10^{-4} mol) and Z-Phe-OH (**2b**) (0.076 g, 2.5 x 10^{-4} mol), following the same procedure described for **3a**, **3b** was obtained as an orange solid (0.115 g, 81%). Mp = 134.6-136.1°C. TLC (ethyl acetate/*n*-hexane, 3:7): R_f = 0.58. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 3.16 (d, *J* = 6.4 Hz, 2 H, β-CH_2 Phe), 3.98 (s, 3 H, OCH_3), 4.73-4.80 (m, 1 H, α-CH Phe), 5.08-5.14 (m, 2H, CH_2 Z), 5.30 (s, 2H, CH_2), 5.38 (d, *J* = 8.0Hz, 1 H, α-NH Phe), 6.39 (s, 1 H, H-3), 6.53 (s, 1 H, H-5), 7.10-7.32 (m, 10 H, 5 x Ar-H Phe and 5 x Ar-H Z), 7.60-7.68 (m, 2 H, H-8 and H-9), 8.20-8.26 (m, 1 H, H-7), 8.38-8.50 (m, 1 H, H-10) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 36.40 (β-CH_2 Phe), 55.16 (α-CH Phe), 55.81 (OCH_3), 62.35 (CH_2), 67.15 (CH_2 Z), 95.26 (C-5), 112.05 (C-4a), 113.14 (C-3), 122.20 (C-7), 122.27 (C-10), 123.85 (C-10a), 127.28 (C-6a), 127.37 (Ar-C), 127.87 (Ar-C), 128.09 (2 x Ar-C), 128.19 (2 x Ar-C), 128.39 (C-9), 128.46 (2 x Ar-C), 128.71 (2 x Ar-C), 129.01 (C-8), 135.13 (C-1 Phe), 135.95 (C-1 Z), 145.63 (C-10b), 148.53 (C-4), 152.20 (C-6), 160.41 (C-2), 162.50 (CONH), 171.15 (CO_2CH_2) ppm. IR (KBr 1%, cm^{-1}): υ = 3759, 3691, 3583, 3054, 2987, 2686, 2522, 2411, 2306, 1725, 1677, 1602, 1551, 1507, 1422, 1386, 1265, 1163, 1113, 1086, 1029, 896, 740, 705 cm^{-1}. HRMS (ESI): calcd for $C_{32}H_{28}NO_7$ [M^+ + H]: 538.18603; found 538.18639.

5.2.3. L-Valine (6-Methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl)methyl ester hydrobromide, HBr·H-Val-OBbp (4a). A 45% solution of hydrobromic acid in acetic acid (162 µL) and acetic acid (1 mL) were added to **3a** with stirring at room temperature. The reaction mixture was maintained in these conditions for 11 h, and the process was followed by TLC (ethyl acetate/*n*-hexane, 3:7). During this time, additional amounts of 45% solution of hydrobromic acid in acetic acid were added (total volume 972 µL). When the reaction was completed, cold diethyl ether was added (0.5 mL), and the precipitate

collected by filtration and washed with the same solvent to give **4a** as a light brown solid (0.021 g, 79%). Mp = 205.5-207.1 °C. TLC (ethyl acetate/methanol, 1:1): R_f = 0.53. ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H = 0.98-1.05 (m, 6 H, 2 x γ-CH_3 Val), 2.00-2.40 (m, 1 H, β-CH Val), 4.06 (s, 3 H, OCH_3), 4.24 (br. s, 1 H, α-CH Val), 5.60-5.90 (m, 2 H, CH_2), 6.72 (s, 1 H, H-3), 7.03 (s, 1 H, H-5), 7.70-7.80 (m, 2 H, H-8 and H-9), 8.20-8.26 (m, 1 H, H-7), 8.30-8.40 (m, 1 H, H-10), 8.44 (br. s, 3 H, NH_3^+) ppm. ^{13}C NMR (DMSO-d_6, 400 MHz): δ_C = 17.65 (γ-CH_3 Val), 18.16 (γ- CH_3 Val), 29.52 (β-CH Val), 56.18 (OCH_3), 57.33 (α-CH Val), 63.08 (CH_2), 97.50 (C-5), 112.09 (C-3), 112.27 (C-4a), 121.66 (C-10), 122.00 (C-7), 122.97 (C-10a), 126.48 (C-6a), 128.22 (C-8), 128.69 (C-9), 144.56 (C-10b), 150.08 (C-4), 151.26 (C-6), 159.63 (C-2), 168.43 (CO_2CH_2) ppm. IR (KBr 1%, cm^{-1}): υ = 3450, 3072, 3009, 2944, 2883, 1764, 1662, 1386, 1222, 1054, 1008, 823, 760 cm^{-1}. HRMS (ESI): calcd for $C_{20}H_{22}NO_5$ [M^+ + H]: 356.14925; found 356.14906.

5.2.4. L-Phenylalanine (6-methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl)-methyl ester hydrobromide, HBr·H-Phe-OBbp (4b). Starting from **3b** (0.027 g, 5.0 x 10^{-5} mol) and using 45% solution of hydrobromic acid in acetic acid (1.2 mL), **4b** was obtained as a brown solid (0.017 g, 70%). Mp = 216.0-218.2 °C. TLC (ethyl acetate/methanol, 1:1): R_f = 0.61. ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H = 3.10-3.30 (m, 2 H, β-CH_2 Phe), 4.05 (s, 3 H, OCH_3), 4.62 (br s, 1 H, α-CH Phe), 5.50-5.70 (m, 2 H, CH_2), 6.50 (s, 1 H, H-3), 6.96 (s, 1 H, H-5), 7.10-7.30 (m, 5 H, 5 x Ar-H Phe), 7.70-7.90 (m, 2 H, H-8 and H-9), 8.19-8.25 (m, 1 H, H-7), 8.31–8.39 (m, 1 H, H-10), 8.55 (br. s, 3 H, NH_3^+) ppm. ^{13}C NMR (DMSO-d_6, 400 MHz): δ_C = 36.07 (β-CH_2 Phe), 53.25 (α-CH Phe), 56.20 (OCH_3), 62.97 (CH_2), 97.47 (C-5), 112.01 (C-4a), 112.21 (C-3), 121.63 (C-7), 121.99 (C-10), 122.96 (C-6a), 126.46 (C-10a), 127.37 (C-8 and Ar-C Phe), 128.21 (C-9), 128.62 (2 x Ar-C Phe), 129.34 (2 x Ar-C Phe), 134.38 (C-1 Phe), 144.50 (C-10b), 149.71 (C-4), 151.24 (C-6), 159.54 (C-2), 168.48 (CO_2CH_2) ppm. IR (KBr 1%, cm^{-1}): υ = 3446, 3073, 3010, 2970, 2882, 1765, 1663, 1383, 1222, 1031, 881, 823, 761. HRMS (ESI): calcd for $C_{24}H_{21}NO_5$ [M^+ + H]: 404.14925; found 404.14882.

5.3. Photolysis

Photolysis was carried out using a Rayonet RPR-100 chamber reactor equipped with 10 lamps of 350 and 419 ± 10 nm. HPLC analyses were performed using a Licrospher 100 RP18 (5 µm) column with a JASCO HPLC system composed of a PU-2080 pump and a UV-2070 detector with ChromNav software.

5.3.1. General photolysis procedure

A 1×10^{-4} M solution of **3**, **4**, **6** and **7** in mixtures of acetonitrile/HEPES buffer (80:20, 5 mL), and **3** and **4** in methanol/HEPES buffer (80:20) was placed in a quartz tube and irradiated in the reactor at the desired wavelength. HEPES buffer solution was prepared in distilled water with HEPES (10 mM), NaCl (120 mM), KCl (3 mM), $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) and pH adjusted to 7.2. Aliquots of 100 µL were taken at regular intervals and analysed by RP-HPLC. The eluent was acetonitrile/water (3:1) at a flow rate of 1 mL/min (retention time: **3a**, 7.6; **3b**, 8.3; **4a**, 10.9; **4b**, 8.2 min) or 0.8 mL/min (**6a**, 7.1; **6b**, 7.4; **7a** and **7b**, 8.0 min), previously filtered through a Millipore, type HN 0.45 µm filter and degassed by ultrasound for 30 min. The chromatograms were traced by detecting UV absorption at the wavelength of maximum absorption for each conjugate.

6. Acknowledgements

Thanks are due to the Portuguese Fundação para a Ciência e Tecnologia (FCT) for financial support through project PTDC/QUI/69607/2006 (FCOMP-01-0124-FEDER-007449) and a PhD grant to A.M.P. (SFRH/BD/61459/2009). The NMR spectrometer Bruker Avance III 400 is part of the National NMR Network and was purchased in the framework of the National Program for Scientific Re-equipment, under contract REDE/1517/RMN/2005, with funds from the Fundo Europeu de Desenvolvimento Regional (FEDER) (POCI 2010) and from FCT.

7. Supporting information

7.1. Time-resolved fluorescence measurements (decay curves and analysis) and rate constants calculation

The fluorescence decay data obtained from the curves shown in Figure S1 are given in Tables S1 and S2.

Acetonitrile/HEPES (80:20) Methanol/HEPES (80:20)

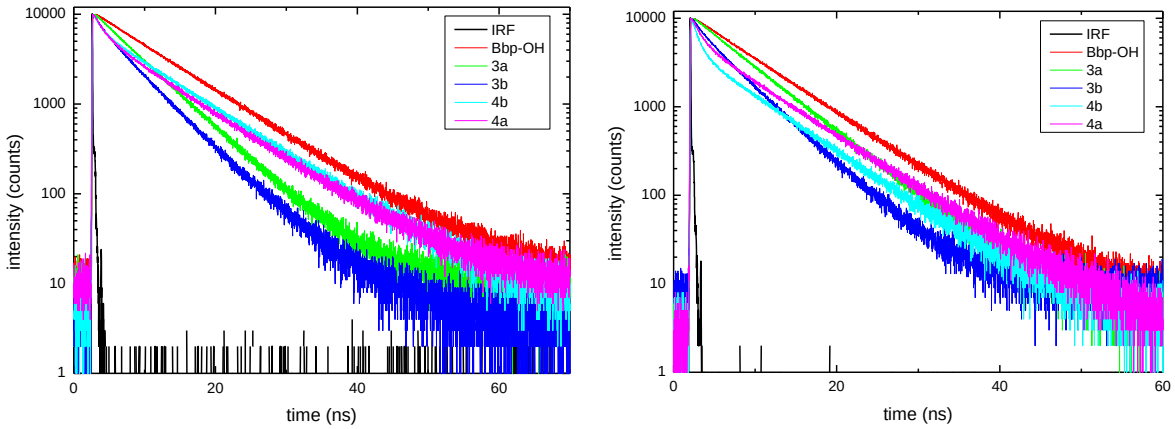


Figure S1. Time-resolved fluorescence decay curves for compounds **3a**, **3b**, **4a**, **4b** and the alcohol product (Bbp-OH) in HEPES containing solvent systems. The instrumental response function (IRF) is also shown.

Table S1. Time-resolved fluorescence analysis (τ in ns) for the compounds in acetonitile/HEPES (80:20).

Cpd	τ_1	τ_2	τ_3	α_1	α_2	α_3	τ_{ave}	χ^2
Bbp-OH	7.14 ± 0.02	--	--	1	--	--	7.14	1.05
3a	6.05 ± 0.03	3.20 ± 1.41	--	0.94	0.06	--	5.89	1.09
3b	5.05 ± 0.02	1.69 ± 0.06	0.22 ± 0.01	0.62	0.19	0.19	3.49	1.14
4a	7.24 ± 0.02	1.41 ± 0.07	0.39 ± 0.01	0.45	0.32	0.23	3.81	1.16
4b	6.90 ± 0.02	0.93 ± 0.03	0.20 ± 0.01	0.31	0.47	0.22	2.61	1.10

Table S2. Time-resolved fluorescence analysis (τ in ns) for the compounds in methanol/HEPES (80:20).

Cpd	τ_1	τ_2	τ_3	α_1	α_2	α_3	τ_{ave}	χ^2
Bbp-OH	8.66 ± 0.01	--	--	1	--	--	8.66	1.05
3a	5.95 ± 0.02	2.61 ± 0.99	--	0.94	0.06	--	5.75	1.07
3b	7.19 ± 0.15	4.43 ± 0.12	0.58 ± 0.06	0.18	0.58	0.24	4.03	1.18
4a	8.49 ± 0.02	1.71 ± 0.06	0.26 ± 0.06	0.47	0.32	0.21	4.62	1.05
4b	8.62 ± 0.02	1.29 ± 0.06	0.25 ± 0.09	0.53	0.27	0.20	4.99	1.11

Time-resolved emission spectra were obtained subsequent to the usual time-resolved decays and the decay associated spectra obtained for the different compounds are shown normalised, along with the steady state spectrum for the alcohol product (Bbp-OH, obtained using the same equipment and excitation source) for comparison. These spectra can help elucidate the origin of the decay components.

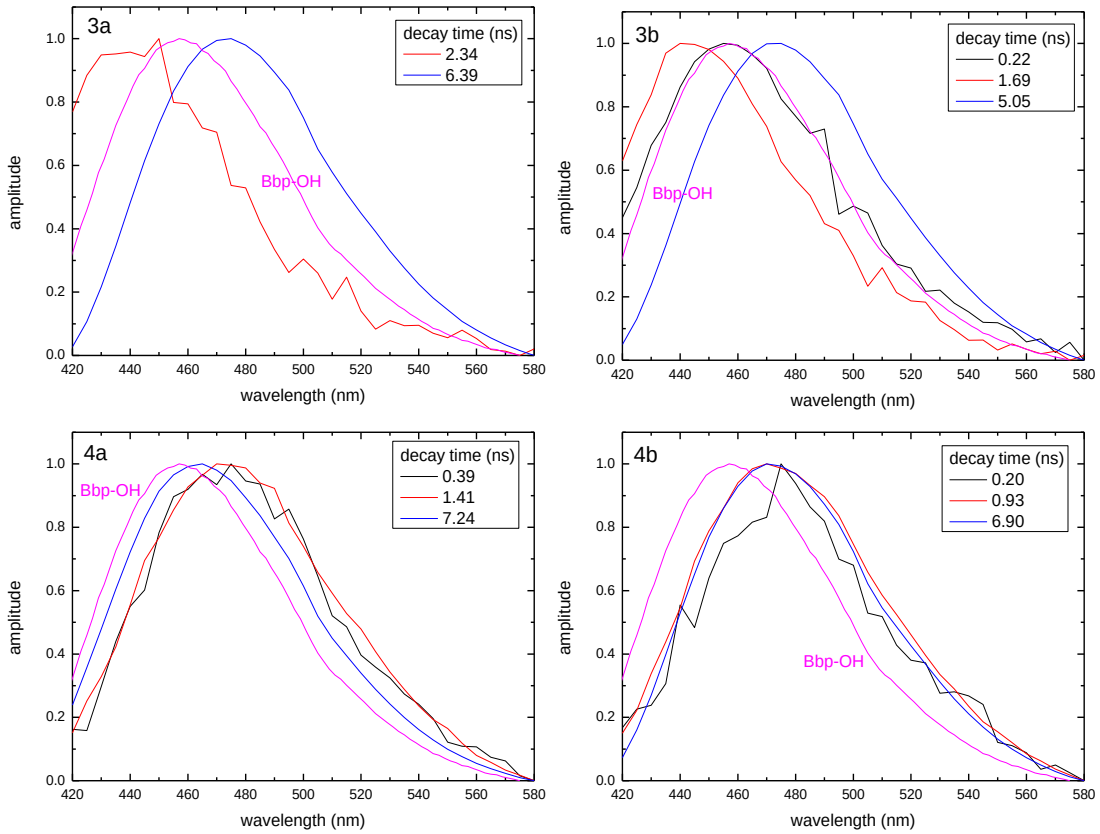


Figure S1. Decay associated spectra for the compounds in acetonitrile/HEPES (80:20).

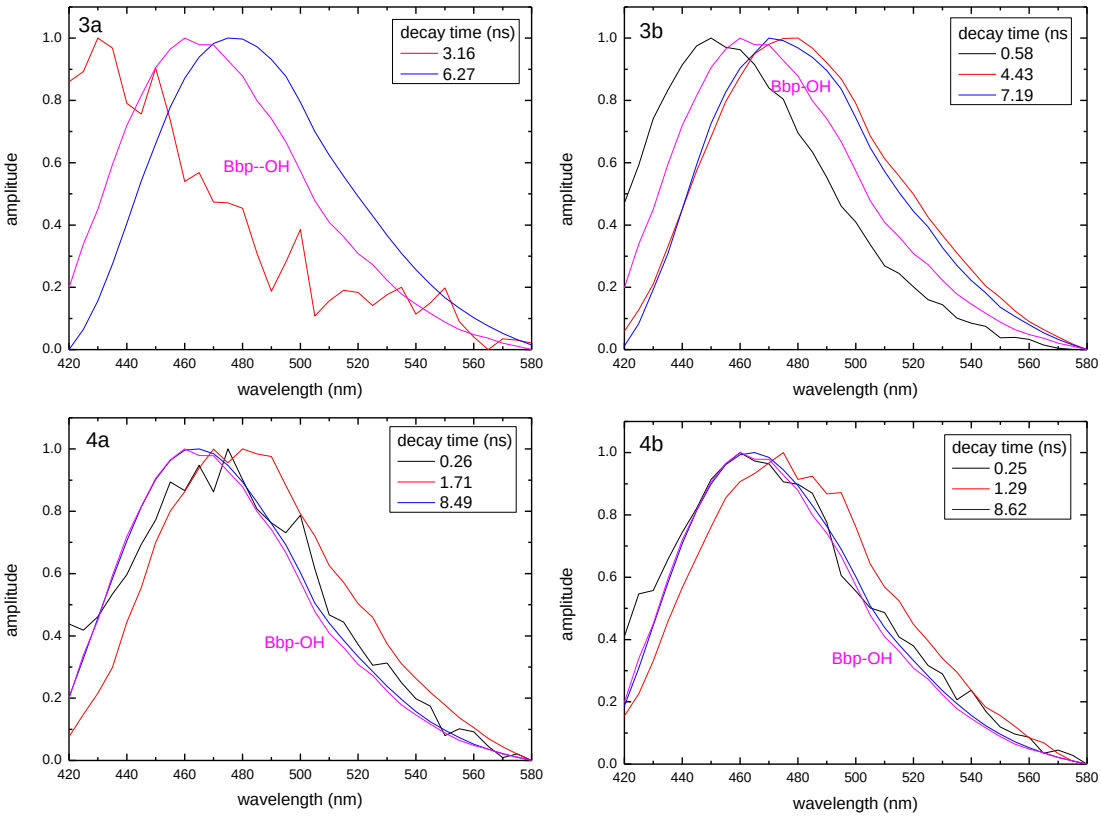


Figure S2. Decay associated spectra for the compounds in methanol/HEPES (80:20).

7.1.3. Rate constants

The fluorescence quantum yield is defined as

$$\Phi_F = k_r / (k_r + k_{nr})$$

with the fluorescence lifetime given as

$$\tau = 1 / (k_r + k_{nr})$$

where k_r is the radiative rate constant and k_{nr} the non-radiative rate constant.

According to reference 23, regarding coumarin derivatives, the radiative and non-radiative rates for both the initial ester and the alcohol product can be considered equal. Making the same assumption, the rate constant for the initial bond cleavage k_c can be obtained (with knowledge of the fluorescence quantum yield and lifetime of the alcohol product to give k_r and k_{nr}) from measurement of the ester fluorescence quantum yield (Φ_e) and lifetime (τ_e).

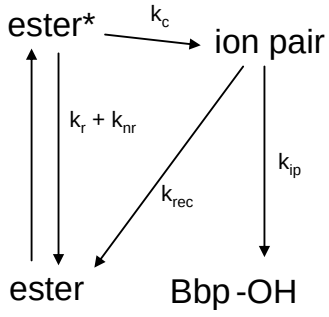
$$\Phi_e = k_r / (k_r + k_{nr} + k_c)$$

$$\tau_e = 1 / (k_r + k_{nr} + k_c)$$

Continuing with the analysis of Schmidt *et al.*[23] the photochemical quantum yield Φ_{Phot} of the ester can be expressed

$$\Phi_{Phot} = [k_c / (k_r + k_{nr} + k_c)] \times [k_{ip} / (k_{rec} + k_{ip})]$$

where k_{ip} is the rate constant for the decay of the ion pair leading to formation of the alcohol and k_{rec} that for recombination of this pair, returning to the initial ester. The associated simplified scheme for the coumarin species is given below.



Scheme S1. Simplified representation of the expected coumarin species and rate constants for de-excitation.

7.1.4. Experimental

Measured using a HORIBA Scientific UltraFast-01 equipped with a DeltaDiode laser excitation source emitting at 374 nm, at a repetition rate of 8 MHz and a Hamamatsu R3809-50 microchannel plate detector. The simple fluorescence decay curves were measured at 475 nm, with a slit width of 8 nm. The instrumental full width at half maximum was <75ps. Decay associated spectra were calculated by multiplying the pre-exponential functions by the related lifetime obtained from the global analysis of the time-resolved emission data. These were analysed using HORIBA Scientific DAS6 software from decay curves taken for equal dwell times at 5 nm wavelength intervals at a slit width of 6 nm. The decays were modelled as a sum of exponentials of the form,

$$I(t) = \sum_{i}^{n} \alpha_i \exp(-t/\tau_i)$$

with the average lifetime given by

$$\tau_{ave} = \sum_{i=1}^{n} \alpha_i \tau_i$$

Goodness of fit was judged in terms of a χ^2 value and weighted residuals.

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2.3

Photolytic release of butyric acid from oxygen- and nitrogen-based heteroaromatic cages

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INDEX

1. Abstract

In order to develop butyric acid photoactive prodrugs, new heteroaromatic conjugates based on oxygen and nitrogen were synthesized and evaluated under irradiation at 254, 300, 350 and 419 nm. Light-triggered uncaging of butyric acid from the corresponding heterocyclic cages was achieved with complete release of the drug in short times. Naphtho[2,3-*d*]oxazole, naphtho[1,2-*d*]oxazole, 3-oxo-3*H*-benzo[*f*]benzopyran, 2-oxo-2*H*-benzo[*h*]benzopyran and 6-oxo-6*H*-benzopyrano[6,7-*d*]oxazole conjugates were readily photolysed, and the best results were obtained for naphtho-oxazoles at 254 and 300 nm, and for 3-oxo-3*H*-benzo[*f*]benzopyran, 2-oxo-2*H*-benzo[*h*]benzopyran and 2-methyl-6-oxo-6*H*-benzopyrano[6,7-*d*]oxazole at 350 nm. 3-Oxo-3*H*-benzo[*f*]benzopyran also afforded good results at 419 nm. The photophysical processes involved were further elucidated by the use of time-resolved fluorescence techniques.

2. Introduction

Several strategies for the development of pharmacologically inert chemical derivatives, prodrugs, which can be converted into active drug molecules, have been explored to overcome common drawbacks such as low oral drug absorption, lack of site specificity, chemical instability, toxicity and poor patient acceptance.[1-3] Photoactive prodrugs with a suitable photolabile group, whose reactivity can be controlled by selecting the wavelength of the excitation light, could be an alternative to the molecular design of prodrugs. The timed and accurate delivery of a drug at a specific location *in vivo* or *in vitro* presents a challenging goal that can be achieved by using photoactive prodrugs that are able to decompose rapidly upon photoirradiation. In recent reports in the field of prodrugs, there have been some examples of light as the triggering agent for the optimisation of drug delivery.[4-6] Given the promising results obtained, this strategy could allow for a more patient-friendly drug delivery and availability scheme, which involves less complex dosing schedules and a lower potential for side effects.

Drugs that contain ionisable polar groups such as carboxylic acids are poorly absorbed from the gastrointestinal tract due to lipophilicity/solubility issues. The

absorption can be improved by masking the carboxyl group by forming derivatives through different covalent linkages.[1] Butyric acid is a naturally occurring short chain fatty acid involved in the regulatory mechanisms for gene expression known to promote markers of cell differentiation, apoptosis and cell-growth control.[7,8] Prodrugs based on butyric acid, upon intracellular hydrolytic degradation, release acids and aldehydes, modulate gene expression, and induce histone hyperacetylation, differentiation, and apoptosis of cancer cells. Prodrugs that release formaldehyde increase apoptosis of cancer cells, at concentrations about 10-fold lower than butyric acid and 100 times faster. The formaldehyde released from these prodrugs has been shown to be a critical antiproliferative factor that induces differentiation and cell death.[8-11]

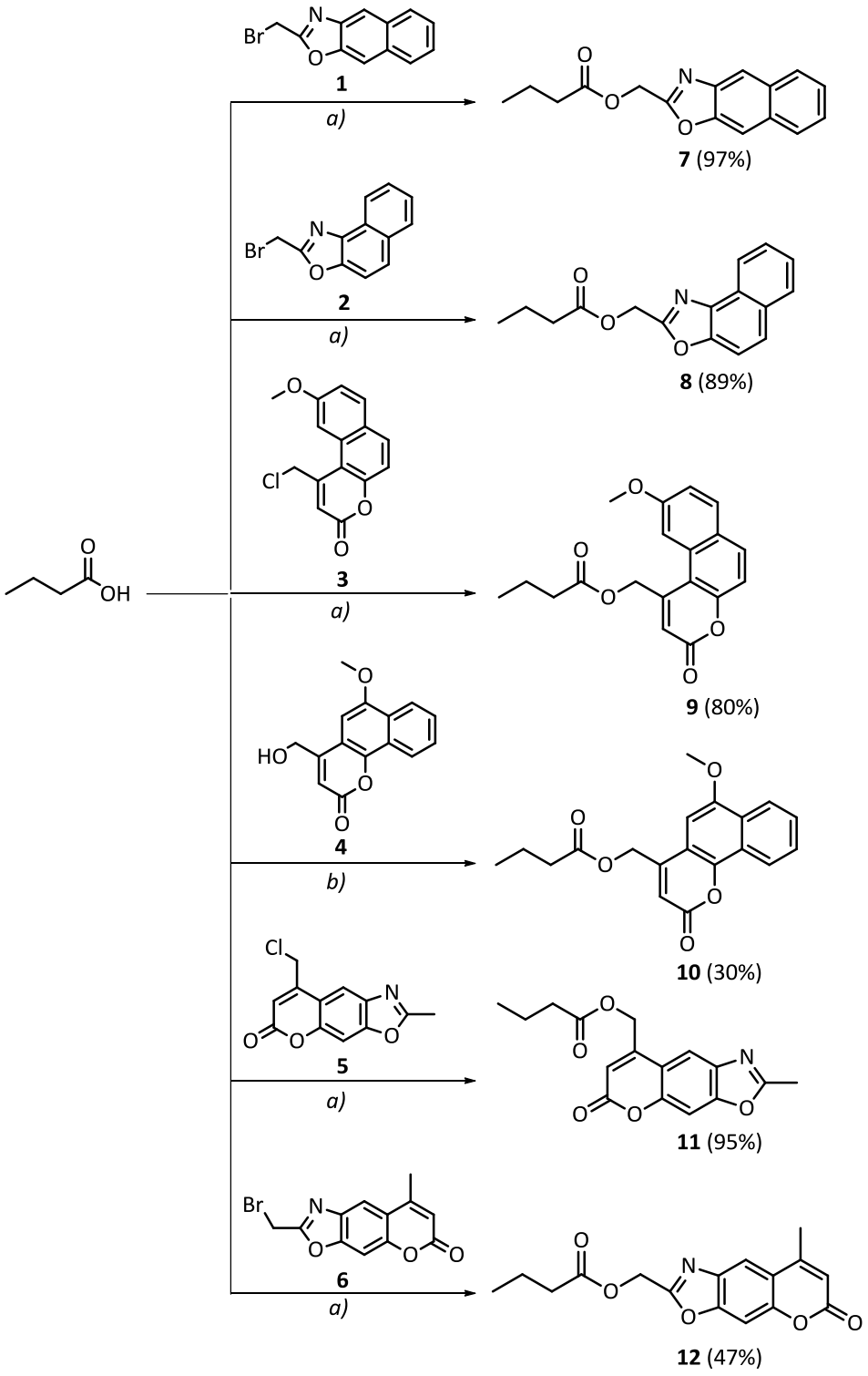
Our recent research has focussed on the synthesis and application of new oxygen and nitrogen heterocycles as photosensitive groups for the protection of carboxylic acid and amino groups.[12-18] In order to extend the scope of applications of such groups to drug-delivery research in the form of photoactive prodrugs, this work evaluates the use of naphtho[2,3-*d*]oxazole, naphtho[1,2-*d*]oxazole, 3-oxo-3*H*-benzo[*f*]benzopyran, 2-oxo-2*H*-benzo[*h*]benzopyran and 6-oxo-6*H*-benzopyrano[6,7-*d*]oxazoles in the light-triggered release of butyric acid from the corresponding heterocyclic cages. The stability to irradiation of the ester bond between butyric acid and the caging group has been evaluated in a photochemical reactor at 254, 300, 350 and 419 nm, and uncaging data have been acquired. The photophysical processes involved have been further elucidated by the use of time-resolved fluorescence techniques.

3. Results and discussion

The synthesis of bromomethylated naphtho[1,2-*d*]oxazole (**2**) was achieved by a condensation reaction between 1-aminonaphthalen-2-ol and bromoethanoic acid mediated by polyphosphoric acid according to a known procedure.[16] We have previously reported the syntheses of naphtho[2,3-*d*]oxazole (**1**), 3-oxo-3*H*-benzo[*f*]benzopyran (**3**), 2-oxo-2*H*-benzo[*h*]benzopyran (**4**) and 6-oxo-6*H*-benzopyrano[6,7-*d*]oxazoles **5** and **6**.[12,16,17]

Compounds **1**-**6** were used in the derivatisation of butyric acid in the presence of potassium fluoride (for **1**-**3**, **5** and **6**) or *N,N'*-dicyclohexylcarbodiimide (DCC)/1-

hydroxybenzotriazole (HOBt, for **4**) in *N*,*N*-dimethylformamide (DMF) at room temperature,[14,19] which resulted in the ester prodrugs **7**-**12** in good yields (Table 1, Scheme 1).



Scheme 1. Synthesis of butyric acid prodrugs **7**-**12**. (a) KF/DMF, rt; (b) DCC/HOBt, DMF, rt.

All compounds synthesized were fully characterised by HRMS, IR, ^{1}H and ^{13}C NMR spectroscopy. The IR spectra of **7**-**12** showed bands attributable to the stretching vibrations of the ester carbonyl group from 1718 to 1749 cm^{-1}. ^{1}H NMR spectra showed signals of the butyric acid moiety, the methyl (δ = 0.93-1.02 ppm) and two methylene groups (δ = 1.72 and 2.49 ppm). The heterocycle methylene groups were also visible for all compounds (δ = 4.14-5.69 ppm). The confirmation of the presence of the newly formed ester linkages was also supported by ^{13}C NMR spectra signals of the carbonyl group, which were found at δ = 172.68-173.59 ppm.

UV/vis spectroscopic characterisation was carried out to obtain the parameters needed for monitoring the uncaging process. Absorption spectra of degassed 10^{-5} M solutions in absolute ethanol and in a methanol/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (80:20) solution of **7**-**12** were measured and compared to those of precursors **1**-**6**. Absorption maxima and molar extinction coefficients are reported in Table 1. By comparison of the absorption maxima for all compounds in both solvents, no significant changes were observed. The differences between the pairs of compounds were examined. For the naphtho-oxazole compounds **7** and **8**, the latter has a more defined band structure and an absorption maximum *ca*. 20 nm red-shifted compared to that of **7**. A similar bathochromic shift was seen between oxobenzopyrans **9** and **10**, and no difference was found between **11** and **12**, which incorporate an oxobenzopyran chromophore fused with an oxazole. Replacement of the benzene ring in **7** by an oxopyran in **12** also resulted in a comparable absorption red-shift.

The fluorescence spectra were also measured as the use of fluorescent labelling with its increased sensitivity over UV/vis absorption techniques makes it well suited for analytical purposes. The outcome of these steady-state measurements is also given in Table 1. In recent years, the direction of improvement of photoreleasable groups has been towards the application of polycyclic aromatic structures, which are fluorophores in most cases. However, there can be the added complication of fluorescence deactivation in some photochemical processes.

Molecules of biological importance such as butyric acid are not easily detected by UV/vis absorption measurements and have no or low intrinsic fluorescence, and thus the introduction of a fluorophore is an appropriate strategy to enhance the photophysical

properties of the resulting conjugates and to provide a means to monitor photoinduced processes. By considering the obtained values for the relative fluorescence quantum yields (Φ_F) for **7**-**12** in absolute ethanol and methanol/HEPES buffer (80:20) solution determined with 9,10-diphenylanthracene in ethanol as standard,[20] it is evident that the processes in these compounds can be monitored by fluorescence techniques. The Stokes's shifts were in the range 2506-9174 and 2984-9271 cm^{-1} in ethanol and methanol/HEPES buffer (80:20) solution, respectively, which is an advantageous property in fluorescence techniques as it will minimize self-quenching phenomena.

Table 1. Yields, UV/vis absorption and emission data in absolute ethanol and methanol/HEPES buffer (80:20) solutions for **1**-**12**.

	Yield (%)	Ethanol					Methanol/HEPES (80:20)				
		λ_{abs} [a]	log ε	λ_{em} [a]	Φ_F	$\Delta\lambda$ [b]	λ_{abs} [a]	log ε	λ_{em} [a]	Φ_F	$\Delta\lambda$ [b]
1 [c]	13	334	3.67	380	0.28	3624	334	3.60	380	0.02	3624
2	98	326	3.94	355	0.22	2506	310	4.61	354	0.01	4009
3 [d]	83	354	4.10	472	0.03	7062	355	3.47	458	0.10	6335
4 [e]	47	365	3.69	460	0.45	5658	371	3.73	469	0.45	5632
5 [c]	34	326	3.98	404	0.06	5922	325	3.94	397	0.005	5580
6 [c]	23	325	3.80	424	0.01	7184	325	3.54	396	0.001	5517
7	97	300	3.52	383	0.42	7224	302	3.99	355	0.03	4944
8	89	322	3.90	355	0.11	2887	321	4.26	355	0.21	2984
9	80	350	3.92	466	0.02	7112	352	4.22	484	0.46	7748
10	30	375	3.88	474	0.38	5570	375	3.85	482	0.28	5920
11	95	325	3.96	425	0.11	7240	326	3.83	424	0.10	7090
12	47	322	3.93	457	0.03	9174	321	4.14	457	0.019	9271

[a] In nm. [b] In cm^{-1}. [c] Yield and absorption data reported from ref. 16. [d] Yield and absorption data in ethanol reported from ref. 21. [e] Yield and absorption data reported from ref. 17.

The photophysics of the different fluorophores in **7**-**12** in methanol/HEPES buffer (80:20) were investigated to help elucidate the processes involved upon light irradiation. Time-resolved fluorescence decays were measured at one emission wavelength (Figure 1).

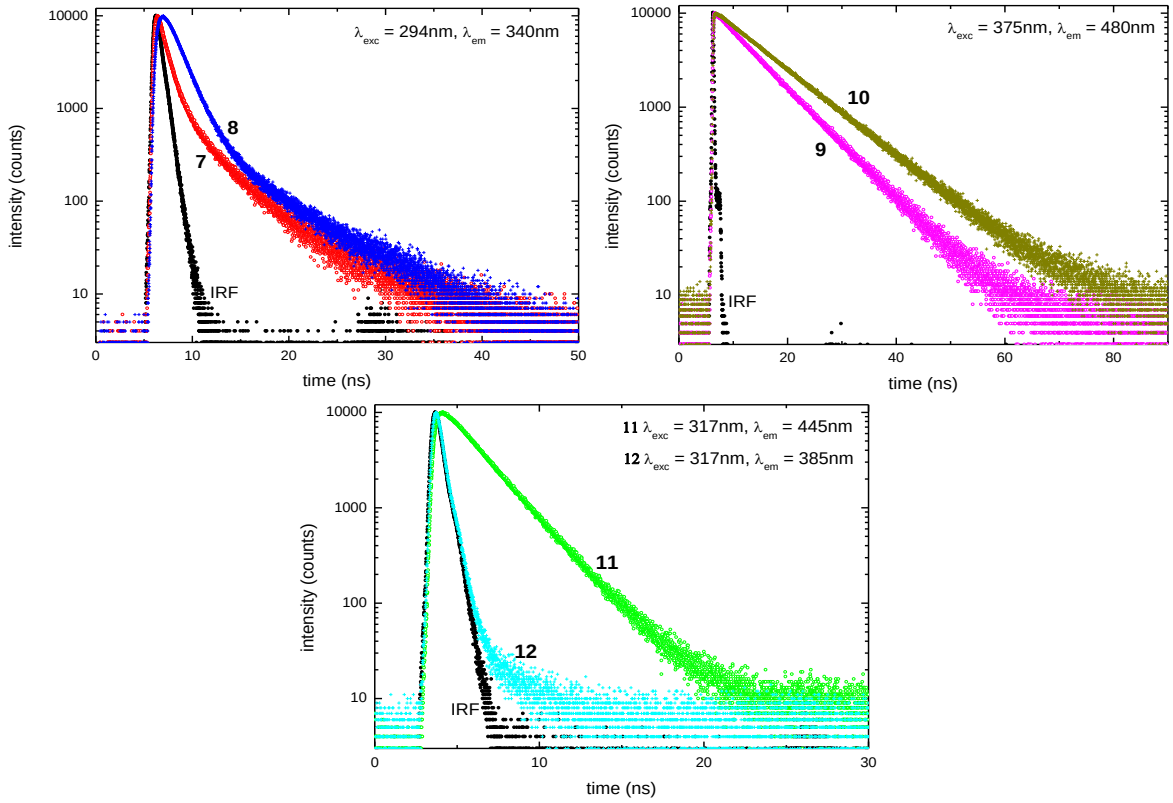


Figure 1. Fluorescence decay curves for compounds **7**-**12**. The instrumental response function (IRF) is also shown.

Figure 1 shows the different decay behaviour for the different sets of fluorophores **7**-**12**. Compounds **7** and **8** exhibit quite complex kinetic behaviour, which is indicative of the presence of several excited states. The other compounds appear to decay in a simpler manner, although the emission from **12** is highly quenched, which is in keeping with the low quantum yield given in Table 1. The associated lifetime values extracted from analysis of these decay curves generally reflect the trend in the quantum yields (longer lifetimes with higher quantum yields, Table 2).

To provide further insight into the processes that could be occurring in the excited state, time-resolved fluorescence measurements that monitored the emission at different wavelengths were performed to allow time-resolved emission spectra (TRES) and, by the use of global analysis, decay associated spectra (DAS) were calculated. Because of the nature of time-correlated single-photon counting, which uses very modest excitation energies (pJ per pulse), it was not envisaged that significant photocleavage would occur during the measurements. TRES for all compounds, except **9**

and **10** (which bear an angular coumarin, 3-oxo-3*H*-benzo[*f*]benzopyran and 2-oxo-2*H*-benzo[*h*]benzopyran, respectively), showed changes in the spectral shape with time after excitation (Supporting information) that hint at the presence of several species. This is in keeping with the simple time-resolved fluorescence measurements (Figure 1 and Table 2). The spectral shape of **9** and **10** appeared relatively constant, consistent with their simpler decay kinetic dominant decays of 7.4 and 9.4 ns, respectively.

Table 2. Fluorescence lifetimes (τ) for **7**-**12** obtained from reconvolution analysis of the data presented in Figure 1.[a]

Cpd	Lifetime (ns)				Relative amplitude (%)			
	τ_1	τ_2	τ_3	τ_4	f_1	f_2	f_3	f_4
7	8.04 ± 0.48	3.13 ± 0.06	0.80 ± 0.02	0.052 ± 0.009	9	23	23	45
8	6.58 ± 0.06	1.44 ± 0.01	--	--	84	16	--	--
9	7.41 ± 0.02	3.68 ± 0.12	--	--	96	4	--	--
10	9.41 ± 0.02	--	--	--	10	--	--	--
11	5.47 ± 0.31	2.12 ± 0.01	0.11 ± 0.01	--	4	87	9	--
12	1.93 ± 0.15	0.02 ± 0.01	--	--	2	98	--	--

[a] The curves were fitted to a sum of exponentials of the form $I(t) = \sum_i^n \alpha_i \tau_i$, where α_i are normalised preexponential factors and the relative amplitude or factional (f_i) is given by $f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i}$.

Upon photolysis, it was expected that an ion pair could form, which would either undergo recombination to the ester or photocleavage.[22] Knowledge of the fluorescent species present and their kinetics may allow the rate of the photocleavage reaction to be estimated. The DAS can be useful to provide a framework to assist in this determination by providing both spectral and dynamic information. The DAS for **7**-**12** are shown in Figure 2 and were obtained from the global analysis of the time-resolved decays taken from the TRES measurements with decays measured at 5 nm intervals. Consistent with the TRES, the behaviour of **9** and **10** appear slightly different with their emission dominated by the deexcitation of one excited state. In the case of the other compounds, global analysis shows the presence of a very short-lived decay component (and associated spectrum, because of the recovery of a clear, sensible spectral shape it is

unlikely that scattered light is the origin of this decay component; a fitting artefact, although unlikely as the datasets consist of > 40 decays and the spectral shape appears sensible, cannot be completely ruled out). The actual value of the shortest-lived decay should be treated with caution as it is at or beyond the resolution of the equipment and hence ought to be considered as a very short-lived decay component. The complexity of the decay behaviour of **7** and **8** may be indicative that these compounds undergo photoinduced processes more readily than the others, which can be seen later in the photolysis data and offers the possibility to monitor the whole uncaging process. The complete and rigorous attribution of the spectra and associated decay kinetics requires the synthesis of appropriate model compounds for each of the chromophores. Because of the complexity seen in the fluorescence emission it can only be speculated that the shorter-lived species are those initially formed with longer lifetimes associated with the products. The complete attribution of the spectra will be left for a further study, and the results presented here demonstrate the ability of this technique to elucidate information.

Compounds **11** and **12** exhibit higher energy (shorter wavelength) spectra associated with a very fast decay and longer-lived species at longer wavelengths. This can be indicative of the longer-lived species formed from the initial formation of the species associated with the shorter-lived fluorescence. In the case of **11**, a further spectrum (*ca*.1 ns) was recovered that exhibits negative amplitude at longer wavelengths, which corresponds to the position of the 2.2 ns spectrum. Negative amplitudes for preexponentials (rise times) are commonly associated with the formation of a species in the excited state. To clearly identify the different spectra, they were normalized, and in that of **12**, the emission is completely dominated by the shorter-lived fluorescence (Table 2).

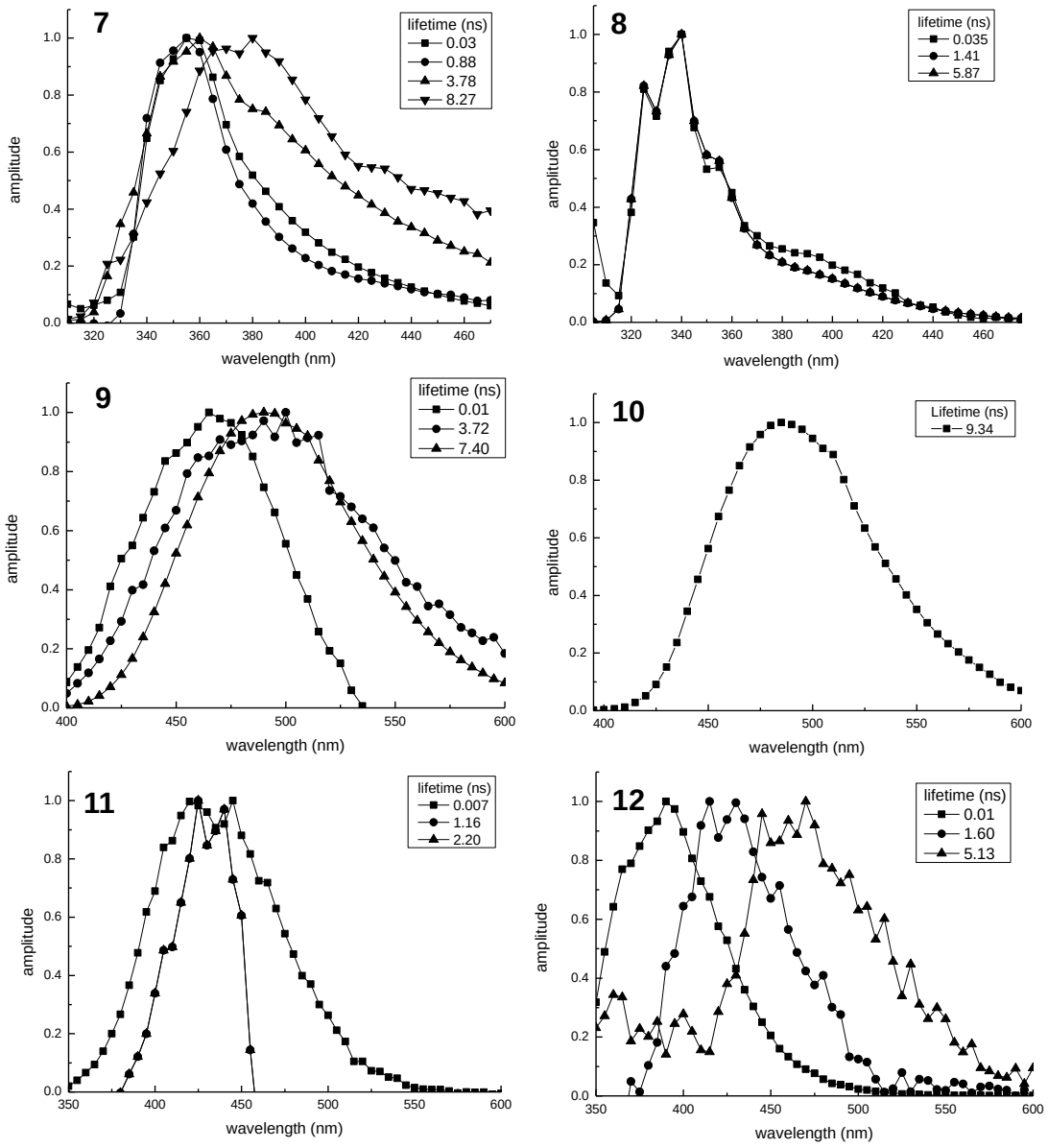


Figure 2. Normalised DAS for **7**-**12** in methanol/HEPES buffer (80:20) solution.

Our research into the development of alternative photosensitive groups for the protection of bifunctional molecules has involved the successful use of oxazole-based structures for the first time.[16] In addition, 2-oxo-2*H*-benzopyrans (coumarins) are well-established light-activated protecting groups, and we have been engaged in the application of their related fused derivatives, 3-oxo-3*H*-benzo[*f*]benzopyrans and 2-oxo-2*H*-benzobenzopyrans (benzocoumarins).[12,14,17,18] Both systems are suitable for photorelease purposes, their choice depends on the required wavelengths and have encouraged us to explore their use in other applications, namely, light-triggered drug delivery.

The evaluation of photoactive butyric acid prodrugs **7**-**12**, which possess naphtho[2,3-*d*]oxazole, naphtho[1,2-*d*]oxazole, 3-oxo-3*H*-benzo[*f*]benzopyran, 2-oxo-2*H*-benzo[*h*]benzopyran and 6-oxo-6*H*-benzopyrano[6,7-*d*]oxazole, in which the latter has the linkage between the heterocycle and the active molecule through oxopyran or oxazole moieties, was carried out by photolysis studies under irradiation at different wavelengths. Solutions of **7**-**12** (1×10^{-4} M) in methanol/HEPES buffer (80:20) solution were irradiated in a Rayonet RPR-100 reactor at 254, 300, 350 and 419 nm in order to determine the most favourable uncaging conditions. The course of the photocleavage reaction was monitored by reverse-phase-(RP)-HPLC with UV detection. The plots of peak area (*A*) of the starting material *versus* irradiation time (t_{irr}) were obtained for each compound at the considered wavelengths. Peak areas were determined by HPLC, which revealed a gradual decrease with time, and were the average of three runs. The determined irradiation time represents the time necessary for the consumption of the starting materials until less than 5% of the initial area was detected. Based on HPLC data for each compound, the plot of ln *A versus* t_{irr} showed a linear correlation for the disappearance of the starting material, which suggests a first-order reaction and was obtained by the linear least-squares method for a straight line with good correlation coefficients. The corresponding rate constants (*k*) were calculated and are presented in Table 3.

By comparison of caged butyric acids **7** and **8**, which bear naphtho-oxazole units that differ in the ring fusion, a linear naphtho[2,3-*d*]oxazole and an angular naphtho[1,2-*d*]oxazole, respectively, it was found that, although both cleaved readily at 254 and 300 nm, the irradiation times obtained for the release of butyric acid from **8** was less than 1 min.

Concerning the photolysis of compounds **7** and **8** at 350 nm, the photocleavage was not achieved in a practical period of time, which was predicted from the values of wavelengths of maximum absorption in the solvent used with the lowest irradiation time of 519 min (*ca*. 9 h) for **8**. Concerning **9** and **10**, which bear angular coumarins 3-oxo-3*H*-benzo[*f*]benzopyran and 2-oxo-2*H*-benzo[*h*]benzopyran, respectively, it was found that cleavage was faster at all the wavelengths of irradiation for **10**, especially at 350 nm.

Table 3. Values of t_{irr} (min) and k ($\times 10^{-2}$ min^{-1}) for the complete photolysis (> 95%) of **7**-**12** at different wavelengths in methanol/HEPES buffer (80:20) solution.

Cpd	254 nm		300 nm		350 nm	
	t_{irr}	k	t_{irr}	k	t_{irr}	k
7	4.9	65.22	5.6	53.42	> 2500	-
8	0.1	2918	0.8	400	519	0.58
9	178	1.70	253	1.19	263	1.14
10	107	2.86	170	2.86	96	3.15
11	45	6.59	33	6.59	285	1.04
12	76	3.95	94	3.16	> 2500	-

With this in mind and in order to achieve the best release conditions of butyric acid, **11** and **12**, which are based on the combination of 2-oxo-2*H*-benzopyran and oxazole in a fused system with ester linkage to the active molecule through a methylene spacer directly connected to the pyran or oxazole rings, respectively, were studied under irradiation under the same conditions as **7**-**10**. At 254 and 300 nm, the irradiation times of **11** and **12** were longer than for **7** and **8**, whereas at 350 nm their photolytic behaviour was inferior to that of **10**, but superior to that of **7** and **8**. Comparison between the irradiation times of **11** and **12** revealed that cleavage of the ester linkage was improved for compound **11**, which bears butyric acid connected to the pyran ring, at all irradiation wavelengths tested. These data revealed the contribution of both heterocyclic rings when combined in a single system for the time required for the complete release of butyric acid from the ester cages. They are also consistent with the time-resolved fluorescence data, which indicate that the compounds that exhibit the more complex decay kinetics have the fastest rate of photocleavage.

As butyric acid is an organic molecule of biological relevance, cleavage and release could benefit from being carried out by irradiation at longer wavelengths in practical times. As a result, the behaviour of **9** and **10**, which were the most promising at 350 nm (263 min for **9** and 96 min for **10**), were also evaluated at 419 nm, which resulted in an expected increased, but still useful, irradiation time for **10** (187 min) and an impractical result for **9** (3442 min, *ca*. 57 h). In addition, the photolytic process at 300 nm was monitored by ^{1}H NMR in a methanol-d_4/D_2O (80:20) solution at concentration of

9.0×10^{-3} M (several times larger than that used in the experiments followed by HPLC), which led to an increase in the photolysis time for the complete release of the acid. During irradiation, the signals related to the linked acid decreased gradually, with concomitant increase of its signals in the released form, as well as signals due to aromatic byproducts related to the heterocyclic cage (see Figure 3 for **10** at 350 nm as a representative example). Variable irradiation times were required for the quantitative release of the butyric acid, which depended on the structure of the heterocycle.

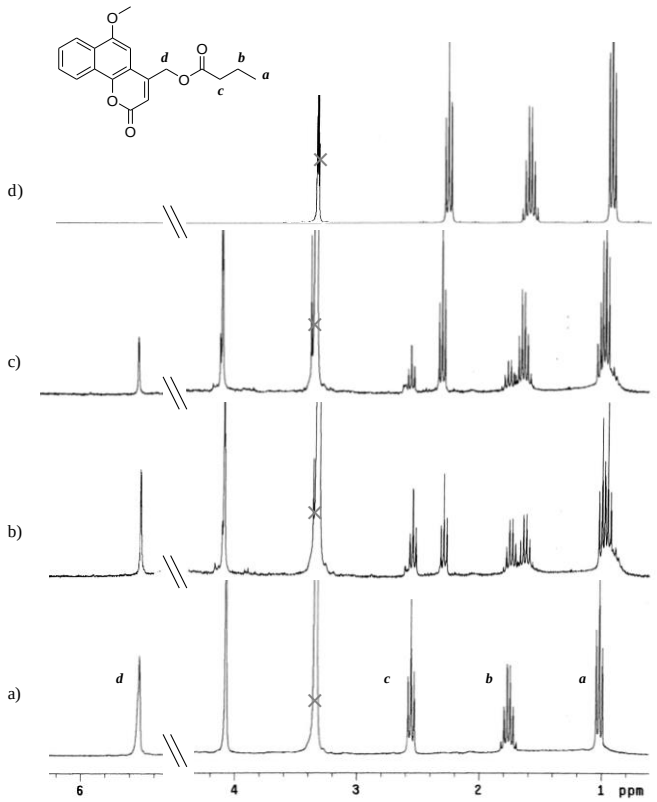


Figure 3. ^{1}H NMR spectra in methanol-d_4/D_2O (80:20) for the photolysis of **10** at 350 nm: (a) before irradiation, (b) after irradiation for 60 min, (c) after irradiation for 140 min, (d) free butyric acid.

4. Conclusions

Naphtho[2,3-*d*]oxazole, naphtho[1,2-*d*]oxazole, 3-oxo-3*H*-benzo[*f*]benzopyran, 2-oxo-2*H*-benzo[*h*]benzopyran and 6-oxo-6*H*-benzopyrano[6,7-*d*]oxazole (with a linkage between the heterocycle and the active molecule through oxopyran or oxazole moieties) were used in the synthesis of caged butyric acid through an ester linkage. Photolytic

cleavage studies at 254, 300, 350 and 419 nm in methanol/HEPES buffer (80:20) solution revealed the possibility of using these heterocyclic systems in the light-triggered release of the active molecule in reasonable times. The use of time-resolved fluorescence techniques contributed to the elucidation of the dynamic behaviour of these systems. Overall, these results suggest that the heterocyclic cages studied may be considered as promising alternatives as photoactive prodrugs for butyric acid as a model carboxylic acid drug.

5. Experimental section

5.1. General

All melting points were measured on a Stuart SMP3 melting point apparatus. TLC analyses were carried out with 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$), and spots were visualised under UV light. Chromatography on silica gel was carried out with Merck Kieselgel (230-240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer. UV/vis absorption spectra (200-700 nm) were obtained using a Shimadzu UV/2501PC spectrophotometer. NMR spectra were obtained on a Varian Unity Plus spectrometer at an operating frequency of 300 MHz for ^{1}H and 75.4 MHz for ^{13}C or a Bruker Avance III 400 instrument at an operating frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C by using the solvent peak as internal reference at 25 °C. All chemical shifts are given in ppm by using δ_H (Me_4Si) = 0 ppm as a reference, and *J* values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and two-dimensional heteronuclear correlation techniques. Low- and high-resolution mass spectrometry was performed at the C.A.C.T.I. - Unidad de Espectrometria de Masas, at the University of Vigo, Spain. Commercially available reagents were used as received.

5.1.1. Synthesis of 2-(bromomethyl)naphtho[1,2-*d*]oxazole (2). To a solution of 1-aminonaphthalen-2-ol (0.300 g, 1.53×10^{-3} mol) in polyphosphoric acid (1.53 g) was added bromoacetic acid (0.320 g, 2.30×10^{-3} mol), and the mixture was stirred at 130 °C

for 5 h. The reaction mixture was poured into iced water and stirred for 1 h to give a fine grey precipitate. The solid was collected by filtration, washed with cold water and dried in a vacuum oven. After purification by chromatography, using ethyl acetate/*n*-hexane (1:1) as eluent, **2** was obtained as a grey solid (0.400 g, 98%). Mp = 106.2-107.4 °C. TLC (ethyl acetate/*n*-hexane, 1:1): R_f = 0.97. ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H = 4.73 (s, 2 H, CH_2), 7.53 (dt, *J* = 6.9 and 1.2 Hz, 1 H, 6-H), 7.68 (dt, *J* = 6.9 and 1.2 Hz, 1 H, 5-H), 7.70 (d, *J* = 6.6 Hz, 1 H, 9-H), 7.86 (d, *J* = 9.0 Hz, 1 H, 7-H), 7.98 (d, *J* = 9.0 Hz, 1 H, 4-H), 8.48 (d, *J* = 6.6 Hz, 1 H, 8-H) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C = 20.88 (CH_2), 110.81 (C-9), 121.99 (C-8), 125.67 (C-6), 126.44 (C-3a), 127.12 (C-7), 127.39 (C-5), 128.62 (C-4), 131.21 (C-3b), 136.51 (C-7a), 148.62 (C-9a), 159.95 (C-2) ppm. IR (KBr 1%, cm^{-1}): υ = 3100, 3055, 2974, 2924, 1700, 1656, 1600, 1500, 1392, 1309, 1275, 1254, 1218, 1102, 1049, 926, 888, 813, 743, 687, 666. HRMS (EI): calcd for $C_{12}H_8NO^{79}Br$ [M^+]: 260.9789; found 260.9778; calcd for $C_{12}H_8NO^{81}Br$ [M^+]: 262.9769; found 262.9772.

5.2. Synthesis of 7-12

5.2.1. General procedure for the synthesis of 7-9, 11 and 12. To a solution of the bromo- or chloromethyl precursor **1**-**3**, **5** and **6** (1 equiv.) in dry DMF (2 or 3 mL) were added potassium fluoride (3 equiv.) and butyric acid (1 equiv.). The reaction mixture was stirred at room temperature for 5 h or 2 d (**9**, **11** and **12**). Potassium fluoride was removed by filtration, the solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using mixtures of ethyl acetate and *n*-hexane as eluent.

5.2.1.1. (Naphtho[2,3-*d*]oxazol-2-yl)methyl butyrate (7). From the reaction of **1** (0.030 g, 1.15 × 10^{-4} mol) in DMF (2 mL), potassium fluoride (0.020 g, 3.45 × 10^{-4} mol) and butyric acid (0.010 g, 1.15 × 10^{-4} mol), **7** was obtained as a yellow solid (0.030 g, 97%). Mp = 125.7-126.5 °C. TLC (ethyl acetate/*n*-hexane, 1:1): R_f = 0.93. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.01 (t, *J* = 7.6 Hz, 3 H, CH_3), 1.75 (sext, *J* = 7.6 Hz, 2 H, CH_2), 2.48 (t, *J* = 7.6 Hz, 2 H, CH_2), 5.40 (s, 2 H, CH_2 Het), 7.46-7.53 (m, 2 H, H-6 and H-7), 7.91 (s, 1 H, H-9), 7.95 (dd, *J* = 6.8 and 2.7 Hz, 1 H, H-8), 7.99 (dd, *J* = 6.9 and 2.7 Hz, 1 H, H-5), 8.18 (s, 1 H, H-4) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.55 (CH_3), 18.28 (CH_2), 35.66 (CH_2), 58.08

(CH_2 Het), 106.66 (C-9), 117.89 (C-4), 124.80 (C-7), 125.71 (C-6), 127.88 (C-8), 128.53 (C-5), 131.26 (C-8a), 131.70 (C-4a), 140.54 (C-3a), 149.50 (C-9a), 163.01 (C-2), 172.71 (C=O) ppm. IR (KBr 1%, cm^{-1}): υ = 2958, 2932, 2875, 1740, 1694, 1644, 1626, 1579, 1505, 1470, 1443, 1415, 1305, 1260, 1242, 1165, 1096, 1050, 1018, 956, 938, 900, 882, 867, 799, 767, 666. HRMS (EI): calcd for $C_{16}H_{15}NO_3$ [M^+]: 269.1052; found 269.1058.

5.2.1.2. (Naphtho[1,2-*d*]oxazol-2-yl)methyl butyrate (8). From the reaction of **2** (0.109 g, 4.18×10^{-4} mol) in DMF (4 mL), potassium fluoride (0.073 g, 1.25×10^{-3} mol) and butyric acid (0.037 g, 4.18×10^{-4} mol), **8** was obtained as a brown oil (0.100 g, 89%). TLC (ethyl acetate/*n*-hexane, 1:1): R_f = 0.98. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 0.98 (t, *J* = 7.6 Hz, 3 H, CH_3), 1.72 (sext, *J* = 7.6 Hz, 2 H, CH_2), 2.43 (t, *J* = 7.6 Hz, 2 H, CH_2), 5.43 (s, 2 H, CH_2 Het), 7.53 (dt, *J* = 6.8 and 0.8 Hz, 1 H, H-6), 7.65 (dt, *J* = 6.8 and 0.8 Hz, 1 H, H-5), 7.66 (d, *J* = 9.2 Hz, 1 H, H-9), 7.79 (d, *J* = 9.2 Hz, 1 H, H-7), 7.94 (d, *J* = 9.0 Hz, 1 H, H-4), 8.46 (d, *J* = 7.6 Hz, 1 H, H-8) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.51 (CH_3), 18.25 (CH_2), 35.67 (CH_2), 58.07 (CH_2 Het), 110.75 (C-9), 121.91 (C-8), 125.42 (C-6), 126.41 (C-3a), 126.57 (C-7), 127.12 (C-5), 128.46 (C-4), 131.05 (C-3b), 136.09 (C-7a), 148.25 (C-9a), 159.55 (C-2), 172.72 (C=O) ppm. IR (KBr 1%, cm^{-1}): υ = 3067, 2966, 2936, 2876, 1747, 1704, 1640, 1592, 1572, 1529, 1448, 1374, 1308, 1273, 1259, 1164, 1101, 1046, 1029, 1005, 939, 880, 804, 749, 697, 665. HRMS (EI): calcd for $C_{16}H_{15}NO_3$ [M^+]: 269.1052; found 269.1051.

5.2.1.3. (9-Methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl)methyl butyrate (9). From reaction of **3** (0.03 g, 1.09×10^{-4} mol) in DMF (2 mL), potassium fluoride (0.019 g, 3.27×10^{-4} mol) and butyric acid (0.011 g, 1.20×10^{-4} mol), **9** was obtained as a brown solid (0.027 g, 80%). Mp = 123.7-124.9 °C. TLC (ethyl acetate/light petroleum, 3:7): R_f = 0.58. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.01 (t, *J* = 7.6 Hz, 3 H, CH_3), 1.74 (sext, *J* = 7.2 Hz, 2 H, CH_2), 2.49 (t, *J* = 7.6 Hz, 2 H, CH_2), 3.97 (s, 3 H, OCH_3), 5.69 (d, *J* = 1.6 Hz, 2 H, CH_2 Het), 6.68 (d, *J* = 1.6 Hz, 1 H, H-2), 7.21 (dd, *J* = 8.8 and 2.4 Hz, 1 H, H-8), 7.33 (d, *J* = 8.8 Hz, 1 H, H-5), 7.48 (d, *J* = 2.4 Hz, 1 H, H-10), 7.82 (d, *J* = 9.2 Hz, 1 H, H-7), 7.91 (d, *J* = 8.8 Hz, 1 H, H-6) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.64 (CH_3), 18.29 (CH_2), 35.96 (CH_2), 55.43 (OCH_3), 64.01 (CH_2 Het), 105.73 (C-10), 111.98 (C-4b), 112.76 (C-2), 115.30 (C-5), 116.65 (C-8), 126.38 (C-6a), 130.64 (C-6b), 131.30 (C-7), 133.74 (C-6), 151.17 (C-1), 155.54 (C-4a), 159.66 (C-9), 160.31 (C-3), 172.76 (C=O) ppm. IR (KBr 1%, cm^{-1}): υ = 3416,

2964, 2931, 2252, 1718, 1625, 1595, 1518, 1461, 1444, 1425, 1406, 1363, 1335, 1299, 1277, 1231, 1198, 1105, 1060, 1023, 988, 949, 911, 846, 821, 732, 665, 646, 601. HRMS (EI): calcd for $C_{19}H_{18}O_5$ [M^+]: 326.1154; found 326.1171.

5.2.1.4. (2-Methyl-6-oxo-6*H*-benzopyrano[6,7-*d*]oxazol-8-yl)methyl butyrate (11). From the reaction of **5** (0.029 g, 1.15 × 10^{-4} mol) in DMF (2 mL), potassium fluoride (0.020 g, 3.45 × 10^{-4} mol) and butyric acid (0.010 g, 1.15 × 10^{-4} mol), **11** was obtained as a yellow oily solid (0.032 g, 95%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.00 (t, *J* = 7.2 Hz, 3 H, CH_3), 1.74 (sext, *J* = 7.2 Hz, 2 H, CH_2), 2.43 (t, *J* = 7.2 Hz, 2 H, CH_2), 2.7 (s, 3 H, CH_3 Het), 5.36 (s, 2 H, CH_2 Het), 6.50 (s, 1 H, H-7), 7.50 (s, 1 H, H-4), 7.79 (s, 1 H, H-9) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.65 (CH_3), 14.62 (CH_3 Het), 18.34 (CH_2), 35.88 (CH_2), 61.18 (CH_2 Het), 99.58 (C-4), 112.13 (C-7), 113.44 (C-9), 114.36 (C-8a), 138.62 (C-9a), 149.55 (C-8), 151.58 (C-4a), 152.67 (C-3a), 160.37 (C-6), 166.02 (C-2), 172.71 (C=O) ppm. IR (KBr 1%, cm^{-1}): υ = 3077, 3048, 3010, 2928, 1743, 1722, 1642, 1606, 1578, 1477, 1437, 1430, 1394, 1382, 1354, 1283, 1273, 1253, 1218, 1149, 1131, 1037, 1016, 962, 915, 901, 879, 869, 814, 734, 699, 681, 609. HRMS (EI): calcd for $C_{16}H_{15}NO_5$ [M^+]: 301.0951; found 301.0953.

5.2.1.5. (8-Methyl-6-oxo-6*H*-benzopyrano[6,7-*d*]oxazol-2-yl)methyl butyrate (12). From the reaction of **6** (0.031 g, 1.05 × 10^{-4} mol) in DMF (2 mL), potassium fluoride (0.018 g, 3.15 × 10^{-4} mol) and butyric acid (0.010 g, 1.15 × 10^{-4} mol), **12** was obtained as a white solid (0.015 g, 47%). Mp = 118.1-119.3 °C. TLC (ethyl acetate/light petroleum, 3:7): R_f = 0.45. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.00 (t, *J* = 7.6 Hz, 3 H, CH_3), 1.74 (sext, *J* = 7.2 Hz, 2 H, CH_2), 2.48 (t, *J* = 7.6 Hz, 2 H, CH_2), 2.52 (d, *J* = 1.2 Hz, 3 H, CH_3 Het), 5.37 (s, 2 H, CH_2 Het), 6.34 (d, *J* = 1.2 Hz, 1 H, H-7), 7.52 (d, *J* = 0.4 Hz, 1 H, H-4), 7.96 (s, 1 H, H-9) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.58 (CH_3), 18.29 (CH_2), 19.21 (CH_3), 35.65 (CH_2), 57.75 (CH_2 Het), 99.64 (C-4), 114.27 (C-7), 115.63 (C-9), 117.89 (C-8a), 137.74 (C-9a), 152.0 (C-4a), 152.32 (C-8), 152.38 (C-3a), 160.40 (C-6), 162.52 (C-2), 172.68 (C=O) ppm IR (KBr 1%, cm^{-1}): υ = 3054, 2960, 2931, 2878, 1788, 1737, 1717, 1631, 1569, 1437, 1404, 1385, 1368, 1341, 1316, 1280, 1241, 1210, 1167, 1130, 1053, 1028, 950, 925, 908, 877, 858, 811, 782, 755, 742, 697, 665, 642. HRMS (EI): calcd for $C_{16}H_{15}NO_5$ [M^+]: 301.0951; found 301.0931.

5.2.2. Synthesis of (6-methoxy-2-oxo-2*H*-benzo[*h*]benzopyran-4-yl)methyl butyrate (10). To a solution of **4** (0.022 g, 8.83×10^{-5} mol) in dry DMF (2 mL) at 0 °C was added HOBt (0.024 g, 2.56×10^{-4} mol) followed by DCC (0.053 g, 2.57×10^{-4} mol) after stirring for 10 min. After 10 min, butyric acid (1.6×10^{-2} mL, 1.77×10^{-4} mol) was added and the reaction mixture was stirred at room temperature for 5 d. The solvent was evaporated and the crude residue dissolved in dichloromethane (10 mL). The organic phase was washed with sodium carbonate (3 × 10 mL) and dried with magnesium sulphate. Purification by chromatography in dichloromethane/methanol (100:1) gave **10** as a yellow solid (0.009 g, 30%). Mp = 124.6-125.3 °C. TLC (dichloromethane/methanol, 100:1): R_f = 0.88. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.02 (t, J = 7.2 Hz, 3 H, CH_3), 1.72-1.81 (m, 2 H, CH_2), 2.48 (t, J = 7.2 Hz, 2 H, CH_2), 4.04 (s, 3 H, OCH_3), 5.38 (d, J = 1.2 Hz, 2 H, CH_2 Het), 6.59 (s, 1 H, H-3), 6.65 (s, 1 H, H-5), 7.65-7,70 (m, 2 H, H-8 and H-9), 8.27-8.29, (m, 1 H, H-7), 8.51-8.54 (m, 1 H, H-10) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.68 (CH_3), 18.41 (CH_2), 35.98 (CH_2), 55.87 (OCH_3), 61.33 (CH_2 Het), 95.27 (C-5), 112.22 (C-4a), 112.47 (C-3), 122.24 (C-7), 122.42 (C-10), 124.01 (C-10a), 127.36 (C-6a), 127.94 (C-9), 128.43 (C-8), 145.72 (C-10b), 149.86 (C-4), 152.30 (C-6), 160.80 (C-2), 172.84 (C=O) ppm. IR (KBr 1%, cm^{-1}): υ = 2965, 2925, 2853, 1749, 1727, 1613, 1595, 1564, 1505, 1472, 1455, 1422, 1384, 1353, 1308, 1272, 1248, 1167, 1142, 1118, 1084, 1031, 990, 956, 858, 818, 778, 735, 666. HRMS (EI): calcd for $C_{19}H_{18}O_5$ [M^+]: 326.1154; found 326.1157.

5.3. Time-resolved fluorescence measurements

Time-resolved fluorescence measurements were performed using a HORIBA Scientific FluoroCube-01 equipped with DeltaDiode excitation sources (running either at 8 MHz or 10 MHz) and a TBX-07C detector. The excitation wavelengths employed were 295 nm (LED source) for **7** and **8**, 375 nm (laser source) for **9** and **10** and 317 nm (LED source) for **11** and **12**. Data were analysed using DAS6 software and the decays reconvoluted with the instrumental response and fitted to the sum of exponentials. The goodness of fit was judged in terms of a chi-squared value and weighted residuals. DAS, associating the spectra with a decay time, were obtained from the global analysis (using DAS6 software) of the TRES measurements, which involved the collection of

fluorescence decays for a fixed collection time over a selected wavelenght range (at 5 nm intervals).

5.4. Photolysis general

Photolyses were carried out using a Rayonet RPR-100 chamber reactor equipped with 10 lamps of 254, 300, 350 and 419 nm. HPLC analyses were performed using a Licrospher 100 RP18 (5 µm) column in a JASCO HPLC system composed by a PU-2080 pump and a UV-2070 detector with ChromNav software.

5.4.1. Photolysis procedure

A 1×10^{-4} M methanol/HEPES buffer (80:20) solution of **7-12** (5 mL) were placed in a quartz tube and irradiated in the reactor at the desired wavelength. HEPES buffer solution was prepared in distilled water with HEPES (10 mM), sodium chloride (120 mM), potassium chloride (3 mM), calcium chloride (1 mM) and magnesium chloride (1mM) and pH adjusted to 7.2. Aliquots of 100 µL were taken at regular intervals and analysed by RP-HPLC. The eluent was acetonitrile/water (3:1) at a flow rate of 0.8 (**7**-**9**,**12**) or 1.0 mL/min (**10**,**11**) previously filtered through a Millipore type HN 0.45 µm filter and degassed by ultrasound for 30 min. The chromatograms were traced by detecting UV absorption at the wavelength of maximum absorption for each conjugate (retention time: **7**, 7.4; **8**, 7.7; **9**, 6.6; **10**, 8.6; **11**, 6.4; **12**, 4.2 min).

6. Acknowledgements

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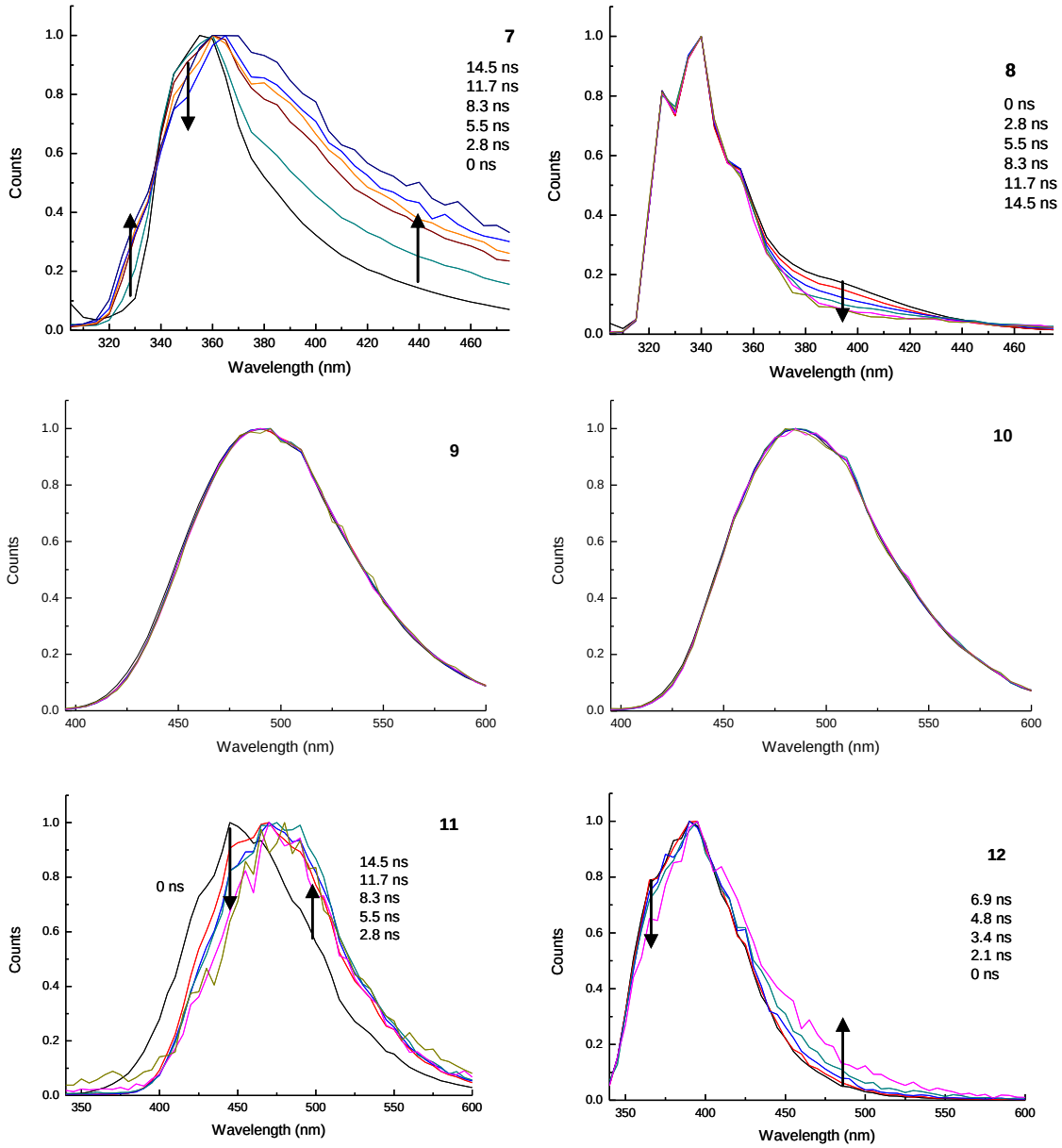
7. Supporting information

Time-resolved emission spectra (TRES) for **7** to **12** in methanol/HEPES buffer (80:20) solution. The excitation wavelengths were: 295 nm for **7** and **8**, 375 nm for **9** and **10**, 317 nm for **11** and **12**. The representative spectra are shown at times relative to the excitation pulse and consist of "time slices" of 0.68 ns and 1.39 ns, for **7**, **8**, **11**, **12** and **9**, **10** respectively. The change in spectral shape with time is indicated.



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2.4

Acridinyl methyl esters as photoactive precursors in the release of neurotransmitter amino acids

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Photochemical and Photobiological Sciences, **2013**, *12*, 339-347

INDEX

1. Abstract

An investigation of the use of an azaheterocycle, acridine, as an alternative photochemically removable protecting group for the carboxylic function of neurotransmitter amino acids was carried out. 9-Bromomethylacridine was used in the reaction with glycine, alanine, glutamic acid, β-alanine and γ-aminobutyric acid, to obtain model ester derivatives, which were irradiated at different wavelengths in a photochemical reactor. The process was followed by HPLC/UV, resulting in the release of the active molecule in short irradiation times. The results obtained using 419 nm irradiation show promise (35-98 min) for practical purposes. The compounds were further characterised *via* time-resolved fluorescence to elucidate their photophysical properties and determine the decay kinetics.

2. Introduction

In recent years, photolabile protecting groups have been the subject of interesting research which has resulted in the emergence of different structures with aromatic and/or heterocyclic skeletons associated with a diversity of applications, not only in the protection of functional groups for synthetic applications, but also as phototriggers for the study of numerous processes in biotechnology and cell biology.[1–3]

Phototriggers allow rapid release of bioactive molecules from their inactive caged precursors upon photolysis.[4–6] In several cases, the biological activity is blocked by the presence of a covalently linked fluorophore, that upon irradiation cleaves the fluorophore and restores function. The use of fluorescent photolabile protecting groups allows the visualization, quantification, and the follow-up of spatial distribution, localization, and depletion of the active compound through the monitoring of its fluorescent caged precursor using fluorescent techniques, in addition to the time and space controlled delivery of the target molecule.[7] Among these groups are pyrene,[8,9] phenanthrene,[8] anthracene,[10] perylene,[11] anthraquinone,[8] coumarin,[12–14] benzocoumarin,[15–17] thiobenzocoumarin,[18] quinolone,[14] and benzoxazole[19] moieties, which can be used in the protection of different functional groups.

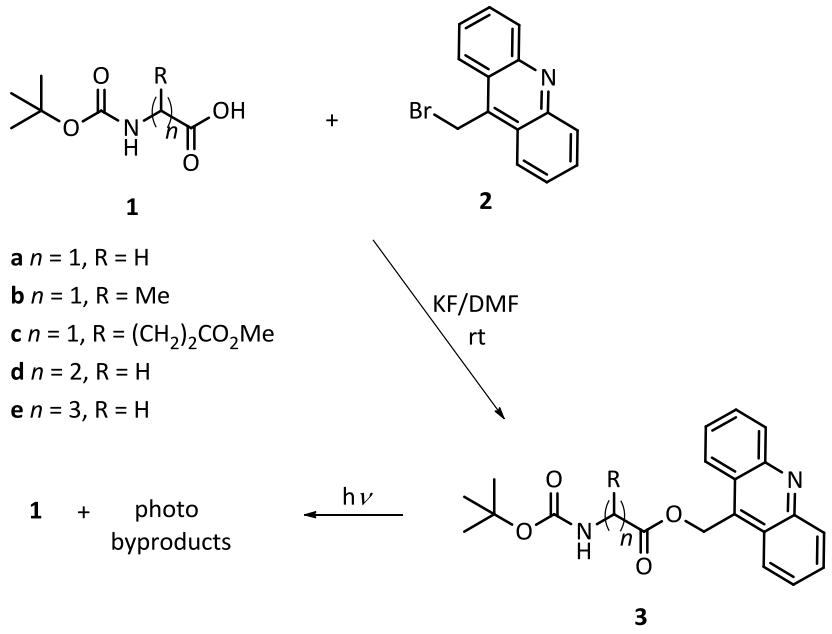
Acridine and its derivatives are planar tricyclic aromatic molecules which fluoresce at the shorter wavelength end of the visible region with a high fluorescence quantum yield.[20,21] Over time, these compounds have found application as derivatising agents, being recently reported, for example, as fluorescent chemosensors for metal ions.[22–26]

The photocontrolled release in time and space for the study of the kinetics of bioactive molecules is an established technique for the study of fast biological processes involving neurotransmitters, such as amino acids, nucleotides and physiological amines. As a result, there is a strong interest in the search for more efficient photolabile protecting groups with the ability to cleave under irradiation at higher wavelengths, in order to minimise cellular damage.

As a continuation of our research interests,[13–19] the synthesis of fluorescent acridinyl conjugates of glycine **3a**, alanine **3b**, glutamic acid **3c**, β-alanine **3d** and γ-aminobutyric acid (GABA) **3e** is now reported. The study of their photorelease under different conditions, in a photochemical reactor equipped with lamps of 254, 300, 350 and 419 nm, was carried out. Cleavage kinetic data was obtained by HPLC/UV monitoring and a time-resolved fluorescence study was performed.

3. Results and discussion

A series of *N*-(*tert*-butoxycarbonyl)-neurotransmitter amino acids **1** (glycine, alanine, glutamic acid, β-alanine and γ-aminobutyric acid) were reacted with 9-bromomethylacridine **2**[25] to afford the corresponding heterocyclic ester derivatives **3**, *via* potassium fluoride coupling in *N*,*N*-dimethylformamide, at room temperature (Scheme 1 and Table 1).[27] The acridin-9-yl methylene group will be designated in this report by a three-letter code (Acm) for simplicity of naming the various fluorescent derivatives, as indicated in Table 1.



Scheme 1. Synthesis of acridinyl methyl ester conjugates **3a**-**e**.

All new compounds were fully characterised by high resolution mass spectrometry, IR, ^{1}H and ^{13}C NMR spectroscopy. ^{1}H NMR spectra showed signals of the amino acid residues, such as the α-CH (δ = 4.26-4.43 ppm), side chain β-CH_2, β-CH_3 and γ-CH_2 (δ = 1.21-2.40 ppm), main chain α-, β-, and γ-CH_2 (δ = 1.75-3.40 ppm). The heterocycle methylene group was also visible for all conjugates (δ = 6.00-6.40 ppm). Also, the ^{13}C NMR spectra showed the corresponding signals for α-CH (δ = 44.93 or 49.26 ppm), side chain β-CH_2, β-CH_3 and γ-CH_2 (δ = 17.89-21.30 ppm), main chain α-, β-, and γ-CH_2 (δ = 25.28-42.35 ppm). The heterocycle methylene group was also visible for all conjugates (δ 49.67-58.32 ppm). The confirmation of the presence of the new ester bond linking the amino acid to the acridinyl moiety was also supported by ^{13}C NMR spectra signals at δ = 170.29-173.19 ppm and IR spectroscopy with a band between 1731 and 1738 cm^{-1}. The UV/vis absorption and emission spectra of degassed 10^{-5} M solutions in absolute ethanol and in a methanol/HEPES buffer (80:20) solution of ester conjugates **3a**-**e** were measured: absorption and emission maxima, molar absorption coefficients and relative fluorescence quantum yields are also reported (Table 1). Relative fluorescence quantum yields were calculated using 9,10-diphenylanthracene as a standard (Φ_F = 0.95 in ethanol).[28] For the Φ_F determination, the fluorescence standard was excited at the wavelengths of maximum absorption found for each one of the

compounds to be tested and in all fluorimetric measurements the absorbance of the solution did not exceed 0.1. For all compounds the maximum wavelength of absorption, due to the presence of the heterocyclic chromophore, showed no variation in both solvents (λ_{abs} at about 360 nm), and was not influenced by the amino acid residue. Derivatives **3a**-**e** displayed emission maxima between 411-435 nm in both solvents, with moderate Stokes's shifts ($\Delta\lambda$, 50-75 nm), and exhibited higher fluorescence quantum yields in a methanol/HEPES buffer solution ($0.11 < \Phi_F < 0.15$).

Table 1. Yields, UV/vis and fluorescence data for conjugates **3a**-**e** in absolute ethanol and methanol/HEPES buffer (80:20) solution.

	Compound	Yield (%)	EtOH					MeOH/HEPES (80:20)				
			λ_{abs}[a]	log ε	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]	λ_{abs}[a]	log ε	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]
3a	Boc-Gly-OAcm	69	360	3.90	435	0.09	75	362	3.48	423	0.12	61
3b	Boc-Ala-OAcm	93	361	3.93	411	0.11	50	360	3.69	412	0.15	52
3c	Boc-Glu-OAcm	19	361	4.04	433	0.08	72	362	3.57	433	0.11	71
3d	Boc-β-Ala-OAcm	72	361	3.91	412	0.11	51	361	3.79	417	0.14	56
3e	Boc-GABA-OAcm	49	361	3.97	424	0.04	63	360	3.94	424	0.11	64

[a] in nm.

In order to evaluate the applicability of the (acridin-9-yl)methyl unit as a potential photolabile protecting group for neurotransmitter amino acids, methanol/HEPES buffer (80:20) solutions of derivatives **3a**-**e** were irradiated at different wavelengths (254, 300, 350 and 419 nm) in a Rayonet RPR-100 reactor (Scheme 1, Table 2). The photolysis reaction was monitored by reverse phase HPLC with UV detection, peak areas of the starting material (*A*, average of three runs for each compound) revealed a gradual decrease with time. Plots of *A versus* irradiation time were obtained for each compound, at the considered wavelengths. The determined irradiation time represents the time necessary for the consumption of the starting materials until less than 5% of the initial area was detected (Table 2). For each compound and based on HPLC data, the plot of ln *A versus* irradiation time showed a linear correlation for the

disappearance of the starting material, which suggested a first order reaction, obtained by the linear least squares methodology for a straight line. The photochemical quantum yields (Φ_{Phot}) were calculated based on half-lives ($t_{1/2}$), molar extinction coefficients (ε) and the incident photon flux (I_0), which was determined by potassium ferrioxalate actinometry.[29] The photocleavage process was not as efficient as desirable (see Φ_{Phot} in Table 2), possibly by deactivation *via* fluorescence pathways that compete with the photochemical reaction, as well as the type of reactor used (open chamber reactor).

Table 2. Irradiation times (t_{irr}, in min), and photochemical quantum yields (Φ_{Phot}, × 10^{-3}) for the photolysis of conjugates **3a**-**e** at 300, 350 and 419 nm in methanol/HEPES buffer (80:20) solution.

Compound	300 nm		350 nm		419 nm	
	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}
3a	25	0.23	7	0.79	35	0.16
3b	18	0.33	10	0.61	81	0.07
3c	22	0.16	8	0.46	77	0.04
3d	22	0.27	10	0.62	98	0.06
3e	14	0.29	3	1.24	89	0.05

The release of *N*-*tert*-butyloxycarbonyl neurotransmitters was accomplished in practicable irradiation times in all the tested wavelengths, the fastest release occurring at 350 nm (3-10 min), as expected according to the maximum wavelengths of absorption. Concerning the irradiation at 300 nm it was observed that photolysis occurred between 14 and 25 min; although 254 nm is not the most suitable wavelength for biological applications, cleavage of the ester bond required 5-14 min.

Regarding the amino acid residue, it was found that its influence on the irradiation times was not marked, as these values were quite similar for all wavelengths of irradiation, but the release of GABA was slightly faster than the remaining amino acids (except at 419 nm, with glycine being faster).

Additionally to monitoring the photolysis process by HPLC/UV detection, the release of Boc-protected alanine, as the expected product of the photolysis of conjugate

3b, was also followed by ^{1}H NMR in a methanol-d_4/D_2O (80:20) solution (Figure 1).

Upon irradiation at 350 nm, the signals due to the alanine in the conjugate form disappeared progressively and were replaced by the corresponding set of signals of Boc-Ala-OH, with the α-CH and β-CH_3 appearing at δ = 4.20 and 1.45 ppm, respectively. The decrease of the benzylic-type CH_2 at position 9 of the acridine ring was visible at δ = 6.30 ppm, thus confirming the release of the amino acid, and accompanied by the appearance of a new signal at about 4.30 ppm relating to the Acm-OH. Moreover, signals due to byproducts related to the acridine ring were also detected in the ^{1}H NMR spectra at higher δ (Fig. 1).

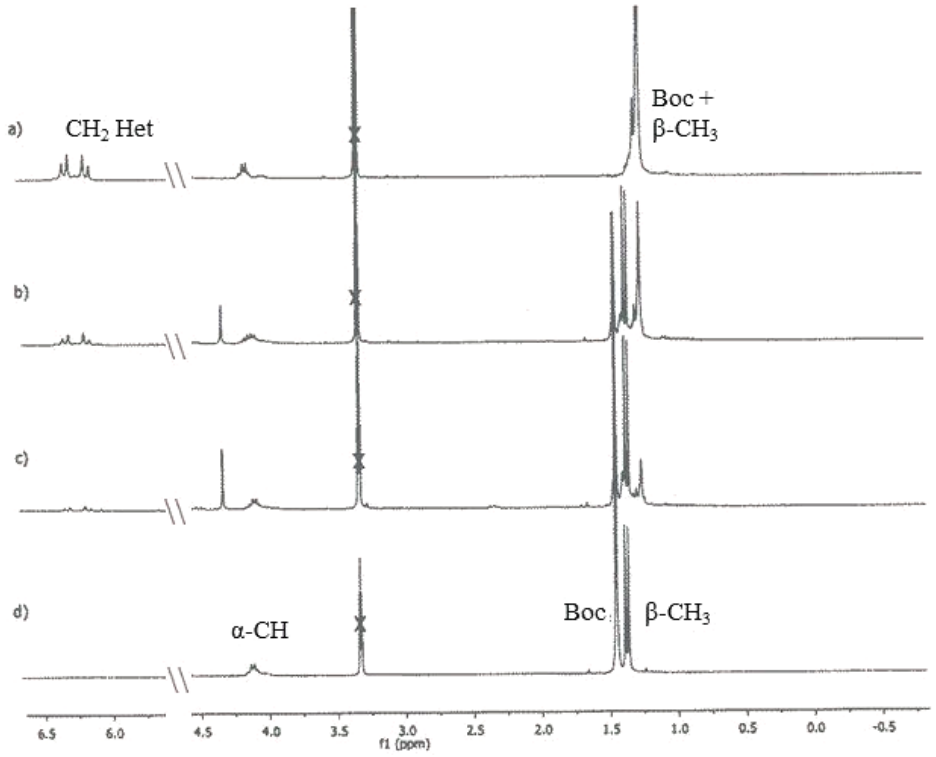


Figure 1. ^{1}H NMR spectra in methanol-d_4/D_2O (80:20) of the photolysis of conjugate **3b** (C = 1.1 × 10^{-4} M) at 350 nm: (a) before irradiation; (b) after irradiation for 20 min; (c) after irradiation for 40 min; (d) Boc-Ala-OH **1b**.

Bearing in mind the importance of the development of novel protecting groups cleavable with UV A or even visible radiation, the results indicated that the release of the amino acids at 419 nm from acridinyl methyl ester derivatives is practicable for organic synthesis and also for caging applications, considering the short irradiation times

(35-98 min), and the fact that it minimises the side reactions for the remaining functionalities of the molecule. In view of the current use of 9-bromomethylacridine as the HPLC derivatizing agent for fluorescent labelling of carboxylic acids,[25,26] the present work represents a novel application, indicative of its versatility and as a result this azaheterocycle can be regarded as an addition to the collection of photolabile protecting groups for the carboxylic acid function.

In order to contribute to the characterisation of these compounds and to elucidate the photophysics associated with the cleavage process observed in the photolysis study, time-resolved fluorescence measurements were performed. An excitation towards the longer wavelength part of the range used in the photolysis study of 375 nm was chosen. This wavelength may also be useful for future studies involving two-photon excitation, as it is obtainable using femtosecond laser systems. The fluorescence emission of acridine derivatives has, in the main, been reported to decay monoexponentially with the lifetime showing a dependency on solvent[20,30] and substituent groups.[31,32] In strong hydrogen bonding solvents, such as water, lifetimes over 10 ns have been reported.[20,21,30,33,34] The photocleavage is expected to proceed *via* the formation of an ion pair, which can undergo either a recombination to the initial ester or complete the cleavage with formation of photo- products (Scheme 1).[35] These processes should influence the fluorescence decay of the Acm moiety and as the different species can be spectrally different, the ability to obtain time-resolved emission spectra is a powerful technique by which to characterise and elucidate the processes occurring in this type of system.

As well as the conjugates, a model (acridin-9-yl)methyl alcohol (Acm-OH) was employed that should be indicative of the photocleaved product. Time-resolved measurements, with the decay monitored at 445 nm, were performed on this compound in different solvents to give an idea of the influence of solvent dielectric constant and hydrogen bonding. The decay kinetics were clearly non-exponential, unlike other studies on acridine derivatives.[30–32] The sum of three exponentials was required to provide a good fit to the data and is indicative of the more complex decay kinetics, even within our model for the photocleaved product. The outcome of the analysis is presented in Table 3. This shows that the presence of alcohols has a greater effect on the lifetime values than the dielectric constant, as seen by comparing the methanol and

acetonitrile data. Also, the influence is on the lifetime rather than the relative proportion of the fluorescing components. The presence of the longer-lived decay, ~10 ns, is in keeping with that reported for acridine in strongly hydrogen bonding solvents.[20] It should be noted that the average lifetime in methanol is only marginally longer than that reported for acridine.[30] From these results, it is clear that our substituent group, even in the model, is exerting an influence. As the objective is to harness the photophysics of the acridine moiety to the uncaging of the conjugate system, these data are indicative that this group expresses activity in the excited state, which is not unsurprising as this state has also been reported as a stronger proton acceptor with emission from both neutral and cationic forms possible.[36] Time-resolved fluorescence decays were obtained at 5 nm wavelength intervals over a range of the fluorescence emission for all of the compounds. The overall fluorescence decay in the methanol/HEPES solution (obtained for each dataset by summing the individual decays obtained at each wavelength) is shown normalised in Figure 2.

Table 3. Fluorescence decay parameters for Acm-OH in four solvents of different dielectric constant (α_i are the normalised pre-exponential factors and the average lifetime (τ_{ave}) is obtained from $\tau_{ave} = \Sigma \alpha_i \tau_i$).

Solvent (ε)	τ_1 (ps)	τ_2 (ns)	τ_3 (ns)	α_1	α_2	α_3	τ_{ave} (ns)	χ^2
Chloroform (5)	150 ± 1	1.51 ± 0.12	5.86 ± 0.01	0.72	0.08	0.20	1.39	1.12
Acetonitrile (38)	97 ± 2	2.85 ± 0.90	5.39 ± 0.09	0.65	0.09	0.26	1.74	1.05
2-Propanol (18)	401 ± 8	2.23 ± 0.45	10.14 ± 0.03	0.70	0.04	0.26	3.04	1.06
Methanol (34)	483 ± 9	1.57 ± 0.08	10.33 ± 0.01	0.68	0.10	0.23	2.81	1.06

In all cases the sum of three exponentials was used to fit the data, returning a longer-lived decay of ~12.5 ns, a short-lived subnanosecond fluorescence and a decay component in between the order of a couple of nanoseconds, which is indicative of the presence of different excited state species. Qualitatively it can be seen that the decay curves for **3d** and **3e** appear to be similar, with a higher proportion of the longer-lived fluorescence, while **3a** exhibits relatively more of the short-lived fluorescence. It is interesting to note that here is an apparent correlation of the proportion of longest-lived

fluorescence to the irradiation time at 419 nm given in Table 2. From these data it appears that processes occurring after excitation are comparable. The main difference is in the relative ratio between these processes. It should also be remembered that the model Acm-OH also exhibits a three exponential decay, thus the influence of the group coupled to the oxygen appears to be a dominant factor.

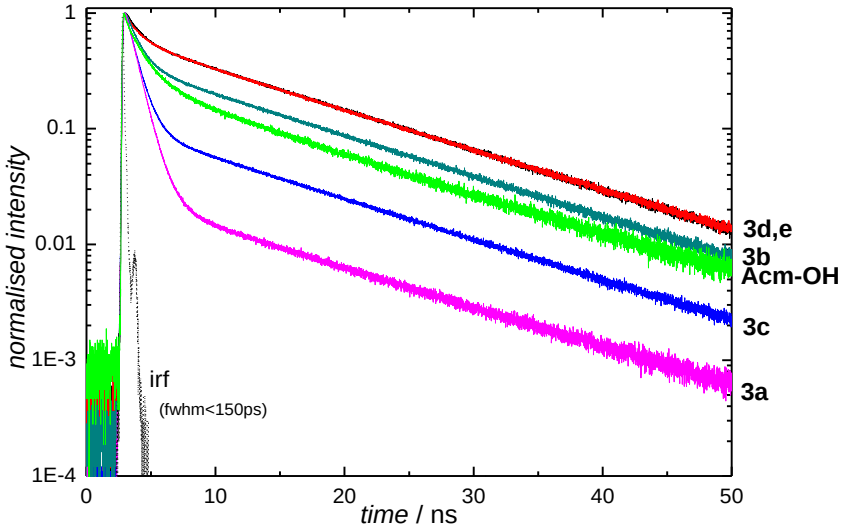


Figure 2. Time-resolved fluorescence decays for Acm-OH and conjugates **3a**-**e**, showing the overall decay (summed over the whole wavelength range) and the instrumental response function (IRF)

A preliminary molecular modelling study was performed, in which the difference between the HOMO and LUMO (to give an indication of the orbitals involved) was calculated for PM3 geometry optimised structures, see Figure 3. This modelling indicates that in all compounds, with the exception of **3d** and **3e**, the oxygen in the linkage between the acridine and the conjugate is involved. This supports the difference in the decay behaviour of **3d** and **3e** in terms of less of the shorter-lived component in comparison to the other compounds.

From the global analysis of each dataset it was possible to obtain the decay associated spectra (DAS), shown in Figure 4, which can be used to produce spectra linked to the decay times. This can be helpful in identifying the species present, especially related to charge transfer that would be expected as part of the uncaging

process. It can clearly be seen that the spectrum associated with the longer-lived fluorescence, as commonly seen in acridine fluorescence, has structure with peak emissions at approximately 415 nm and 435 nm. The spectrum associated with the shortest-lived fluorescence has a peak emission at ~425 nm (see Supporting information for normalised spectra). The spectrum associated with the middle decay time is not as well defined, which can relate to the fact that generally its contribution to the overall fluorescence emission is small and may not be so well resolved in the analysis. However, from this figure it seems that common species are present for all of the Acm conjugates. It is also clear from Figure 4 that for the conjugates **3b**, **3d** and **3e** the major contribution to the overall fluorescence emission is from the longest-lived species, in keeping with the model, Acm-OH. However, its concentration (as indicated by the normalised pre-exponential factor) is inferior to that of the shortest-lived component, which is the dominant process.

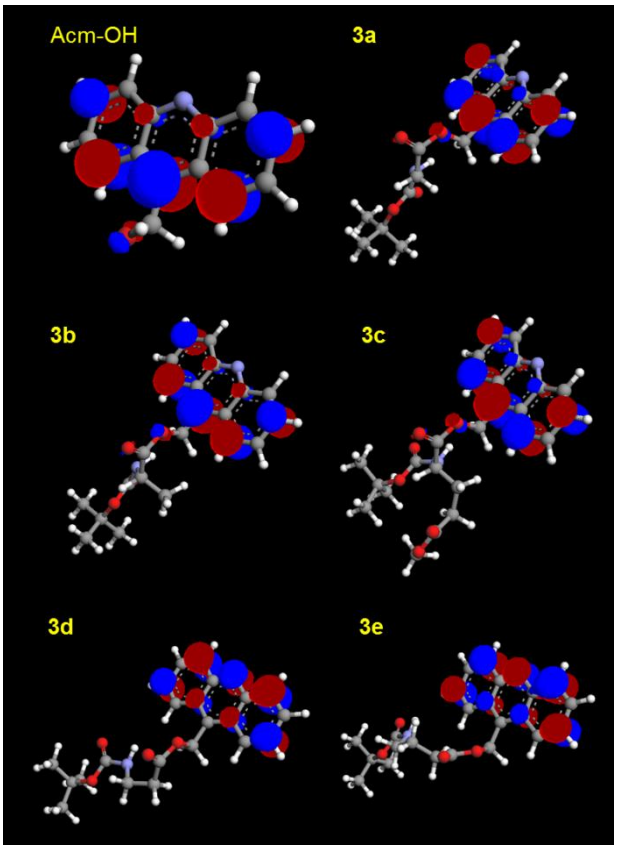


Figure 3. Differences in electron density between the calculated HOMO and LUMO for Acm-OH and conjugates **3a**-**e** (red lobes - negative, blue lobes - positive).

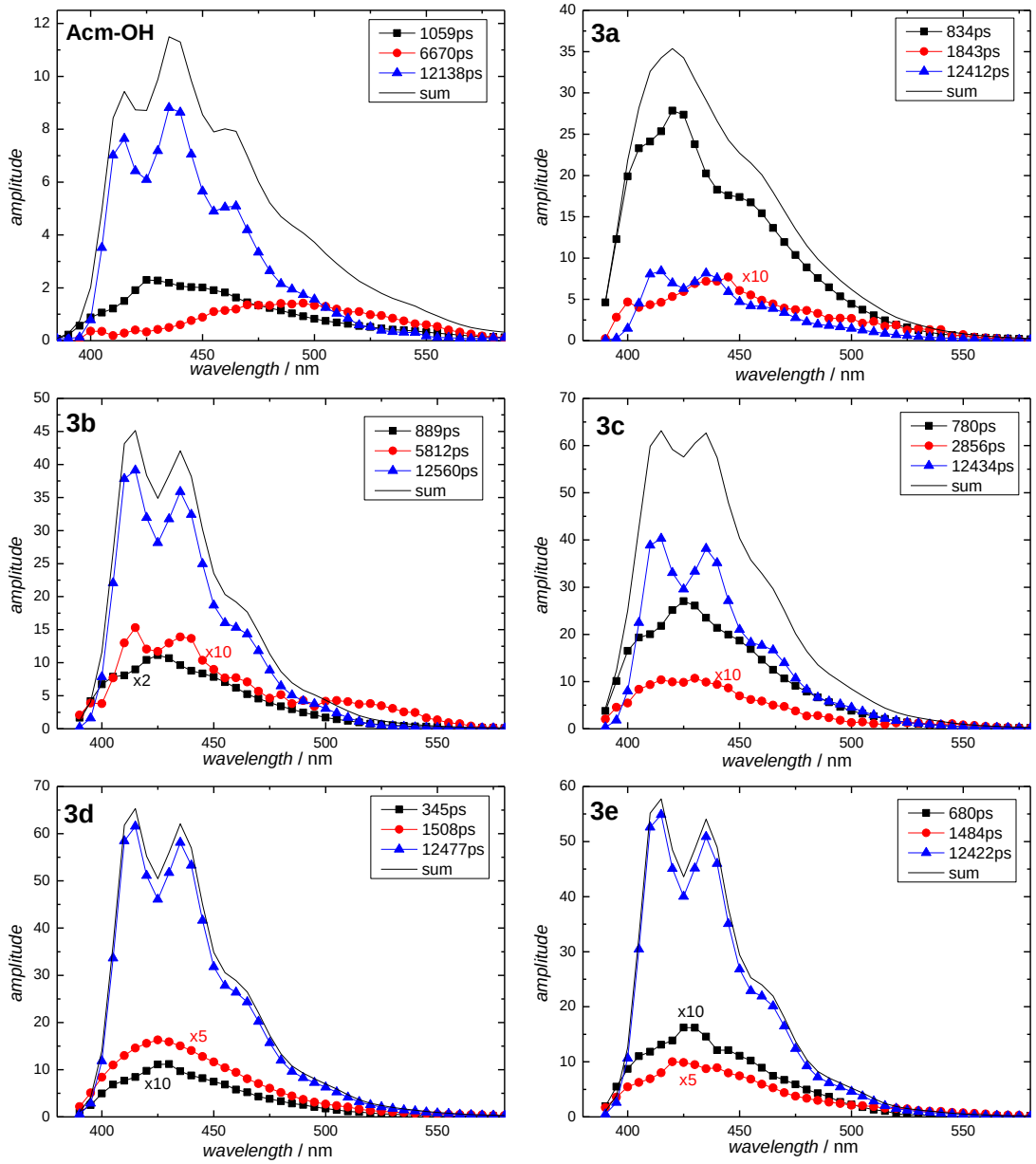


Figure 4. Decay associated spectra for Acm-OH and conjugates **3a**-**e**. The spectra obtained for each decay time (given in ps) are shown along with the sum, which is indicative of the steady state spectrum.

The structured spectrum and the lifetime of the longer-lived component correspond well to that of acridine in a strong hydrogen bonding environment.[37] This can relate to the methanol/HEPES solvent mixture used. The lack of structure in the spectra of the shortest-lived decay can be indicative of charge transfer.[37] The spectrum

associated with the middle lifetime is, in the main, lacking in structure, pointing to the fact that it is also from an intermediate form. The fact that different species can be elucidated using this technique shows that complementary information concerning the photocleavage process can be obtained to support the photolysis study. The fact that the decay of Acm-OH is also complex means that a full characterisation of these conjugates, i.e. determination of rate constants, is not straightforward. However, the spectrum associated with the intermediate decay time for Acm-OH is notably red-shifted in relation to the other compounds, which may relate to its lack of conjugation. Although the peak emission of this spectrum is similar to that of the shortest-lived decay, a longer wavelength shoulder is also present and clearly seen for **3a** and **3b** (see Supporting information). A broadening towards longer wavelengths is present for all compounds.

Considering the two shorter-lived components, which may be more indicative for charge transfer processes involved in the photocleavage process, the values obtained for the model compound (Acm-OH) are significantly longer than those for the conjugated compounds. It is possible to estimate relative quenching rates for these lifetimes for the compounds (using Acm-OH as a reference) and this is shown in Figure 5.

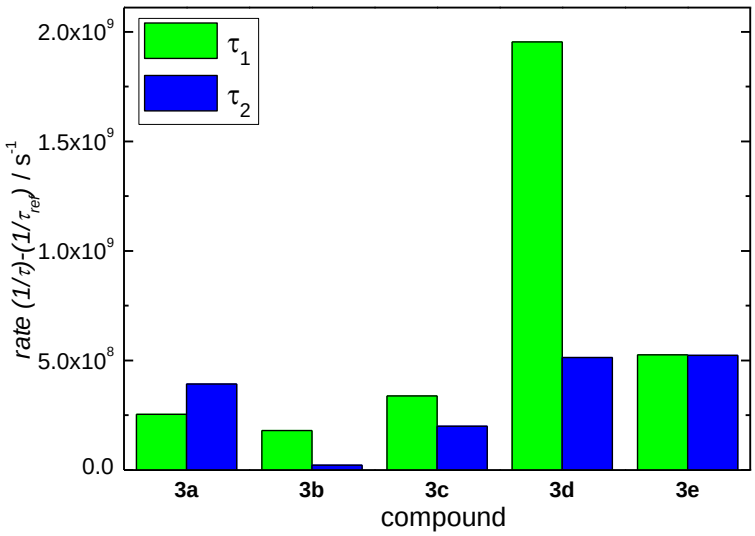


Figure 5. Rates of quenching for the shorter-lived decays for conjugates **3a**-**e** (τ_{ref} from model compound Acm-OH).

From this, it is clear that the reduction in lifetime is greatest for the GABA and alanine containing compounds (**3d**, **3e**), although the amounts of these emissions

contribute less to the overall fluorescence. The closest resemblance to the model decay times is present in compound **3b**, which also expresses some spectral similarities to Acm-OH. It is clear that the conjugate to which the acridine group is attached is influencing its non-radiative properties.

Thus, it is apparent from this study that the use of this form of fluorescence measurement can help elucidate the presence of different emitting species, although a complete characterisation is hampered by the complex decay of the model for the photocleaved fluorophore. It can be seen that an emission from the acridine moiety in a hydrogen bonded environment is present and that the use of decay associated spectra can elucidate the presence of intermediate species. Thus, it should be possible, by comparison to the model, in terms of both spectral shape and decay time to gain an insight into the photophysics of this class of molecule with their use as photocleavable labels in mind.

4. Conclusions

Novel C-protected neurotransmitter amino acids bearing the acridinyl methyl ester were obtained by potassium fluoride mediated coupling between 9-bromomethylacridine and *N*-*tert*-butyloxycarbonylated glycine, alanine, glutamic acid, β-alanine and γ-aminobutyric acid. The photorelease of these amino acids from the corresponding fluorescent derivatives in methanol/HEPES buffer (80:20) solutions was achieved by irradiation, with the best results obtained at 350 nm (3-10 min). However, the results obtained using 419 nm irradiation show promise (35-98 min) for practical purposes. Time-resolved fluorescence, enabling the calculation of decay associated spectra, showed the presence of the different expected species, in keeping with the results from longer wavelength irradiation.

Given these findings, the acridinyl methyl ester can be considered a suitable option for the photochemical release of functional molecules bearing a carboxylic acid group, for organic synthesis and eventually in caging applications.

5. Experimental section

5.1. General

All melting points were measured on a Stuart SMP3 melting point apparatus. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230-240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer. UV/vis absorption spectra (200-700 nm) were obtained using a Shimadzu UV/2501PC spectrophotometer. NMR spectra were obtained on a Varian Unity Plus Spectrometer at an operating frequency of 300 MHz for ^{1}H and 75.4 MHz for ^{13}C or a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C using the solvent peak as an internal reference at 25 °C. All chemical shifts are given in ppm using δ_H Me_4Si = 0 ppm as a reference and *J* values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. Mass spectrometry analyses were performed at the "C.A.C.T.I. – Unidad de Espectrometria de Masas", at University of Vigo, Spain. Fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer. All reagents were used as received.

5.2. General procedure for the synthesis of compounds 3a-e

9-Bromomethylacridine **2** (1 equiv.) was dissolved in dry DMF (2 mL), potassium fluoride (3 equiv.) and the corresponding *N-tert*-butyloxycarbonyl amino acid (1.1 equiv.) were added. The reaction mixture was stirred at room temperature from 10 to 18 h. Potassium fluoride was filtered and the solvent was removed by rotary evaporation under reduced pressure, and the crude residue was purified by column chromatography using mixtures of dichloromethane and light petroleum of increasing polarity as the eluent.

5.2.1. *N*-(*tert*-Butyloxycarbonyl)-glycine (acridin-9-yl)methyl ester, Boc-Gly-OAcm (3a). From the reaction of compound **2** (0.064 g, 2.4 × 10^{-4} mol), in DMF (2 mL), potassium fluoride (0.041 g, 7.08 × 10^{-3} mol) and *N*-*tert*-butyloxycarbonylglycine **1a** (0.045 g, 2.6 × 10^{-4} mol), compound **3a** was obtained as a pale brown solid (0.059 g, 69%). Mp = 160.4-162.8 °C. TLC (ethyl acetate/*n*-hexane, 3:7): R_f = 0.74. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H =1.41 (s, 9 H, $C(CH_3)_3$), 3.93 (d, *J* = 5.6Hz, 2 H, CH_2 Gly), 5.02 (br s, 1 H, NH Gly), 6.21 (s, 2 H, CH_2 Acm), 7.66 (dt, *J* = 7.2 and 0.8 Hz, 2 H, H-2 and H-7), 7.84 (t, *J* = 7.8 and 1.2 Hz, 2 H, H-3 and H-6), 8.30-8.40 (m, 4 H, H-1, H-4, H-5 and H-8) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 28.32 ($C(CH_3)_3$), 42.35 (CH_2 Gly), 58.19 (CH_2 Acm), 80.20 ($C(CH_3)_3$), 124.00 (C-1 and C-8), 125.32 (C-8a and C-9a), 127.13 (C-2 and C-7), 129.14 (C-4 and C-5), 130.80 (C-3 and C-6), 147.58 (C-4a and C-10a), 155.67 (CONH), 170.29 (CO_2CH_2) ppm. IR (KBr 1%, cm^{-1}): υ = 3332, 2979, 2934, 1758, 1738, 1712, 1660, 1602, 1573, 1520, 1451, 1392, 1367, 1318, 1281, 1250, 1162, 1104, 1056, 967, 862, 845, 751, 703, 666. HRMS (ESI): calcd for $C_{21}H_{24}N_2O_4$ [M^++ H]: 367.16589; found 367.16592.

5.2.2. *N*-(*tert*-Butyloxycarbonyl)-L-alanine (acridin-9-yl)methyl ester, Boc-Ala-OAcm (3b). From the reaction of compound **2** (0.116 g, 4.26 × 10^{-4} mol), in DMF (2 mL), potassium fluoride (0.074 g, 1.28 × 10^{-3} mol) and *N*-*tert*-butyloxycarbonyl)-L-alanine **1b** (0.089 g, 4.69 × 10^{-4} mol), compound **3b** was obtained as a pale yellow solid (0.150 g, 93%). Mp = 160.1-162.5 °C. TLC (ethyl acetate/*n*-hexane, 3:7): R_f = 0.81. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.27 (d, *J* = 7.2 Hz, 3 H, β-CH_3 Ala), 1.37 (s, 9 H, $C(CH_3)_3$), 4.29-4.40 (m, 1 H, α-CH Ala), 5.05 (br s, 1 H, NH Ala), 6.00-6.30 (m, 2 H, CH_2 Acm), 7.57-7.63 (m, 2 H, H-2 and H-7), 7.75-7.80 (m, 2 H, H-3 and H-6), 8.25 (d, *J* = 8.8 Hz, 2 H, H-4 and H-5), 7.29 (d, *J* = 8.8 Hz, 2 H, H-1 and H-8). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 18.31 (β-CH_3 Val), 28.17 ($C(CH_3)_3$), 49.26 (α-CH Ala), 58.32 (CH_2 Acm), 79.85 ($C(CH_3)_3$), 123.80 (C-8 and C-9), 125.21 (C-8a and C-9a), 126.73 (C-2 and C-7), 129.88 (C-3 and C-6), 130.28 (C-4 and C-5), 136.29 (C-9), 148.68 (C-4a and C-10a), 155.00 (CONH), 173.19 (CO_2CH_2). IR (KBr 1%, cm^{-1}): υ = 3349, 2980, 2934, 1731, 1681, 1629, 1608, 1557, 1520, 1418, 1392, 1367, 1327, 1252, 1218, 1163, 1110, 1069, 1021, 970, 864, 846, 801, 784, 751, 737, 700. HRMS (ESI): calcd for $C_{22}H_{25}N_2O_4$ [M^++ H]: 381.18088; found 381.18086.

5.2.3. 1-(Acridin-9-ylmethyl) 5-methyl 2-((*tert*-butyloxycarbonyl)-amino)pentanedioate, Boc-Glu(OMe)-OAcm (3c). From the reaction of compound **2** (0.062 g, 2.3×10^{-4} mol), in DMF (2 mL), potassium fluoride (0.038 g, 6.5×10^{-4} mol) and 2-((*tert*-butoxycarbonyl)amino)-5-methoxy-5-oxopentanoic acid **1c** (0.063 g, 2.4×10^{-4} mol), compound **3c** was obtained as a pale yellow orange solid (0.020 g, 19%). Mp = 157.5-160.2 °C. TLC (ethyl acetate/*n*-hexane, 3:7): R_f = 0.53. ^{1}H NMR (CD_3OD, 300 MHz): δ_H = 1.30 (s, 9 H, $C(CH_3)_3$), 1.79-2.11 (2 × m, 2 H, β-CH_2 Glu), 2.23-2.40 (m, 2 H, γ-CH_2 Glu), 3.57 (s, 3 H, OCH_3), 4.10–4.24 (m, 1 H, α-CH Glu), 6.16-6.40 (m, 2 H, CH_2 Acm), 7.71 (dt, *J* = 8.0 and 0.9 Hz, 2 H, H-2 and H-7), 7.89 (dt, *J* = 8.1 and 1.2 Hz, 2 H, H-3 and H-6), 8.20 (d, *J* = 8.7 Hz, 2 H, H-4 and H-5), 8.49 (d, *J* = 9.0 Hz, 2 H, H-1 and H-8) ppm. ^{13}C NMR (CD_3OD, 75.4 MHz): δ_H = 17.89 (β-CH_2 Glu), 19.04 ($C(CH_3)_3$), 21.30 (γ-CH_2 Glu), 42.60 (OCH_3), 44.93 (α-CH Glu), 49.67 (CH_2 Acm), 71.10 ($C(CH_3)_3$), 116.30 (C-1 and C-8), 117.18 (C-8a and C-9a), 118.54 (C-2 and C-7), 120.24 (C-4 and C-5), 122.46 (C-3 and C-6), 130.60 (C-4a and C-10b), 139.91 (C-9), 163.94 (CONH), 165.14 (CO_2CH_2), 167.01 (CO_2CH_3) ppm. IR (KBr 1%, cm^{-1}): υ = 3345, 2977, 2931, 2855, 1738, 1715, 1659, 1603, 1574, 1519, 1451, 1392, 1368, 1320, 1249, 1203, 1164, 1051, 966, 845, 750. HRMS (ESI): calcd for $C_{25}H_{29}N_2O_6$ [M^+ + H]: 453.20201; found 453.20203.

5.2.4. *N*-(*tert*-Butyloxycarbonyl)-β-alanine (acridin-9-yl) methylester, Boc-β-Ala-OAcm (3d). From the reaction of compound **2** (0.062 g, 2.3×10^{-4} mol), in DMF (2 mL), potassium fluoride (0.040 g, 6.9×10^{-4} mol) and *N-(tert*-butyloxycarbonyl)-β-alanine **1d** (0.051 g, 2.5×10^{-4} mol), compound **3d** was obtained as a pale brown solid (0.063 g, 72%). Mp = 160.3-162.6 °C. TLC (ethyl acetate/*n*-hexane, 3:7): R_f = 0.83. ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H = 1.39 (s, 9 H, $C(CH_3)_3$), 2.55 (t, *J* = 6.3 Hz, 2 H, α-CH_2 β-Ala), 3.30-3.40 (m, 2 H, β-CH_2 β-Ala), 4.98 (br s, 1 H, NH), 6.12 (s, 2 H, CH_2 Acm), 7.62 (dt, *J* = 7.65 and 1.2 Hz, 2 H, H-2 and H-7), 7.79 (dt, *J* = 7.4 and 1.2 Hz, 2 H, H-3 and H-6), 8.27 (dt, *J* = 8.7 Hz, 2 H, H-4 and H-5), 8.32 (dt, *J* = 8.7 Hz, 2 H, H-1 and H-8) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ_C = 28.28 ($C(CH_3)_3$), 34.52 (α-CH_2 β-Ala), 36.02 (β-CH_2 β-Ala), 57.72 (CH_2 Acm), 79.38 ($C(CH_3)_3$), 123.85 (C-1 and C-8), 125.22 (C-8a and C-9a), 126.74 (C-2 and C-7), 129.93 (C-3 and C-6), 130.26 (C-4 and C-5), 136.66 (C-9), 148.67 (C-4a and C-10a), 155.66 (CONH), 172.14 (CO_2CH_2) ppm. IR (KBr 1%, cm^{-1}): υ = 3353, 3052, 2978, 2933, 1731, 1711, 1630,

1603, 1521, 1458, 1443, 1392, 1367, 1250, 1166, 1066, 974, 823, 755, 737, 666. HRMS (ESI): calcd for $C_{22}H_{25}N_2O_4$ [M^+ + H]: 381.18088; found 381.18086.

5.2.5. Acridin-9-ylmethyl 4-((*tert*-butyloxycarbonyl)amino)butanoate, Boc-GABA-OAcm (3e). From the reaction of compound **2** (0.060 g, 2.2 × 10^{-4} mol), in DMF (2 mL), potassium fluoride (0.038 g, 6.6 × 10^{-4} mol) and 4-((*tert*-butoxycarbonyl)amino)butanoic acid **1e** (0.049 g, 2.4 × 10^{-4} mol), compound **3e** was obtained as a white solid (0.049 g, 49%). Mp = 160.4-163.1 °C. TLC (ethyl acetate/*n*-hexane, 3:7): R_f = 0.85. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.41 (s, 9 H, $C(CH_3)_3$), 1.75-1.88 (m, 2 H, β-CH_2 GABA), 2.39 (t, *J* = 7.2 Hz, 2 H, α-CH_2 GABA), 3.10-3.20 (m, 2 H, γ-CH_2 GABA), 4.55 (br s, 1 H, NH GABA), 6.15 (s, 2 H, CH_2 Acm), 7.65 (dt, *J* = 7.6 and 1.2 Hz, 2 H, H-2 and H-7), 7.83 (dt, *J* = 8.2 and 1.2Hz, 2 H, H-3 and H-6), 8.32 (d, *J* = 8.4 Hz, 2 H, H-4 and H-5), 8.36 (d, *J* = 8.8 Hz, 2 H, H-1 and H-8) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_H = 25.28 (β-CH_2 GABA), 28.35 ($C(CH_3)_3$), 31.27 (α-CH_2 GABA), 39.71 (γ-CH_2 GABA), 57.59 (CH_2 Acm), 79.29 ($C(CH_3)_3$), 124.06 (C-1 and C-8), 125.35 (C-8a and C-9a), 126.90 (C-2 and C-7), 129.83 (C-4 and C-5), 130.37 (C-3 and C-6), 137.94 (C-9), 148.20 (C-4a and C-10a), 155.89 (CONH), 172.94 (CO_2CH_2) ppm. IR (KBr 1%, cm^{-1}): υ = 3371, 2977, 1736, 1682, 1628, 1611, 1557, 1519, 1417, 1392, 1366, 1284, 1248, 1163, 1091, 1005, 957, 866, 821, 782, 751, 637. HRMS (ESI): calcd for $C_{23}H_{27}N_2O_4$ [M^+ + H]: 395.19653; found 395.19652.

5.3. Time-resolved fluorescence measurements

Time-resolved fluorescence measurements were performed using a HORIBA Scientific FluoroCube-01 equipped with a DeltaDiode laser excitation source,[38] emitting at 375 nm (running at 10 MHz), and a TBX-850 detector. Data were acquired by stepping the emission monochromator through 5 nm increments, over the range 390 to 580 nm, and measuring the decay for a fixed time period. The instrumental response (full width at half maximum < 150 ps) was also measured. This resulted in the acquisition of 31 time-resolved decays and the instrumental response in each dataset analysis was performed using DAS6 software and the decays reconvoluted with the instrumental response and fitted globally (producing common decay times, τ_i) to the sum of

exponentials (equation (1)) for each dataset.

$$I(t) = \sum_{i=1}^{n} \alpha_i exp^{-t/\tau_i} \qquad (1)$$

The goodness of fit was judged in terms of a chi-squared value and weighted residuals. The software also allowed for the summing of all the decay data to produce an overall decay, fitting to which assisted in determining the number of decay times to be included in the analysis. Decay associated spectra (DAS), allowing the spectra to be obtained and related to decay times, were recovered from the global analysis. The pre-exponential values for the individual decays were weighted by the appropriate lifetime value and plotted against wavelength. The sum of the individual DAS for each data was compared to the steady state spectra, and in all cases agreement was seen.

5.4. Molecular modeling

Semi-empirical quantum chemical calculations were performed using ArgusLab 4.0.1 from Mark Thompson, Plenaria Software LLC, Seattle. Structure geometry was optimised using a PM3 model and the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) determined. The difference between them was calculated within the software.

5.5. Photolysis general

Photolysis was carried out using a Rayonet RPR-100 chamber reactor equipped with 10 lamps of 254, 300, 350 and 419 ± 10 nm. HPLC analyses were performed using a Licrospher 100 RP18 (5 µm) column in a JASCO HPLC system composed of a PU-2080 pump and a UV-2070 detector with ChromNav software.

5.5.1. Photolysis procedure

A 1×10^{-4} M methanol/HEPES buffer (80:20) solution of compounds **3a**-**e** (5 mL) was placed in a quartz tube and irradiated in the reactor at the desired wavelength. The

HEPES buffer solution was prepared in distilled water with HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (10 mM), sodium chloride (120 mM), potassium chloride (3 mM), calcium chloride (1 mM) and magnesium chloride (1 mM) and pH adjusted to 7.2 with aqueous 1 M sodium hydroxide solution. Aliquots of 100 µL were taken at regular intervals and analysed by RP-HPLC. The eluent was acetonitrile/water, 3:1, at a flow rate of 1.0 mL min^{-1}, previously filtered through a Millipore, type HN 0.45 µm filter and degassed by ultra-sound for 30 min. The chromatograms were traced by detecting UV absorption at the wavelength of maximum absorption for each compound (retention time: **3a**, 4.3; **3b**, 5.0; **3c**, 4.5; **3d**, 4.9 and **3e**, 5.1 min).

6. Acknowledgements

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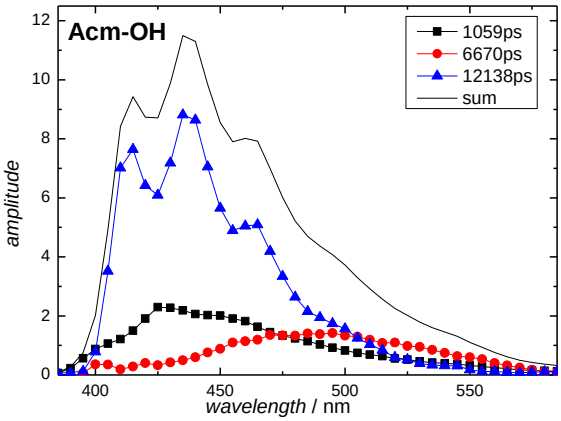
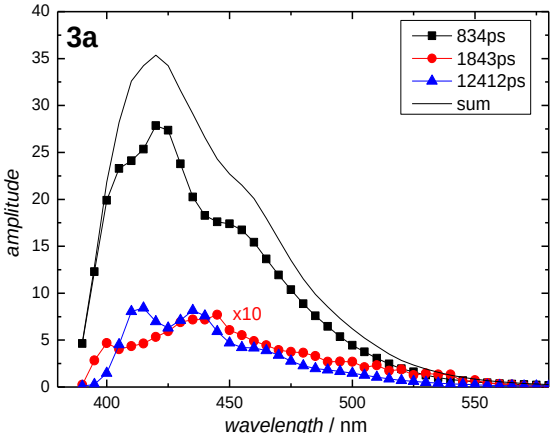
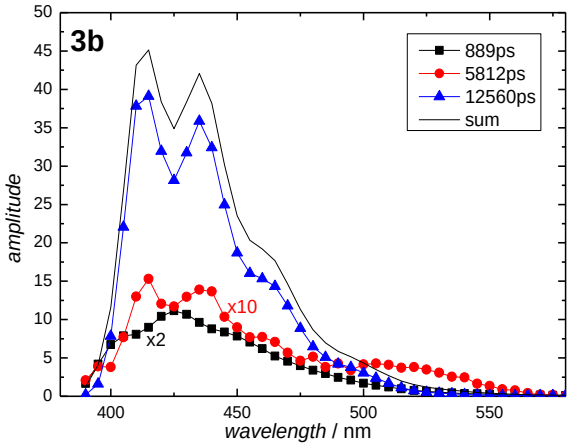
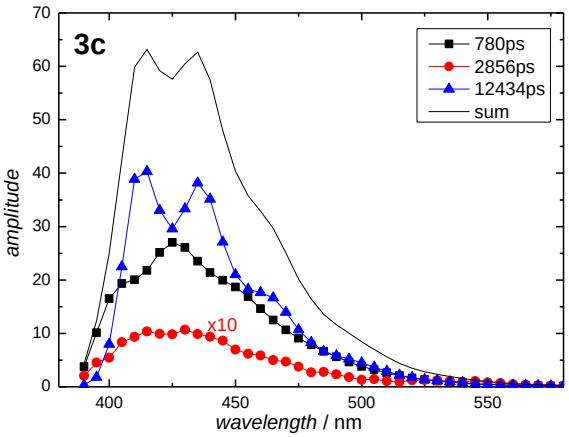
7. Supporting information

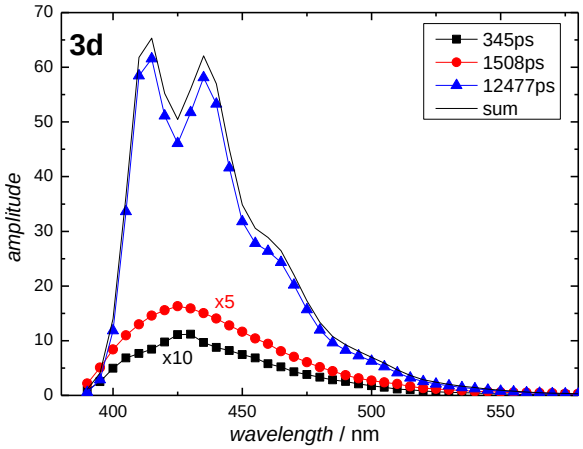
Normalised decay associated spectra for Acm-OH and conjugates **3a**-**e**.

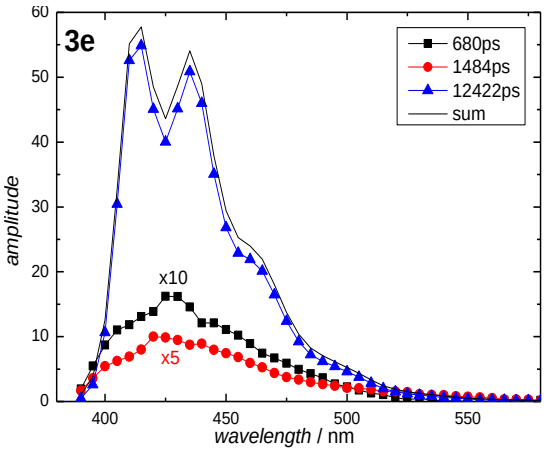












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2.5

Photoinduced release of neurotransmitter amino acids from novel coumarin fused julolidine ester cages

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INDEX

1. Abstract

The photoinduced release of several neurotransmitter amino acids (glycine, alanine, glutamic acid, β-alanine and γ-aminobutyric acid) was accomplished from ester cages based on a new photoremovable protecting group consisting of a coumarin built on the julolidine nucleus, namely a (11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl)methyl moiety. Photolysis and steady-state sensitization studies revealed that release of the active molecule occurred in short irradiation times at long wavelengths, with a very promising performance at 419 nm. Given the interest in the development of novel protecting groups cleavable with UV A or even visible radiation, it was found that a structural modification in the coumarin ring by assembly of a fused julolidine lead to a promising photolabile protecting group for organic synthesis and also for bioapplications.

2. Introduction

Amino acids play important roles in neuronal communication in the central nervous system (CNS), with glycine (Gly) and γ-aminobutyric acid (GABA) acting as the main inhibitory transmitters within the mammalian CNS.[1,2] β-Alanine (β-Ala) is the only naturally occurring β-amino acid and acts as a physiological transmitter, while alanine (Ala) is an inhibitory neurotransmitter and an agonist of ionotropic glycinergic and GABA receptors.[3,4]

There has been growing interest in photoremovable protecting groups (PPGs), known as phototriggers or cages in biochemistry, because they allow controlled spatial and temporal release, based on sensitivity to light, of numerous chemically and biologically relevant entities. Examples are calcium ions,[5,6] amino acids,[7] nucleotides,[8,9] peptides,[10] proteins,[11] neurotransmitters,[12,13] and other cell-signalling molecules.[14] Their applications include the caging of several functional groups (e.g. phosphates,[9] thiols,[15] amines,[9] alcohols,[16,17] and carboxylic acids[17]) and it is possible to find photolabile protecting groups with a large variety of chemical structures, which have been developed to fulfil the requirements of increasingly ambitious applications.[18] To be suitable for biological purposes, PPGs are required to exhibit large molar extinction

coefficients above 350 nm and high photolysis efficiency. Coumarin phototriggers possess these desirable properties, which combined with improved stability and fluorescence properties provide a mean for useful monitoring of the reaction course. These facts have attracted researcher's interest in the development of this promising class of PPGs.[19,20] Furthermore, efforts are being made to tailor caging groups to shift absorption into the visible region, since only a few examples have been reported in the literature.[21-24]

Heteropolycycles based on julolidine are extremely valuable and are applied in industry and also in biological systems, such as photoconductive materials,[25] nonlinear optical materials,[26,27] highly fluorescent molecular rotors,[28,29] chemiluminescent dyes,[30,31] chromogenic substrates in analytical redox reactions,[32] potential antidepressants and tranquilizers.[33] Considering the importance of the julolidine structural motif, the synthesis of new structurally diverse julolidine derivatives that can benefit from its properties, is challenging[27,34,35] and recently the first synthesis of a julolidine fused heteroaromatic with coumarin has been reported for application as a fluorescent label.[36]

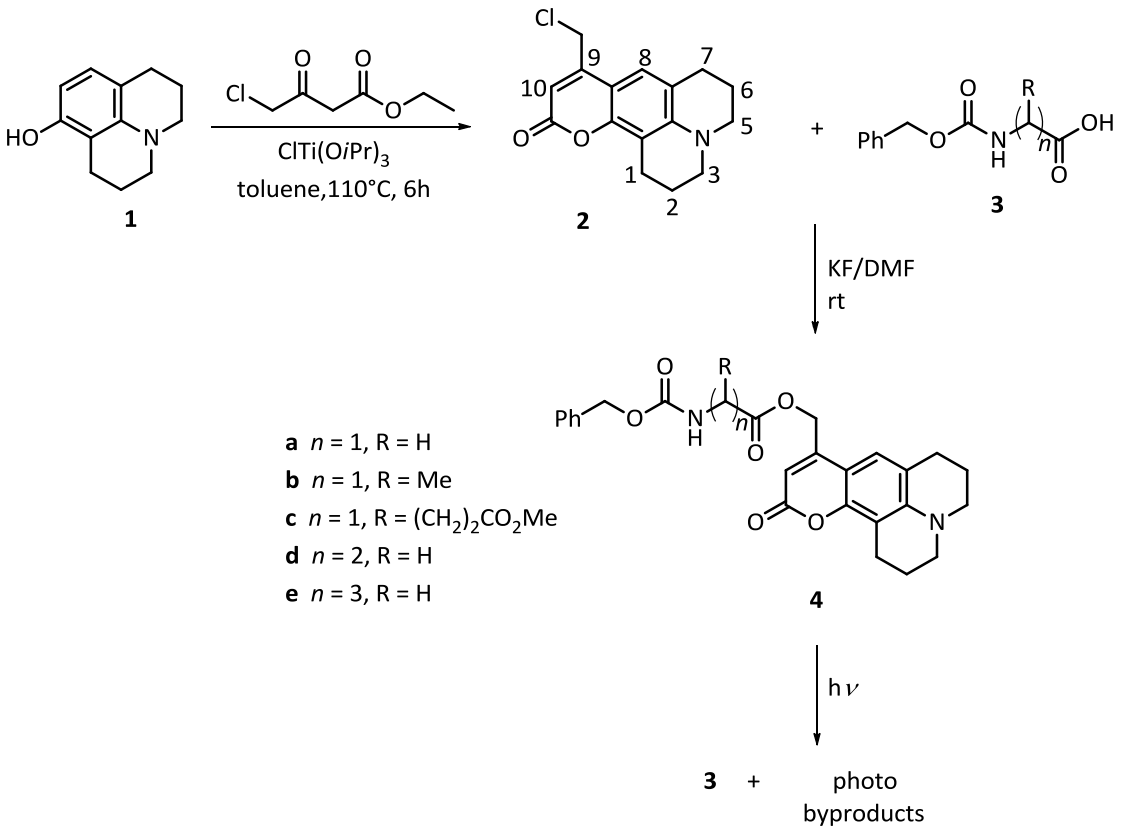
Previous work by the authors in the field of photoremovable protecting groups has included the use of different fluorescent skeletons, such as pyrene,[37,38] benzoxazoles,[39] acridines,[13] quinolones,[40] coumarins,[40-42] and their polyaromatic[38,43-46] and thionated analogues.[47,48] The present work aims to provide a new contribution to the collection of photoremovable protecting groups, with possible application as phototriggers, through the design, synthesis and evaluation of new fused motifs. To the best of our knowledge, the synthesis of a chloromethyl coumarin built on the julolidine nucleus and its use as a photocleavable protecting group has not been reported. For the light sensitivity evaluation of the new (11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl)methyl group, several ester cages were prepared with neurotransmitter amino acids as models of carboxylic target molecules and irradiated at 254, 300, 350 and 419 nm in two solvent systems with an organic solvent and water in 80:20 proportion (methanol or acetonitrile and aqueous HEPES buffer at pH 7.2) in a photochemical reactor. In addition, the compounds were further characterised by using time-resolved fluorescence with time-correlated single-photon counting (TCSPC) to elucidate their photophysical properties and determine the decay kinetics.

3. Results and discussion

3.1. Synthesis of julolidine fused coumarin 2 and amino acid conjugates 4

Assembly of a fused coumarin on the julolidine skeleton, resulting in 9-(chloromethyl)-2,3,6,7-tetrahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-11(5*H*)-one **2** was achieved by reaction of commercially available 1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinolin-8-ol **1** (trivially named 8-hydroxyjulolidine) with ethyl 4-chloro-3-oxobutanoate catalysed by triisopropoxytitanium (IV) chloride in 71% yield. Reaction of the functionalised coumarin **2** with model neurotransmitter amino acids **3** protected at their *N-terminus* with a benzyloxycarbonyl group, namely glycine (**3a**), alanine (**3b**), glutamic acid (**3c**), β-alanine (**3d**) and γ-aminobutyric acid (**3e**) in the presence of potassium fluoride in *N*,*N*-dimethylformamide (DMF), at room temperature, gave rise to the corresponding ester cages **4a**-**e** in moderate to good yields (Scheme 1, Table 1).

All compounds were fully characterised by high resolution mass spectrometry, IR, ^{1}H and ^{13}C NMR spectroscopy. ^{1}H NMR spectra showed signals of the amino acid residues, such as the α-CH (δ = 4.40-4.55 ppm), side chain β-CH_3, β-CH_2, and γ-CH_2 (δ = 1.48-2.52 ppm), main chain α-, β-, and γ-CH_2 (δ = 1.84-4.10 ppm). Regarding the heterocycle, the methylene group was visible for all conjugates (δ = 5.16- 5.24 ppm) and also the aromatic protons H-8 (δ = 6.84- 6.87 ppm) and H-10 (δ = 6.04- 6.07 ppm). In addition, the ^{13}C NMR spectra showed the corresponding signals for α-CH (δ = 49.67 or 53.47 ppm), side chain β-CH_3, β-CH_2, and γ-CH_2 (δ = 18.30-29.87 ppm), main chain α-, β-, and γ-CH_2 (δ = 25.09-42.71 ppm). For the heterocycle, the methylene group (δ = 61.43-62.53 ppm) and the aromatic C-8 (δ = 120.36-120.43 ppm) and C-10 (δ = 105.52-105.92 ppm) were visible for all conjugates. Confirmation of the presence of the new ester bond linking the amino acid to the coumarinyl moiety was also supported by ^{13}C NMR spectra signals at δ = 169.48-172.42 ppm and IR spectroscopy with a broad band centred between 1712 and 1720 cm^{-1}.



Scheme 1. Synthesis of julolidine fused coumarin **2** and amino acid conjugates **4a**-**e**.

Table 1. Yields, UV/vis and fluorescence data for compounds **2** and **4a**-**e** in absolute ethanol, methanol/HEPES buffer (80:20) solution and acetonitrile/HEPES buffer (80:20) solution.

	Compound	Yield (%)	Ethanol					Methanol/HEPES (80:20)					Acetonitrile/HEPES (80:20)				
			λ_{abs} (nm)	log ε	λ_{em} (nm)	Φ_F	$\Delta\lambda$ (nm)	λ_{abs} (nm)	log ε	λ_{em} (nm)	Φ_F	$\Delta\lambda$ (nm)	λ_{abs} (nm)	log ε	λ_{em} (nm)	Φ_F	$\Delta\lambda$ (nm)
2	Het-CH_2Cl	71	400	3.93	496	0.38	96	409	3.86	499	0.18	90	405	3.94	482	0.10	77
4a	Z-Gly-OHet	37	392	4.41	490	0.13	98	400	4.28	502	0.37	102	397	4.40	468	0.05	71
4b	Z-Ala-OHet	54	389	4.28	489	0.11	100	400	4.27	499	0.12	99	390	4.28	454	0.06	64
4c	Z-Glu(OMe)-OHet	47	389	4.40	483	0.14	94	402	4.36	497	0.19	95	391	4.27	463	0.06	72
4d	Z-β-Ala-OHet	81	397	4.27	488	0.16	91	402	4.32	502	0.23	100	391	4.50	465	0.05	74
4e	Z-GABA-OHet	29	395	4.39	495	0.18	100	401	4.37	502	0.22	101	391	4.47	465	0.06	74

3.2. Evaluation of the photophysical properties of amino acid conjugates 4

The UV/vis absorption and emission spectra of degassed 10^{-5} M solutions in absolute ethanol, methanol/HEPES and acetonitrile/HEPES buffer (80:20) solutions of ester conjugates **4a**-**e** were measured. Absorption and emission maxima, molar extinction coefficients and relative fluorescence quantum yields are reported in Table 1. Relative fluorescence quantum yields were calculated using a 0.05 M solution of quinine in sulfuric acid as standard (Φ_F = 0.546).[49] For the Φ_F determination, the fluorescence standard was excited at the wavelengths of maximum absorption found for each one of the compounds to be tested and in all fluorimetric measurements the absorbance of the solution did not exceed 0.1.

Absorption maxima for all compounds in the solvents used occurred at about 400 nm, revealing that no significant differences were observed due to the nature of the solvent or the amino acid residue. Concerning the fluorescence spectra, it was observed that the emission maxima in ethanol and methanol/HEPES buffer (80:20) solutions occurred at longer wavelengths (in the range 483-502 nm) than those using acetonitrile/HEPES buffer (80:20) solutions (in the range 454-468 nm). The Stokes's shifts ($\Delta\lambda$) were larger in ethanol and methanol/HEPES buffer (80:20) (*ca*. 100 nm) than in acetonitrile/HEPES buffer (80:20) (*ca*. 70 nm). The same trend was observed for the fluorescence quantum yields, with conjugates **4a**-**e** displaying very low values in acetonitrile/HEPES buffer (80:20) solution and in the range 0.11-0.37 in the other solvents (Table 1).

3.3. Photolysis studies of amino acid conjugates 4

Keeping in mind the importance of developing photoremovable groups with improved properties, namely the lability at longer wavelengths of irradiation, in recent years several attempts have been made in order to fine-tune the behaviour of the light sensitive molecule in terms of structure and solvent system used in the photolytic reaction. Taking advantage of the knowledge gathered concerning coumarins and their derivatives, plus the attractive properties of the julolidine unit, it was envisaged that the combination of both systems would result in a good candidate for a photocleavable

protecting group. As no report of such a system was available in literature, it was decided to evaluate the new (11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl)methyl group under photolytic conditions by exposing compounds **4a**-**e** in mixtures of organic solvent and water, since the title compounds displayed limited solubility in water. Thus, methanol/HEPES and acetonitrile/HEPES buffer (80:20) solutions of **4a**-**c** were irradiated in a Rayonet RPR-100 reactor at 254, 300, 350 and 419 nm (Scheme 1). The course of the photocleavage reaction was followed by reverse phase HPLC with UV detection. The plots of peak area (*A*) of the starting material *versus* irradiation time were obtained for each compound, at the considered wavelengths, which revealed a gradual decrease of the peak areas with time (average of three runs). The irradiation time determined represents the time necessary for the consumption of the starting materials until less than 5% of the initial area was detected (Table 2). For each compound, based on HPLC data, the plot of ln *A versus* irradiation time showed a linear correlation for the disappearance of the starting material. The photochemical quantum yields (Φ_{Phot}) were calculated based on half-lives ($t_{1/2}$), molar extinction coefficient (ε) and the incident photon flux (I_0), which was determined by potassium ferrioxalate actinometry.[46,50]

Table 2. Irradiation times, t_{irr}, and photochemical quantum yields, Φ_{Phot}, for the photolysis of conjugates **4a**-**e** at 254, 300, 350 and 419 nm in methanol/HEPES buffer (80:20) solution and at 350 and 419 nm in acetonitrile/HEPES buffer (80:20) solution.

	Compound	Methanol/HEPES (80:20)								Acetonitrile/HEPES (80:20)			
		254 nm		300 nm		350 nm		419 nm		350 nm		419 nm	
		t_{irr} (min)	Φ_{Phot}	t_{irr} (min)	Φ_{Phot}	t_{irr} (min)	Φ_{Phot}	t_{irr} (min)	Φ_{Phot}	t_{irr} (min)	Φ_{Phot}	t_{irr} (min)	Φ_{Phot}
4a	Z-Gly-OHet	4	1.46×10^{-3}	8	3.62×10^{-4}	7	3.71×10^{-4}	7	3.63×10^{-4}	4	4.35×10^{-4}	5	3.97×10^{-4}
4b	Z-Ala-OHet	9	6.79×10^{-4}	10	2.73×10^{-4}	12	2.18×10^{-4}	18	1.50×10^{-4}	11	2.59×10^{-4}	8	3.49×10^{-4}
4c	Z-Glu(OMe)-OHet	7	6.63×10^{-4}	13	1.80×10^{-4}	15	1.43×10^{-4}	9	2.44×10^{-4}	11	2.34×10^{-4}	8	3.50×10^{-4}
4d	Z-β-Ala-OHet	12	4.40×10^{-4}	16	1.58×10^{-4}	14	1.64×10^{-4}	20	1.29×10^{-4}	8	2.91×10^{-4}	10	1.48×10^{-4}
4e	Z-GABA-OHet	8	5.76×10^{-4}	9	2.54×10^{-4}	10	2.09×10^{-4}	10	2.05×10^{-4}	10	2.18×10^{-4}	7	2.51×10^{-4}

The release of *N*-benzyloxycarbonyl amino acids **3** in methanol/HEPES buffer (80:20) solutions under irradiation at 254 and 300 nm occurred rapidly with irradiation times between 4 and 16 min, and among the various amino acids, glycine was found to be the fastest to cleave.

The evaluation of the behaviour of (11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl)methyl group at longer wavelengths of irradiation, namely 350 and 419 nm, was also carried out to assess its potential applicability as a caging group in addition to its use as a benzyl ester type protecting group in organic synthesis. The results indicated a very slight increase in the irradiation times, which were in the range 7 to 20 min. Although in methanol/HEPES buffer (80:20) solutions the values obtained were very promising, it was decided to perform a similar study by using HEPES buffer combined with an organic solvent of a different character; acetonitrile. Comparison of the irradiation times necessary for the release of the active molecules in both solvent systems revealed that the cleavage was faster in acetonitrile/HEPES buffer (80:20) solutions (4-11 min), especially at 419 nm. This is a particularly suitable wavelength that minimises side reactions for the remaining functionalities of the molecule and in the future may allow for two-photon excitation to be used.

The release of *N*-benzyloxycarbonyl amino acids from coumarinyl ester conjugates was previously reported by the authors, and taking the release of β-alanine in methanol/HEPES buffer (80:20) solutions at 350 nm as an example, the earlier irradiation times were between 242-513 min (*ca*. 4-9 h) and at 419 nm the values were too long to be practicable (longer than 58 h). These previous results were obtained for coumarin derivatives with angular skeletons with fusions to benzene or oxazole rings.[39,43,44] The rapid release from the newly reported coumarin cages can be related to the innovative fusion to a julolidine unit, which resulted in a much faster cleavage within a few minutes (*ca*. 35 times faster). An even more drastic difference was observed at 419 nm, because photocleavage became practicable in very short irradiation times, in contrast to previously reported coumarin derivatives. Regarding the structures of the neurotransmitter amino acids, it was seen that its influence on the irradiation times was not significant; these values were quite similar for all wavelengths of irradiation. In addition to monitoring the photolysis process by HPLC/UV detection, the release of Z-

protected amino acids **3**, as the expected product of the photolysis of conjugates **4**, was also followed by ^{1}H NMR in a acetonitrile-d_3/D_2O (80:20) solution (see Figure 1 for alanine conjugate **4b** at 419 nm, as a representative example).

Upon irradiation at 419 nm, the signals due to alanine in the conjugate form disappeared progressively and were replaced by the corresponding set of signals of Z-Ala-OH, with the α-CH and β-CH_3 appearing at δ 4.14 and 1.35 ppm, respectively. The decrease of the benzylic-type CH_2 at position 9 of the heterocyclic ring was visible at δ = 5.10 ppm, thus confirming the release of the amino acid; this was accompanied by the appearance of a new signal at about 4.70 ppm relating to the hydroxylated byproduct from the heterocycle (Figure 1).

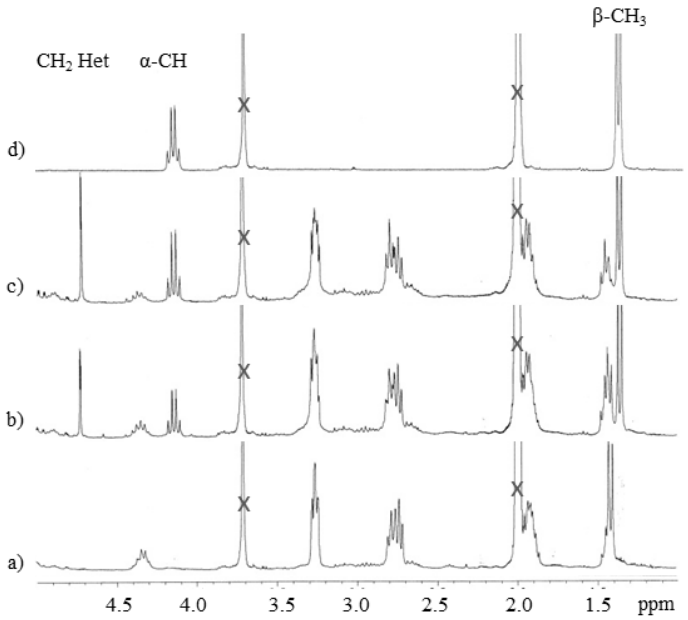
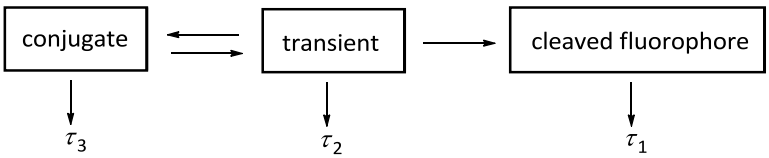


Figure 1. Partial ^{1}H NMR spectra in acetonitrile-d_3/D_2O (80:20) of the photolysis of alanine conjugate **4b** (C = 1.11 × 10^{-2} M) at 419 nm: (a) before irradiation; (b) after irradiation for 45 min; (c) after irradiation for 90 min; (d) Z-Ala-OH **3b**.

The promising results by using the 419 nm wavelength irradiation and acetonitrile/HEPES buffer (80:20) solutions was further investigated in order to elucidate the kinetic behaviour by monitoring the time-resolved fluorescence from the coumarinyl moiety. Upon excitation there is the possibility of the formation of an ion pair (see Scheme 2), which can either recombine or separate to form the photocleaved products, which we have previously reported.[46] The conjugation of the amino acid to the

coumarinyl unit influences its fluorescence properties by providing another route for energy to be dissipated, *i.e.* through charge transfer. To evaluate the fluorescence lifetimes, 9-(hydroxymethyl)-2,3,6,7-tetrahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-11(5*H*)-one **5**, an expected cleavage product (equivalent to **2**, but with -OH replacing -Cl), was also measured, to serve as a reference to follow the photocleavage reaction. The outcome of these measurements is shown in Figure 2. From this and the analysis given in Table 3, it is evident that the decay kinetics are not single exponential in nature and that the conjugated compounds exhibit a short-lived (*ca*. 100 ps) component accounting for about a third of the emission, which is not present in compound **5**. The trend seen in the cleavage rates (k_c, Table 3) is in keeping with that for the equivalent photochemical quantum yield (Table 2). Compounds **4d** and **4e** exhibit significantly lower values, with the higher attributed to **4a** and **4c**.



Scheme 2. Simplified photophysical schematic after photoexcitation and de-excitation paths.

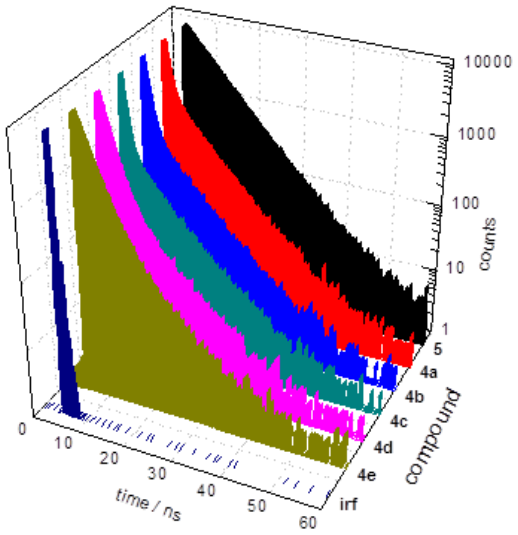


Figure 2. Time-resolved fluorescence from compounds **4a**-**e** and **5** monitored close to the peak emission. The excitation was at 418 nm. The instrumental response function (IRF) is also shown.

Table 3. Time-resolved fluorescence parameters for compounds **4a**-**e** and **5** in acetonitrile/HEPES buffer (80:20) solution. The excitation wavelength was at 418 nm. The cleavage rate, k_c, was calculated assuming that τ_1 relates to cleaved fluorophore and τ_2 to charge transfer transient, [$k_c = 1/\tau_2 - 1/\tau_1$].

Compound	λ_{em} (nm)	τ_1 (ps)	τ_2 (ps)	τ_3 (ps)	α_1	α_2	α_3	τ_{ave} (ps)	χ^2	k_c (s^{-1})
4a	500	5681 ± 39	736 ± 15	86 ± 13	0.12	0.64	0.24	1164	1.03	1.18×10^9
4b	500	5559 ± 54	805 ± 12	100 ± 46	0.06	0.63	0.30	897	0.97	1.06×10^9
4c	500	5357 ± 57	619 ± 12	122 ± 45	0.05	0.58	0.37	690	1.05	1.43×10^9
4d	500	5351 ± 93	1496 ± 16	91 ± 57	0.05	0.72	0.23	1369	0.97	4.8×10^8
4e	500	5034 ± 112	2140 ± 30	56 ± 31	0.05	0.59	0.35	1159	1.02	2.7×10^8
5	495	5775 ± 15	619 ± 210		0.88	0.12		5174	1.09	

It is clear that monitoring the fluorescence lifetime is indicative of the cleavage status of the conjugates, so a kinetic TCSPC measurement was performed, with the sample constantly irradiated by the excitation laser (in keeping with TCSPC conditions this provides less energy than the photoreactor, implying slower photocleavage rates).

The outcome of this experiment using alanine conjugate **4b** is summarised in Figure 3, from which it can clearly be seen that an increase in both intensity and average lifetime (mainly due to an increase in the amount of the longer-lived, τ_1, component), occurred with irradiation of the sample. A tentative explanation is the formation of photocleaved fluorophore (in keeping with the longer-lived emission from the compound **5**), which results in concomitant reduction of the proportion emanating from the transient species (e.g. ion pair). The fact that the concentration of the shorter-lived species is more or less constant can be indicative of the presence of conjugated fluorophore implying that complete photocleavage was not achieved.

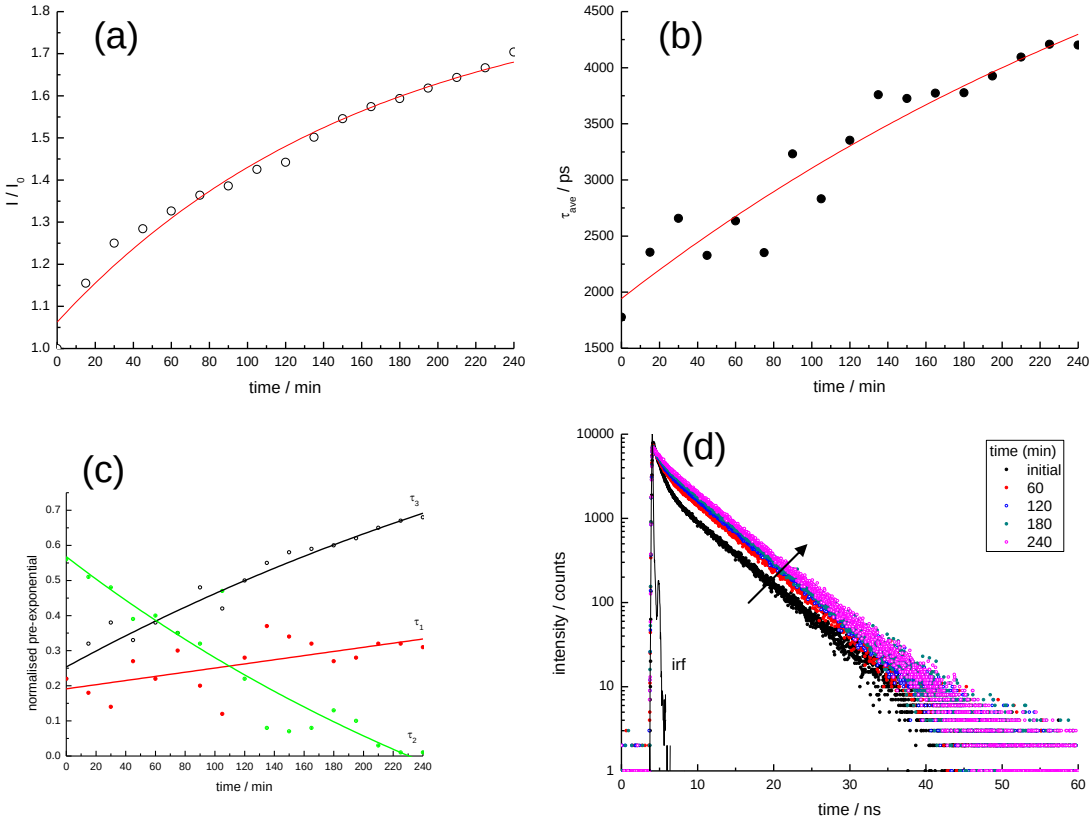


Figure 3. Analysis of the time-resolved fluorescence from alanine conjugate **4b** showing the effect of irradiation time on the time-resolved fluorescence: (a) change in the emission intensity; (b) the average lifetime; (c) the evolution of the pre-exponential quantity of the longest-lived decay; (d) representative time-resolved decays along with the instrumental response (IRF).

In a similar manner, glycine conjugate **4a** was irradiated to attempt a fuller photocleavage. To gain further information, time-resolved emission spectral (TRES) measurements were performed at the beginning and end of the experiment and the decay associated spectra obtained; these are shown in Figure 4, along with the decay associated spectra obtained from compound **5**. Initially it is necessary to fit the data to the sum of three exponentials (Table 3) and results in the presence of three decay associated spectra. However, at the end of the experiment, the dataset can be adequately fitted using the sum of two exponentials, resulting in two decay associated spectra. This is principally at the expense of the spectrum associated with the shorter-lived decay confirming that the longer-lived decay can be attributed to the photocleaved fluorophore and the shorter decay to deactivation mediated by the linkage to the amino acid. The intermediate decay time can relate to another transient species in the photocleavage process.

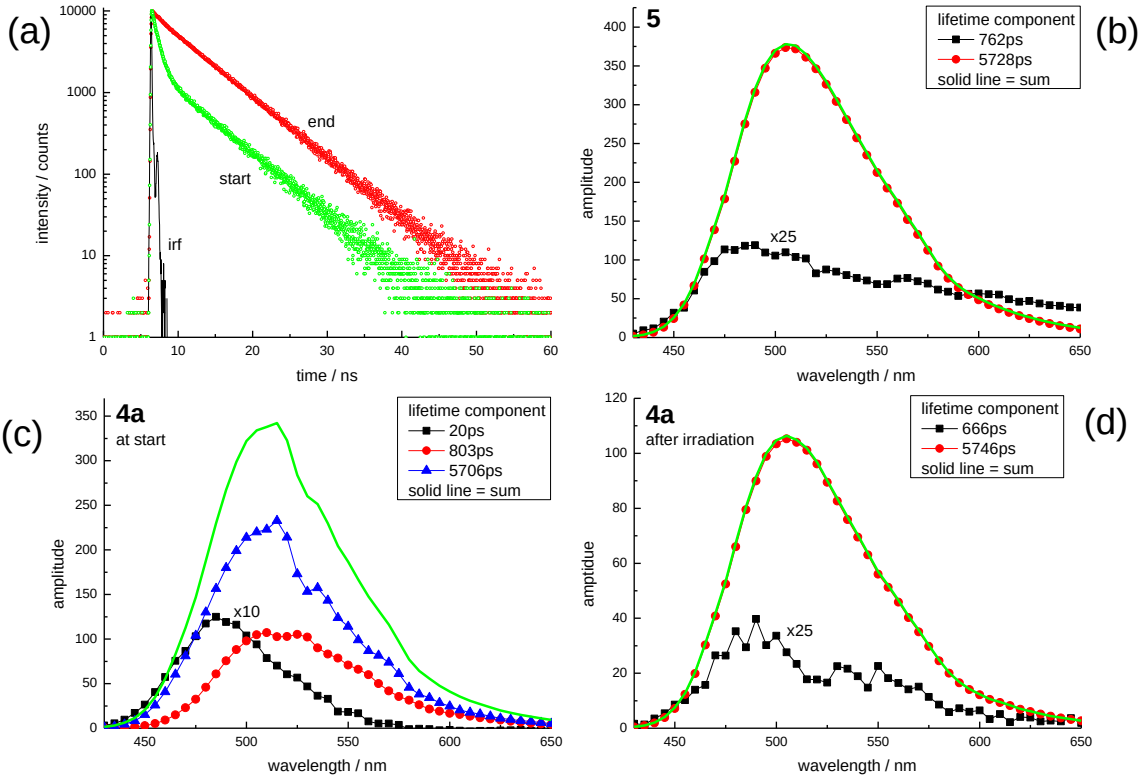


Figure 4. Analysis of the time-resolved fluorescence from glycine conjugate **4a** showing the effect of prolonged irradiation: (a) fluorescence decays, along with IRF. Decay associated spectra for (b) compound **5**, (c) compound **4a** before irradiation, (d) compound **4a** after irradiation.

4. Conclusions

A new chloromethyl coumarin built on a julolidine unit was synthesized and covalently linked to the carboxylic *terminus* of several amino acid neurotransmitters. To evaluate the behaviour of the resulting ester conjugates towards light, photolysis experiments were carried out at different irradiation wavelengths (254, 300, 350 and 419 nm) in methanol/HEPES and acetonitrile/HEPES buffer (80:20) solutions. The longer wavelength measurement was supported by time-resolved fluorescence, for which the presence of transient species relating to the charge transfer process was elucidated. The photolysis results revealed that the release of the active molecule occurred in short times (4-20 min, or in the range 5-10 min upon irradiation at 419 nm) in acetonitrile/HEPES buffer (80:20) solutions. The results obtained for the newly synthesized (11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl)methyl group are a very notable innovation in relation to previous reports using fused coumarin derivatives as photoremovable protecting groups for application at long wavelengths. The significant reduction in photocleavage time makes these compounds more practical for use in two photon excitation applications and in biological environments in which shorter wavelength excitation may be absorbed.

5. Experimental section

5.1. General

All melting points were measured on a Stuart SMP3 melting point apparatus. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel 60F_{254}) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230-240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer. UV/vis absorption spectra (200-700 nm) were obtained using a Shimadzu UV-2501PC spectrophotometer. NMR spectra were obtained on a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C using the solvent peak as internal reference at 25 °C. All chemical shifts are given in ppm using δ_H Me_4Si = 0 ppm as reference and *J* values are given in Hz. Assignments

were made by comparison of chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. Mass spectrometry analyses were performed at the "C.A.C.T.I. - Unidad de Espectrometria de Masas", at University of Vigo, Spain. Fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer. Commercially available reagents were used as received.

5.1.1. Synthesis of 9-(chloromethyl)-2,3,6,7-tetrahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-11(5*H*)-one (2). To a suspension of 1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinolin-8-ol **1** (0.058 g, 3.1×10^{-4} mol) and ethyl 4-chloro-3-oxobutanoate (0.042 mL, 3.1×10^{-4} mol) in toluene (5 mL), triisopropoxytitanium (IV) chloride 1M solution (0.62 mL, 6.2×10^{-4} mol) was added and the mixture was heated at reflux for 6 h. The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography with silica gel using ethyl acetate/light petroleum mixtures of increasing polarity as eluent, to give compound **2** as a green solid (0.064 g, 71%). Mp = 176.3-177.4 °C. TLC (ethyl acetate/light petroleum, 1:2): R_f = 0.48. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.92-2.03 (m, 4 H, H-2 and H-6), 2.79 (t, *J* = 6.0 Hz, 2 H, H-7), 2.89 (t, *J* = 6.4 Hz, 2 H, H-1), 3.21-3.33 (m, 4 H, H-3 and H-5), 4.55 (s, 2 H, CH_2Cl), 6.14 (s, 1 H, H-10), 7.03 (s, 1 H, H-8) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 20.42 (C-2), 20.53 (C-1), 21.44 (C-6), 27.75 (C-7), 41.55 (CH_2Cl), 49.49 (C-3), 49.91 (C-5), 106.00 (C-8a), 107.00 (C-12b), 108.28 (C-10), 118.28 (C-7a), 121.07 (C-8), 146.09 (C-7b), 149.96 (C-9), 151.49 (C-12a), 162.07 (C-11) ppm. IR (KBr 1%, cm^{-1}): υ = 3019, 2932, 2852, 2401, 1703, 1605, 1557, 1522, 1430, 1378, 1312, 1216, 1123, 1074, 928, 850, 769, 667. HRMS (ESI): calcd for $C_{16}H_{17}{}^{37}ClNO_2$ [M^++H] 292.09128, found 292.09423; calcd for $C_{16}H_{17}{}^{35}ClNO_2$ [M^++H]: 290.09423, found 290.09407.

5.2. General procedure for the synthesis of amino acid conjugates 4a-e

Compound **2** (1 equiv.) was dissolved in dry DMF and potassium fluoride (3 equiv.) and *N*-benzyloxycarbonylated amino acid **3** (1.1 equiv.) were added. The reaction mixture was stirred at room temperature for 16-19 h and followed by TLC (ethyl acetate/light petroleum, 1:2). The solvent was removed by rotary evaporation under

reduced pressure and the crude residue was purified by column chromatography with silica gel using ethyl acetate/light petroleum mixtures of increasing polarity as eluent, to give compounds **4a**-**e**.

5.2.1. *N*-(Benzyloxycarbonyl)-glycine [11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl]methyl ester (4a). Starting from compound **2** (0.053 g, 1.8 $\times$ 10^{-4} mol) in dry DMF (3 mL), potassium fluoride (0.032 g, 5.5 $\times$ 10^{-4} mol) and *N*-(benzyloxycarbonyl)-glycine, Z-Gly-OH **3a** (0.042 g, 2.0 $\times$ 10^{-4} mol), compound **4a** was isolated as an oil (0.031 g, 37%). TLC (ethyl acetate/light petroleum, 1:2): R_f = 0.78. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.90-2.02 (m, 4 H, H-2 and H-6), 2.76 (t, *J* = 6.0 Hz, 2 H, H-7), 2.87 (t, *J* = 6.4 Hz, 2 H, H-1), 3.20-3.32 (m, 4 H, H-3 and H-5), 4.10 (d, *J* = 6.8 Hz, 2 H, CH_2 Gly), 5.14 (s, 2 H, CH_2 Z), 5.24 (s, 2 H, CH_2), 5.41 (d, *J* = 8.0 Hz, 1 H, NH), 6.06 (s, 1 H, H-10), 6.85 (s, 1 H, H-8), 7.30-7.37 (m, 5 H, 5 $\times$ Ar-H Z) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 20.35 (C-2), 20.48 (C-1), 21.38 (C-6), 27.64 (C-7), 42.71 (CH_2 Gly), 49.44 (C-3), 49.86 (C-5), 62.23 (CH_2), 67.18 (CH_2 Z), 105.58 (C-8a), 105.81 (C-10), 106.92 (C-12b), 118.28 (C-7a), 120.38 (C-8), 128.07 (2 $\times$ Ar-C Z), 128.17 (2 $\times$ Ar-C Z), 128.49 (Ar-C Z), 136.07 (C-1 Z), 146.02 (C-7b), 148.62 (C-9), 151.17 (C-12a), 156.30 (C=O urethane), 162.02 (C-11), 169.48 (C=O ester) ppm. IR (neat, cm^{-1}): υ = 3335, 3064, 3030, 3011, 2939, 2849, 1712, 1603, 1556, 1524, 1437, 1387, 1344, 1326, 1312, 1277, 1178, 1124, 1054, 1020, 924, 885, 852, 754, 698, 665. HRMS (ESI): calcd for $C_{26}H_{27}N_2O_6$ [M^++H]: 463.18636; found 463.18623.

5.2.2. *N*-(Benzyloxycarbonyl)-L-alanine [11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl]methyl ester (4b). Starting from compound **2** (0.093 g, 3.2 $\times$ 10^{-4} mol) in dry DMF (3 mL), potassium fluoride (0.056 g, 9.6 $\times$ 10^{-4} mol) and *N*-(benzyloxycarbonyl)-L-alanine, Z-Ala-OH **3b** (0.079 g, 3.5 $\times$ 10^{-4} mol), compound **4b** was isolated as an oil (0.082 g, 54%). TLC (ethyl acetate/light petroleum, 1:2): R_f = 0.84. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.48 (d, *J* = 7.2 Hz, 3 H, β-CH_3 Ala), 1.82-2.05 (m, 4 H, H-2 and H-6), 2.74 (t, *J* = 6.4 Hz, 2 H, H-7), 2.85 (t, *J* = 6.4 Hz, 2 H, H-1), 3.15-3.38 (m, 4 H, H-3 and H-5), 4.40-4.55 (m, 1 H, α-CH Ala), 5.02-5.16 (m, 2 H, CH_2 Z), 5.17-5.34 (m, 2 H, CH_2), 5.46 (d, *J* = 8.0 Hz, 1 H, NH), 6.04 (s, 1 H, H-10), 6.84 (s, 1 H, H-8), 7.21-7.39 (m, 5 H, 5 $\times$ Ar-H Z) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 18.30 (β-CH_3 Ala), 20.30 (C-2),

20.43 (C-1), 21.33 (C-6), 27.58 (C-7), 49.39 (C-3), 49.67 (α-CH Ala), 49.81 (C-5), 62.28 (CH_2), 66.94 (CH_2 Z), 105.53 (C-8a), 105.61 (C-10), 106.81 (C-12b), 118.24 (C-7a), 120.38 (C-8), 128.01 (2 × Ar-C Z), 128.08 (2 × Ar-C Z), 128.43 (Ar-C Z), 136.07 (C-1 Z), 145.98 (C-7b), 148.80 (C-9), 151.11 (C-12a), 155.62 (C=O urethane), 162.01 (C-11), 172.36 (C=O ester) ppm. IR (neat, cm^{-1}): υ = 3325, 3014, 2941, 2851, 1715, 1608, 1557, 1526, 1452, 1380, 1342, 1312, 1255, 1207, 1178, 1124, 1071, 956, 885, 854, 822, 754, 699, 666. HRMS (ESI): calcd for $C_{27}H_{29}N_2O_6$ [M^++H]: 477.20201; found 477.20195.

5.2.3. (11-Oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl)methyl 5-methyl 2-[(benzyloxycarbonyl)amino]pentanedioate (4c). Starting from compound **2** (0.035 g, 1.2 × 10^{-4} mol) in dry DMF (3 mL), potassium fluoride (0.021 g, 3.6 × 10^{-4} mol) and 2-(benzyloxycarbonyl)amino-5-methoxy-5-oxopentanoic acid, Z-Glu(OMe)-OH **3c** (0.039 g, 1.3 × 10^{-4} mol), compound **4c** was isolated as an oil (0.031 g, 47%). TLC (ethyl acetate/light petroleum, 1:2): R_f = 0.83. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.90-2.00 (m, 4 H, H-2 and H-6), 2.00-2.04 (m, 1 H, β-CH_2 Glu), 2.24-2.34 (m, 1 H, β-CH_2 Glu), 2.36-2.52 (m, 2 H, γ-CH_2 Glu), 2.74 (t, *J* = 6.0 Hz, 2 H, H-7), 2.85 (t, *J* = 6.4 Hz, 2 H, H-1), 3.19-3.29 (m, 4 H, H-3 and H-5), 3.64 (s, 3 H, OCH_3), 4.45-4.55 (m, 1 H, α-CH Glu), 5.10 (s, 2 H, CH_2 Z), 5.21 (s, 2 H, CH_2), 5.59 (d, *J* = 8.0 Hz, 1 H, NH), 6.06 (s, 1 H, H-10), 6.85 (s, 1 H, H-8), 7.28-7.40 (m, 5 H, 5 × Ar-H Z) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 20.31 (C-2), 20.44 (C-1), 20.95 (C-6), 27.15 (β-CH_2 Glu), 27.58 (C-7), 29.87 (γ-CH_2 Glu), 49.40 (C-3), 49.82 (C-5), 51.79 (OCH_3), 53.47 (α-CH Glu), 62.53 (CH_2), 67.08 (CH_2 Z), 105.57 (C-8a), 105.92 (C-10), 106.84 (C-12b), 118.26 (C-7a), 120.43 (C-8), 128.03 (2 × Ar-C Z), 128.11 (2 × Ar-C Z), 128.43 (Ar-C Z), 136.00 (C-1 Z), 145.99 (C-7b), 148.53 (C-9), 151.14 (C-12a), 155.93 (C=O urethane), 161.95 (C-11), 172.27 (C=O ester), 172.95 (C=O methyl ester) ppm. IR (neat, cm^{-1}): υ = 3336, 2949, 2849, 1720, 1604, 1556, 1525, 1439, 1380, 1343, 1313, 1263, 1205, 1180, 1125, 1054, 1028, 960, 850, 754, 699, 665. HRMS (ESI): calcd for $C_{30}H_{33}N_2O_8$ [M^++H]: 549.22314; found 549.22322.

5.2.4. *N*-(Benzyloxycarbonyl)-β-alanine [11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl]methyl ester (4d). Starting from compound **2** (0.044 g, 1.5 × 10^{-4} mol) in dry DMF (3 mL), potassium fluoride (0.026 g, 4.6 × 10^{-4} mol)

and *N*-(benzyloxycarbonyl)-β-alanine, Z-β-Ala-OH **3d** (0.037 g, 1.7×10^{-4} mol), compound **4d** was isolated as an oil (0.059 g, 81%). TLC (ethyl acetate/light petroleum, 1:2): R_f = 0.85. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.90-2.03 (m, 4 H, H-2 and H-6), 2.69 (t, *J* = 5.6 Hz, 2 H, β-CH_2 β-Ala), 2.75 (t, *J* = 6.4 Hz, 2 H, H-7), 2.87 (t, *J* = 6.4 Hz, 2 H, H-1), 3.20-3.31 (m, 4 H, H-3 and H-5), 3.47-3.60 (m, 2 H, α-CH_2 β-Ala), 5.09 (s, 2 H, CH_2 Z), 5.20 (s, 2 H, CH_2), 5.27 (d, *J* = 8.0 Hz, 1 H, NH), 6.05 (s, 1 H, H-10), 6.86 (s, 1 H, H-8), 7.28-7.40 (m, 5 H, 5 × Ar-H Z) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 20.37 (C-2), 20.51 (C-1), 21.40 (C-6), 27.67 (C-7), 34.37 (β-CH_2 β-Ala), 36.45 (α-CH_2 β-Ala), 49.46 (C-3), 49.87 (C-5), 61.66 (CH_2), 66.77 (CH_2 Z), 105.68 (C-8a), 105.74 (C-10), 106.96 (C-12b), 118.25 (C-7a), 120.36 (C-8), 128.09 (2 × Ar-C Z), 128.10 (2 × Ar-C Z), 128.49 (Ar-C Z), 136.35 (C-1 Z), 146.00 (C-7b), 148.07 (C-9), 151.19 (C-12a), 155.23 (C=O urethane), 162.09 (C-11), 171.63 (C=O ester) ppm. IR (neat, cm^{-1}): υ = 3336, 3030, 2940, 2852, 1715, 1604, 1556, 1525, 1438, 1380, 1343, 1313, 1252, 1174, 1075, 1012, 854, 753, 698, 665. HRMS (ESI): calcd for $C_{27}H_{29}N_2O_6$ [M^++H]: 477.20201; found 477.20224.

5.2.5. *N*-(Benzyloxycarbonyl)-γ-aminobutyric acid [11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl]methyl ester (4e). Starting from compound **2** (0.091 g, 3.1×10^{-4} mol) in dry DMF (3 mL), potassium fluoride (0.055 g, 9.4×10^{-4} mol) and *N*-(benzyloxycarbonyl)-γ-aminobutyric acid, Z-GABA-OH **3e** (0.082 g, 3.4×10^{-4} mol), compound **4e** was isolated as an oil (0.044 g, 29%). TLC (ethyl acetate/light petroleum, 1:2): R_f = 0.82. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.84-2.02 (m, 6 H, β-CH_2 GABA, H-2 and H-6), 2.50 (t, *J* = 7.2 Hz, 2 H, γ-CH_2 GABA), 2.76 (t, *J* = 6.4 Hz, 2 H, H-7), 2.88 (t, *J* = 6.4 Hz, 2 H, H-1), 3.20-3.33 (m, 6 H, α-CH_2 GABA, H-3 and H-5), 4.96 (br s, 1 H, NH), 5.10 (s, 2 H, CH_2 Z), 5.22 (s, 2 H, CH_2), 6.07 (s, 1 H, H-10), 6.86 (s, 1 H, H-8), 7.30-7.40 (m, 5 H, 5 × Ar-H Z) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 20.36 (C-2), 20.51 (C-1), 20.99 (C-6), 25.09 (β-CH_2 GABA), 27.65 (C-7), 31.20 (γ-CH_2 GABA), 40.24 (α-CH_2 GABA), 49.45 (C-3), 49.85 (C-5), 61.43 (CH_2), 66.65 (CH_2 Z), 105.52 (C-10), 105.70 (C-8a), 106.92 (C-12b), 118.20 (C-7a), 120.36 (C-8), 128.04 (2 × Ar-C Z), 128.29 (2 × Ar-C Z), 128.45 (Ar-C Z), 136.46 (C-1 Z), 145.95 (C-7b), 149.38 (C-9), 151.15 (C-12a), 156.41 (C=O urethane), 162.16 (C-11), 172.42 (C=O ester) ppm. IR (neat, cm^{-1}): υ = 3336, 3063, 3009, 2931, 2855, 1712, 1603, 1556, 1524, 1439, 1382, 1343, 1313, 1255, 1209, 1165, 1126, 1091, 1020, 962, 924, 885,

851, 820, 754, 699, 665. HRMS (ESI): calcd for $C_{28}H_{31}N_2O_6$ [M^++H]: 491.21766; found 491.21756.

5.3. Photolysis general

A 1×10^{-4} M methanol or acetonitrile/HEPES (80:20) solution of compounds **4a**-**e** (5 mL) were placed in a quartz tube and irradiated in a Rayonet RPR-100 reactor at the desired wavelength. The lamps used for irradiation were of 254, 300, 350 and 419 ± 10 nm. HEPES buffer solution was prepared in distilled water with HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (10 mM), sodium chloride (120 mM), potassium chloride (3 mM), calcium chloride (1 mM) and magnesium chloride (1mM) and pH adjusted to 7.2 with aqueous 1 M sodium hydroxide solution.

Aliquots of 100 µL were taken at regular intervals and analysed by RP-HPLC using a Licrospher 100 RP18 (5 µm) column in a JASCO HPLC system composed by a PU-2080 pump and a UV-2070 detector with ChromNav software. The eluent was acetonitrile/water, 75:25 at a flow rate of 1.0 mL/min, previously filtered through a Millipore, type HN 0.45 µm filter and degassed by ultra-sound for 30 min. The chromatograms were traced by detecting UV absorption at the wavelength of maximum absorption for each compound (retention time: **4a**, 5.7; **4b**, 6.4; **4c**, 6.1; **4d**, 6.3; **4e**, 6.8 min).

For determination of the photochemical quantum yields (Φ_{Phot}), the half-lives ($t_{1/2}$) were calculated from the plot of ln area (directly correlated with concentration) *versus* the irradiation time and correspond to the time required for a 50% conversion by photocleavage. The quantum yields were calculated by using Equation (1)[46]

$$\Phi_{Phot} = (0.693)\times(6.02\times10^{20})/(2.303\times t_{1/2}\times\varepsilon_\lambda\times I_0) \qquad (1)$$

where I_0 = incident photon flux (photon.s^{-1}.cm^{-2}), ε_λ = molar extinction coefficient at the irradiation wavelength (M^{-1}.cm^{-1}), and $t_{1/2}$ = half-lives (s).

5.4. Time-resolved fluorescence measurements

Time-resolved fluorescence measurements made use of the time-correlated single-photon counting (TCSPC) technique and were performed using a HORIBA Scientific DeltaFlex, equipped with a DeltaDiode laser excitation source emitting at 418 nm (running at 8 MHz) and a TBX-850 detector. Time-resolved emission spectral (TRES) data were acquired by stepping the emission monochromator through 5 nm increments and measuring the decay for equal time periods. The instrumental response (full width at half maximum < 200 ps) was also measured. Analysis of each dataset was performed using DAS6 software and the decays reconvoluted with the instrumental response and fitted globally (producing common decay times, τ_i) to the sum of exponentials (Equation 2).

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp -t/\tau_i \qquad (2)$$

The average lifetime was obtained from Equation (3)

$$\tau_{ave} = \Sigma \alpha_i \tau \qquad (3)$$

The goodness of fit was judged in terms of a chi-squared value and weighted residuals. Decay associated spectra, allowing the spectra to be obtained and related to decay times, were recovered from the global analysis. The pre-exponential values for the individual decays were weighted by the appropriate lifetime value and plotted against wavelength. The sum of the individual decay associated spectra for each dataset was compared to the steady state spectra, and in all cases an agreement was seen. Kinetic TCSPC measurements involved sequential recording the time-resolved decay histograms (collected for 30 seconds) with constant irradiation from the DeltaDiode laser over a four hour period. Data were analysed using DAS6 software in a batch mode recovering the lifetime and intensity values. For simplicity, data points are displayed at fifteen minute intervals. Further analysis of these data was performed using Origin software and fitting involved the use of the exponential growth model provided.

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2.6

Photoactivable heterocyclic cages in a comparative release study of butyric acid as a model drug

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INDEX

1. Abstract

Aiming at the improvement of the photorelease of butyric acid - a model carboxylic acid drug, a set of heteroaromatic compounds based on acridine, naphtho[2,1-*b*]pyran, 3*H*-benzopyran fused julolidine and thioxo-naphtho[2,1-*b*]pyran were evaluated as benzyl-type phototriggers, in comparison with the well-known *o*-nitrobenzyl group. The corresponding ester cages were irradiated in a photochemical reactor at 254, 300, 350 and 419 nm in two solvent systems (methanol or acetonitrile in 80:20 mixtures with HEPES buffer). Photolysis studies showed that, for some of the cages, the release of the active molecule occurred with short irradiation times at 419 nm. Time-resolved fluorescence was used to elucidate their photophysical properties and determine the decay kinetics.

2. Introduction

The use of several drugs in clinical practice is dependent on the improvement of their therapeutic effects, bioavailability, physicochemical properties and the minimizing of other undesirable side effects. Prodrugs, pharmacologically latent derivatives of active agents, can be designed to undergo activation through a specific stimulus. In addition to chemical and/or enzymatic triggers, light is an appealing tool to use for conversion of prodrugs to active agents in a spatially and temporally controlled manner.[1-8] Butyric acid, a saturated unbranched monocarboxylic acid, is one of the short chain fatty acids. The effects of butyric acid include the disruption of cell proliferation and induction of apoptosis; modification of cell morphology and alteration of gene expression.[9] The presence of carboxylic acids or other ionisable polar groups in drugs can result in poor absorption from the gastrointestinal tract owing to lipophilicity/solubility issues.

Light triggerable benzyl or heterocyclic benzyl esters represent a large family of photolabile protecting groups. *o*-Nitrobenzylic derivatives have been widely used in various applications, as they combine a satisfactory photosensitivity with a stability for handling and synthesis purposes.[10-12] Nevertheless, *o*-nitrobenzyl cages exhibit some limitations, since the wavelength of excitation required for the uncanging is not necessarily the most suitable for bioapplications. The search for protecting groups with

improved photochemical properties and even displaying fluorescence has motivated the incorporation of heterocyclic moieties in their design, with coumarinyl methyl as relevant examples. It is possible to find a wide range of derivatives possessing different combinations of substituents and/or fusion ring units for the release of various active molecules.[13-15] Recently, acridinyl methyl esters have also proved to possess the required photosensitivity for the release of carboxylic acid compounds.[16]

Considering the know-how of the authors in the field of fluorescent photoactivable molecules based on aromatic and heteroaromatic skeletons for the release of bioanalytes,[17-24] in connection with the interest in the development of alternative light sensitive prodrugs, and following the previous work regarding butyric acid, a new set of oxygen and nitrogen heterocyclic cages were synthesised. The use of these heterocyclic moieties can produce longer maximum wavelengths of absorption, allowing the photorelease of butyric acid at longer wavelengths, not detrimental to bioapplications. Thus, the present work evaluates the behaviour of (acridin-9-yl)methyl, (5-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methyl, (8-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methyl, and [11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl]methyl groups, in comparison with the well-known *o*-nitrobenzyl group in the light-induced release of butyric acid. Additionally, bearing in mind that the replacement of a carbonyl by a thiocarbonyl group results in an improvement in the photolytic release,[23,24] thionated groups namely (5-methoxy-3-thioxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methyl, (8-methoxy-3-thioxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methyl, and (9-methoxy-3-thioxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methyl were tested. The ester cages, in two solvent systems (methanol or acetonitrile in 80:20 mixtures with HEPES buffer), were irradiated at 254, 300, 350 and 419 nm in a photochemical reactor. The fact that the groups exhibit fluorescence enabled time-resolved fluorescence measurements to be employed to elucidate their photophysical properties and determine the decay kinetics.

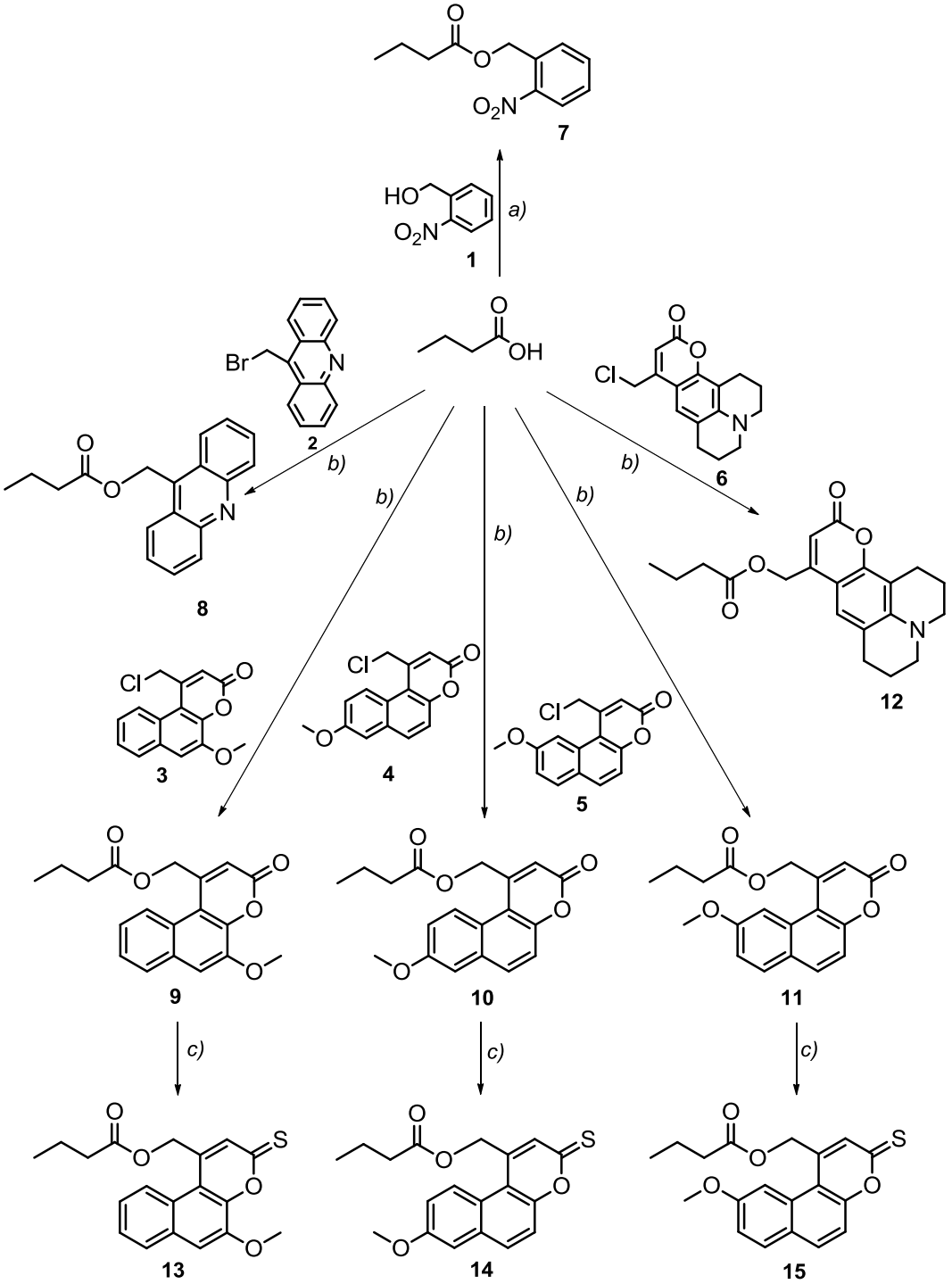
3. Results and discussion

3.1. Synthesis of butyric acid conjugates 7-15

Butyric acid was chosen as a model carboxylic acid drug to study the behaviour towards irradiation of the corresponding cages with several light sensitive *o*-nitrobenzyl or heterocyclic benzyl type esters **7**-**12**. Their synthesis started with the preparation of the 2-nitrobenzyl ester cage **7**, by a *N,N'*-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) mediated coupling of butyric acid with (2-nitrophenyl)methanol **1** in DMF at room temperature. For the assembly of the heterocyclic benzyl type ester cages **8**-**12**, several bromo- or chloromethylated heteroaromatics based on acridine **2**, naphtho[2,1-*b*]pyran **3**-**5** and a 3*H*-benzopyran fused julolidine **6** were reacted with the model acid in the presence of potassium fluoride in DMF at room temperature (Scheme 1, Table 1). Fused pyrans **3**-**6** resulted from a Pechmann condensation between ethyl 4-chloro-3-oxobutanoate and 3-methoxy-2-naphthol, 6-methoxy-2-naphthol, 7-methoxy-2-naphthol[25] and 1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinolin-8-ol (trivially named 8-hydroxyjulolidine),[26] respectively. With the purpose of replacing the carbonyl by a thiocarbonyl group at the naphtho[2,1-*b*]pyran conjugates **9**-**11**, these compounds were reacted with Lawesson's reagent in toluene, under reflux conditions,[27] affording the corresponding thionaphthopyran conjugates **13-15**.

All compounds were fully characterised by high resolution mass spectrometry, IR, ^{1}H and ^{13}C NMR spectroscopy. The IR spectra of compounds **7**-**15** displayed stretching vibration bands of the ester carbonyl group from 1711 to 1743 cm^{-1}. ^{1}H NMR spectra showed signals of butyric acid, the methyl (δ 0.89-1.02 ppm) and two methylenes (δ 1.58-1.81 and 2.32-2.50 ppm). The heterocycle methylene group, adjacent to the ester link, was visible for all compounds (δ 5.20-6.12 ppm). The newly formed ester linkages were confirmed by ^{13}C NMR spectra signals of the carbonyl group, at about δ 172.52-173.42 ppm. The thiocarbonyl group in compounds **13-15** affected the chemical shift of the pyran proton H-2, which appeared downfield in the range δ 7.51-7.59 ppm, while in the precursor compounds **9-11**, with a carbonyl group, it occurred at δ 6.58-6.70 ppm. The presence of the new C=S bond (C-3) at the heterocyclic ring was also confirmed by ^{13}C NMR spectra signals at δ 194.49-195.27 ppm, when compared to the carbonyl group,

which occurred at δ 159.43-160.31 ppm. The chemical shift of the pyran carbon C-2 was also influenced by the carbon-sulphur double bond, being in the range δ 126.97-127.80 ppm for compounds **13**-**15**, and δ 112.72 or 113.14 ppm for compounds **9**-**11**.



Scheme 1. Synthesis of ester cages of butyric acid **7**-**15**. Reagents and conditions: *a)* DCC/HOBt, DMF, rt; *b)* KF, DMF, rt; *c)* Lawesson's reagent, toluene, reflux.

Table 1. Yields, UV/visible absorption and fluorescence data for compounds **7**-**15** in absolute ethanol, methanol/HEPES buffer (80:20) and acetonitrile/HEPES buffer (80:20) solutions.[a] in nm. [b] Data in ethanol and methanol/HEPES buffer (80:20) solution was previously reported.[20] [c] The excitation was carried out at 326 nm.

Cpd	Yield (%)	Ethanol					Methanol/HEPES (80:20)					Acetonitrile/HEPES (80:20)				
		λ_{abs}[a]	log ε	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]	λ_{abs}[a]	log ε	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]	λ_{abs}[a]	log ε	λ_{em}[a]	Φ_F	$\Delta\lambda$[a]
7	29	256	3.77	303	0.08	47	259	3.73	304	0.07	45	260	3.74	304	0.13	44
8	69	360	3.71	410	0.34	50	360	4.12	427	0.32	67	360	4.15	427	0.27	67
9	70	344	3.54	464	0.18	120	343	3.93	479	0.21	136	343	3.96	472	0.20	129
10	71	316, 365	4.07, 3.85	459	0.53	94	316, 366	3.99, 3.80	470	0.49	104	314, 364	4.18, 3.97	464	0.45	100
11[b]	80	350	3.92	466	0.45	116	352	4.22	484	0.46	132	345	3.86	474	0.40	129
12	34	394	4.28	485	0.19	91	399	4.13	497	0.16	98	397	4.06	494	0.18	97
13	5	310, 404	3.83, 3.70	447	0.03	43	308, 406	3.94, 3.86	448	0.03	42	308, 405	4.04, 3.95	437	0.03	32
14[c]	80	283, 326, 431	4.19, 4.28, 4.21	464	0.06	138	326, 428	4.15, 4.12	477	0.05	151	326, 430	4.26, 4.23	466	0.01	140
15	70	407	4.20	462	0.002	55	412	3.97	470	0.001	58	412	4.21	470	0.001	58

3.2. Evaluation of the photophysical properties of butyric acid conjugates 7-15

Fundamental UV/visible photophysical characterisation was carried out to obtain the parameters required for monitoring the photolytic process. The absorption and emission spectra of degassed 10^{-5} M solutions in absolute ethanol, a methanol/HEPES and acetonitrile/HEPES buffer (80:20) solutions of ester conjugates **7**-**15** were measured and the corresponding data, absorption and emission maxima, molar extinction coefficients and relative fluorescence quantum yields are reported in Table 1. Relative fluorescence quantum yields were calculated using 9,10-diphenylanthracene in ethanol (Φ_F 0.95)[28] or a 0.05 M solution of quinine in sulphuric acid (Φ_F 0.546)[29] as standards. For the Φ_F determination, the fluorescence standard was excited at the wavelengths of maximum absorption found for each compound to be tested and in all fluorimetric measurements the absorbance of the solution did not exceed 0.1.

Regarding the maximum absorption wavelengths (λ_{abs}) of conjugates **7**-**15**, in all the tested solvents, it was found that the nitrobenzyl conjugate **7** displayed the lowest values (at about 260 nm), in comparison with the polycyclic compounds **8**-**12** (344-394 nm), whereas the thiocarbonyl conjugates **13**-**15**, displayed even larger bathochromic shifts (404-431 nm). For naphtho[2,1-*b*]pyrans **9**-**11**, which differ in the relative position of the electron donor methoxy group, the λ_{abs} was longer for substitution in position 8 (compound **10**), with a bathochromic shift between 15 to 23 nm, when compared with substitution in positions 5 and 9 (compounds **9** and **11**, respectively). Nevertheless, the largest shift was observed for the 3*H*-benzopyran fused julolidine **12**, with maximum absorption wavelengths in the range 394-399 nm, in the various solvents. Furthermore, the replacement of the carbonyl group in the pyran ring (compounds **9**-**11**) by a thiocarbonyl (compounds **13**-**15**) also resulted in a shift in the λ_{abs}, by tuning absorption to values in the visible region (λ_{abs} 404-431 nm).

Concerning the fluorescence spectra, in all the tested solvents, it was observed that emission maxima (λ_{em}) of conjugates **8**-**15** occurred in the range (410-497 nm), being the 3*H*-benzopyran fused julolidine **12** associated to the longer wavelengths, and the naphtho[2,1-*b*]pyran **10** the most emissive (Φ_F 0.45 to 0.53). The nitrobenzyl conjugate **7** displayed the lowest values (λ_{em} at about 300 nm), as was expected due to its structure. The emission of thiocarbonyl conjugates **13**-**15** was hypsochromically

shifted with lower fluorescent quantum yields, in comparison with the corresponding carbonyl precursors.

3.3. Photolysis studies of butyric acid conjugates 7-15

The present work intended to profit from our gathered knowledge in the application of different heteroaromatic benzyl-type systems as photoremovable protecting groups for a variety of relevant biomolecules. In particular, our previous findings in the photolytic release of butyric acid from different naphtho-oxazole, naphthopyran and oxazole fused pyran cages, revealed that these heterocyclic systems were promising mainly at short wavelengths. In order to develop a system that allows the delivery of butyric acid at longer wavelengths, heterocyclic compounds that showed interesting results with other biomolecules, as previously reported,[16,18,24] as well as new naphthopyrans methoxylated at different positions of the polycyclic ring, and the corresponding thionated derivatives were used in the caging of butyric acid. Photolytic studies of all the ester cages **7**-**15** were carried out in a Rayonet RPR-100 reactor at 254, 300, 350 and 419 nm in mixtures of methanol or acetonitrile with aqueous HEPES buffer in 80:20 solutions, with collection of kinetic data. The course of the photocleavage reaction was followed by reverse phase HPLC with UV detection. The plots of peak area (*A*) of the starting material *versus* irradiation time were obtained for each compound, at the considered wavelengths. Peak areas were determined by HPLC, which revealed a gradual decrease with time, and were the average of three runs. The determined irradiation time represents the time necessary for the consumption of the starting materials until less than 5% of the initial area was detected (Table 2). For each compound and based on HPLC data, the plot of ln *A versus* irradiation time showed a linear correlation for the disappearance of the starting material, which suggested a first order reaction, obtained by the linear least squares methodology for a straight line. The photochemical quantum yields (Φ_{Phot}) were calculated based on half-lives ($t_{1/2}$), molar extinction coefficients (ε) and the incident photon flux (I_0), which was determined by potassium ferrioxalate actinometry.[30]

The results at various wavelengths of irradiation revealed the significant influence of the photoactive unit structure in the irradiation time (t_{irr}) necessary to

release butyric acid (Table 2). Although a study at short wavelengths (254 and 300 nm) was carried out for comprehensiveness and the results are included in Table 2, the main focus of the present work was the performance at 350 and 419 nm. At these wavelengths, considering the nitrogen heterocyclic system, namely acridine cage **8**, it was found that the release occurred with very short t_{irr} in methanol/HEPES when compared to acetonitrile/HEPES, which suggested the influence of the organic solvent character in this photoreaction. For the remaining oxygen heterocyclic conjugates, naphtho[2,1-*b*]pyrans **9**-**11**, irradiation times were comparable in both solvent systems, with compound **9** cleaving faster at 350 nm and compound **11** at 419 nm, which can be related to the position of the methoxy substituent.

However, the best results were obtained with the 3*H*-benzopyran fused julolidine **12**, at both wavelengths and solvent systems, as butyric acid was completely released within 4 to 6 min of irradiation. The bathochromic shift in the maximum absorption wavelengths related to the presence of the thiocarbonyl group in cages **13**-**15** lead to significantly shorter irradiation times at 419 nm due to an increase in the efficiency of the photolysis, in comparison to the carbonyl precursors **9**-**11**, as expected.

Additionally to monitoring the photolysis process by HPLC/UV detection, the release of butyric acid was also followed by ^{1}H NMR in an acetonitrile-d_3/D_2O (80:20) solution. Upon irradiation at 419 nm of a solution of 3*H*-benzopyran fused julolidine cage **12**, the signal due to the benzylic-type CH_2 at position 9 of the heterocyclic ring, visible at about δ 5.17 ppm gradually decreased with time. The same observation occurred with the signals related to the butyric acid in the conjugated form at about δ 2.45, 1.65 and 0.95 ppm, giving rise to a close set of signals corresponding to butyric acid in its free form at about δ 2.10, 1.55 and 0.90 ppm, respectively (Figure 1). NMR monitoring was carried out with a 1.17×10^{-2} M solution, which led to an expected increase in the photolysis time for the complete release of the molecule, when compared to the irradiation times in Table 2 obtained with dilute solutions.

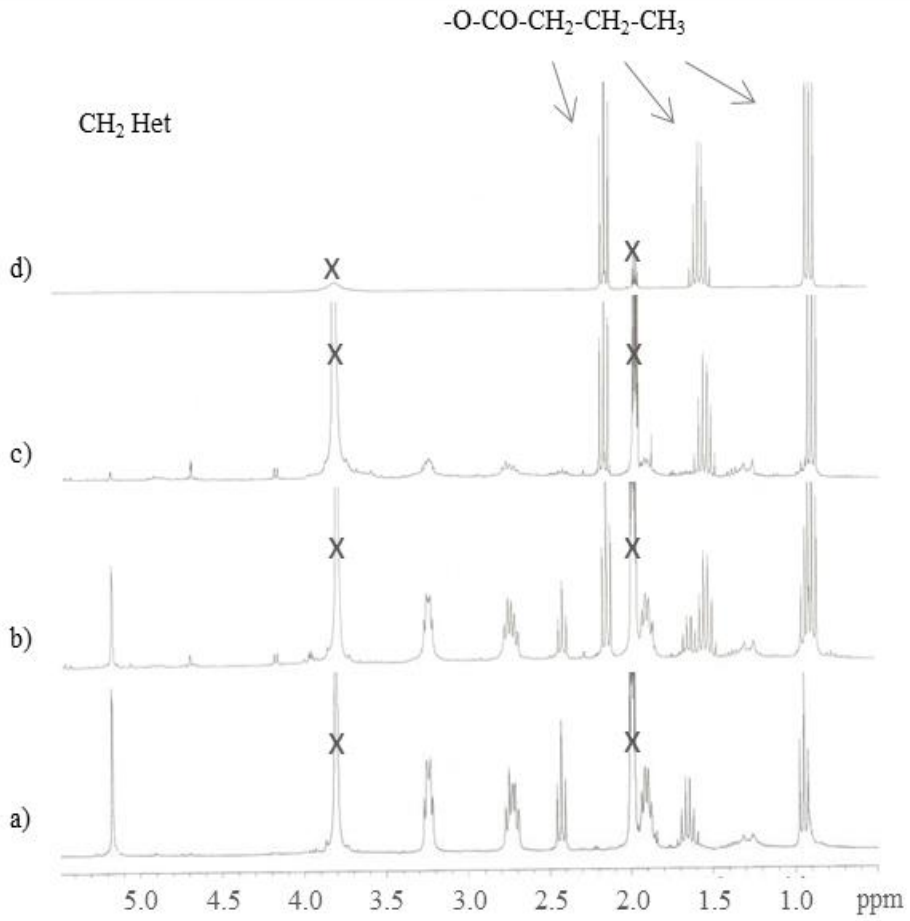


Figure 1. Partial ^{1}H NMR spectra in acetonitrile-d_3/D_2O (80:20) of the photolysis of 3*H*-benzopyran fused julolidine **12** (C = 1.17 × 10^{-2} M) at 419 nm: (a) before irradiation; (b) after irradiation for 4 h; (c) after irradiation for 10 h; (d) butyric acid.

Table 2. Irradiation times (t_{irr} in min), and photochemical quantum yields ($\Phi_{Phot} \times 10^{-3}$) for the photolysis of conjugates **7**-**15** at 254, 300, 350 and 419 nm in methanol/HEPES buffer (80:20) and acetonitrile/HEPES buffer (80:20) solutions. [a]Data in ethanol and methanol/HEPES buffer (80:20) solution was previously reported.[20]

Cpd	Methanol/HEPES (80:20)								Acetonitrile/HEPES (80:20)							
	254 nm		300 nm		350 nm		419 nm		254 nm		300 nm		350 nm		419 nm	
	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}	t_{irr}	Φ_{Phot}
7	11	1.71	26	0.37	76	0.12	2217	0.004	14	1.49	41	0.225	106	0.085	1198	0.008
8	4	1.88	7	0.60	2	1.64	83	0.044	58	0.129	120	0.030	328	0.011	2499	0.001
9	192	0.063	230	0.027	182	0.033	5985	0.001	186	0.062	184	0.033	172	0.031	4288	0.001
10	432	0.039	546	0.015	555	0.014	3324	0.0024	457	0.025	481	0.012	493	0.012	9989	0.001
11[a]	178	0.034	253	0.011	263	0.011	490	0.006	278	0.054	294	0.024	270	0.026	520	0.013
12	6	1.41	9	0.426	6	0.79	5	0.790	5	1.87	7	0.691	4	1.04	4	1.03
13	55	0.262	178	0.039	210	0.032	142	0.047	53	0.202	129	0.044	292	0.018	193	0.029
14	212	0.040	371	0.001	526	0.007	351	0.010	153	0.042	585	0.005	308	0.010	296	0.010
15	90	0.129	184	0.029	296	0.018	98	0.129	57	0.114	111	0.020	203	0.023	76	0.040

3.4. Time-resolved studies of butyric acid conjugates 8-15

To help elucidate the photophysical processes occurring in these compounds, time-resolved fluorescence measurements were performed on samples that were freshly prepared (after an initial check of the absorption and emission steady state spectra). To facilitate future (accessible using two-photon excitation) studies, an excitation wavelength of 378 nm for the compounds in acetonitrile/HEPES buffer (80:20) solution was chosen. This wavelength is between the two longer ones employed in the photoreactor and provides an excitation into the main absorption bands for each of the compounds. Measurements were made close to the peak emission and in every case the decay was found to require the sum of three exponential components (apart from two exponentials for compound **8**). This is indicative of the presence of different excited state species and the outcome of these measurements is presented in Table 3. The behaviour seen is in keeping with our previous observations[19,20,26] and the longer-lived emission can relate to unquenched fluorophore. The shorter-lived decays are attributed to charge transfer states, caused by the presence of the conjugate upon excitation, with the possibility of the formation of an ion pair that can either recombine or progress to a cleaved pair. Thus, the contribution of each of the fluorescing components gives an insight into the species present.

Here we aim to ascertain if the fluorescence lifetime can help provide complementary information to that of the photolysis study and assume that the amount of photocleavage during the measurement is not too significant. From Table 3 it can be noted that the compound giving the highest photochemical yield (**12**) also exhibits the shortest average lifetime, showing that overall the non-radiative processes, ie charge transfer, can be higher in this compound. On the other hand, compound **8** considering the 350 nm values in Table 2, has one of the lowest photochemical yields and exhibits the longest-lived average lifetime. It is a moot point in this case whether the quantity of the longest-lived component relates to cleaved photoproduct, as we have previously shown using decay associated spectra and a kinetic measurement,[26] or can reflect on the strength of coupling to the conjugate.

Table 3. Time-resolved fluorescence decay parameters, lifetime (τ_i, in ps), and normalised pre-exponential values (α_i), calculated average lifetime and goodness of fit (χ^2), for the compounds **8**-**15** in acetonitrile/HEPES buffer (80:20). The excitation wavelength was 378 nm and the emission wavelengths (λ_{em}) are also in nm.

Compound	λ_{em}	τ_1	τ_2	τ_3	α_1	α_2	α_3	τ_{ave}	χ^2
8	410	511 ± 60	10791 ± 27	--	0.31	0.69	--	7655	0.98
9	475	113 ± 39	2631 ± 507	3591 ± 75	0.24	0.34	0.42	2416	1.14
10	460	155 ± 150	4954 ± 174	10379 ± 67	0.13	0.53	0.34	6121	1.08
11	470	65 ± 39	3522 ± 543	7208 ± 33	0.38	0.09	0.53	4142	1.09
12	470	240 ± 30	2241 ± 99	5108 ± 60	0.46	0.35	0.19	1835	1.20
13	470	216 ± 54	2991 ± 75	5831 ± 175	0.28	0.64	0.08	2434	1.16
14	470	91 ± 39	4812 ± 162	10065 ± 93	0.42	0.35	0.23	4100	1.08
15	470	118 ± 129	2445 ± 339	6893 ± 24	0.25	0.11	0.64	4701	1.10

This will be the basis of further work, although the fact that **12** exhibits a high photochemical yield, while displaying the shortest average lifetime may indicate the latter. Here the photophysics will be used to assess the effect of treatment with Lawesson's reagent (*ie* comparison of **9**, **10**, **11** with **13**, **14**, **15** respectively). Considering the data in Table 3, it does not appear that treatment dramatically affects the lifetime values, minor changes are observed and their trend between compounds similar before and after treatment with Lawesson's reagent. However there are some differences in the contribution of the lifetimes to the overall emission. Generally it would appear that the use of Lawessons´s reagent has had the desired effect of enabling longer wavelength irradiation to be employed, without significantly altering the photophysics of these compounds.

4. Conclusions

Acridine, naphtho[2,1-*b*]pyran, 3*H*-benzopyran fused julolidine and thioxo-naphtho[2,1-*b*]pyran ester cages of butyric acid were studied for the controlled delivery of the active molecule by photolysis at selected wavelengths (254, 300, 350 and 419 nm), in comparison with the well-known *o*-nitrobenzyl photolabile group. Irradiation was carried out in a Rayonet RPR-100 photochemical reactor in mixtures of organic solvents (methanol or acetonitrile) with aqueous HEPES buffer and monitored by HPLC/UV and ^{1}H NMR. Overall, the obtained results, combined with time-resolved fluorescence data, showed that for naphtho[2,1-*b*]pyrans the attachment position of the methoxy substituent as well as the presence of the thiocarbonyl group influenced the behaviour towards light of the corresponding cage. Furthermore, the release of butyric acid was faster in the case of using (acridin-9-yl)methyl, and [11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl]methyl (3*H*-benzopyran fused julolidine) groups as phototriggers.

5. Experimental

5.1. General

All melting points were measured on a Stuart SMP3 melting point apparatus. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230-240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer. UV/visible absorption spectra (200.700 nm) were obtained using a Shimadzu UV/2501PC spectrophotometer. NMR spectra were obtained on a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C using the solvent peak as internal reference at 25 °C. All chemical shifts are given in ppm using δ_H Me_4Si = 0 ppm as reference and *J* values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. Mass spectrometry analyses were performed at the "C.A.C.T.I. - Unidad de Espectrometria de Masas", at University of Vigo, Spain. Fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer. All reagents were used as received. Compounds **5**, **6** and **11** were synthesised as previously reported.[20,25,26]

5.1.1. Synthesis of 1-chloromethyl-5-methoxy-3-oxo-3H-naphtho[2,1-b]pyran (3). To a solution of 3-methoxy-2-naphthol (0.104 g, 5.97×10^{-4} mol) in 70% aqueous sulphuric acid (5 mL), ethyl 4-chloro-3-oxobutanoate (0.089 mL, 6.57×10^{-4} mol) was added. The reaction was followed by TLC (ethyl acetate/*n*-hexane, 1:4), and stirred at room temperature for 96 h. The mixture was poured into ice water and stirred for 2 h to give a fine pale precipitate. The solid was collected by filtration, washed with cold water, dried and purified by column chromatography, using ethyl acetate/ light petroleum as eluent, with mixtures of increasing polarity. Compound **3** was obtained as pale brown solid (0.012 g, 7%). Mp = 169.5-171.8 °C. TLC (ethyl acetate/*n*-hexane, 1:4): R_f = 0.50. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 4.03 (s, 3 H, OCH_3), 5.05 (s, 2 H, CH_2), 6.75 (s, 1 H, H-2), 7.36 (s, 1 H, H-6), 7.54-7.57 (m, 2 H, H-8 and H-9), 7.82-7.84 (m, 1 H, H-7), 8.30-8.32 (m, 1 H, H-10) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 45.79 (CH_2), 56.16 (OCH_3), 111.17 (C-6), 113.51

(C-4b), 117.74 (C-2), 123.69 (C-6b), 124.71 (C-10), 125.95 (C-9), 126.17 (C-8), 128.46 (C-7), 131.25 (C-6a), 146.75 (C-5), 147.21 (C-4a), 151.33 (C-1), 159.22 (C-3) ppm. IR (KBr 1%, cm^{-1}): υ = 1728, 1627, 1599, 1556, 1513, 1486, 1462, 1439, 1422, 1378, 1348, 1325, 1264, 1217, 1168, 1140, 1122, 1046, 1024, 1001, 938, 896, 866, 844, 778, 737, 704. HRMS (ESI): calcd for $C_{15}H_{12}{}^{37}ClO_3$ $[M^+ + H]$: 277.04456; found 277.04478; calcd for $C_{15}H_{12}{}^{35}ClO_3$ $[M^+ + H]$: 275.04756; found 275.04770.

5.1.2. Synthesis of 1-chloromethyl-8-methoxy-3-oxo-3H-naphtho[2,1-b]pyran (4). To a solution of 6-methoxy-2-naphthol (0.248 g, 1.42×10^{-3} mol) in 70% aqueous sulphuric acid (5 mL), ethyl 4-chloro-3-oxobutanoate (0.288 mL, 2.13×10^{-3} mol) was added. The reaction was followed by TLC (ethyl acetate/*n*-hexane, 1:4), and stirred at room temperature for 72 h. The mixture was poured intoe ice water and stirred for 2 h to give a fine pale precipitate. The solid was collected by filtration, washed with cold water, dried and purified by column chromatography, using ethyl acetate/light petroleum as eluent, with mixtures of increasing polarity. Compound **4** was obtained as pale yellow solid (0.145 g, 37%). Mp = 207.3-209.9 °C. TLC (ethyl acetate/*n*-hexane, 1:4): R_f = 0.50. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 3.97 (s, 3 H, OCH_3), 5.03 (s, 2 H, CH_2), 6.72 (s, 1 H, H-2), 7.26 (d, *J* = 2.8 Hz, 1 H, H-7), 7.36 (dd, *J* = 9.6 and 2.8 Hz, 1 H, H-9), 7.46 (d, *J* = 8.8 Hz, 1 H, H-5), 7.92 (d, *J* = 8.8 Hz, 1 H, H-6), 8.33 (d, *J* = 9.2 Hz, 1 H, H-10) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 45.84 (CH_2), 55.41 (OCH_3), 108.61 (C-7), 112.70 (C-4b), 117.51 (C-2), 118.18 (C-5), 120.09 (C-9), 123.44 (C-6b), 126.36 (C-10), 132.97 (C-6a), 133.16 (C-6), 150.98 (C-1), 153.76 (C-4a), 157.13 (C-8), 160.09 (C-3) ppm. IR (KBr 1%, cm^{-1}): υ = 1722, 1611, 1553, 1514, 1468, 1422, 1362, 1318, 1264, 1213, 1182, 1154, 1118, 1034, 1015, 998, 912, 897, 878, 858, 816, 737, 704. HRMS (ESI): calcd for $C_{15}H_{12}{}^{37}ClO_3$ $[M^+ + H]$: 277.04456; found 277.04449; calcd for $C_{15}H_{12}{}^{35}ClO_3$ $[M^+ + H]$: 275.04756; found 275.04751.

5.1.3. Synthesis of 2-nitrobenzyl butyrate (7). To a solution of butyric acid (0.263 mL, 2.88×10^{-3} mol) in dry DMF (4 mL) at 0 °C, 1-hydroxybenzotriazole (HOBt) (0.072 g, 5.33×10^{-4} mol) was added. After stirring for 10 min, *N,N'*-dicyclohexylcarbodiimide (DCC) (0.114 g, 5.52×10^{-4} mol) was added, followed by (2-nitrophenyl)methanol **1** (0.402 g, 2.62×10^{-3} mol). The reaction mixture was stirred at room temperature for 72 h and followed by TLC (ethyl acetate/light petroleum, 1:4). The solid was filtered and the

residue was evaporated under vacuum. Cold acetone was added and the dicyclohexylurea precipitate was filtered. The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using ethyl acetate/light petroleum, with mixtures of increasing polarity as eluent. Compound **7** was obtained as an orange oily solid (0.169 g, 29 %). TLC (ethyl acetate/light petroleum, 1:4): R_f = 0.88. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 0.92 (t, *J* = 7.6 Hz, 3 H, C*H*$_3$-CH$_2$-CH$_2$), 1.65 (sext, *J* = 7.2 Hz, 2 H, CH$_3$-C*H*$_2$-CH$_2$), 2.36 (t, *J* = 7.2 Hz, 2 H, CH$_3$-CH$_2$-C*H*$_2$), 5.47 (s, 2 H, CH$_2$), 7.44 (dt, *J* = 7.4 and 1.6 Hz, 1 H, H-4), 7.55 (dd, *J* = 7.6 and 0.8 Hz, 1 H, H-6), 7.62 (dt, *J* = 7.4 and 0.8 Hz, 1 H, H-5), 8.02 (dd, *J* = 8.0 and 0.8 Hz, 1 H, H-3) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.43 (*C*H$_3$-CH$_2$-CH$_2$), 18.19 (CH$_3$-*C*H$_2$-CH$_2$), 35.80 (CH$_3$-CH$_2$-*C*H$_2$), 62.54 (CH$_2$), 124.79 (C-3), 128.56 (C-4), 128.84 (C-6), 132.06 (C-1), 133.54 (C-5), 147.39 (C-2), 172.80 (C=O) ppm. IR (KBr 1%, cm^{-1}): υ = 2964, 2931, 2851, 1711, 1613, 1574, 1525, 1476, 1445, 1434, 1366, 1338, 1305, 1251, 1187, 1144, 1085, 1037, 989, 858, 792, 726. HRMS (ESI): calcd for $C_{11}H_{14}NO_4$ [M$^+$+H]: 224.09232; found 224.09240.

5.1.4. Synthesis of (acridin-9-yl)methyl butyrate (8). To a solution of 9-(bromomethyl)acridine **2** (0.038 g, 1.39 × 10^{-4} mol) in dry DMF (3 mL) potassium fluoride (0.073 g, 4.17 × 10^{-4} mol) and butyric acid (0.014 mL, 1.52 × 10^{-4} mol) were added. The reaction was followed by TLC (ethyl acetate/light petroleum, 1:1), and stirred at room temperature for 15 h. The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using ethyl acetate/light petroleum, mixtures of increasing polarity as eluent. Compound **8** was obtained as a brown oily solid (0.027 g, 69%). TLC (ethyl acetate/light petroleum, 1:1): R_f = 0.48. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 0.89 (t, *J* = 7.2 Hz, 3 H, C*H*$_3$-CH$_2$-CH$_2$), 1.63 (sext, *J* = 7.2 Hz, 2 H, CH$_3$-C*H*$_2$-CH$_2$), 2.32 (t, *J* = 7.2 Hz, 2 H, CH$_3$-CH$_2$-C*H*$_2$), 6.12 (s, 2 H, CH$_2$), 7.62 (dt, *J* = 7.6 and 1.2 Hz, 2 H, H-2 and H-7), 7.79 (dt, *J* = 7.8 and 1.2 Hz, 2 H, H-3 and H-6), 8.28 (d, *J* = 8.6 Hz, 2 H, H-4 and H-5), 8.34 (d, *J* = 9.0 Hz, 2 H, H-1 and H-8) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.54 (*C*H$_3$-CH$_2$-CH$_2$), 18.34 (CH$_3$-*C*H$_2$-CH$_2$), 35.96 (CH$_3$-CH$_2$-*C*H$_2$), 57.31 (CH$_2$), 124.03 (C-1 and C-8), 125.32 (C-8a and C-9a), 126.68 (C-2 and C-7), 129.97 (C-4 and C-5), 130.08 (C-3 and C-6), 137.53 (C-9), 148.46 (C-4a and C-4b), 173.42 (C=O) ppm. IR (KBr 1%, cm^{-1}): υ = 3067, 2965, 2934, 2875, 1737, 1692, 1629, 1603, 1557, 1519,

1498, 1461, 1441, 1416, 1382, 1352, 1303, 1286, 1249, 1165, 1100, 1058, 1040, 1018, 976, 911, 862, 753, 733, 643. HRMS (ESI): calcd for $C_{18}H_{18}NO_2$ [M^++H]: 280.13384; found 280.13403.

5.1.5.Synthesis of (5-methoxy-3-oxo-3H-naphtho[2,1-b]pyran-1-yl)methyl butyrate (9). To a solution of 1-chloromethyl-5-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran **3** (0.100 g, 3.64×10^{-4} mol) in dry DMF (4 mL), potassium fluoride (0.063 g, 1.09×10^{-3} mol) and butyric acid (0.033 mL, 3.64×10^{-4} mol) were added. The reaction was followed by TLC (ethyl acetate/light petroleum, 1:4), and stirred at room temperature for 42 h. The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using mixtures of ethyl acetate/light petroleum as eluent. Compound **9** was obtained as an oily solid (0.083 g, 70%). TLC (ethyl acetate/light petroleum, 1:4): R_f = 0.22. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.01 (t, *J* = 7.2 Hz, 3 H, C*H*$_3$-CH$_2$-CH$_2$), 1.70-1.80 (m, 2 H, CH$_3$-C*H*$_2$-CH$_2$), 2.48 (t, *J* = 7.2 Hz, 2 H, CH$_3$-CH$_2$-C*H*$_2$), 4.04 (s, 3H, OCH$_3$), 5.64 (d, *J* = 1.2 Hz, 2 H, CH$_2$), 6.70 (t, *J* = 1.2 Hz, 1 H, H-2), 7.30 (s, 1 H, H-6), 7.47-7.53 (m, 2 H, H-8 and H-9), 7.76-7.81 (m, 1 H, H-10), 7.95-8.10 (m, 1 H, H-7) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.62 (*C*H$_3$-CH$_2$-CH$_2$), 18.32 (CH$_3$-*C*H$_2$-CH$_2$), 35.95 (CH$_3$-CH$_2$-*C*H$_2$), 56.06 (OCH$_3$), 63.94 (CH$_2$), 110.81 (C-6), 113.14 (C-2), 113.43 (C-4b), 123.94 (C-6b), 124.40 (C-7), 125.89 (C-8), 125.98 (C-9), 128.42 (C-10), 131.09 (C-6a), 146.65 (C-4a), 146.81 (C-5), 151.46 (C-1), 159.43 (C-3), 172.65 (C=O) ppm. IR (KBr 1%, cm^{-1}): υ = 3426, 3060, 2964, 2878, 2836, 1733, 1627, 1599, 1559, 1513, 1460, 1425, 1346, 1322, 1264, 1147, 1121, 1085, 1005, 932, 864, 835, 780, 738, 703. HRMS (ESI): calcd for $C_{19}H_{19}O_5$ [M^++H]: 327.12327; found 327.12356.

5.1.6. Synthesis of (8-methoxy-3-oxo-3H-naphtho[2,1-b]pyran-1-yl)methyl butyrate (10). To a solution of 1-chloromethyl-8-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran **4** (0.305 g 1.11×10^{-3} mol) in dry DMF (5 mL), potassium fluoride (0.193 g, 3.33×10^{-3} mol) and butyric acid (0.101 mL, 1.11×10^{-3} mol) were added. The reaction was followed by TLC (ethyl acetate/light petroleum, 1:4), and stirred at room temperature for 44 h. The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using mixtures of ethyl acetate/light petroleum as eluent. Compound **10** was obtained as oil (0.257 g, 71%). TLC

(ethyl acetate/light petroleum, 1:4): R_f = 0.28. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 0.99 (t, *J* = 7.2 Hz, 3 H, C*H*$_3$-CH$_2$-CH$_2$), 1.69-1.78 (m, 2 H, CH$_3$-C*H*$_2$-CH$_2$), 2.45 (t, *J* = 7.2 Hz, 2 H, CH$_3$-CH$_2$-C*H*$_2$), 3.90 (s, 3H, OCH$_3$), 5.52 (d, *J* = 1.2 Hz, 2 H, CH$_2$), 6.58 (t, *J* = 1.2 Hz, 1H, H-2), 7.14 (d, *J* = 2.4 Hz, 1 H, H-7), 7.22 (dd, *J* = 9.2 and 2.8 Hz, 1 H, H-9), 7.31 (d, *J* = 9.2 Hz, 1 H, H-10), 7.78 (d, *J* = 8.8 Hz, 1 H, H-5), 7.87 (d, *J* = 9.6 Hz, 1H, H-6) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.54 (*C*H$_3$-CH$_2$-CH$_2$), 18.22 (CH$_3$-*C*H$_2$-CH$_2$), 35.82 (CH$_3$-CH$_2$-*C*H$_2$), 55.22 (OCH$_3$), 63.78 (CH$_2$), 108.43 (C-7), 112.43 (C-4b), 112.72 (C-2), 117.89 (C-10), 119.86 (C-9), 123.50 (C-6b), 125.86 (C-6), 132.62 (C-5), 132.66 (C-1), 150.91 (C-4a), 153.14 (C-6a), 156.82 (C-8), 160.05 (C-3), 172.52 (C=O) ppm. IR (neat, cm^{-1}): υ = 3425, 2965, 2877, 2840, 1725, 1614, 1556, 1519, 1468, 1364, 1308, 1249, 1205, 1170, 1122, 1086, 1035, 999, 916, 857, 812, 734, 700, 675. HRMS (ESI): calcd for $C_{19}H_{19}O_5$ [M$^+$+H]: 327.12327; found 327.12319.

5.1.7.Synthesis of (11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl)methyl butyrate (12). To a solution of 9-(chloromethyl)-2,3,6,7-tetrahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-11(5*H*)-one **6** (0.042 mg, 1.45 × 10^{-4} mol) in dry DMF (4 mL), potassium fluoride (0.025 g, 4.35 × 10^{-4} mol) and butyric acid (0.014 mL, 1.59 × 10^{-4} mol) were added. The reaction was followed by TLC (ethyl acetate/light petroleum, 1:4), and stirred at room temperature for 14 h. The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using mixtures of ethyl acetate/light petroleum as eluent. Compound **12** was obtained as oil (0.017 g, 34 %). TLC (ethyl acetate/light petroleum, 1:4): R_f = 0.58. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 0.99 (t, *J* = 7.2 Hz, 3 H, C*H*$_3$-CH$_2$-CH$_2$), 1.73 (sext, *J* = 7.2 Hz, 2 H, CH$_3$-C*H*$_2$-CH$_2$), 1.91-2.03 (m, 4H, H-2 and H-6), 2.42 (t, *J* = 7.2 Hz, 2 H, CH$_3$-CH$_2$-C*H*$_2$), 2.76 (t, *J* = 6.4 Hz, 2 H, H-7), 2.89 (t, *J* = 6.4 Hz, 2 H, H-1), 3.18-3.33 (m, 4 H, H-3 and H-5), 5.20 (s, 2 H, CH$_2$), 6.10 (s, 1 H, H-10), 6.88 (s, 1 H, H-8) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.66 (*C*H$_3$-CH$_2$-CH$_2$), 18.37 (CH$_3$-*C*H$_2$-CH$_2$), 20.39 (C-2), 20.55 (C-1), 21.45 (C-6), 27.67 (C-7), 36.00 (CH$_3$-CH$_2$-*C*H$_2$), 49.48 (C-3), 49.88 (C-5), 61.17 (CH$_2$), 105.49 (C-10), 105.80 (C-8a), 106.96 (C-12b), 118.20 (C-7a), 120.40 (C-8), 145.95 (C-7b), 149.68 (C-9), 151.17 (C-12a), 162.26 (C-11), 172.93 (C=O) ppm. IR (neat, cm^{-1}): υ = 2958, 2933, 2873, 2856, 1720, 1605, 1558, 1522, 1437, 1382, 1343, 1312,

1203, 1174, 1124, 1075, 1019, 884, 851, 753, 738, 701. HRMS (ESI): calcd for $C_{20}H_{24}NO_4$ [M^++H]: 342.17062; found 342.17031.

5.1.8. Synthesis of (5-methoxy-3-thioxo-3H-naphtho[2,1-b]pyran-1-yl)methyl butyrate (13). Lawesson's reagent (0.268 g, 6.62 × 10^{-4} mol) was added to a solution of (5-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methyl butyrate **9** (0.072 g, 2.20 × 10^{-4} mol) in toluene (8 mL). The reaction mixture was refluxed for 9 h and the process was followed by TLC (ethyl acetate/light petroleum, 1:4). The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using dichlorometane/light petroleum mixtures of increasing polarity as eluent. Compound **13** was obtained as oil (0.004 g, 5 %). TLC (ethyl acetate/light petroleum, 1:4): R_f = 0.71. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.02 (t, *J* = 7.2 Hz, 3 H, C*H*$_3$-CH_2-CH_2), 1.70-1.81 (m, 2 H, CH_3-C*H*$_2$-CH_2), 2.50 (t, *J* = 7.2 Hz, 2 H, CH_3-CH_2-C*H*$_2$), 4.10 (s, 3 H, OCH_3), 5.66 (d, *J* = 1.2 Hz, 2 H, CH_2), 7.39 (s, 1 H, H-6), 7.52-7.62 (m, 3 H, H-2, H-8 and H-9), 7.81-7.86 (s, 1 H, H-7), 8.03-8.10 (m, 1 H, H-10) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 13.66 (*C*H_3-CH_2-CH_2), 18.39 (CH_3-*C*H_2-CH_2), 35.99 (CH_3-CH_2-*C*H_2), 56.24 (OCH_3), 63.76 (CH_2), 111.27 (C-6), 116.07 (C-4b), 123.76 (C-6b), 125.02 (C-10), 126.24 (C-9), 126.73 (C-8), 127.80 (C-2), 128.57 (C-7), 131.63 (C-6a), 142.49 (C-1), 146.33 (C-5), 150.29 (C-4a), 172.78 (C=O), 194.49 (C-3) ppm. IR (neat, cm^{-1}): υ = 2964, 2932, 2875, 1738, 1623, 1593, 1545, 1513, 1460, 1425, 1323, 1297, 1256, 1233, 1218, 1164, 1125, 1104, 1004, 913, 866, 835, 778, 739. HRMS (ESI): calcd for $C_{19}H_{19}O_4S$ [M^++H]: 343.10047; found 343.10079.

5.1.9.Synthesis of (8-methoxy-3-thioxo-3H-naphtho[2,1-b]pyran-1-yl)methyl butyrate (14). Lawesson's reagent (0.383 g, 9.47 × 10^{-4} mol) was added to a solution of (8-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methyl butyrate **10** (0.103 g, 3.16 × 10^{-4} mol) in toluene (8 mL). The reaction mixture was refluxed for 23 h and followed by TLC (ethyl acetate/light petroleum, 1:4). The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using ethyl acetate/light petroleum mixtures of increasing polarity as eluent. Compound **14** was obtained as an orange solid (0.087 g, 80%). Mp = 144.9-146.5 °C. TLC (ethyl acetate/light petroleum, 1:4): R_f = 0.64. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.02 (t, *J* = 7.2

Hz, 3 H, C*H*$_3$-CH$_2$-CH$_2$), 1.70-1.80 (m, 2 H, CH$_3$-C*H*$_2$-CH$_2$), 2.49 (t, *J* = 7.2 Hz, 2 H, CH$_3$-CH$_2$-C*H*$_2$), 3.96 (s, 3 H, OCH$_3$), 5.57 (d, *J* = 0.8 Hz, 2 H, CH$_2$), 7.24 (d, *J* = 2.8 Hz, 1 H, H-7), 7.29-7.35 (m, 1 H, H-9), 7.51 (s, 1 H, H-2), 7.55 (d, *J* = 9.2 Hz, 1 H, H-10), 7.91 (d, *J* = 9.2 Hz, 1 H, H-5), 8.02 (d, *J* = 9.6 Hz, 1 H, H-6) ppm. ^{13}C NMR (CDCl$_3$, 100.6 MHz): δ_C = 13.63 (*C*H$_3$-CH$_2$-CH$_2$), 18.34 (CH$_3$-*C*H$_2$-CH$_2$), 35.93 (CH$_3$-CH$_2$-*C*H$_2$), 55.43 (OCH$_3$), 63.66 (CH$_2$), 108.83 (C-7), 115.09 (C-4b), 117.77 (C-10), 120.24 (C-9), 123.26 (C-6b), 126.60 (C-6), 127.22 (C-2), 133.23 (C-6a), 133.39 (C-5), 142.29 (C-1), 156.87 (C-4a), 157.56 (C-8), 172.71 (C=O), 195.02 (C-3) ppm. IR (KBr 1%, cm^{-1}): υ = 3061, 2961, 2877, 1903, 1743, 1609, 1542, 1524, 1468, 1454, 1426, 1367, 1292, 1266, 1208, 1163, 1139, 1097, 1035, 978, 936, 908, 858, 810, 736 cm^{-1}. HRMS (ESI): calcd for $C_{19}H_{19}O_4S$ [M$^+$+H]: 343.10047; found 343.10096.

5.1.10. Synthesis of (9-methoxy-3-thioxo-3H-naphtho[2,1-b]pyran-1-yl)methyl butyrate (15). Lawesson's reagent (0.166 g, 4.11 × 10^{-4} mol) was added to a solution of (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl)methyl butyrate, **11** (0.067g, 2.05 × 10^{-4} mol) in toluene (5 mL). The reaction mixture was heated at reflux for 48 h and the process was followed by TLC (ethyl acetate/ light petroleum, 1:4). The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using ethyl acetate/light petroleum, with mixtures of increasing polarity as eluent. Compound **15** was obtained as an orange solid (0.049 g, 70%). Mp = 132.5-134.7 °C. TLC (ethyl acetate/light petroleum, 1:4): R_f = 0.86. ^{1}H NMR (CDCl$_3$, 400 MHz): δ_H = 1.01 (t, *J* = 7.2 Hz, 3 H, C*H*$_3$-CH$_2$-CH$_2$), 1.75 (sext, *J* = 7.2 Hz, 2 H, CH$_3$-C*H*$_2$-CH$_2$), 2.49 (t, *J* = 7.6 Hz, 2 H, CH$_3$-CH$_2$-C*H*$_2$), 3.97 (s, 3 H, OCH$_3$), 5.60 (s, 2 H, CH$_2$), 7.24 (dd, *J* = 8.8 and 2.4 Hz, 1 H, H-8), 7.43 (d, *J* = 9.2 Hz, 1 H, H-5), 7.48 (d, *J* = 2.4 Hz, 1 H, H-10), 7.49 (s, 1 H, H-2), 7.83 (d, *J* = 8.8 Hz, 1 H, H-7), 7.93 (d, *J* = 8.8 Hz, 1 H, H-6) ppm. ^{13}C NMR (CDCl$_3$, 100.6 MHz): δ_C = 13.63 (*C*H$_3$-CH$_2$-CH$_2$), 18.28 (CH$_3$-*C*H$_2$-CH$_2$), 35.92 (CH$_3$-CH$_2$-*C*H$_2$), 55.47 (OCH$_3$), 63.69 (CH$_2$), 106.25 (C-10), 114.30 (C-4b), 114.90 (C-5), 117.22 (C-8), 126.56 (C-6a), 126.97 (C-2), 130.29 (C-6b), 131.38 (C-7), 134.25 (C-6), 142.39 (C-1), 158.69 (C-4a), 159.86 (C-9), 172.76 (C=O), 195.27 (C-3) ppm. IR (KBr 1%, cm^{-1}): υ= 3074, 2964, 2935, 2875, 2836, 1742, 1623, 1606, 1592, 1538, 1485, 1461, 1443, 1431, 1364, 1296, 1229, 1216, 1163, 1139, 1096, 1056, 1025, 1008, 982, 917, 870, 838, 803, 706 cm^{-1}. HRMS (ESI): calcd for $C_{19}H_{19}O_4S$ [M$^+$+H]: 343.10047; found 343.10074.

5.2. Photolysis general

A 1×10^{-4} M methanol or acetonitrile/HEPES (80:20) solution of compounds **7**-**15** (5 mL) were placed in a quartz tube and irradiated in a Rayonet RPR-100 reactor at the desired wavelength. The lamps used for irradiation were of 254, 300, 350 and 419 ± 10 nm. HEPES buffer solution was prepared in distilled water with HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (10 mM), sodium chloride (120 mM), potassium chloride (3 mM), calcium chloride (1 mM) and magnesium chloride (1mM) and pH adjusted to 7.2 with aqueous 1 M sodium hydroxide solution.

Aliquots of 100 µL were taken at regular intervals and analysed by RP-HPLC using a Licrospher 100 RP18 (5 µm) column in a JASCO HPLC system composed by a PU-2080 pump and a UV-2070 detector with ChromNav software. The eluent was acetonitrile/water, 75:25 at a flow rate of 0.8 mL/min, previously filtered through a Millipore, type HN 0.45 µm filter and degassed by ultra-sound for 30 min. The chromatograms were traced by detecting UV absorption at the wavelength of maximum absorption for each compound (retention time: **7**, 5.2; **8**, 8.7; **9**, 6.1; **10**, 6.6; **11**, 6.5; **12**, 8.4; **13**, 9.4; **14**, 10.4; **15**, 8.5 min).

5.3. Time-resolved fluorescence measurements

Time-resolved fluorescence measurements made use of time-correlated single-photon counting (TCSPC) technique and were performed using a HORIBA Scientific DeltaFlex, equipped with aDeltaDiode laser excitation source emitting at 378 nm and a PPD-850 detector. Time-resolved emission spectral (TRES) data were acquired by stepping the emission monochromator through 5 nm increments and measuring the decay for equal time periods. Analysis of each dataset was performed using DAS6 software and the decays reconvoluted with the instrumental response and fitted globally (producing common decay times, τ_i) to the sum of exponentials (equation 1).

$$I(t) = \sum_{i=1}^{n} \alpha_i exp^{-t/\tau_i} \qquad (1)$$

The average lifetime was obtained from

$$\tau_{ave} = \Sigma \alpha_i \tau_i \quad (2)$$

The goodness of fit was judged in terms of a chi-squared value and weighted residuals.

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7. References

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2.7

Wavelength-selective cleavage of *o*-nitrobenzyl and polyheteroaromatic benzyl protecting groups

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INDEX

1. Abstract

Evaluation of the wavelength-selective cleavage of five photolabile protecting groups from two different families has been performed. Alanine, as a model bifunctional target molecule was masked at the amine terminal with *o*-nitrobenzyl group and at the carboxylic terminal with benzyl-type nitrogen and oxygen polyheteroaromatics, namely acridine, (thioxo)benzocoumarin and a coumarin built on the julolidine nucleus. The photosensitivity of the corresponding alanine conjugates was studied at selected wavelengths with HPLC/UV and ^{1}H NMR monitoring. The release of the fully deprotected molecule could be achieved by sequential irradiation in variable irradiation times, which were dependent on the heteroaromatic group used.

2. Introduction

Photoremovable protecting groups (PPGs) defined as systems that contain a chromophore which is sensitive to light, but relatively stable to most chemical reagents, have been used to mask various functional groups. Their importance has been proved by their potential applications in synthetic organic chemistry,[1-3] biochemistry,[4-6] and materials science.[4,7]

In the last two decades, the intense development of fluorescence techniques along with fluorescent reagents and strategies for various purposes, including bioapplications, has also evolved the research concerning PPGs to include fluorescent molecules. Fluorescent photoremovable protecting groups present advantages over non-fluorescent groups, since in addition to releasing molecules of interest at desired location for a specific period of time, they also allow the visualization, quantification and monitoring of the spatial distribution, localization, and depletion of the released molecules.[8] The above strategy of using fluorescent PPGs has been successfully employed on the temporally and spatially controlled delivery of bioactive molecules in the study of numerous processes in biological and medical research fields. Also, in organic synthetic methodologies, the use of fluorescent PPGs is important, since it can facilitate the following up of experimental procedures involved in synthesis and deprotection reaction steps.

Among the fluorescent PPGs, polyaromatics including (anthracen-9-yl)methyl,[9] (pyren-1-yl)methyl,[10-12] (perylen-3-yl)methyl,[13] and (phenanthren-9-yl)methyl[10] have been introduced. Heteroaromatics that can also be considered analogues of the benzyl protecting group have emerged in the last years, following the report on (coumarin-4-yl)methyl group photosensitivity in the release of phosphate ester by Givens in 1984,[14] and includes quinolines,[15,16] quinolones,[17,18] thiocoumarins and thioquinolones,[18] fused oxazoles,[19,20] and acridine.[21,22]

Structural modifications at the coumarin ring by the introduction of substituents, particularly at C-6 and C-7 resulted in an improvement of photochemical and photophysical properties increasing the range of applications of this class of protecting groups.[23] An extension of the aromatic ring of coumarins, resulting in polyaromatic analogues has led to the enhancement of fluorophoric properties.[24-27] Also the exchange of the carbonyl function to thiocarbonyl affording the corresponding thioxo(benzo)coumarin results in an increase in the photosensitivity.[18,28]

The advantages of fluorescence protecting groups mentioned do not diminish the importance of the remaining groups, including the most well-known the *o*-nitrobenzyl group and its derivatives, which have been widely used in the caging of different molecules.[3]

The variety of PPGs, working by different mechanisms and possessing different types of chromophores/fluorophores, allows the possibility for wavelength-selective deprotection, a type of orthogonality referred to as chromatic orthogonality. This concept has been considered in certain research works, including in peptide solid phase synthesis with selective release using the nitroveratryl/pivaloylglycol pair.[29,30] Also, in bioapplications, some examples have been reported, involving amino acids,[31,32] peptides[33] and (poli)nucleotides,[34,35] using different pairs of protecting groups, such as coumarins, nitrophenethyl, 6-nitroveratryl and *p*-methoxy-phenacyl groups. These studies involved photolysis of mixtures of the caged molecules bearing in each case only one photolabile protecting group, whereas a single report[33] refers to the use of two different groups in the same molecule.

Considering these facts and taking advantage of our knowledge in fluorescent PPGs, especially benzyl-based heteroaromatic groups, the present work aims to evaluate the possibility of wavelength-selective photolysis between four heteroaromatic

photolabile protecting groups, previously introduced by our research team, and *o*-nitrobenzyl group. Given our promising earlier findings with (acridin-9-yl)methyl, (9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methyl (trivially named benzocoumarin), [11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl]methyl (commonly called coumarin, based on the julolidine nucleus), and (9-methoxy-3-thioxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methyl (trivially designated (thioxo)benzocoumarin) groups, these systems were selected for the present study. Alanine was used as model of a bifunctionalised molecule, possessing different functional groups, an amine and a carboxylic acid. Conjugates were prepared bearing the *o*-nitrobenzyl and the heterocyclic group as protections of the *N*- and *C-terminus* through carbamate and ester linkages, respectively. Photolysis of these conjugates at selected wavelengths was carried out and the process course was monitored by HPLC/UV and ^{1}H NMR.

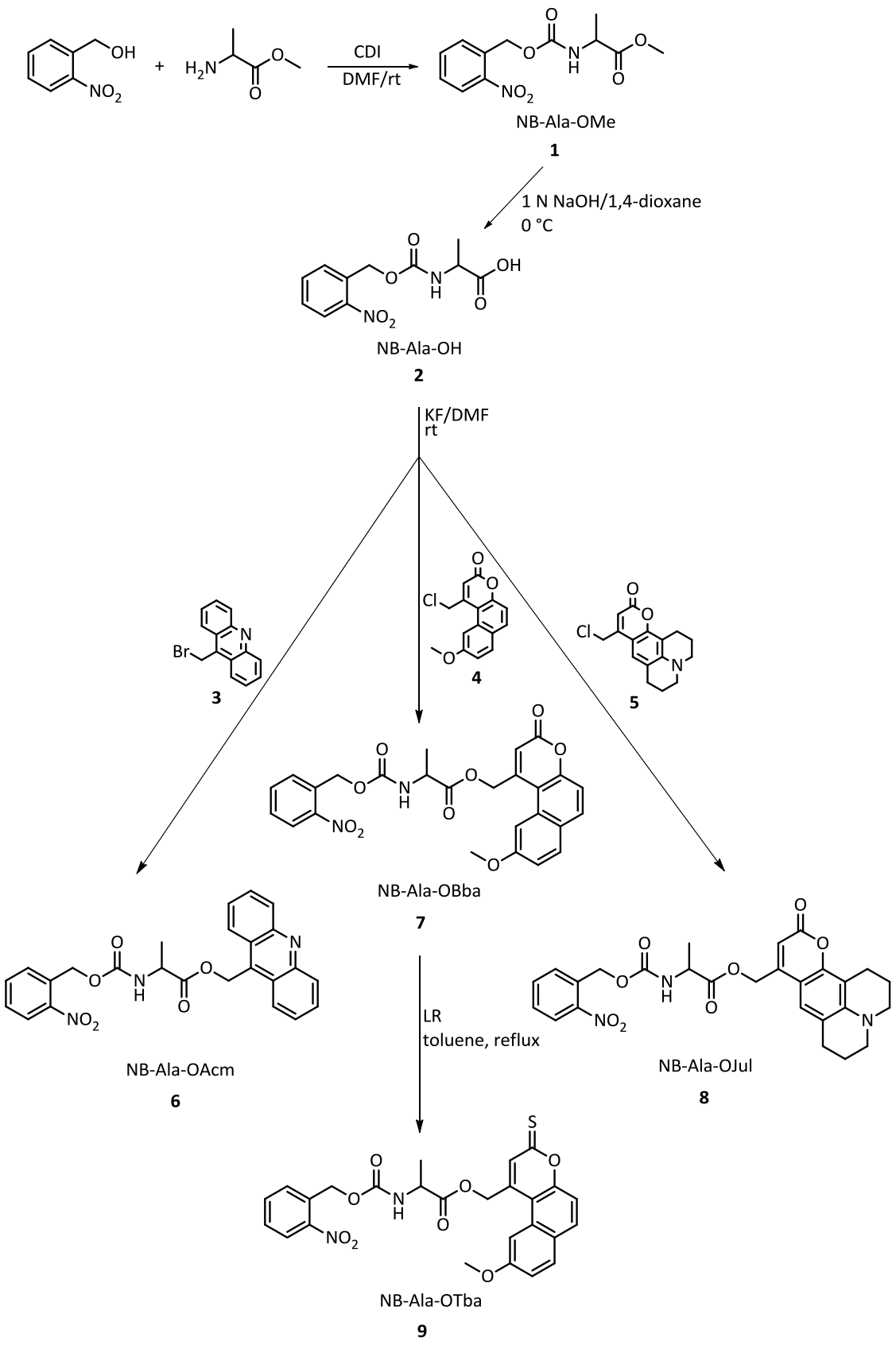
3. Results and discussion

3.1. Synthesis of alanine conjugates 1,2 and 6-9

(2-Nitrophenyl)methanol was reacted with L-alanine methyl ester via a *N*,*N*´-carbonyldiimidazole (CDI) mediated carbonyl transfer reaction in *N*,*N*-dimethylformamide, at room temperature to give *N*-(2-nitrobenzyloxycarbonyl)-L-alanine methyl ester **1**. Subsequent basic hydrolysis of the methyl ester with aqueous sodium hydroxide in 1,4-dioxane, at low temperature gave *N*-(2-nitrobenzyloxycarbonyl)-L-alanine **2**. This carbamate conjugate was used in the reaction with bromo- or chloromethylated heterocycles, namely 9-(bromomethyl)acridine **3**, 1-chloromethyl-9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran[36] **4** and 9-(chloromethyl)-2,3,6,7-tetrahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-11(5*H*)-one[37] **5** (trivially named 8-hydroxyjulolidine), in the presence of potassium fluoride in *N*,*N*-dimethylformamide, at room temperature (Scheme 1, Table 1),[38] to afford the corresponding conjugates **6**-**8**. In order to exchange the carbonyl by a thiocarbonyl group at the naphtho[2,1-*b*]pyran conjugate **7**, this compound was reacted with Lawesson's reagent in toluene, under reflux conditions,[39] yielding the corresponding thionaphthopyran conjugate **9** (Scheme 1, Table 1).

For simplicity of naming the various conjugates in this report, the photocleavable protecting groups will be designated by a two- or three-letter code, as follows: 2-nitrobenzyloxycarbonyl (NB), (acridin-9-yl)methylene (Acm), (9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methylene (Bba), 11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl)methylene (Jul) and (9-methoxy-3-thioxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)methylene (Tba), as indicated in Schemes 1 and 2 and Tables 1 and 2.

All new compounds were fully characterised by high resolution mass spectrometry, IR, ^{1}H and ^{13}C NMR spectroscopy. ^{1}H NMR spectra showed signals of the alanine residue, such as the α-CH (δ 3.50-4.60 ppm) and β-CH_3 (δ 1.17-1.54 ppm). The 2-nitrobenzyloxycarbonyl (conjugates **1**, **2**, **6**-**9**) and heterocycle (conjugates **6**-**9**) methylene groups were also visible (δ 5.32-5.60 ppm, and δ 5.17-6.20 ppm, respectively). The confirmation of the presence of the carbamate bond linking the *N*-terminal of alanine to 2-nitrobenzyl group, as well as the ester linkage between the *C*-terminal of *N*-(2-nitrobenzyloxycarbonyl)-L-alanine and the heterocyclic moieties were also supported by ^{13}C NMR spectra signals at δ 154.46-155.55 ppm, and δ 172.17-172.69 ppm, respectively.



Scheme 1. Synthesis of photosensitive conjugates **2** and **6**-**9**.

Table 1. Yields, UV/vis and fluorescence data for conjugates **2** and **6**-**9** in absolute ethanol and methanol/HEPES buffer (80:20) solution.

	Compound	Yield	Ethanol					Methanol/HEPES (80:20)				
		(%)	λ_{abs} (nm)	log ε	λ_{em} (nm)	Φ_F	$\Delta\lambda$ (nm)	λ_{abs} (nm)	log ε	λ_{em} (nm)	Φ_F	$\Delta\lambda$ (nm)
2	NB-Ala-OH	92	257	3.91	304	0.064	47	263	3.78	304	0.073	41
6	NB-Ala-OAcm	32	254	5.61				254	5.42			
			354	4.46	412	0.113	58	362	4.60	413	0.157	51
7	NB-Ala-OBba	55	259	4.65				257	4.96			
			347	4.59	466	0.439	119	350	4.92	480	0.222	130
8	NB-Ala-OJul	44	254	4.68				259	4.66			
			395	4.50	486	0.184	91	403	4.67	496	0.207	93
9	NB-Ala-OTba	28	250	4.48				261	4.65			
			307	3.98				309	4.11			
			400	3.96	475	0.012	75	414	4.09	480	0.003	66

In order to evaluate the possibility of wavelength-selective photolysis and to obtain the parameters required for monitoring the processes, fundamental UV/vis photophysical characterisation was carried out. The UV/vis absorption and emission spectra of degassed 10^{-5} or 10^{-6} M solutions in absolute ethanol and in a methanol/HEPES buffer (80:20) solution of conjugates **2** and **6**-**9** were measured; absorption and emission maxima, molar extinction coefficients and relative fluorescence quantum yields are also reported (Table 1). Relative fluorescence quantum yields were calculated using naphthalene (Φ_F = 0.23 in cyclohexane),[40] 9,10-diphenylanthracene (Φ_F = 0.95 in ethanol),[41] or a 0.05 M solution of quinine in sulfuric acid (Φ_F = 0.54)[42] as standards. For the determination of Φ_F, the fluorescence standard was excited at the wavelengths of maximum absorption found for each one of the compounds to be tested and in all fluorimetric measurements the absorbance of the solution did not exceed 0.1.

Regarding the absorption data of conjugates **6**-**9**, in both solvents, it was found that all conjugates displayed two maxima absorption wavelengths (λ_{abs}), one of them at about 260 nm, which is related to the presence of 2-nitrobenzyloxycarbonyl group, as it was confirmed by the spectra of *N*-(2-nitrobenzyloxycarbonyl)-L-alanine **2** (λ_{abs} about 260 nm), and the other associated to the heterocycle moiety, which differs accordingly to its structure. Conjugate **6** bearing the nitrogen heterocycle acridine displayed absorption maxima at about 360 nm in both solvents. Concerning conjugate **7**, possessing the naphtho[2,1-*b*]pyran skeleton showed the lowest value of the set (λ_{abs} about 347 nm), but the replacement of the carbonyl group in the pyran ring by a thiocarbonyl (conjugate **9**) resulted in a bathochromic shift, tuning absorption maxima to values in the visible region (λ_{abs} 400 or 414 nm). In addition, it was observed that for conjugate **8** with a 3*H*-benzopyran fused julolidine, the maximum absorption wavelengths were at about 400 nm in both solvents (λ_{abs} 395 or 403 nm). For easier comparison, Figure 1 shows the UV/vis spectra of the photosensitive compounds **2**, **6**-**9** in methanol/HEPES buffer (80:20) solutions.

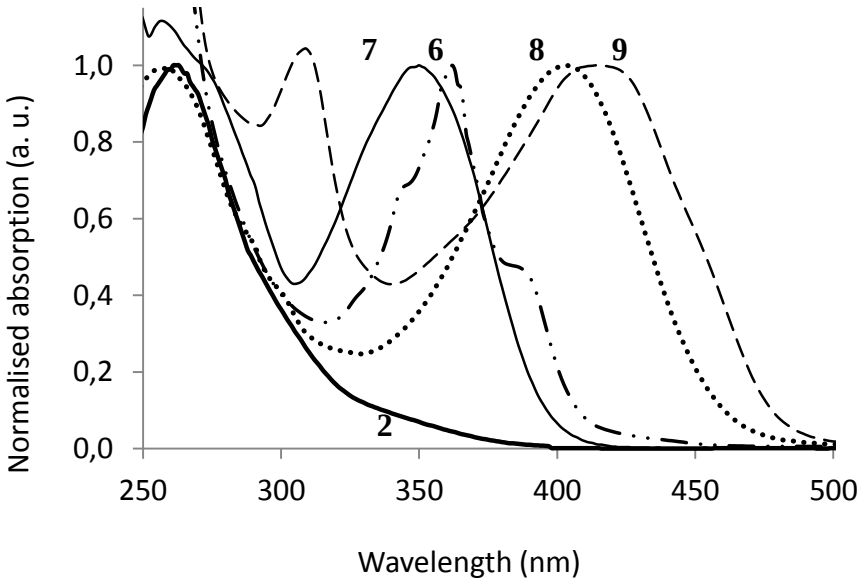


Figure 1. Normalised UV/vis absorption spectra of conjugates **2** and **6**-**9** in methanol/HEPES buffer (80:20).

Concerning the fluorescence spectra, in both tested solvents, it was observed that emission maxima (λ_{em}) of conjugates **6**-**9** occurred in the range (412-496 nm), being the conjugate possessing 3*H*-benzopyran fused julolidine **8** associated to the longer wavelengths, and the *N*-(2-nitrobenzyloxycarbonyl)-L-alanine **2** displaying the lowest value (λ_{em} at 304 nm), as expected due to its less conjugated structure. As for the fluorescence quantum yields, naphtho[2,1-*b*]pyran **7** was the most emissive (Φ_F 0.44 and 0.22, depending on the solvent). The emission of thiocarbonyl conjugate **9** was bathochromically shifted in ethanol with lower fluorescent quantum yields, in comparison with the corresponding carbonyl precursor (compound **7**). Stokes's shifts ($\Delta\lambda$) occurred in a br range (41-130 nm), being the lowest values associated to conjugate **2**, and the highest values corresponding to conjugates **7** and **8**, bearing coumarin moieties.

3.2. Photolysis studies of alanine conjugates 2 and 6-9

The possibility of selective deprotection of either *terminus* of a bifuntionalised molecule by choosing the appropriate wavelength was the main goal of the present work. As mentioned before, alanine was chosen as model target, and protected at its *N*-

terminal with the well-known *o*-nitrobenzyl group *via* a carbamate linkage, and at its *C*-terminal with benzyl-type nitrogen and oxygen polyheteroaromatics, namely acridine, (thioxo)benzocoumarin and a coumarin built on the julolidine nucleus, through an ester bond. The later groups were selected from the collection of photoremovable protecting groups developed by our research group taking into consideration the photophysical properties, namely the wavelengths of maximum absorption displayed when linked to amino acid residues or butyric acid,[17-21,24-28,36,37] and also the promising photolytic results especially at longer wavelengths, which provided potential orthogonal behaviour to the *o*-nitrobenzyl group. Nevertheless, the fundamental photophysical studies of conjugates **2** and **6**-**9** were obviously essential to ascertain the properties of the selected groups and the *o*-nitrobenzyl group together in the same molecule.

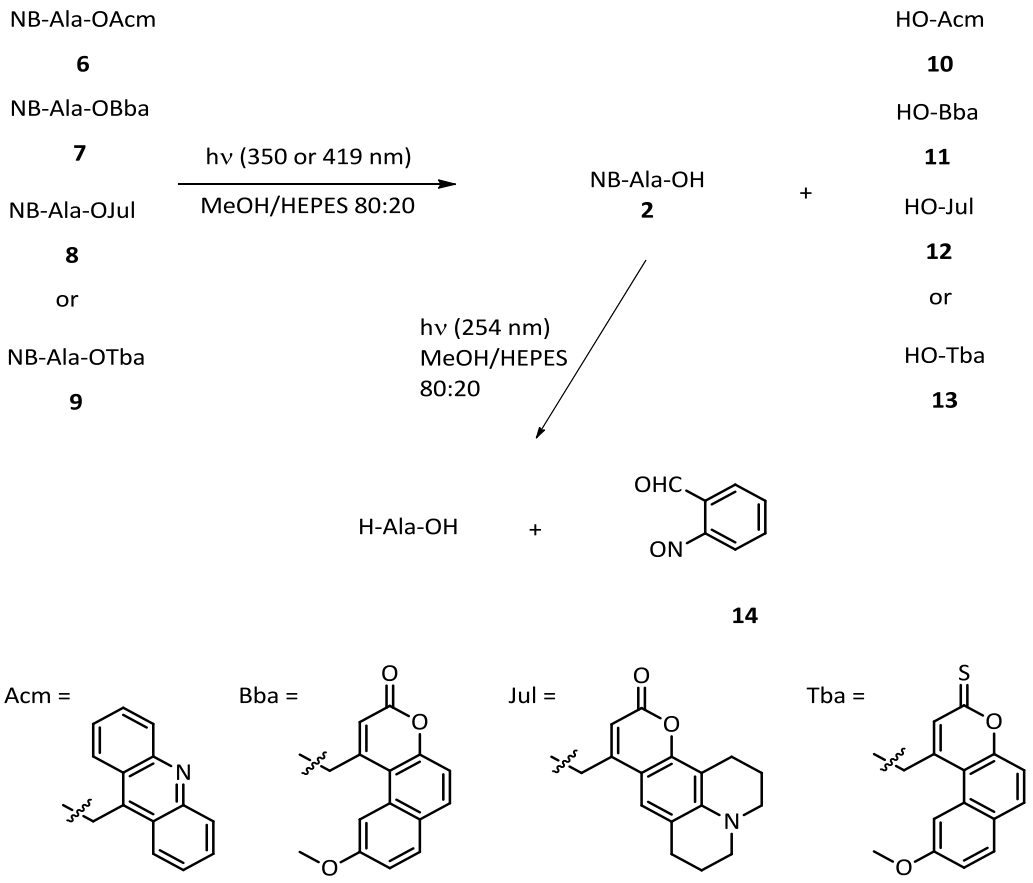
Overall, owing to the wavelengths of maximum absorption of conjugates **6**-**9** in methanol/HEPES buffer (80:20), it was found that photolysis at 350 nm would probably be suitable for the deprotection of the *C*-terminal of alanine in conjugates **6** and **7**, whereas at 419 nm would be adequate in the case of conjugates **8** and **9**. In order to check this and also to verify the stability to other nearby wavelengths, solutions of conjugates **2** and **6**-**9** were irradiated at different wavelengths (254, 300, 350 and 419 nm) in a Rayonet RPR-100 reactor (Scheme 2, Table 2). The photolysis reaction was monitored by reverse phase HPLC with UV detection, peak areas of the starting material (*A*, average of three runs for each compound) revealing a gradual decrease with time and plots of *A versus* irradiation time were obtained for each compound, at the considered wavelengths. The determined irradiation time represents the time necessary for the consumption of the starting materials until less than 5% of the initial area was detected (Table 2). For each compound and based on HPLC data, the plot of ln *A versus* irradiation time showed a linear correlation for the disappearance of the starting material, which suggested a first order reaction, obtained by the linear least squares methodology for a straight line. The photochemical quantum yields (Φ_{Phot}) were calculated based on half-lives ($t_{1/2}$), molar extinction coefficients (ε) and the incident photon flux (I_0), which was determined by potassium ferrioxalate actinometry.[43]

Table 2. Irradiation times, t_{irr}, and photochemical quantum yields, Φ_{Phot}, for the photolysis of conjugates **2**, **6**-**9** at 254, 300, 350 and 419 nm in methanol/HEPES buffer (80:20) solution.

	Compound	254 nm		300 nm		350 nm		419 nm	
		t_{irr} (min)	Φ_{Phot}	t_{irr} (min)	Φ_{Phot}	t_{irr} (min)	Φ_{Phot}	t_{irr} (min)	Φ_{Phot}
2	NB-Ala-OH	153	1.17×10^{-4}	256	3.33×10^{-5}	469	1.72×10^{-5}	647	1.29×10^{-5}
6	NB-Ala-OAcm	25	2.28×10^{-4}	35	1.04×10^{-4}	18	7.90×10^{-5}	108	1.32×10^{-4}
7	NB-Ala-OBba	48	3.53×10^{-5}	98	6.38×10^{-6}	60	$1.01 \quad 10^{-5}$	243	2.51×10^{-6}
8	NB-Ala-OJul	12	1.94×10^{-4}	13	8.53×10^{-5}	21	5.20×10^{-5}	17	6.25×10^{-5}
9	NB-Ala-OTba	38	1.09×10^{-4}	41	3.63×10^{-5}	51	6.89×10^{-5}	31	1.11×10^{-5}

Conjugates **6** and **7** were exposed to light at 350 nm and results revealed that cleavage of the ester bond with release of *N*-(2-nitrobenzyloxycarbonyl)-L-alanine **2** occurred with an irradiation time (t_{irr}) of 18 and 60 min, respectively. On the other hand, irradiation at 419 nm of conjugates **8** and **9** cleaved the carboxylic protecting group in 17 and 31 min, respectively (Scheme 2, Table 2). Conjugates **6**-**9** possess benzyl-type nitrogen and oxygen heterocycles connected through an ester bond which have been found to cleave by formation of a ion pair that can recombine or proceed to the products after nucleophilic attack by the solvent molecules.[3] In this case, the formation of the corresponding heterocyclic alcohols **10**-**13** as byproducts, respectively, was expected.

In order to estimate the stability of 2-nitrobenzyloxycarbonyl group in the above conditions, *N*-(2-nitrobenzyloxycarbonyl)-L-alanine **2** was irradiated at 350 and 419 nm. The results showed that 2-nitrobenzyloxycarbonyl group cleaved in 11% (after 18 min), 31% (after 60 min) at 350 nm, and in 9% (after 17 min), 15% (after 31 min) at 419 nm. Considering these results, the tested protecting groups cannot be considered totally orthogonal but rather quasi-orthogonal since selective cleavage can only be achieved in a particular order of irradiation: in order to minimize simultaneous cleavage at both terminals, the molecule should always be irradiated first at the longer wavelength of irradiation.



Scheme 2. Photocleavage reactions of conjugates **2** and **6**-**9**.

Considering that after deprotection of the *C*-terminal, cleavage of protecting group of *N*-terminal is required, *N*-(2-nitrobenzyloxycarbonyl)-L-alanine **2** was exposed to light of 254 nm, and it was found that cleavage of the carbamate linkage occurred in 153 min. The photolytic process performed at 300, 350 and 419 nm with conjugate **2**, revealed that the irradiation times increased considerably (t_{irr} between 256 and 647 min), thus confirming the expected behaviour, with 254 nm being the most adequate wavelength for the cleavage of *o*-nitrobenzyl group. This group is known to cleave by an intramolecular proton abstraction mechanism yielding the released product (in the present case involving a carbamate linkage, accompanied by spontaneous decarboxylation to the free amine) and a nitroso compound **14**.[3]

Bearing in mind the above findings, the release of the fully deprotected alanine residue by sequential irradiation was attempted, by irradiating first at the longer wavelength of irradiation (350 or 419 nm, depending on the heteroaromatic group) followed by the shorter wavelength of irradiation (254 nm). It was found that the release

was possible in irradiation times very similar to the sum of the irradiation times presented in Table 2 (relative to the cleavage of each individual group), revealing that the byproducts of the first irradiation (which remained in the solution being irradiated) did not influence the overall outcome of the second irradiation.

Additionally to monitoring the photolysis process by HPLC/UV detection, the photolysis of conjugates **2** (NB-Ala-OH) and **8** (NB-Ala-OJul)**,** as representative examples of the tested set of compounds, was also followed by ^{1}H NMR in a methanol-d_4/D_2O (80:20) solution. In the case of conjugate **2**, upon irradiation at 254 nm, the signals related to the alanine α-CH and β-CH_3 in the conjugated form at about δ 4.20 and 1.45 ppm, gave rise to a close set of signals corresponding to alanine in its free form at about δ 3.75 and 1.50 ppm, respectively (Figure 2). This conversion was also accompanied by the decrease with time of the integration of the signal due to the benzylic CH_2 at about δ 5.5 ppm. In the case of conjugate **8**, irradiation was carried out first at 419 nm, to cleave the heterocyclic C-terminal protecting group (the coumarin built on the julolidine nucleus). The alanine α-CH and β-CH_3 signals in the conjugated form, at about δ 4.35 and 1.50 ppm, were replaced by the new set of signals corresponding to the released compound, *N*-(2-nitrobenzyloxycarbonyl)-L-alanine **2**, at about δ 4.20 and 1.45 ppm, respectively (Figure 3). The multiplets corresponding to the benzylic CH_2 of the *N*- and *C*-termini protecting groups of conjugate **8**, between δ 5.30-5.50 ppm, were substituted by a singlet due to the remaining *N*-(2-nitrobenzyloxycarbonyl) group. After irradiation at 419 nm for 11 h, ensuring almost complete conversion of conjugate **8** to conjugate **2**, the same solution was further irradiated at 254 nm to cleave the N-terminal nitrobenzyl protecting group. The same observations were made concerning the shifts of the alanine α-CH and β-CH_3 signals, as in the above referred photolysis for the pure conjugate **2**.

The NMR monitoring was carried out with a 2×10^{-2} M solution, which led to an expected increase in the photolysis time for the complete release of the molecule, when compared to the irradiation times in Table 2 obtained with dilute solutions.

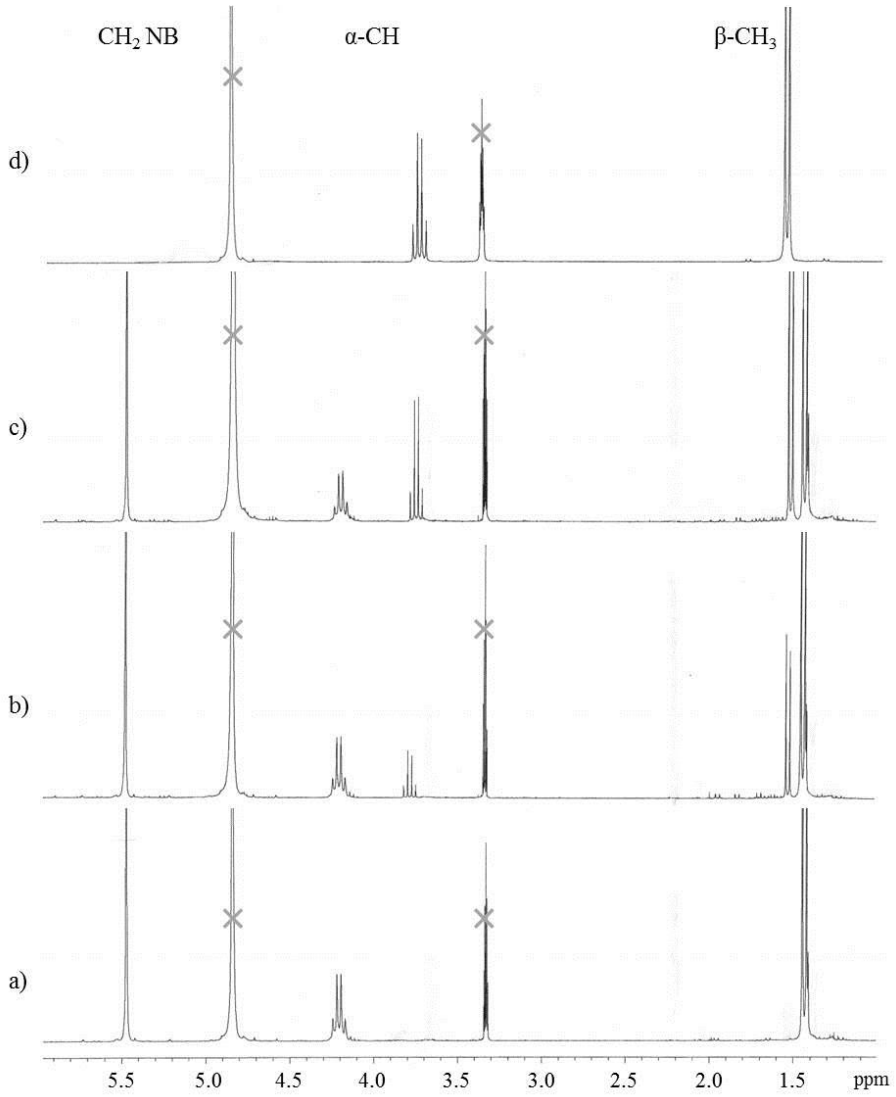


Figure 2. Partial ^{1}H NMR spectra in methanol-d_3/D_2O (80:20) of the photolysis of conjugate **2**, NB-Ala-OH (C = 2 × 10^{-2} M) at 254 nm: a) before irradiation; b) after irradiation for 18h; c) after irradiation for 34h; d) free H-Ala-OH.

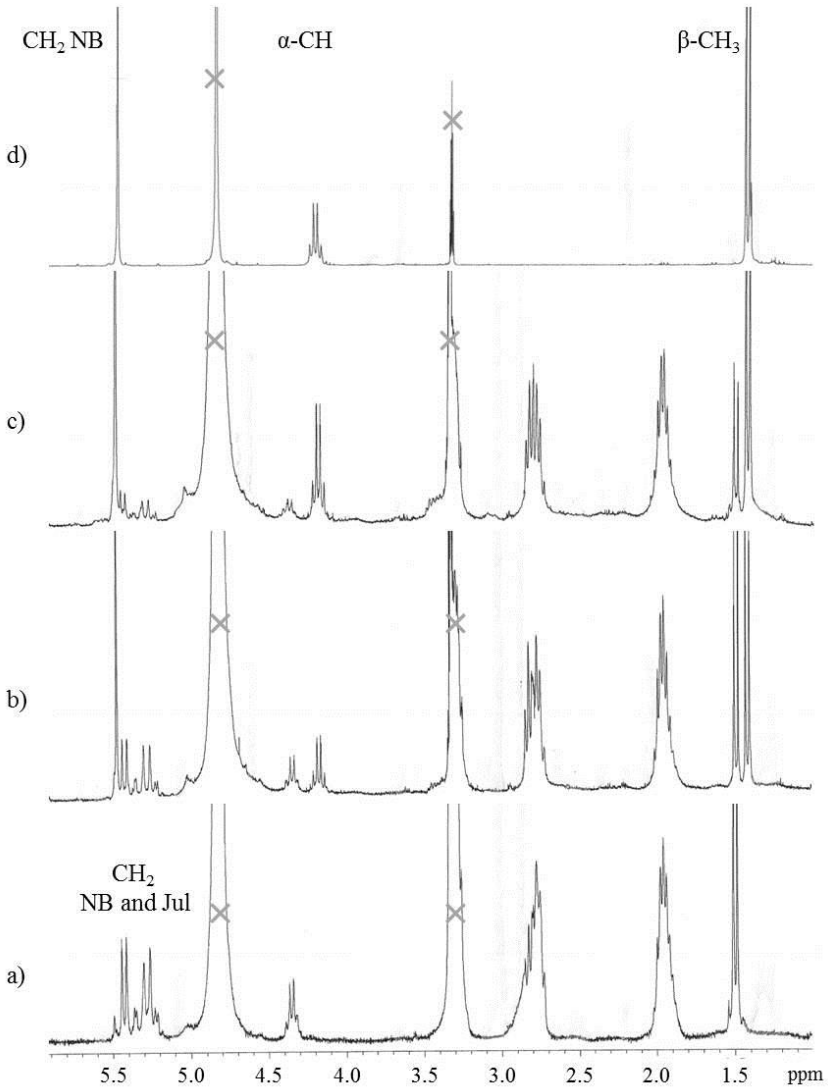


Figure 3. Partial ^{1}H NMR spectra in methanol-d_3/D_2O (80:20) of the photolysis of conjugate **8**, NB-Ala-OJul (C = 2 × 10^{-2} M) at 419 nm: (a) before irradiation; (b) after irradiation for 2h; (c) after irradiation for 10h; (d) free NB-Ala-OH **2**.

4. Conclusions

It was investigated the photolysis of four conjugates bearing in the same molecule two photolabile groups, the *o*-nitrobenzyloxycarbonyl and a benzyl-type polyheteroaromatics, namely acridine, (thioxo)benzocoumarin and a coumarin built on the julolidine nucleus, and identified the wavelengths where the chromophores show different sensitivities. It was demonstrated that selective cleavage of the photolabile groups was possible by choosing the most appropriated wavelength, being for the tested

heterocyclic chromophores 350 and 419 nm, and for the *o*-nitrobenzyl group 254 nm. Overall, the results showed that the combinations of photolabile protecting groups used in conjugates **6**-**9** are wavelength-selective; however, a specific irradiation sequence is required, meaning that the heterocyclic chromophore should always be cleaved before the *o*-nitrobenzyl group.

The possibility of using other classes of photolabile protecting group, together with other photolysis conditions, and even the use of two-photon excitation are possibilities under study in our research group.

5. Experimental section

5.1. General

All melting points were measured on a Stuart SMP3 melting point apparatus. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230-240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer using KBr discs. UV/vis absorption spectra (200-700 nm) were obtained using a Shimadzu UV/2501PC spectrophotometer. NMR spectra were obtained on a Varian Unity Plus Spectrometer at an operating frequency of 300 MHz for ^{1}H NMR or a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H NMR and 100.6 MHz for ^{13}C NMR using the solvent peak as internal reference at 25 °C. All chemical shifts are given in ppm using δ_H Me_4Si = 0 ppm as reference and *J* values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear correlation techniques. Mass spectrometry analyses were performed at the “C.A.C.T.I. - Unidad de Espectrometria de Masas”, at University of Vigo, Spain. Fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer. Photolysis was carried out using a Rayonet RPR-100 chamber reactor equipped with 10 lamps of 254, 300, 350 and 419 ± 10 nm. HPLC analyses were performed using a Licrospher 100 RP18 (5 μm) column in a JASCO HPLC system composed by a PU-2080

pump and a UV-2070 detector with ChromNav software. All reagents were used as received.

5.2. Synthesis of compounds 1, 2 and 6-9

5.2.1. *N*-(2-Nitrobenzyloxycarbonyl)-L-alanine methyl ester (1). *N*,*N*´-carbonyldiimidazole (1.078 g, 6.65 × 10^{-3} mol) was stirred with (2-nitrophenyl)methanol (1.018 g, 6.65 × 10^{-3} mol) in dry DMF (4 mL) at 0 °C for 30 min. L-Alanine methyl ester hydrochloride (0.928 g, 6.65 × 10^{-3} mol), previously treated with triethylamine (1.01 mL, 7.32 × 10^{-3} mol) in dry DMF (2 mL) was added to the reaction mixture and the mixture was kept stirring for 18 h. The precipitate was filtered, the solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using ethyl acetate and light petroleum, with mixtures of increasing polarity as eluent. Compound **1** was obtained as a pink oily solid (0.430 g, 23%). TLC (ethyl acetate/light petroleum, 1:1): R_f = 0.48. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.44 (d, *J* 7.2 Hz, 3 H, β-CH_3), 3.76 (s, 3 H, OCH_3), 4.30-4.44 (m, 1 H, α-CH), 5.49 (br s, 1 H, α-NH), 5.53 (s, 2 H, CH_2), 7.47 (dt, *J* 7.4 and 1.6 Hz, 1 H, H-4), 7.62 (dd, *J* = 7.2 and 1.2 Hz, 1 H, H-6), 7.65 (dt, *J* = 6.8 and 0.8 Hz, 1 H, H-5), 8.10 (dd, *J* = 8.0 and 0.8 Hz, 1 H, H-3) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 18.54 (β-CH_3), 49.63 (α-CH), 52.49 (OCH_3), 63.41 (CH_2), 124.92 (C-3), 128.49 (C-4), 128.56 (C-6), 132.98 (C-1), 133.74 (C-5), 147.22 (C-2), 155.01 (C=O carbamate), 173.32 (C=O ester) ppm. IR (KBr 1%, cm^{-1}): υ = 3345, 3113, 3044, 2991, 2956, 2880, 1729, 1613, 1578, 1526, 1453, 1344, 1302, 1250, 1216, 1177, 1151, 1117, 1086, 1071, 980, 859, 816, 791, 733, 703, 672. HRMS (EI): calcd for $C_{12}H_{14}N_2O_6$ [M^+]: 282.08522; found 282.08543.

5.2.2. *N*-(2-Nitrobenzyloxycarbonyl)-L-alanine (**2**). To a suspension of compound **1** (0.403 g, 1.44 × 10^{-3} mol) in 1,4-dioxane (4.0 mL) at 0 °C, 1 M aqueous sodium hydroxide (1.44 mL, 1.44 × 10^{-3} mol) was added. The solution was stirred at low temperature for 18 h and acidified to pH 2-3 with 1 M aqueous potassium hydrogensulphate. The reaction mixture was reduced to half the initial volume in a rotary evaporator and extracted with dichloromethane (3 × 10 mL). The organic extracts were combined, dried with anhydrous magnesium sulfate and after solvent evaporation, compound **2** was obtained

as a colorless oil (0.355 g, 92%). TLC (ethyl acetate): R_f = 0.39. ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H = 1.17 (d, *J* 6.8 Hz, 3 H, β-CH_3), 3.50-3.59 (m, 1 H, α-CH), 5.32 (s, 2 H, CH_2), 6.64 (d, *J* 6.0 Hz, 1 H, α-NH), 7.58 (dt, *J* = 7.6 and 1.2 Hz, 1 H, H-4), 7.68 (dd, *J* = 7.6 and 0.8 Hz, 1 H, H-6), 7.77 (dt, *J* = 7.2 and 0.8 Hz, 1 H, H-5), 8.08 (dd, *J* = 8.4 and 1.2 Hz, 1 H, H-3) ppm. ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ_C = 19.41 (β-CH_3), 51.40 (α-CH), 61.76 (CH_2), 124.73 (C-3), 128.74 (C-6), 128.86 (C-4), 133.22 (C-1), 134.20 (C-5), 147.12 (C-2), 154.46 (C=O carbamate), 174.33 (C=O acid) ppm. IR (film, cm^{-1}): υ = 3369, 2987, 1692, 1614, 1576, 1533, 1451, 1343, 1295, 1264, 1215, 1120, 1067, 1020, 982, 936, 896, 860, 802, 738, 704. HRMS (EI): calcd for $C_{11}H_{12}N_2O_6$ [M^+]: 268.06956; found 268.06986.

5.2.3. *N*-(2-Nitrobenzyloxycarbonyl)-L-alanine (acridin-9-yl)methyl ester (6). To a solution of compound **2** (0.073 g, 2.73 × 10^{-4} mol) in dry DMF (3 mL), potassium fluoride (0.047 g, 8.16 × 10^{-4} mol) and 9-(bromomethyl)acridine **3** (0.074 g, 2.72 × 10^{-4} mol) were added. The reaction mixture was stirred at room temperature for 30 h. Potassium fluoride was removed by filtration, the solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using mixtures of ethyl acetate and light petroleum of increasing polarity as eluent. Compound **6** was obtained as light brown oil (0.040 g, 32%). TLC (ethyl acetate/light petroleum, 1:1): R_f = 0.51. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.33 (d, *J* = 7.2 Hz, 3 H, β-CH_3), 4.30-4.40 (m, 1 H, α-CH), 5.45 (s, 2 H, CH_2 NB), 5.58 (d, = *J* 7.6 Hz, 1 H, NH), 6.08 (d, *J* = 6.8 Hz, 1 H, CH_2 OAcm), 6.20 (d, *J* = 6.8 Hz, 1 H, CH_2 OAcm), 7.35-7.45 (m, 1 H, H-4´), 7.46-7.55 (m, 2 H, H-5´and H-6´), 7.56-7.62 (m, 2 H, H-2 and H-7), 7.73-7.79 (m, 2 H, H-3 and H-6), 8.043 (dd, *J* = 6.4 and 1.6 Hz, 1 H, H-3´), 8.20-8.30 (m, 4 H, H-1, H-8, H-4 and H-5) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 18.14 (β-CH_3), 49.77 (α-CH), 58.57 (CH_2 OAcm), 63.37 (CH_2 NB), 123.74 (C-1 and C-8), 124.83 (C-3´), 125.16 (C-8a and C-9a), 126.82 (C-2 and C-7), 128.34 (C-4´), 128.41 (C-5´), 129.85 (C-4 and C-5), 130.11 (C-3 and C-6), 132.78 (C-1´), 133.65 (C-6´), 136.49 (C-9), 147.06 (C-2´), 148.32 (C-4a and C-10a), 155.04 (C=O carbamate), 172.69 (C=O ester) ppm. IR (film, cm^{-1}): υ = 3420, 3331, 3056, 2985, 2941, 2878, 1727, 1630, 1611, 1579, 1526, 1452, 1421, 1344, 1305, 1266, 1209, 1170, 1115, 1068, 966, 910, 897, 859, 819, 790, 738, 704, 674, 642. HRMS (EI): calcd for $C_{25}H_{21}N_3O_6$ [M^+]: 459.14313; found 459.14292.

5.2.4. *N*-(2-Nitrobenzyloxycarbonyl)-L-alanine [(9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)]methyl ester (7). To a solution of compound **2** (0.134 g, 4.99×10^{-4} mol) in dry DMF (3 mL), potassium fluoride (0.087 g, 1.49×10^{-3} mol) and 1-chloromethyl-9-methoxy-3-oxo-3*H*-naphtho[2,1-*b*]pyran **4** (0.137 g, 4.99×10^{-4} mol) were added. The reaction mixture was stirred at room temperature for 48 h. Potassium fluoride was removed by filtration, the solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using ethyl acetate and light petroleum, with mixtures of increasing polarity as eluent. Compound **7** was obtained as a light yellow solid (0.139 g, 55%). Mp = 145.0-146.6°C. TLC (ethyl acetate/light petroleum, 1:1): R_f = 0.40. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.54 (d, *J* = 7.6 Hz, 3 H, β-CH_3), 3.96 (s, 3 H, OCH_3), 4.52-4.58 (m, 1 H, α-CH), 5.53 (s, 2 H, CH_2 NB), 5.55 (br s, 1 H, α-NH), 5.60-5.79 (m, 2 H, CH_2 OBba), 6.64 (s, 1 H, H-2), 7.22 (dd, *J* = 8.8 and 2.0 Hz, 1 H, H-8), 7.30 (d, *J* = 9.2 Hz, 1 H, H-5), 7.36-7.42 (m, 1 H, H-10), 7.45 (dt, *J* = 7.2 and 2.0 Hz, 1 H, H-4´), 7.55-7.69 (m, 2 H, H-5´and H-6´), 7.82 (d, *J* = 8.8 Hz, 1 H, H-7), 7.90 (d, *J* = 8.8 Hz, 1 H, H-6), 8.08 (d, *J* = 8.0 Hz, 1 H, H-3´) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 18.12 (β-CH_3), 49.86 (α-CH), 55.46 (OCH_3), 63.64 (CH_2 NB), 64.98 (CH_2 OBba), 105.72 (C-10), 111.70 (C-4b), 112.93 (C-2), 115.23 (C-5), 116.53 (C-8), 124.93 (C-3´), 126.32 (C-6a), 128.60 (C-4´), 128.74 (C-5´), 130.48 (C-6b), 131.35 (C-7), 132.66 (C-1´), 133.75 (C-6´), 133.86 (C-6), 147.28 (C-2´), 150.25 (C-1), 155.15 (C-4a), 155.55 (C=O carbamate), 159.71 (C-9), 160.07 (C-3), 172.17 (C=O ester) ppm. IR (KBr 1%, cm^{-1}): υ = 3406, 3057, 2987, 2959, 2942, 1718, 1624, 1579, 1553, 1523, 1460, 1425, 1345, 1232, 1214, 1171, 1142, 1121, 1073, 1026, 983, 946, 894, 862, 844, 822, 789, 731, 703, 674, 608. HRMS (EI): calcd for $C_{26}H_{22}N_2O_9$ [M^+]: 506.13256; found 506.13268.

5.2.5. *N*-(2-Nitrobenzyloxycarbonyl)-L-alanine [11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl]methyl ester (8). To a solution of compound **2** (0.027 g, 1.01×10^{-4} mol) in dry DMF (3 mL), potassium fluoride (0.017 g, 3.02×10^{-4} mol) and 9-(chloromethyl)-2,3,6,7-tetrahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-11(5*H*)-one **5** (0.029 g, 1.01×10^{-4} mol) were added. The reaction mixture was stirred at room temperature for 46 h. Potassium fluoride was removed by filtration, the solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using ethyl acetate and light petroleum, with

mixtures of increasing polarity as eluent. Compound **8** was obtained as brown oil (0.023 g, 44%). TLC (ethyl acetate/light petroleum, 1:1): R_f = 0.44. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.52 (d, *J* = 7.2 Hz, 3 H, β-CH_3), 1.90-2.00 (m, 4 H, H-2 and H-6), 2.73-2.78 (m, 2 H, H-7), 2.83-2.89 (m, 2 H, H-1), 3.20-3.30 (m, 4 H, H-3 and H-5), 4.45-4.55 (m, 1 H, α-CH), 5.17-5.32 (m, 2 H, CH_2 Jul), 5.40-5.50 (m, 1 H, α-NH), 5.51-5.60 (m, 2 H, CH_2 NB), 6.06 (s, 1 H, H-10), 6.86 (s, 1 H, H-8), 7.47 (dt, *J* 7.2 and 1.6 Hz, 1 H, H-4´), 7.59-7.68 (m, 2 H, H-5´and H-6´), 8.10 (dd, *J* = 8.4 and 0.8 Hz, 1 H, H-3´) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 18.34 (β-CH_3), 20.36 (C-2), 20.47 (C-1), 21.37 (C-6), 27.63 (C-7), 49.47 (C-3), 49.82 (α-CH), 49.89 (C-5), 62.49 (CH_2 Jul), 63.53 (CH_2 NB), 105.66 (C-10), 105.90 (C-8a), 106.97 (C-12b), 118.35 (C-7a), 120.48 (C-8), 124.93 (C-3´), 128.52 (C-4´), 128.57 (C-5´), 132.88 (C-1´), 133.81 (C-6´), 145.99 (C-7b), 147.19 (C-2´), 148.70 (C-9), 151.19 (C-12a), 155.08 (C=O carbamate), 162.01 (C-11), 172.27 (C=O ester) ppm. IR (film, cm^{-1}): υ = 3318, 3060, 2940, 2851, 1717, 1603, 1556, 1524, 1440, 1381, 1343, 1312, 1255, 1206, 1177, 1122, 1071, 1015, 960, 885, 858, 820, 790, 732, 700. HRMS (EI): calcd for $C_{27}H_{27}N_3O_8$ [M^+]: 521.17991; found 521.17989.

5.2.6. *N*-(2-Nitrobenzyloxycarbonyl)-L-alanine [(9-methoxy-3-thioxo-3*H*-naphtho[2,1-*b*]pyran-1-yl)]methyl ester (9). Lawesson´s reagent (0.172 g, 4.26 × 10^{-4} mol) was added to a solution of compound **7** (0.072 g, 1.42 × 10^{-4} mol) in toluene (5 mL). The reaction mixture was refluxed for 9 h and the process was followed by TLC (ethyl acetate/light petroleum, 1:4). The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography using dichloromethane and light petroleum, with mixtures of increasing polarity as eluent. Compound **9** was obtained as an orange oil (0.021 g, 28%). TLC (ethyl acetate/light petroleum, 1:1): R_f = 0.67. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H = 1.54 (d, *J* = 7.2 Hz, 3 H, β-CH_3), 3.97 (s, 3 H, OCH_3), 4.50-4.60 (m, 1 H, α-CH), 5.35-5.45 (m, 1 H, α-NH), 5.54 (s, 2 H, CH_2 NB), 5.65-5.80 (m, 2 H, CH_2 OTba), 7.25 (d, *J* = 2.0 Hz, 1 H, H-2), 7.41-7.53 (m, 4 H, H-4´, H-10, H-5 and H-8), 7.54-7.70 (m, 2 H, H-5´and H-6´), 7.85 (d, *J* = 8.8 Hz, 1 H, H-7), 7.96 (d, *J* = 8.8 Hz, 1 H, H-6), 8.03 (d, *J* = 8.0 Hz, 1 H, H-3´) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ_C = 18.21 (β-CH_3), 49.87 (α-CH), 55.55 (OCH_3), 63.70 (CH_2 NB), 64.79 (CH_2 OTba), 106.29 (C-10), 114.17 (C-4b), 114.93 (C-5), 117.17 (C-2), 124.97 (C-3´), 126.61 (C-6a), 127.39 (C-8), 128.62 (C-4´), 128.77 (C-5´), 130.23 (C-6b), 131.49 (C-7), 132.68 (C-1´), 133.77 (C-6´),

134.42 (C-6), 141.34 (C-1), 147.32 (C-2´), 155.12 (C=O carbamate), 158.81 (C-4a), 159.97 (C-9), 172.21 (C=O ester), 195.12 (C-3) ppm. IR (film, cm^{-1}): υ = 3338, 3065, 2938, 2852, 1727, 1622, 1591, 1535, 1453, 1343, 1297, 1231, 1174, 1141, 1096, 1069, 1024, 976, 858, 840, 790, 732, 700, 688, 671. HRMS (EI): calcd for $C_{26}H_{22}N_2O_8S$ [M^+]: 522.10976; found 522.10995.

5.3. General photolysis procedure

A 1×10^{-4} M methanol/HEPES (80:20) solution of compounds **2** and **6**-**9** (5 mL) were placed in a quartz tube and irradiated in a Rayonet RPR-100 reactor at the desired wavelength. The lamps used for irradiation were of 254, 300, 350 and 419 ± 10 nm.

Aliquots of 100 µL were taken at regular intervals and analysed by RP-HPLC. The eluent was acetonitrile/water, 75:25 with 0.1% trifluoroacetic acid, at a flow rate of 0.8 mL/min, previously filtered through a Millipore, type HN 0.45 µm filter and degassed by ultra-sound for 30 min. The chromatograms were traced by detecting UV absorption at the wavelength of maximum absorption for each compound (retention time: 2, 3.5; 6, 5.0; 7, 6.3; 8, 7.5; 9, 9.5 min).

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Chapter 3

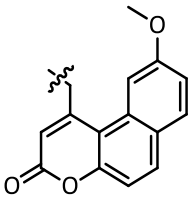
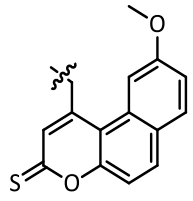
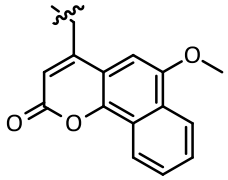
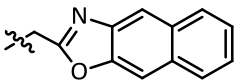
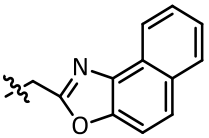
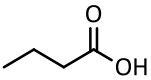
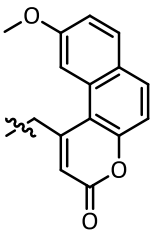
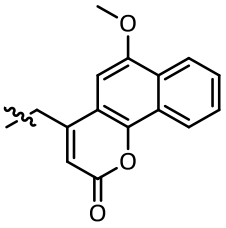
Overview of results

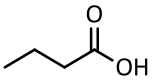
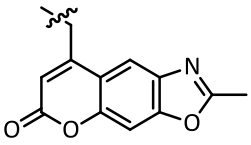
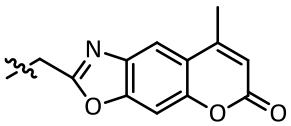
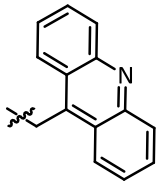
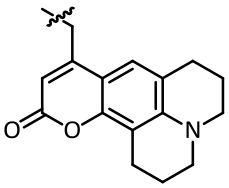
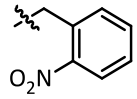
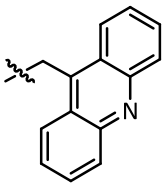
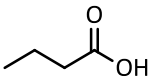
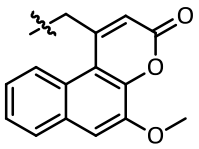
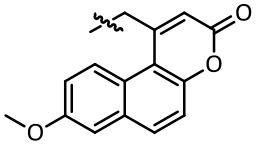
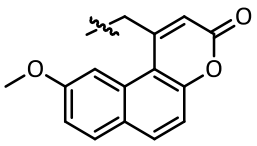
In this chapter, the numbering system of the compounds was updated for quick reference by adding the subchapter number to the number used in each article, in order to avoid confusion between different compounds with the same number but belonging to different subchapters: for example compound **1a** in subchapter 2.1 is now compound **2.1-1a**.

The synthesized compounds that were studied as photocleavable protecting groups/phototriggers presented in Chapter 2 of this dissertation were organised into subchapters in Table 1 for easier comparison of results. The corresponding synthesis precursors were not included.

In Table 2, the photolysis data for the conjugates, namely the wavelength of irradiation, solvent mixture, maximum wavelength of absorption, irradiation time, rate constant and photochemical quantum yield, were compiled into a table for quick reference and easier comparison of results.

Table 1. List of the studied conjugates, organized into subchapters according to the protecting group or bioactive molecule.

New number	Bioactive molecule	Protecting group
2.1-1a **2.1-1b** **2.1-4a** **2.1-4b**	Z-Val-OH Z-Phe-OH H-Val-OH H-Phe-OH	
2.1-2a **2.1-2b** **2.1-3a** **2.1-3b**	Z-Val-OH Z-Phe-OH H-Val-OH H-Phe-OH	
2.2-3a **2.2-3b** **2.2-4a** **2.2-4b**	Z-Val-OH Z-Phe-OH H-Val-OH H-Phe-OH	
2.3-7		
2.3-8		
2.3-9		
2.3-10		

New number	Bioactive molecule	Protecting group
2.3-11		
2.3-12		
2.4-3a	Boc-Gly-OH	
2.4-3b	Boc-Ala-OH	
2.4-3c	Boc-Glu(OMe)-OH	
2.4-3d	Boc-β-Ala -OH	
2.4-3e	Boc-GABA-OH	
2.5-4a	Z-Gly-OH	
2.5-4b	Z-Ala-OH	
2.5-4c	Z-Glu(OMe)-OH	
2.5-4d	Z-β-Ala-OH	
2.5-4e	Z-GABA-OH	
2.6-7		
2.6-8		
2.6-9		
2.6-10		
2.6-11		

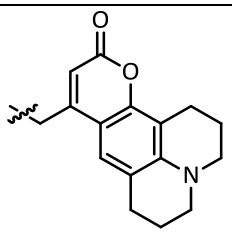
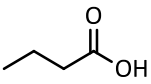
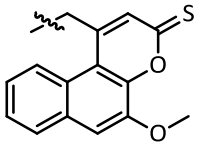
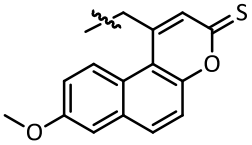
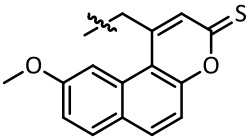
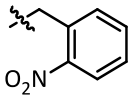
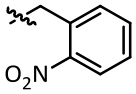
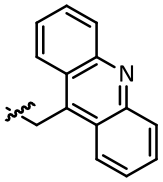
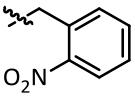
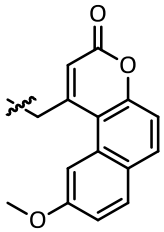
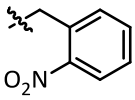
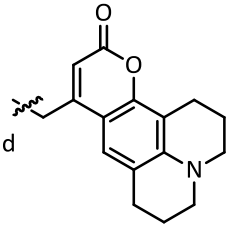
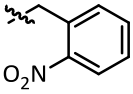
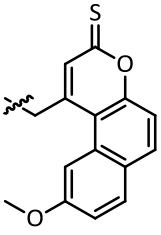
New number	Bioactive molecule	Protecting group
2.6-12		
2.6-13		
2.6-14		
2.6-15		
2.7-2		
2.7-6		 and 
2.7-7	H-Ala-OH	 and 
2.7-8		 and 
2.7-9		 and 

Table 2. Photolysis data for the bioactive molecule conjugates (solvent mixture, maximum wavelength of absorption, irradiation time (min), rate constants (k, × 10^{-2} min^{-1}) and photochemical quantum yield (Φ_{Phot}, × 10^{-3}).

Compound	Solvent	λ_{abs} (nm)	254 nm			300 nm			350 nm			419 nm		
			t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}
2.1-1a	MeOH/HEPES 80:20	351	41	6.84	0.167	84	3.44	0.046	33	9.08	0.098	462	0.66	0.079
2.1-1b		349	79	3.89	0.109	46	6.23	0.108	38	8.28	0.093	301	1.0	0.144
2.1-2a		417	137	2.23	0.057	112	2.80	0.029	66	4.76	0.130	30	9.68	0.119
2.1-2b		417	87	3.40	0.080	224	1.33	0.015	354	0.84	0.020	52	5.80	0.062
2.1-3a		417	101	3.00	0.038	161	1.90	0.011	162	1.97	0.015	48	6.07	0.041
2.1-3b		416	246	1.23	0.032	250	1.22	0.015	146	2.12	0.043	49	6.49	0.078
2.1-4a		348	152	2.00	0.032	672	0.45	0.003	607	0.49	0.004	3304	0.09	0.006
2.1-4b		345	781	1.93	0.068	658	0.46	0.009	520	0.58	0.011	7478	0.04	0.004
2.2-3a	MeOH/HEPES 80:20	376	151	1.96	0.059	86	3.51	0.087	59	5.06	0.243	270	1.10	0.164
2.2-3b		375	78	3.89	0.062	62	4.86	0.065	47	6.41	0.176	148	2.03	0.158
2.2-4a		375	887	0.34	0.009	845	0.28	0.006	231	1.30	0.055	1253	0.24	0.047
2.2-4b		374	275	1.10	0.031	613	0.47	0.012	632	0.53	0.023	1153	0.26	0.042

Compound	Solvent	λ_{abs} (nm)	254 nm			300 nm			350 nm			419 nm		
			t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}
2.2-3a	ACN/HEPES 80:20	373	69	4.36	0.102	154	1.94	0.042	86	3.46	0.121	77	3.91	0.645
2.2-3b		373	43	7.02	0.111	79	3.70	0.051	35	8.47	0.210	53	5.87	0.512
2.2-4a		371	21	14.40	0.038	40	7.57	0.013	35	8.51	0.338	23	12.83	2.439
2.2-4b		373	17	17.23	0.402	84	3.56	0.070	33	9.03	0.304	45	6.58	0.802
2.3-7	MeOH/HEPES 80:20	302	4.9	65.22	ND	5.6	53.42	ND	>2500	--	ND	ND	ND	ND
2.3-8		321	0.1	2918	ND	0.8	400	ND	519	0.58	ND	ND	ND	ND
2.3-9		352	178	1.70	ND	253	1.19	ND	263	1.14	ND	ND	ND	ND
2.3-10		375	107	2.86	ND	170	2.86	ND	96	3.15	ND	ND	ND	ND
2.3-11		326	45	6.59	ND	33	6.59	ND	285	1.04	ND	ND	ND	ND
2.3-12		321	76	3.95	ND	94	3.16	ND	>2500	--	ND	ND	ND	ND
2.4-3a	MeOH/HEPES 80:20	362	13	23.18	0.933	25	12.01	0.227	7	42.03	0.788	35	8.63	0.155
2.4-3b		360	9	32.82	1.333	18	16.67	0.326	10	30.80	0.613	81	3.68	0.071
2.4-3c		362	14	21.49	0.552	22	13.44	0.161	8	39.35	0.455	77	3.89	0.044
2.4-3d		361	9	34.93	1.482	22	13.27	0.266	10	31.25	0.619	98	3.05	0.060
2.4-3e		360	5	56.44	0.579	14	21.25	0.289	3	96.45	1.244	89	3.33	0.045

Compound	Solvent	λ_{abs} (nm)	254 nm			300 nm			350 nm			419 nm		
			t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}
2.5-4a	MeOH/HEPES 80:20	400	4	75.96	1.46	8	39.29	0.362	7	44.54	0.371	7	41.27	0.363
2.5-4b		400	9	33.40	0.68	10	30.90	0.273	12	24.60	0.218	18	17.11	0.150
2.5-4c		402	7	42.54	0.66	13	23.44	0.180	15	19.57	0.143	9	32.50	0.244
2.5-4d		402	12	24.65	0.440	16	18.15	0.158	14	20.83	0.164	20	14.55	0.129
2.5-4e		401	8	37.00	0.576	9	34.36	0.254	10	28.56	0.209	10	29.14	0.205
2.5-4a	ACN/HEPES 80:20	397	ND	ND	ND	ND	ND	ND	4	71.09	0.435	5	57.70	0.397
2.5-4b		390	ND	ND	ND	ND	ND	ND	11	27.86	0.259	8	38.63	0.349
2.5-4c		391	ND	ND	ND	ND	ND	ND	11	27.44	0.234	8	38.45	0.350
2.5-4d		391	ND	ND	ND	ND	ND	ND	8	39.08	0.291	10	29.31	0.148
2.5-4e		391	ND	ND	ND	ND	ND	ND	10	31.10	0.218	7	42.88	0.251
2.6-7	MeOH/HEPES 80:20	259	11	26.64	1.71	26	11.4	0.37	76	3.94	0.12	2217	0.135	0.004
2.6-8		360	4	71.64	1.88	7	45.51	0.60	2	127.39	1.64	83	3.65	0.044
2.6-9		343	192	1.59	0.063	230	1.29	0.027	182	1.64	0.033	5985	0.05	0.001
2.6-10		366	432	0.70	0.039	546	0.55	0.015	555	0.54	0.014	3324	0.09	0.002
2.6-11		352	178	1.70	0.034	253	1.19	0.011	263	1.14	0.011	490	0.61	0.006
2.6-12		399	6	51.70	1.41	9	33.31	0.426	6	64.49	0.79	5	66.09	0.790
2.6-13		406	55	5.51	0.262	178	1.67	0.039	210	1.43	0.032	142	2.11	0.047
2.6-14		428	212	1.41	0.040	371	0.81	0.001	526	0.57	0.007	351	0.86	0.010
2.6-15		412	90	3.35	0.129	184	1.64	0.029	296	1.01	0.018	98	3.09	0.129

Compound	Solvent	λ_{abs} (nm)	254 nm			300 nm			350 nm			419 nm		
			t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}	t_{irr}	k	Φ_{Phot}
2.6-7	ACN/HEPES 80:20	260	14	21.92	1.49	41	7.29	0.225	106	2.82	0.085	1198	0.25	0.008
2.6-8		360	58	5.24	0.129	120	2.50	0.030	328	0.91	0.011	2499	0.12	0.001
2.6-9		343	186	1.63	0.062	184	1.61	0.033	172	1.75	0.031	4288	0.07	0.001
2.6-10		364	457	0.66	0.025	481	0.62	0.012	493	0.61	0.012	9989	0.03	0.001
2.6-11		345	278	1.08	0.054	294	1.02	0.024	270	1.11	0.026	520	0.58	0.013
2.6-12		397	5	61.47	1.87	7	46.43	0.691	4	73.25	1.04	4	74.01	1.03
2.6-13		405	53	5.67	0.202	129	2.33	0.044	292	1.02	0.018	193	1.56	0.029
2.6-14		430	153	1.95	0.042	585	0.51	0.005	308	0.97	0.010	296	1.02	0.010
2.6-15		412	57	5.29	0.114	111	2.72	0.020	203	1.47	0.023	76	3.94	0.040
2.7-2	MeOH/HEPES 80:20	263	153	1.96	0.117	256	1.17	0.033	469	0.63	0.0172	647	0.46	0.013
2.7-6		362	25	11.88	0.228	35	8.68	0.104	18	16.71	0.079	108	2.8	0.132
2.7-7		350	48	8.01	0.035	98	3.04	0.006	60	4.96	0.010	243	1.23	0.002
2.7-8		403	12	25.09	0.194	13	22.12	0.085	21	14.41	0.052	17	17.87	0.062
2.7-9		414	38	8.02	0.109	41	7.22	0.036	51	5.88	0.069	31	9.49	0.011

ND: not determined.

Photocleavage studies of (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl)methyl ester conjugates (compounds **2.1**-**1a**,**b** and **2.1**-**4a**,**b**) in methanol/HEPES buffer (80:20) solution at 350 nm revealed that the amino acid-heterocycle ester bond was efficiently photolised, releasing the *N*-protected and free amino acids from both types of precursors. However, the presence of the thiocarbonyl in thioxoconjugates, (9-methoxy-3-thioxo-3*H*-benzo[*f*]benzopyran-1-yl)methyl esters (compounds **2.1**-**2a**,**b** and **2.1**-**3a**,**b**) clearly improved the photolysis rates at 419 nm (practicable irradiations times 30-52 min).

The newly reported thioxo-protecting group emerges as an innovation for the release at longer wavelengths, since it photolyses with short irradiation times at wavelengths that are not detrimental to a variety of applications.

The new valine and phenylalanine (6-methoxy-2-oxo-2*H*-naphtho[1,2-*b*]pyran-4-yl)methyl esters **2.2**-**3a**,**b** and **2.2**-**4a**,**b** were photolysed faster in a mixture of acetonitrile/HEPES buffer (80:20), at 350 and 419 nm, compared to the previously reported 3-oxo-3*H*-naphtho[2,1-*b*]pyran with a different ring fusion, releasing the *N*-protected and free amino acids. The most interesting results were also obtained at 419 nm in practical irradiations times (23-77 min), with better performance on photochemical quantum yields, which is an interesting improvement, thus confirming the suitability of oxonaphtho[1,2-*b*]pyran as a photocleavable protecting group for release at longer wavelengths.

The light-triggered release of butyric acid from photoactive prodrugs **2.3**-**7** to **2.3**-**12** was evaluated by photochemical cleavage of the corresponding heterocyclic cages of naphtho[2,3-*d*]oxazole, naphtho[1,2-*d*]oxazole, 3-oxo-3*H*-benzo[*f*]benzopyran, 2-oxo-2*H*-benzo[*h*]benzopyran and 6-oxo-6*H*-benzopyrano[6,7-*d*]oxazoles, through an ester linkage between the heterocycle and the active molecule.

Photolytic cleavage studies at 254, 300 and 350 in methanol/HEPES buffer (80:20) solution were conducted. Compounds **2.3**-**9** and **2.3**-**10** showed the fastest cleavage results at 350 nm (263 min and 96 min, respectively) and were also evaluated at 419 nm, which resulted in an increased, but still useful irradiation time of 187 min for **2.3**-**10**, however an impractical result of *ca*. 57 h for **2.3**-**9**.The use of time-resolved

fluorescence techniques contributed to the elucidation of the dynamic behaviour of these systems. Overall, these results suggest that the heterocyclic cages studied may be considered as promising alternatives as photoactive prodrugs for butyric acid as a model carboxylic acid drug.

The photorelease of the amino acids *N*-*tert*-butyloxycarbonylated glycine, alanine, glutamic acid, β-alanine and γ-aminobutyric acid from the corresponding acridinyl methyl ester fluorescent derivatives **2.4**-**3a**-**e** in methanol/HEPES buffer (80:20) solutions was achieved by irradiation, with the best results obtained at 350 nm (3-10 min). However, the presence of different excited species sustained from decay associated spectra calculations, explained the longer irradiation times obtained at 419 nm (35-98 min), in keeping with promise of the acridinyl methyl ester as photoremovable protecting group of the carboxylic acid function for organic synthesis and eventually in caging applications.

Novel (11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl)methyl ester conjugates **2.5**-**4a**-**e** were synthesized and their photolysis behavior evaluated towards light in methanol/HEPES and acetonitrile/HEPES buffer (80:20) solutions at different irradiation wavelengths (254, 300, 350 and 419 nm). The photolysis results revealed that the release of the amino acid neurotransmitters covalently linked through the carboxylic *terminus* to the coumarin built on julolidine unit occurred in short times (4-20 min), being at 419 nm the fastest releases (5-10 min) in acetonitrile/HEPES buffer (80:20) solutions. The significant reduction in photocleavage time is a very notable innovation in relation to previous reports using fused coumarin derivatives, making these compounds more practical for use in two photon excitation and in biological environments where shorter wavelength excitation may be absorbed.

In order to improve the photorelease of butyric acid, several polyheteroaromatics, namely acridine, (thioxo)benzocoumarin and a coumarin built on the julolidine nucleus, were evaluated as benzyl-type phototriggers. The correspondent conjugates **2.6**-**8** to **2.6**-**15** were studied for the controlled delivery of the active molecule by photolysis at selected wavelengths (254, 300, 350 and 419 nm), in mixtures

of methanol or acetonitrile with aqueous HEPES buffer in 80:20 solutions and compared to conjugate **2.6**-**7** bearing the well-known *o*-nitrobenzyl group. It was found that the release of butyric acid was fastest in the case of using acridine and the coumarin fused julolidine nucleus under all the irradiation conditions used.

Alanine was used as model of a bifunctionalised molecule protected at the N- and C-*terminus* through carbamate and ester linkages, respectively, in four conjugates (**2.7**-**6** to **2.7**-**9**), to evaluate the possibility of wavelength-selective photolysis between *o*-nitrobenzyloxycarbonyl and benzyl-type polyheteroaromatics, namely acridine, benzocoumarin, (thioxo)benzocoumarin and a coumarin built on the julolidine nucleus. It was demonstrated that selective cleavage of the photolabile groups was possible by choosing the most appropriated wavelength, being for the tested heterocyclic chromophores 350 and 419 nm, and for the *o*-nitrobenzyl group 254 nm, however, a specific irradiation sequence is required, meaning that the heterocyclic chromophore should always be cleaved before the *o*-nitrobenzyl group.

Chapter 4

Conclusions and future prospects

The work presented in this dissertation focused on the synthesis and characterisation of new fluorescent heteroaromatics and their study as photocleavable protecting groups/phototriggers for bioactive molecules, namely amino acids, including neurotransmitters, and butyric acid.

The fluorophores used in the synthesis of the biomolecule fluorescent conjugates were benzocoumarins, thioxobenzocoumarins, a coumarin built on the julolidine nucleus, naphtho-oxazoles, benzopyrano-oxazoles and acridine as representatives of oxygen, nitrogen and sulfur polyheteroaromatics. The well-known *o*-nitrobenzyl group was also used in the wavelength-selective studies.

The application of the above mentioned fluorophores as photocleavable protecting groups/phototriggers for bioactive molecules was tested by using amino acids, such as valine and phenylalanine, the neurotransmitter amino acids glycine, alanine, glutamic acid, β-alanine and γ-aminobutyric acid, as well as butyric acid, a naturally occurring short chain fatty acid. For that purpose, the coupling of the fluorophores was carried out through adequate coupling methodologies by an ester bond to the carboxylic acid terminal of the bioactive molecules.

Photocleavage studies of the obtained conjugates, in two solvent systems with an organic solvent and water usually in 80:20 proportion (methanol or acetonitrile and aqueous HEPES buffer at pH 7.2), at 254, 300, 350 and 419 nm, confirmed their photosensitivity, which was influenced by the structure of the fluorophore and the active molecule, the wavelength of irradiation and the solvent used in the photocleavage reaction.

In a more detailed manner, the highligths of the obtained results were:

- chapter 2.1

9-Methoxy-3-thioxo-3*H*-benzo[*f*]benzopyran-L-valine and L-phenylalanine model conjugates were prepared through a thionation reaction of the corresponding oxo-benzobenzopyrans. The photolysis experiments at 350 nm in methanol/HEPES buffer (80:20) solution revealed that the amino acid-heterocycle ester bond was efficiently

photolised, releasing the *N*-protected and free amino acids from both types of precursors. However, the most interesting results were obtained at 419 nm for the thioxoconjugates, revealing that the exchange of the carbonyl by a thiocarbonyl group enhanced the performance of the heterocyclic protecting group at 419 nm by improving the photolysis rates, making it an appropriate group for practical applications at long wavelengths.

- chapter 2.2

N-(benzyloxycarbonyl)-L-valine and L-phenylalanine and the correspondent amino acids free forms protected at the carboxylic acid function with a 6-methoxylated benzo[*f*]benzopyran, yielded the correspondent fluorescent ester conjugates. Photocleavage studies of the ester compounds in methanol/HEPES and acetonitrile/HEPES buffer (80:20) at 350 and 419 nm, revealed that the amino acid-heterocycle ester bond was readily photolysed in the last media, releasing the *N*-protected and free amino acids. The most interesting results were obtained at 419 nm for all compounds confirming the suitability of the 6-methoxylated benzo[*f*]benzopyran as a photocleavable protecting group for release at longer wavelengths in practical irradiations times, which is an interesting improvement compared to the previously reported 9-methoxylated benzo[*f*]benzopyran with a different ring fusion.

- chapter 2.3

Naphtho-oxazole, benzocoumarin, and coumarin-oxazole (with a linkage between the heterocycle and the active molecule through coumarin or oxazole moieties) were used in the synthesis of caged butyric acid through an ester linkage. Photolytic cleavage studies at 254, 300, 350 and 419 nm in methanol/HEPES buffer (80:20) solution revealed the possibility of using these heterocyclic systems for the light-triggered release of the active molecule in reasonable times. The caged butyric acids which bear naphtho-oxazole units cleaved readily at 254 and 300 nm, however at 350 nm, the photocleavage was not achieved in a practical period of time. At this wavelength, the most promising fluorophores were the benzocoumarins, having the 6-methoxylated

coumarin an increased, but still useful, irradiation time while an impractical result was obtained for the 9-methoxylated one.

These results show that butyric acid can act as a model carboxylic acid photoactive prodrug for the heterocyclic cages studied here, being short wavelengths the most appropriate for the uncaging of the mentioned conjugates.

- chapter 2.4

Various *N*-(*tert*-butyloxycarbonyl)-neurotransmitter amino acids (glycine, alanine, glutamic acid, β-alanine and γ-aminobutyric acid) were released by irradiation of the corresponding acridinyl methyl ester conjugates in methanol/HEPES buffer (80:20) solutions at different wavelengths. The assessment of the suitability of the (acridin-9-yl)methyl group as a photolabile protecting group for the amino function of the listed amino acids was carried out by photolysis studies at all wavelengths of irradiation, followed by HPLC/UV and ^{1}H NMR detection. It was found that the photocleavage of the *N*-(*tert*-butyloxycarbonyl)-neurotransmitters was accomplished in practicable times in all the wavelengths, the fastest release occurring at 350 nm (3-10 min), as expected according to the maximum wavelength of absorption and that 419 nm irradiation showed to be promising for practical purposes.

- chapter 2.5

The photoinduced release of several neurotransmitter amino acids (glycine, alanine, glutamic acid, β-alanine and γ-aminobutyric acid) was accomplished from ester cages based on a new photoremovable protecting group consisting of a coumarin built on the julolidine nucleus. The photosensitivity of the ester bond was evaluated by photolysis experiments at 254, 300, 350 and 419 nm in methanol/HEPES and acetonitrile/HEPES (80:20) solution. Photolysis studies obtained at 419 nm are promising for practical purposes (5-10 min), evidencing the suitability of this wavelength to minimise side reactions for the remaining functionalities of the molecule and in the future to allow for two-photon excitation to be used.

- chapter 2.6

Acridine, naphtho[2,1-*b*]pyran, 3*H*-benzopyran fused julolidine and thioxo-naphtho[2,1-*b*]pyran ester cages of butyric acid were evaluated for the controlled release of the active molecule by photolysis at selected wavelengths (254, 300, 350 and 419 nm) towards the *o*-nitrobenzyl photolabile group.

The obtained results, combined with time-resolved fluorescence data, showed that for naphtho[1,2-*b*]pyrans the attachment position of the methoxy substituent as well as the presence of the thiocarbonyl group influenced the behaviour towards light of the corresponding cage. Moreover, the release of butyric acid was faster in the case of using (acridin-9-yl)methyl, and [11-oxo-2,3,5,6,7,11-hexahydro-1*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinolin-9-yl]methyl (3*H*-benzopyran fused julolidine) groups as phototriggers.

- chapter 2.7

Conjugates of five photolabile protecting groups were prepared as protections of the N- and C-*terminus* of alanine, used as a model bifunctional target molecule, through carbamate and ester linkages respectively. The amine terminal was masked with *o*-nitrobenzyl group and the carboxylic terminal with benzyl-type nitrogen and oxygen polyheteroaromatics, namely acridine, (thioxo)benzocoumarin and a coumarin-based julolidine nucleus. Photolysis of these conjugates at all wavelengths was carried out in methanol/HEPES (80:20) solutions and the process course was monitored by HPLC/UV and ^{1}H NMR. Overall, the results showed that photolysis of these conjugates was possible by choosing the most appropriate wavelength to irradiate combinations of these photolabile protecting groups, in a sequencial selective maner.

To the best of our knowledge, the work developed in this thesis resulted in the first literature reports on the use of long-wavelength photolysis of thioxobenzocoumarins, naphtho-oxazoles, benzopyrano-oxazoles, acridine and coumarin-based julolidine photocleavable protecting groups, for the caging of amino acids including neurotransmitters, and butyric acid. Our reports on the photolysis

studies for these conjugates, regarding the short irradiation times obtained at 419 nm, revealed that these fluorophores can potencially be used as photosensitive ester prodrugs of carboxylic acid active compounds.

Also, bearing in mind the pharmacological activities of these type of heterocycles it is intended to perform studies on the cytotoxicity and antitumoural activity of the conjugates and based on the results, in combination with molecular modelling studies, to fine tune the design of new prodrug frameworks towards the optimisation of its pharmacokinetics.