Authors

Filipa C. Santos, Ana M. S. Soares, M. Sameiro T. Gonçalves, Susana P. G. Costa

Title

Phototriggered release of tetrapeptide AAPV from coumarinyl and pyrenyl cages

Affiliations

Centre of Chemistry, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Corresponding author

Susana P. G. Costa

Tel: + 351 253 604054

Fax: + 351 253 604382

email: spc@quimica.uminho.pt

Abstract: Ala-Ala-Pro-Val (AAPV) is a bioactive tetrapeptide that inhibits human neutrophil elastase (HNE), an enzyme involved in skin chronic inflammatory diseases like psoriasis. Caged derivatives of this peptide were prepared by proper N- and C-terminal derivatisation through a carbamate or ester linkage, respectively, with two photoactive moieties, namely 7-methoxycoumarin-2-ylmethyl and pyren-2-ylmethyl groups. These groups were chosen to assess the influence of the photosensitive group and the type of linkage in the controlled photorelease of the active molecule. The caged peptides were irradiated at selected wavelengths of irradiation (254, 300, and 350 nm), and the photolytic process was monitored by HPLC-UV. The results established the applicability of the tested photoactive groups for the release of AAPV, especially for the derivative bearing the carbamate-linked pyrenylmethyl group, which displayed the shortest irradiation times for the release at the various wavelengths of irradiation (*ca*. 4 min at 254 nm, 8 min at 300 nm and 46 min at 350 nm).

Keywords: AAPV; Caged peptide; Coumarin; Pyrene; Photorelease.

Introduction

The tetrapeptide Ala-Ala-Pro-Val (AAPV) has been evaluated in the treatment of psoriasis, a chronic inflammatory disease of the skin, as a competitive inhibitor of human neutrophil elastase (HNE). This enzyme relates physiologically to immune processes and the extracellular matrix remodelling after tissue injury (Wiedow et al 1995; Vasconcelos et al. 2011; Caccetta et al. 2006; Namjoshi et al. 2014; Macdonald et al. 1998; Hassal et al. 1985). In healthy conditions, the proteolytic activity of this enzyme is controlled by endogenous inhibitors; however, in inflammatory processes an unbalance occurs between the levels of HNE, which is produced in high levels, and its endogenous inhibitors, leading to an abnormal tissue destruction (Namjoshi et al. 2008; Benson et al. 2003; Avlonitis et al. 2013).

Several derivatives of AAPV have been proposed with modifications aiming at improving skin absorption and passage through the *stratum corneum* in order to allow efficient transdermal delivery and be able to provide a therapeutic effect with the least possible side effects. Reported modifications include preparing peptide conjugates with PEG possessing higher lipophilicity (Yingyuad et al. 2014), by coupling to short chain lipoaminoacids (Caccetta et al. 2006; Namjoshi et al. 2014; Benson et al. 2003; Toth et al. 1995; Rocco et al. 2016; Benson and Namjoshi 2008), and encapsulating the peptide in hydrophobic carriers improving its absorption through the *stratum corneum* (Toth et al. 1995; Benson and Namjoshi 2008). For example, improved efficacy of this tetrapeptide as an inhibitor of HNE was demonstrated by conjugating the N-terminus with one, two or three lipid groups, wherein the inhibitory potency of the lipopeptides in relation to the skin HNE increased about ten thousand times compared with the unconjugated tetrapeptide (Caccetta et al. 2006; Benson et al. 2003).

Most peptides do not show ideal features to formulate drugs due to their intrinsic physico-chemical and pharmacokinetic profiles and often therapeutic peptides have limitations such as low oral bioavailability, short half-life due to rapid degradation by proteolytic enzymes and blood plasma, rapid elimination from the systemic circulation by hepatic and renal clearance, low capacity to cross physiological barriers due to their hydrophilicity, high conformational flexibility which can cause a lack of selectivity for the target to interact with different receptors, and immunogenic risks. The strategy for chemical optimization of a therapeutic peptide is usually based on structure-activity relationship studies in order to improve bioavailability, reduce or eliminate degradation and increase selectivity or affinity for the corresponding receptor (Vlieghe et al. 2010).

Prodrugs are, thus, inactive forms of a drug that, after efficient transfer to the target site, suffer proper transformation to release the active drug with control over space and time. It is crucial to control the scission of chemical bonds between the active drug and the moiety that masks the activity, and among the various triggering stimuli that can be used, i.e. enzymes, reducing or oxidising agents, temperature or pH, the use of light represents a fast-developing methodology for application in controlled drug delivery (Bildstein et al. 2011; Kratz et al. 2008; Jin et al. 2014).

Photocontrolled triggering and delivery have known an exponential growth in recent years, as it allows the cleavage of specific bonds within a structure with the consequent removal of the photoactive moiety (linked covalently to the active drug molecule) and the on demand/on site release of an active molecule. Among the numerous examples of biological phototriggering applications, reports can be found on caged capsaicin analogues for the in vitro activation of the capsaicin receptor TRPV1(Carr et al. 2006), short oligonucleotide uracil prodrugs for the inhibition of cancer cell proliferation (Fujimoto et al. 2014), protein biosynthesis using a caged aminoacyl-tRNA (Akahoshi et al. 2014), caged hydrogen sulfide for delivery to live cells (Fukushima et al. 2015), photo-uncaging of nitric oxide for targeting to mitochondria (Horinouchi et al. 2011), caged agonists for light-activated gene patterning (Link et al. 2004), molecular release based on auto-degradable polymer-containing organic–inorganic hybrids (Okada et al. 2014), photo-control of binding affinity to phosphopeptide-recognizing protein (Ebisuno et al. 2014), and light-switched inhibitors of protein tyrosine phosphatase PTP1B (Wagner et al. 2015).

In recent years, our research has been focused on the release of bioactive molecules mainly from heterocyclic cages, specifically designed for being triggered by light, to act as photoremovable protecting groups and phototriggers. Coumarin, benzocoumarin and pyrene derivatives, among other moieties, have been designed and applied in the caging of amino acids, neurotransmitters, biogenic amines, butyric acid and 5-aminolevulinic acid (Fernandes et al. 2007; Fernandes et al. 2008; Soares et al. 2010; Hungerford et al. 2010; Fonseca et al. 2010; Soares et al. 2012; Piloto et al. 2013; Fernandes et al. 2013; Piloto et al. 2015; Soares et al. 2015a; Soares et al. 2015b; Soares et al. 2017). Considering our past experience in such phototriggers, and although both photoactive moieties used in this manuscript have been previously reported by us, to the best of our knowledge the present work is the first account on the application of 7-methoxycoumarin-2-ylmethyl and pyren-2-ylmethyl groups in the preparation of caged

tetrapeptide AAPV (a bioactive peptide). The introduction of lipophilic phototriggers might also enhance the caged peptide's lipophilicity and the study of its light-triggered uncaging may lead, in future work, to a novel application scope aided by light, for example, in topical skin treatment of psoriasis and dermatitis.

Experimental Section

General

All melting points were measured on a Stuart SMP3 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). Fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer (Horiba/Jobin-Yvon). 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 ^{o}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 supported by bidimensional heteronuclear correlation techniques. Microwave-assisted solid phase peptide synthesis was carried out on a CEM Discover SPS equipment. Peptide analysis was carried out with analytical 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. Mass spectrometry analyses were performed at the "C.A.C.T.I. - Unidad de Espectrometria de Masas", at University of Vigo, Spain. 4-Chloromethyl-7-methoxycoumarin **1a** was synthesised as reported elsewhere (Piloto et al. 2006). 1-Chloromethylpyrene **1b** was purchased from TCI Chemicals, 1-pyrenemethanol **1c** from Sigma-Aldrich, Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Ala-OH, *N*,*N*′-diisopropylcarbodiimide (DIC), 6-chloro-*N*-hydroxybenzotriazole (6-Cl-HOBt) and 2-chlorotrityl chloride resin (degree of functionalisation 84%) were purchased from AAPPTec and *N*,*N*-diisopropylethylamine (DIPEA) was purchased from ACROS Organics and used as received.

MW-assisted solid phase synthesis of Fmoc-Ala-Ala-Pro-Val-OH 2a

Coupling of Fmoc-Val-OH to resin. Fmoc-Val-OH (0.858 g, 2.53 mmol, 2 equiv) was dissolved in dry DMF (7 mL) and DIPEA was added (878 μL, 5.04 mmol, 4 equiv). The

mixture was stirred for 10 minutes and then it was added to the previously swollen 2-chlorotrityl resin (1.51 g, 1.27 mmol) in dry DMF. The suspension was then subjected to microwave irradiation for 30 minutes (power = 25 W, temperature = 38 °C). The resin was filtered and washed with MeOH (2 × 10 mL), DMF (2 × 10 mL) and MeOH (2 × 10 mL) and this washing cycle was repeated 2 times. The filtered resin was dried in vacuum.

Removal of Fmoc protecting group. The filtered resin was swollen in DMF for 15 min and subjected to an initial deprotection to remove the Fmoc group using a solution of 20% piperidine in DMF (7 mL/g resin) for 30 seconds, under microwave irradiation (power = 28 W, temperature = 75 °C). The resin was washed with DMF (2 × 10 mL), MeOH (2 × 10 mL) and DMF (2 × 10 mL). A fresh solution of 20% piperidine in DMF was added to the resin and allowed to react for 3 minutes under microwave irradiation (power = 28 W, temperature = 75 °C). The resin was washed with DMF (2 × 10 mL) and MeOH (2 × 10 mL), and this washing cycle was repeated 3 times. The loading after the coupling of the first amino acid was determined to be 0.39 mmol/g, by taking a small sample of the resin (≈10 mg), cleaving the Fmoc group as stated and measuring the liberated dibenzofluvene by UV-vis absorption spectroscopy at 290 nm (Chan and White 2000).

Coupling of the remaining amino acids. Fmoc-Pro-OH (0.992 g, 2.94 mmol, 5 equiv) was dissolved in dry DMF (6 mL/g resin) and DIC (512 µL, 2.94 mmol, 5 equiv) and 6-Cl-HOBt (0.397 g, 2.94 mmol, 5 equiv) dissolved in dry DMF (2 mL/g) were added. The reaction mixture was allowed to stir at room temperature for 15 minutes and then was added to the resin, previously swollen in DMF. The suspension was subjected to microwave irradiation for 5 min (power = 28 W, temperature = 75 °C). The resin was washed with DMF (2 × 10 mL) followed by MeOH (2 × 10 mL) and this washing cycle was repeated 3 times. The filtered resin was swollen in dry DMF and the N-terminal Fmoc group was removed as described above.
The same procedure was followed twice for the coupling of Fmoc-Ala-OH, but the final N-deprotection was not carried out, in order to keep the Fmoc group at the N-terminal.

Cleavage from the resin. The filtered resin was swollen in dry DMF and a solution of AcOH/TFE/DCM (1:1:3) (10 mL) was added. The suspension was stirred for 1 hour at

room temperature and then the solvent mixture was collected by filtration. The resin was washed with DMF (2 × 10 mL) and DCM (2 × 10 mL) and the solvents were combined and evaporated to dryness on a rotary evaporator without heating. Cold diethyl ether was added and a colourless solid separated, which was filtered. The purity of the peptide was checked by analytical HPLC. The peptide Fmoc-Ala-Ala-Pro-Val-OH **2a** was obtained as a colourless solid (0.290 g, 85%). Mp = 94.0-95.2 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.88-0.91 (6H, m, 2 × γ-CH_3-Val), 1.35-1.39 (6H, m, 2 × CH_3 Ala), 2.02-2.08 (2H, m, H-3 Pro), 2.18-2.38 (3H, m, β-CH Val and H-4 Pro), 3.71-3.76 (2H, m, H-5 Pro), 4.22 (1H, t, *J* 7.2 Hz, H-9 Fmoc), 4.37-4.41 (3H, m, α-CH Ala and CH_2 Fmoc), 4.52-4.55 (1H, m, α-CH Val), 4.69 (1H, d, *J* 6 Hz, α-CH Pro), 4.83 (1H, t, *J* 6.8 Hz, α-CH Ala), 5.57 (1H, d, *J* 6.8 Hz, NH), 7.31 (2H, t, *J* 7.6 Hz, H-2 and H-7 Fmoc), 7.42 (3H, t, *J* 7.2 and 8.4 Hz, NH, H-3 and H-6 Fmoc), 7.60 (2H, d, *J* 7.6 Hz, H-4 and H-5 Fmoc), 7.63 (1H, d, *J* 6.8 Hz, NH), 7.76 (2H, d, *J* 7.2 Hz, H-1 and H-8 Fmoc).^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 17.56 (γ-CH_3 Val), 17.99 (CH_3 Ala), 18.93 (γ-CH_3 Val), 19.11 (CH_3 Ala), 25.03 (C-4 Pro), 29.68 (C-3 Pro), 31.35 (β-CH Val), 46.64 (α-CH Ala), 47.11 (C-9 Fmoc), 47.61 (C-5 Pro), 50.31 (α-CH Ala), 57.24 (α-CH Val), 60.27 (α-CH Pro), 67.11 (CH_2 Fmoc), 119.97 (C-1 and C-8 Fmoc), 125.11 (C-4 and C-5 Fmoc), 127.07 (C-2 and C-7 Fmoc), 127.71 (C-3 and C-6 Fmoc), 141.23 (C-4a and C-4b Fmoc), 143.75 (C-1a Fmoc), 143.86 (C-8a Fmoc), 155.88 (C=O Fmoc), 170.79 (C=O Pro), 172.37 (C=O Ala), 172.94 (C=O Ala), 174.42 (C=O Val). HRMS (ESI): calcd for $C_{31}H_{39}N_4O_7$ [M^++H]: 579.28207; found: 579.28174.

Synthesis of H-Ala-Ala-Pro-Val-OH 2b. The Fmoc-protected peptide **2a** (0.200 g, 0.35 mmol) was treated with a solution of 20% piperidine in DMF (3 mL), with stirring at room temperature for 3 hours. The mixture was treated with diethyl ether to precipitate the resulting peptide **2b** (0.112 g, 90%) which was filtered and used immediately in the following reactions. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.94-0.98 (6H, m, 2 × γ-CH_3 Val), 1.42 (3H, d, *J* 7.2 Hz, CH_3 Ala), 1.50 (3H, d, *J* 7.2 Hz, CH_3 Ala), 2.00-2.20 (5H, m, β-CH Val, H-3 Pro and H-4 Pro), 3.70-3.75 (2H, m, H-5 Pro), 3.98-4.00 (1H, m, α-CH Ala), 4.19-4.21 (1H, m, α-CH Val), 4.54-4.56 (1H, m, α-CH Pro), 4.67-4.69 (1H, m, α-CH Ala), 5.10 (2H, br s, NH_2), 8.00 (2H, br s, 2 × NH).

Synthesis of Boc-Ala-Ala-Pro-Val-OH 2c. The peptide H-Ala-Ala-Pro-Val-OH **2b** (0.100 g, 0.18 mmol) was dissolved in a 1,4-dioxane (3.6 mL), water (1.8 mL) and sodium hydroxide 1 M aqueous solution (1.8 mL), with stirring in an ice bath. *t*-Butyl pyrocarbonate (0.044 g, 0.20 mmol, 1.1 equiv) was added and the mixture stirred at room temperature for 1 day. The volume was reduced by half in a rotary evaporator under reduced pressure and ethyl acetate (2 mL) was added. The mixture was acidified by addition of aqueous potassium hydrogen sulphate 1 M solution and extracted with ethyl acetate (3 × 5 mL), followed by washing of the organic layer with water (3 × 5 mL). The organic layer was dried with anhydrous magnesium sulphate and evaporated under reduced pressure, resulting an oily off-white solid (0.067 g, 82%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.84-0.88 (6H, m, 2 × γ-CH_3 Val), 1.38 (3H, d, *J* 7.2 Hz, CH_3 Ala), 1.45 (3H, d, *J* 7.2 Hz, CH_3 Ala), 1.48 (9H, s, $C(CH_3)_3$), 1.67-1.71 (2H, m, H-4 Pro), 1.83-2.05 (2H, m, H-3 Pro), 2.23-2.31 (1H, m, β-CH Val), 3.53-3.61 (2H, m, H-5 Pro), 4.27 (1H, br s, α-CH Val), 4.43-4.46 (1H, m, α-CH Pro), 4.65-4.67 (1H, m, α-CH Ala), 4.74 (1H, q, *J* 7.2 Hz, α-CH Ala), 5.21 (1H, d, *J* 7.6 Hz, NH), 7.30 (2H, br s, 2 × NH).

Synthesis of H-Ala-Ala-Pro-Val-OMe 2d. The peptide H-Ala-Ala-Pro-Val-OH **2b** (0.100 g, 0.18 mmol) was added to a previously prepared solution of thionyl chloride (14 µL, 0.18 mmol) in methanol (3 mL), in an ice bath. The solution was stirred for 8 hours at room temperature and the solvent was evaporated under reduced pressure without heating, yielding peptide **2d** in the form of hydrochloride salt as an off-white crystalline solid (0.070 g, quantitative). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.88-0.91 (6H, m, 2 × γ-CH_3 Val), 1.40 (3H, d, *J* 7.2 Hz, CH_3 Ala), 1.47 (3H, d, *J* 7.2 Hz, CH_3 Ala),1.69-1.72 (2H, m, H-4 Pro), 1.82-2.04 (2H, m, H-3 Pro), 2.20-2.30 (1H, m, β-CH Val), 3.54-3.63 (2H, m, H-5 Pro), 3.70 (3H, s, OCH_3), 4.25 (1H, br s, α-CH Val), 4.43-4.45 (1H, m, α-CH Pro), 4.65-4.67 (1H, m, α-CH Ala), 4.69-4.71 (1H, m, α-CH Ala), 5.21 (1H, d, *J* 7.6 Hz, NH), 7.30 (1H, br s, NH), 8.12 (3H, s, NH_3^+).

Synthesis of Fmoc-Ala-Ala-Pro-Val-OMcm 3a. The peptide Fmoc-Ala-Ala-Pro-Val-OH **2a** (0.135 g, 0.23 mmol, 1 equiv) was added to a solution of 4-chloromethyl-7-methoxycoumarin **1a** (0.079 g, 0.35 mmol, 1.5 equiv) and potassium fluoride (0.041 g, 0.70 mmol, 3 equiv) in dry DMF (3 mL). The mixture was stirred at room temperature for 48 h. The solid was filtered and the solvent was evaporated to dryness. The

coumarin labelled peptide **3a** was obtained as a light orange solid (0.174 g, 97%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.86-0.90 (6H, m, 2 × γ-CH_3 Val), 1.36-1.38 (6H, m, 2 × CH_3 Ala), 1.94-2.01 (2H, m, H-4 Pro), 2.09-2.12 (2H, m, H-3 Pro), 2.19-2.22 (1H, m, β-CH Val), 3.65-3.72 (2H, m, H-5 Pro), 3.85 (3H, s, OCH_3), 4.32-4.38 (1H, m, α-CH Ala), 4.45 (1H, t, *J* 6.8 Hz, H-9 Fmoc), 4.47-4.51 (1H, m, α-CH Val), 4.63-4.67 (1H, m, α-CH Pro), 4.68-4-70 (3H, m, α-CH Ala and CH_2 Fmoc), 5.23 (2H, d, *J* 1.6 Hz, CH_2 Mcm), 6.38 (1H, s, H-3 Mcm), 6.78-6.82 (2H, m, H-6 and H-8 Mcm), 7.28 (3H, t, *J* 7.2 Hz, H-2 and H-7 Fmoc and NH), 7.35 (3H, t, *J* 7.2 Hz, H-3 and H-6 Fmoc and NH), 7.42 (1H, d, *J* 7.2 Hz, H-5 Mcm), 7.67 (2H, d, *J* 7.2 Hz, H-4 and H-5 Fmoc), 7.71 (2H, d, *J* 7.6 Hz, H-1 and H-8 Fmoc), 7.61 (1H, br s, NH).

Synthesis of Fmoc-Ala-Ala-Pro-Val-OPym 3b. The peptide Fmoc-Ala-Ala-Pro-Val-OH **2a** (0.135 g, 0.23 mmol, 1 equiv) was added to a solution of 1-chloromethylpyrene **1b** (0.088 g, 0.35 mmol, 1.5 equiv) and potassium fluoride (0.041 g, 0.70 mmol, 3 equiv) in dry DMF (3 mL). The mixture was stirred at room temperature for 48 hours. The solid was filtered and the solvent was evaporated to dryness. The crude solid was purified by silica gel column chromatography using ethyl acetate/petroleum ether (4:6) as eluent. The fractions containing the product were combined and evaporated to dryness and the pyrene-labelled peptide **3b** was obtained as a beige solid (0.118 g, 65%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.78-0.83 (6H, m, 2 × γ-CH_3 Val), 1.33-1.35 (6H, m, 2 × CH_3 Ala), 1.90-2.14 (4H, m, H-4 Pro and H-3 Pro), 2.33 (1H, m, β-CH Val), 3.48-3.55 (2H, m, H-5 Pro), 4.53-4.60 (3H, m, α-CH Val, α-CH Ala and H-9 Fmoc),4.64-4.71 (4H, m, α-CH Pro, α-CH Ala and CH_2 Fmoc), 5.79 (2H, d, *J* 6.8 Hz, CH_2 Pym), 7.27-7.38 (4H, m, H-2, H-3, H-6 and H-7 Fmoc), 7.68-7.75 (4H, m, H-1, H-4, H-5 and H-8 Fmoc), 7.82 (1H, d, *J* 6.8 Hz, NH), 8.01-8.18 (11H, m, 9 × *H*-Pym and 2 × NH).

Synthesis of H-Ala-Ala-Pro-Val-OMcm 4a. The peptide Fmoc-Ala-Ala-Pro-Val-OMcm **3a** (0.170 g, 0.22 mmol) was treated with a solution of 20% piperidine in DMF (5 mL), stirring at room temperature for 3 hours. The mixture was concentrated by evaporation under vacuum without heating and treated with diethyl ether to precipitate the resulting peptide **4a** as a light yellow solid (0.050 g, 41%). Mp = 256.5-258.0 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.95-1.02 (6H, m, 2 × γ-CH_3 Val), 1.37-1.41 (6H, m, 2 ×

CH_3 Ala), 1.88-1.93 (2H, m, H-4 Pro), 1.96-1.99 (2H, m, H-3 Pro), 2.17-2.23 (1H, m, β-CH Val), 3.78-3.79 (3H, m, H-5 Pro and α-CH Ala), 3.90 (3H, s, OCH_3), 4.17 (1H, br d, *J* 4.4 Hz, α-CH Val), 4.55-4.57 (1H, m, α-CH Pro), 4.62-4.64 (1H, m, α-CH Ala), 5.42 (2H, s, CH_2 Mcm), 6.36 (1H, s, H-3 Mcm), 6.92-6.97 (1H, m, H-6 and H-8 Mcm), 7.62 (1H, d, *J* 7.2 Hz, H-5 Mcm) (NH protons not visible). ^{13}C NMR ($CDCl_3$, 100.6 MHz): *δ* 18.37 (CH_3 Ala), 18.64 (CH_3 Ala), 19.57 (γ-CH_3 Val), 20.19 (γ-CH_3 Val), 23.09 (C-3 Pro), 23.76 (C-4 Pro), 31.59 (β-CH Val), 45.71 (C-5 Pro), 56.39 (OCH_3), 56.48 (α-CH Ala), 59.60 (α-CH Val), 61.04 (α-CH Pro), 61.76 (α-CH Ala), 63.02 (CH_2 Mcm), 102.05 (C-8 Mcm), 108.62 (C-4a Mcm), 110.78 (C-3 Mcm), 111.83 (C-6 Mcm), 113.84 (C-5 Mcm), 156.77 (C-4 Mcm), 158.19 (C-8a Mcm), 162.88 (C-2 Mcm), 164.70 (C-7 Mcm), 172.29 (C=O Pro), 173.22 (C=O Ala), 173.53 (C=O Ala), 174.57 (C=O Val). HRMS (ESI): calcd for $C_{27}H_{37}N_4O_8$ [M^++H]: 545.26131; found: 545.26174.

Synthesis of Ala-Ala-Pro-Val-OPym 4b. The peptide Fmoc-Ala-Ala-Pro-Val-OPym **3b** (0.110 g, 0.14 mmol) was treated with a solution of 20% piperidine in DMF (5 mL), stirring at room temperature for 3 hours. The mixture was concentrated by evaporation under vacuum without heating and treated with diethyl ether to precipitate the resulting peptide **4b** as a light yellow oil (0.050 g, 63%). ^{1}H NMR ($CDCl_3$, 400 MHz): *δ* 0.87-0.92 (6H, m, 2 × γ-CH_3 Val), 1.31-1.34 (6H, m, 2 × CH_3 Ala), 1.58-1.63 (2H, m, H-4 Pro), 1.82-1.87 (2H, m, H-3 Pro), 2.12-2.17 (1H, m, β-CH Val), 3.45-3.55 (2H, m, H-5 Pro), 3.58-3.60 (1H, m, α-CH Ala), 4.32-4.33 (1H, m, α-CH Val), 4.38-4.41 (1H, m, α-CH Pro), 4.54-4.56 (1H, m, α-CH Ala), 5.77 (2H, d, *J* 4.8 Hz, CH_2 Pym), 8.04-8.22 (11H, m, 9 × *H*-Pym and 2 × NH) (NH_2 protons not visible). ^{13}C NMR ($CDCl_3$, 100.6 MHz): *δ* 16.88 (CH_3 Ala), 18.52 (γ-CH_3 Val), 19.47 (γ-CH_3 Val), 19.91 (CH_3 Ala), 25.65 (C-4 Pro), 30.02 (C-3 Pro), 31.59 (β-CH Val), 45.74 (C-5 Pro), 50.81 (α-CH Ala), 59.66 (α-CH Ala), 60.87 (α-CH Pro and α-CH Val), 66.16 (CH_2 Pym), 128.17 (C-Pym), 128.34 (C-Pym), 128.38 (C-Pym), 128.76 (C-Pym), 128.80 (C-Pym), 128.96 (C-Pym), 129.10 (C-Pym), 129.23 (C-Pym), 129.33 (C-Pym), 129.37 (C-Pym), 129.85 (C-Pym), 130.00 (C-Pym), 130.85 (C-Pym), 132.03 (C-Pym), 132.55 (C-Pym), 133.23 (C-Pym), 172.75 (C=O Val), 173.12 (C=O Ala), 174.38 (C=O Pro), 175.33 (C=O Ala). HRMS (ESI): calcd for $C_{33}H_{39}N_4O_5$ [M^++H]: 571.29227; found: 571.29193.

Synthesis of Boc-Ala-Ala-Pro-Val-OMcm 4c. The peptide Boc-Ala-Ala-Pro-Val-OH **2c** (0.105 g, 0.23 mmol, 1 equiv) was added to a solution of 4-chloromethyl-7-methoxycoumarin **1a** (0.079 g, 0.35 mmol, 1.5 equiv) and potassium fluoride (0.041 g, 0.70 mmol, 3 equiv) in dry DMF (3 mL). The mixture was stirred at room temperature for 48 hours. The solid was filtered and the solvent was evaporated to dryness. The crude solid was purified by silica gel column chromatography using DCM/MeOH (50:1) as eluent. The fractions were combined and evaporated and the coumarin labelled peptide **4c** was obtained as a light yellow solid (0.101 g, 83%). Mp = 137.9 – 138.3 ºC. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.90-0.94 (6H, m, 2 × γ-CH_3 Val), 1.34-1.39 (6H, m, 2 × CH_3 Ala), 1.44 (9H, s, C(CH_3)$_3$), 1.99-2.10 (2H, m, H-4 Pro), 2.15-2.21 (1H, m, β-CH Val), 2.40-2.45 (1H, m, H-3 Pro), 3.51-3.66 (2H, m, H-5 Pro), 3.89 (3H, s, OCH_3), 4.28 (1H, br s, α-CH Ala), 4.56-4.59 (1H, m, α-CH Pro), 4.69-4.72 (2H, m, α-CH Val), 4.77-4.81 (1H, m, α-CH Ala), 5.19 (1H, br s, NH), 5.30-5.36 (2H, m, CH_2 Mcm), 6.58 (1H, s, H-3 Mcm), 6.86-6.90 (2H, m, H-6 and H-8 Mcm), 7.41 (2H, d, *J* 8.4 and 6.8 Hz, H-5 Mcm and NH), 7.67 (1H, d, *J* 8.0 Hz, NH). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 17.71 (CH_3 Ala), 18.58 (γ-CH_3 Val), 18.89 (CH_3 Ala), 19.09 (γ-CH_3 Val), 25.14 (C-4 Pro), 26.58 (C-3 Pro), 28.32 (C(*C*H_3)$_3$), 31.18 (β-CH Val), 46.65 (α-CH Ala), 47.40 (C-5 Pro), 50.06 (α-CH Ala), 55.82 (OCH_3), 57.74 (α-CH Pro), 59.84 (α-CH Val), 61.86 (CH_2 Mcm), 79.90 (*C*(CH_3)$_3$), 101.29 (C-8 Mcm), 110.34 (C-3 Mcm), 110.48 (C-4a Mcm), 112.74 (C-6 Mcm), 124.37 (C-5 Mcm), 148.53 (C-4 Mcm), 154.86 (C8a Mcm), 155.53 (C=O Boc), 161.04 (C- 2 Mcm), 162.95 (C-7 Mcm), 170.56 (C=O Val), 170.81 (C=O Pro), 172.26 (C=O Ala), 172.94 (C=O Ala). HRMS (ESI): calcd for $C_{32}H_{45}N_4O_{10}$ [M^++H]: 645.31375; found: 645.31351.

Synthesis of Poc-Ala-Ala-Pro-Val-OMe 4d. *N',N'*-Carbonyldiimidazole (0.061 g; 0.38 mmol, 1 equiv) was dissolved in dry DMF (2 mL) in an ice bath, then 1-pyrene methanol **1c** (0.087 g; 0.38 mmol, 1 equiv) was added slowly and the solution was left stirring for 1 hour at low temperature. The peptide H-Ala-Ala-Pro-Val-OMe **2d** in the form of hydrochloride (0.182 g; 0.38 mmol, 1 equiv) was neutralised with triethylamine in dry DMF (2 mL). The triethylammonium chloride was filtered and the solution was added to the previous mixture and left stirring at room temperature for 12 hours. The solvent was evaporated to dryness and the crude residue was purified by silica gel column chromatography using ethyl acetate/petroleum ether (4:6) as eluent. The fractions containing the product were combined and evaporated to yield the pyrene-

labelled peptide **4d** as an off-white solid (0.024 g, 10%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.87-0.88 (6H, m, 2 × γ-CH_3 Val), 1.15-1.18 (6H, m, 2 × CH_3 Ala), 1.87-1.89 (2H, m, H-4 Pro), 1.92-1.98 (1H, m, β-CH Val), 1.99-2.01 (2H, m, H-3 Pro), 3.38-3.41 (2H, m, H-5 Pro), 3.78 (3H, s, OCH_3), 4.25-4.26 (1H, m, α-CH Ala), 4.27-4.28 (1H, m, α-CH Val), 4.30-4.31 (1H, m, α-CH Pro), 4.33-4.35 (1H, m, α-CH Ala), 5.23 (1H, br s, NH), 5.74 (2H, d, CH_2 Pym), 7.93-8.15 (11H, m, 9 × *H*-Pym and 2 × NH). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 16.90 (CH_3 Ala), 18.51 (γ-CH_3 Val), 19.45 (γ-CH_3 Val), 19.92 (CH_3 Ala), 25.68 (C-4 Pro), 30.07 (C-3 Pro), 31.60 (β-CH Val), 45.75 (C-5 Pro), 50.85 (α-CH Ala), 52.51 (OCH_3), 59.75 (α-CH Ala), 60.78 (α-CH Pro), 60.82 (α-CH Val), 61.76 (CH_2 Poc), 128.19 (C-Poc), 128.37 (C-Poc), 128.41 (C-Poc), 128.72 (C-Poc), 128.75 (C-Poc), 128.94 (C-Poc), 129.13 (C-Poc), 129.24 (C-Poc), 129.38 (C- Poc), 129.45 (C-Poc), 129.81 (C-Poc), 130.04 (C-Poc), 130.80 (C-Poc), 132.05 (C-Poc), 132.59 (C-Poc), 133.21 (C-Poc), 155.62 (C=O Poc), 172.77 (C=O Val), 173.22 (C=O Ala), 174.44 (C=O Pro), 175.36 (C=O Ala). HRMS (ESI): calcd for $C_{35}H_{41}N_4O_7$ [M^++H]: 629.29773; found: 629.29810.

Photolysis general procedure

A 1 × 10^{-4} M MeOH/HEPES (80:20) or ACN/HEPES (80:20) solution of compounds **4a-d** (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, and 350 ± 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 ACN/water (3:1) at a flow rate of 0.8 mL/min for compounds **4c**-**d**, or ACN/water (3:1) with 0.1% TFA at a flow rate of 0.6 mL/min for compounds **4a**-**b**, 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 at the corresponding solvent (retention time in minutes: **4a**, 15.5; **4b**, 17.2; **4c**, 6.9; **4d**, 8.5).

Results and discussion

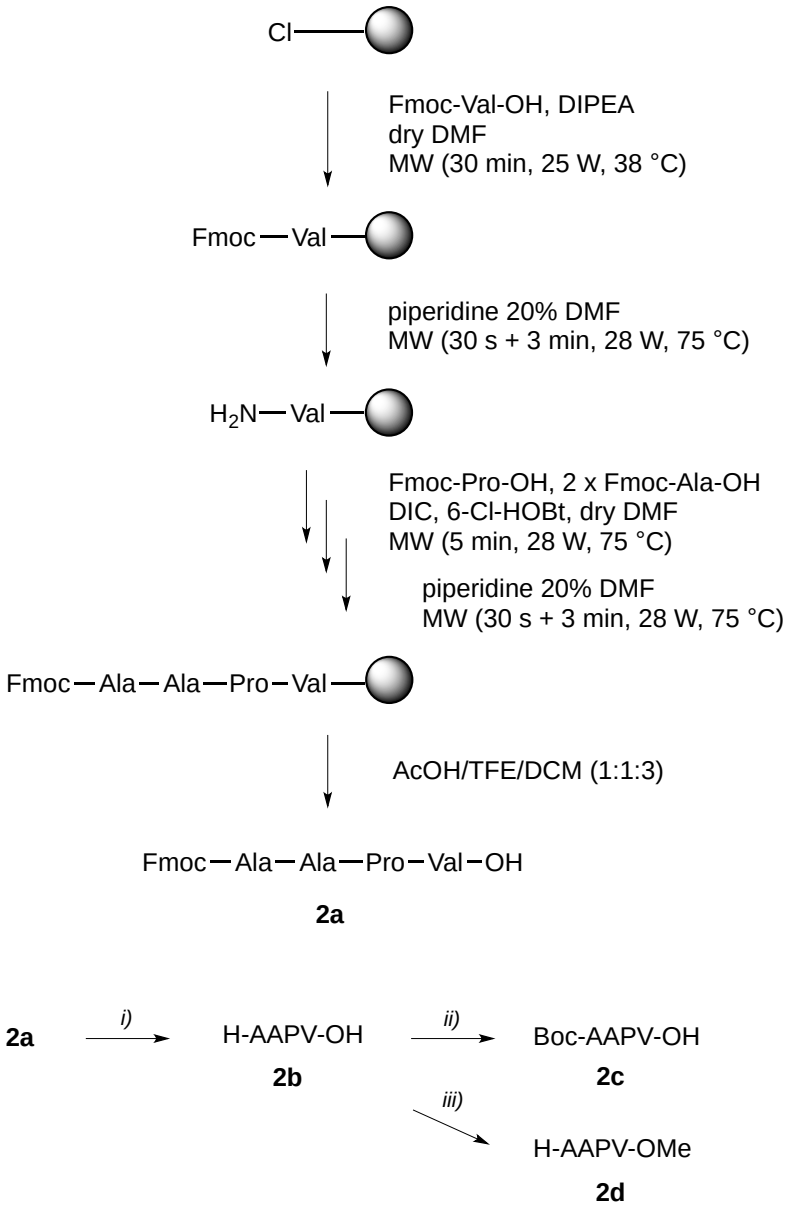
Synthesis of caged AAPV derivatives 4a-d

Coumarin and pyrene derivatives **1a-c** were chosen as photosensitive groups to assess their influence in the uncaging of the model bioactive tetrapeptide Ala-Ala-Pro-Val (AAPV), by introducing these groups at the N- and C-terminals of the peptide through carbamate or ester linkages, respectively.

4-Chloromethyl-7-methoxycoumarin **1a** was obtained by a Pechmann reaction between 3-methoxyphenol and ethyl 4-chloro-3-oxobutanoate, catalysed by acid, in 93% yield, by a previously published procedure (Piloto et al. 2006). This heterocycle has been used previously by us as caging group for amino acids with interesting photosensitivity (Fonseca et al. 2007; Fonseca et al. 2010). Likewise, pyrene-based photolabile groups were also applied by us in the caging of neurotransmitter amino acids and biogenic amines (Fernandes et al. 2007; Fernandes et al. 2008; Fernandes et al. 2013). So, 4-chloromethyl-7-methoxycoumarin **1a**, 1-choloromethylpyrene **1b** and 1-pyrenemethanol **1c** were used in the derivatization of AAPV at the C- and N-terminals.

The synthesis of Fmoc-AAPV-OH **2a** was accomplished by a solid phase synthesis protocol in an acid-labile 2-chlorotritylchloride resin on a manual microwave-assisted peptide synthesizer (CEM Discover) (Scheme 1). The resin and the necessary Fmoc-amino acids were commercially available and the resin had a functionalisation degree of 0.84 mmol/g. Amino acid coupling reactions were carried out in dry DMF in the presence of *N,N'*-diisopropylcarbodiimide (DIC) and 6-chloro-*N*-hydroxybenzotriazole (6-Cl-HOBt) (a 5 equiv excess was used), with a standard MW coupling protocol (5 minutes, 28 W, 75°C). After each coupling, the Fmoc protecting group was removed with 20% piperidine in DMF solution using a standard MW protocol (30 seconds cycle plus a 3 minutes' cycle, 28 W, 75 ^{o}C). The cleavage of the peptide **2a** from the resin was achieved by treatment with a AcOH/TFE/DCM (1:1:3) cocktail and Fmoc-AAPV-OH **2a** was isolated as a solid in 85% yield. The purity was checked by analytical HPLC and the peptide was characterised by ^{1}H and ^{13}C NMR spectroscopy and high-resolution mass spectrometry.

After removal of the Fmoc group from peptide **2a** to give free H-AAPV-OH **2b** (in 90% yield), reaction with *t*-butyl pyrocarbonate afforded Boc-AAPV-OH **2c** in 82% yield, while reaction with methanol and thionyl chloride resulted in the respective methyl ester H-AAPV-OMe **2d** in the form of hydrochloride salt in quantitative yield (Scheme 1).

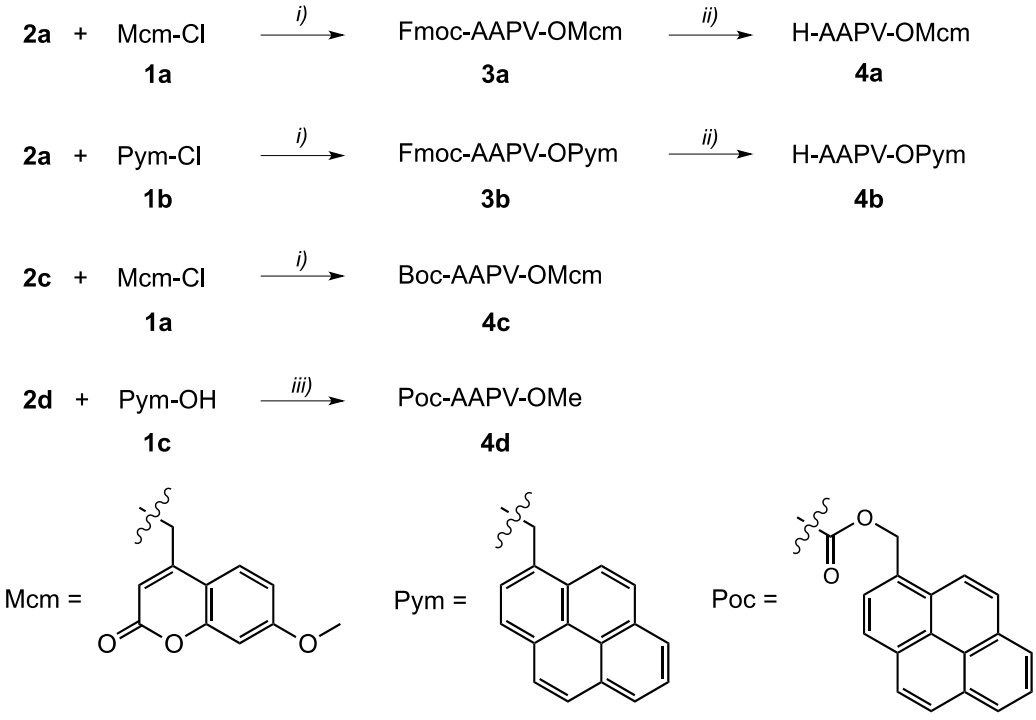


Scheme 1. Synthesis of AAPV derivatives **2a-d** by MW-SPPS on a 2-chlorotrityl chloride resin. Reaction conditions: *i)* 20% piperidine in DMF, rt; *ii)* Boc_2O, aq. 1M NaOH 1,4-dioxane, rt ; *iii)* $SOCl_2$, MeOH, rt.

The chloromethyl precursors **1a**,**b** were reacted with Fmoc-AAPV-OH **2a** in the presence of potassium fluoride in DMF (Tjoeng and Heavner 1981) to afford the corresponding ester conjugate peptides Fmoc-AAPV-OMcm **3a** (97%) and Fmoc-AAPV-OPym **3b** (65%) (Mcm will be used to designate the methoxycoumarinylmethyl group whereas Pym will represent the pyrenylmethyl group) (Scheme 2, Table 1). Considering the aromatic nature of the Fmoc protecting group, which could interfere with the photocleavage process by competitive radiation absorption, it was decided to

remove this group prior to subjecting the peptides to irradiation. Therefore, treatment with 20% piperidine in DMF solution afforded the N-deprotected peptides H-AAPV-OMcm **4a** (in 41% yield) and H-AAPV-OPym **4b** (in 63% yield).

In a similar manner, chloromethylated coumarin **1a** was reacted with Boc-AAPV-OH **2c** in the presence of potassium fluoride in DMF, resulting in Boc-AAPV-OMcm **4c** in 83% yield. As for peptide Poc-AAPV-OMe **4d**, it was obtained by reaction of AAPV-OMe **2d** with 1-pyrenemethanol **1c** mediated by *N',N'*-carbonyldiimidazole (CDI). The product was isolated after column chromatography in lower yield (10%) (Poc will be used to designate the pyrenylmethoxycarbonyl group). The purity of the C-and N-terminal caged peptides **4a-d** was checked by analytical HPLC and the peptides were characterised by ^{1}H and ^{13}C NMR spectroscopy and high-resolution mass spectrometry. ^{1}H NMR spectra showed the characteristic signals of the photosensitive groups in the aromatic zone and the methylene group, adjacent to the ester or carbamate link, was visible between δ 5.30-5.77 ppm. The newly formed ester or carbamate linkages in caged peptide **4a-d** were confirmed by ^{13}C NMR spectra signals of the carbonyl group, at about δ 170.56-174.57 (for the esters) or δ 155.62 ppm (for the carbamate).



Scheme 2. Synthesis of caged AAPV derivatives **4a-d**. Reaction conditions: *i)* KF, DMF, rt; *ii)* 20% piperidine in DMF, rt; *iii)* CDI, DMF, rt.

Photophysical characterisation of caged peptides 4a-d

Given the highly conjugated structure of the photosensitive groups, coumarin and pyrene, which are known for displaying strong absorption and fluorescence in the UV/vis, fundamental UV/vis absorption characterisation was performed for the caged peptides **4a-d**, also considering that their photolysis would be monitored by HPLC with UV detection. For the photolysis studies, it was decided to evaluate the influence of a protic (methanol) and aprotic (acetonitrile) solvent on the photolysis rates.

The absorption spectra of degassed 10^{-4} M solutions in MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) solutions of caged peptides **4a-d** were measured and the absorption maxima (λ_{max}), and molar absorption coefficients (log ε) are reported in Table 1.

Table 1. UV/vis absorption data for caged peptides **4a-d** in MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) solutions.

Compound	UV/vis absorption			
	MeOH/HEPES (80:20)		ACN/HEPES (80:20)	
	λ_{max} (nm)	log ε	λ_{max} (nm)	log ε
H-AAPV-OMcm **4a**	325	3.89	323	3.84
H-AAPV-OPym **4b**	342	4.39	342	4.43
Boc-AAPV-OMcm **4c**	325	4.29	320	4.07
Poc-AAPV-OMe **4d**	342	4.38	341	4.30

From the data in Table 1, it can be seen that the nature of the organic solvent did not influence significantly the absorption maxima: the values were very similar in both solvents, with the exception of peptide **4c**, for which a bathochromic shift of 5 nm was observed when comparing the protic and the aprotic solvent. As for the nature of the photosensitive group, the presence of the pyrene group led to larger λ_{max} (between 17-19 nm) in both solvents for peptides **4a** (bearing a C-terminal coumarin) and **4b** (bearing a a C-terminal pyrene).

Considering the fluorescent nature of the photosensitive groups, the fluorescence of caged peptide **4c**, as representative example, was studied in different organic solvents and aqueous mixtures. Emission maxima (λ_{em}) and relative fluorescence quantum yields (Φ_F) are given in Table 2. Relative fluorescence quantum yields were calculated using 9,10-diphenylanthracene in ethanol (Φ_F 0.95) (Morris et al. 1976) as standard. For the Φ_F determination, the fluorescence standard was excited at the maximum absorption wavelength, in each solvent, and in all fluorimetric measurements the absorbance of the solution did not exceed 0.1. The fluorescence displayed by the caged peptides **4a-d** is an interesting feature since it allows the monitoring of the processes and dynamics in which these peptides could participate by fluorescence spectroscopy.

Table 2. UV/vis absorption and fluorescence data for caged peptide Boc-AAPV-OMe **4c** in different organic solvents and aqueous mixtures.

Solvent	UV/vis absorption		Fluorescence		
	λ_{max} (nm)	log ε	λ_{em} (nm)	Φ_F	Stokes' shift (nm)
EtOH	325	4.05	394	0.34	69
MeOH	319	4.11	399	0.58	80
ACN	319	4.24	393	0.12	74
MeOH/HEPES (80:20)	325	4.29	400	0.76	75
ACN/HEPES (80:20)	320	3.54	397	0.56	77

The collected data revealed similar wavelengths of maximum absorption and emission, but the relative fluorescence quantum yield varied significantly being higher in protic solvents/mixtures (Table 2). The overall trend revealed that caged peptide **4c** was more fluorescent in aqueous mixtures, especially with methanol. This fact is of great significance for future biological applications involving fluorescence based techniques, as it could maximize the response and, consequently, increase the sensitivity of detection of the labelled peptide in aqueous media (Figure 1). The Stokes' shifts varied between 69-80 nm and a large Stokes' shift is an attractive characteristic for a fluorescent probe in biological media that allows an improved separation of the light inherent to the matrix and the light dispersed by the sample.

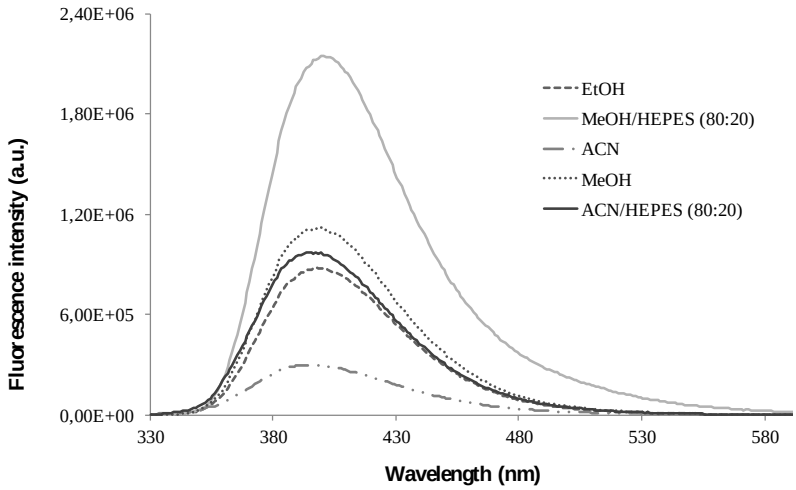
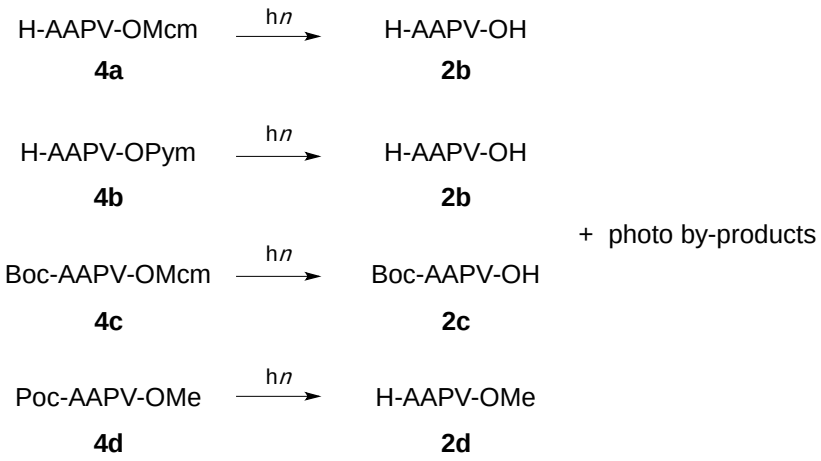


Figure 1. Normalised fluorescence spectra of caged peptide **4c** in different solvents (C = 7.6×10^{-6} M).

Photolysis studies of caged peptides 4a-d

Evaluation of the 7-methoxycoumarinylmethyl and pyrenylmethyl groups as photosensitive units for the caging of peptides, using AAPV as a model bioactive tetrapeptide, was carried out. Accordingly, peptides **4a-d** were irradiated at 254 nm, 300 nm and 350 nm in mixtures of methanol or acetonitrile with aqueous HEPES buffer in 80:20 solutions, in a Rayonet RPR-100 reactor, and kinetic data were collected. The course of the photolytic reaction was followed by reverse phase HPLC with UV detection and the disappearance of the peptide conjugates and the release of the corresponding peptide precursors was monitored (Scheme 3). The plots of peak area (A) of the starting material *versus* irradiation time were obtained for each compound, at chosen wavelengths. The peak areas were determined by HPLC, which revealed a gradual decrease with time and were taken as the average of three runs. The irradiation time (t_{irr}) given 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 (Muller et al. 2001). The rate constants (k) were calculated for a short conversion (30%) of the initial conjugates.



Scheme 3. Photolysis of caged peptides **4a-d** by irradiation at 254, 300, and 350 nm in MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) solutions.

Table 3. Irradiation times (t_{irr} in min), photochemical quantum yields (Φ_{phot}, $\times 10^{-3}$) and rate contants (k, in min^{-1}) for the photolysis of caged peptides **4a-d** by irradiation at 254, 300, and 350 nm in MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) solutions.

Cpd.	Solvent [a]	254 nm			300 nm			350 nm		
		t_{irr}	Φ_{phot}	k	t_{irr}	Φ_{phot}	k	t_{irr}	Φ_{phot}	k
4a	A	40	0.335	0.083	41	0.019	0.114	352	0.018	0.009
	B	46	0.400	0.106	55	0.148	0.081	1108	0.007	0.006
4b	A	18	0.162	0.113	32	0.075	0.129	310	0.006	0.006
	B	81	0.056	0.068	53	0.042	0.115	389	0.004	0.003
4c	A	57	0.087	0.076	347	0.012	0.025	2080	0.001	0.002
	B	74	0.125	0.040	163	0.038	0.017	2169	0.002	0.001
4d	A	4.4	1.050	0.806	8.3	0.274	0.404	46	0.045	0.073
	B	[b]	[b]	[b]	[b]	[b]	[b]	162	0.013	0.018

[a] A = MeOH/HEPES (80:20); B = ACN/HEPES (80:20). [b] Not determined.

The data in Table 3 revealed that, by irradiation at 254 nm, the time necessary for the release of the bioactive peptide from cages **4a-c** was larger in ACN/HEPES, with this difference being more accentuated for caged peptide **4b**. For peptides **4a** and **4c**, the presence of a *N*-butyloxycarbonyl protecting group resulted in a slightly longer irradiation time for the N-protected peptide (**4c**). When considering the different

photosensitive groups linked through an ester bond at this wavelength of irradiation, there was no clear tendency in the irradiation times in the solvents tested: the pyrenylmethyl group (Pym) cleaved faster in MeOH/HEPES while the coumarinylmethyl group (Mcm) cleaved faster in ACN/HEPES. In terms of the influence of the type of linkage between the photosensitive group and the peptide, in the case of pyrene it was observed that a carbamate link, as in Poc-AAPV-OMe **4d**, cleaved much faster (*ca*. 4 times) that the corresponding counterpart with an ester link, as in H-AAPV-OPym **4b**. Irradiation at 300 nm afforded similar observations: once again the carbamate caged peptide **4d** was the fastest to cleave and the influence of the presence of a N-Boc led to a dramatic difference in irradiation times, which occurred in peptide **4c** (Boc-AAPV-OMcm), in comparison with and **4a** (H-AAPV-OMcm).

At 350 nm, the influence of the solvent was clear with photolysis being faster in MeOH/HEPES for all caged peptides. At this wavelength of irradiation, the obtained irradiation times were too large to be of practical use, except for carbamate caged peptide Pym-AAPV-OMe **4d** in MeOH/HEPES, which cleaved readily in *ca*. 46 min.

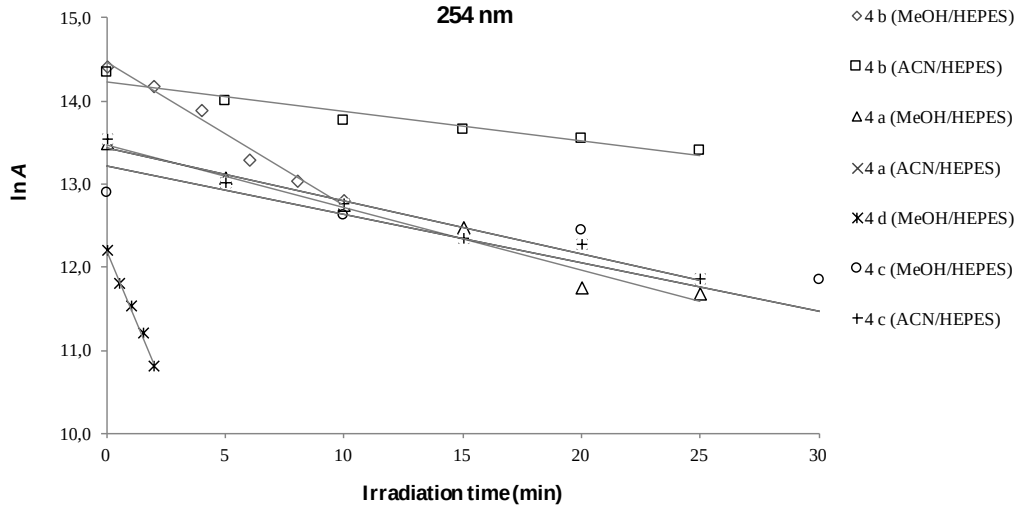
The relationship between the molar extinction coefficient of the conjugates at the wavelengths of irradiation and the photolysis rate constants was confirmed, as larger rate constants were obtained when the molar extinction coefficient was also larger, which was expected due to the higher probability of absorbing radiation and undergoing a photolytic pathway. For compound **4c**, nevertheless, this trend was not followed at 350 nm irradiation, which may be related to its tendency to undergo a radiative pathway (as seen by the high relative fluorescence quantum yields in the studied solvent mixtures).

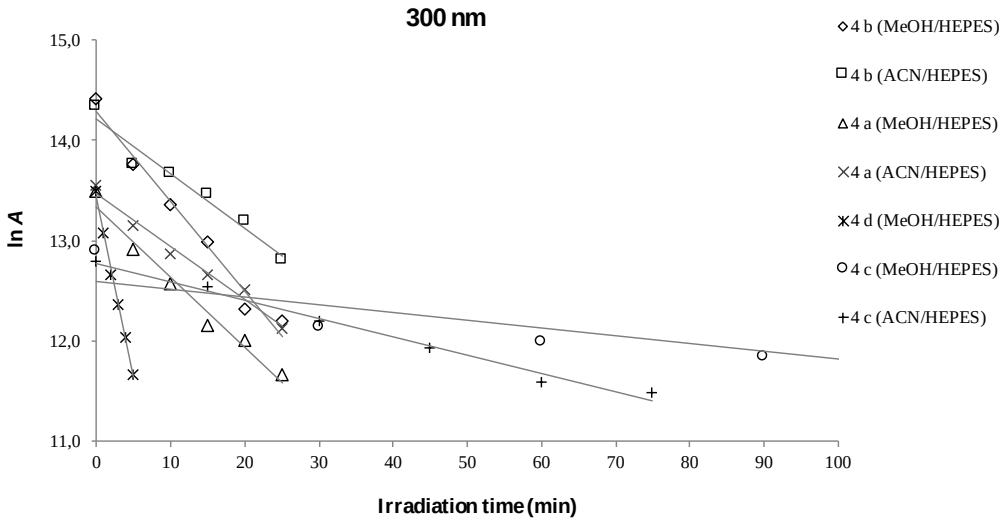
Overall, in the photolysis studies the time necessary to release the active peptide was shorter when photolyses were carried out in the more polar and protic solvent, MeOH/HEPES (80:20) and when the photosensitive group was linked to the peptide by a carbamate bond, as in peptide **4d**. Given the need to minimise cellular damage in the uncaging of molecules with biological activity by using wavelengths longer than 350 nm, the most suitable for biological applications, only the data obtained by irradiation at 350 nm for peptide **4d** constitute a promising result for future development.

Nevertheless, the reported results reflect the uniqueness of the experimental setup and this work intends to be a preliminary assessment of the applicability of the tested photoactive groups in the photorelease of AAPV. As a follow-up of this work, the most promising AAPV cage **4d** will undergo a more detailed study using time-resolved

fluorescence spectroscopy to characterise its photophysical properties. This kind of studies have been previously reported by us, regarding other bioactive targets, in order to relate to the conjugate, the ion pair intermediate and photocleaved species, by the determination of decay associated spectra to enable both spectral (energetic) and decay kinetics to be compared.

For each peptide, based on HPLC data, the plot of ln *A versus* irradiation time showed a linear correlation for the disappearance of the starting caged peptide. Although this fitting suggests a first order reaction, obtained by the linear least squares methodology for a straight line (Figure 2), one should not consider the behaviour of this system unequivocally as such, since deviations may occur considering that after photocleavage there are several absorbing species in solution (the caged peptide, the released photoactive moiety and the peptide).





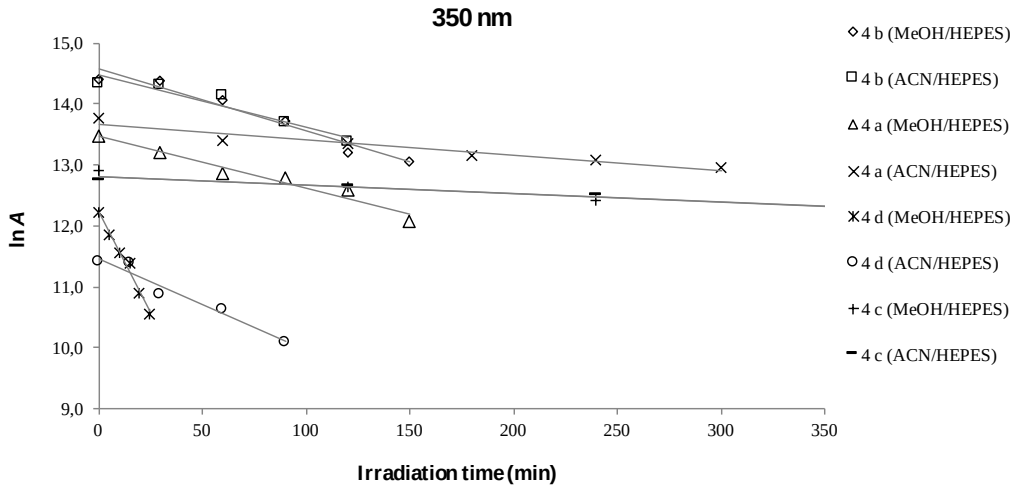


Figure 2. Plot of ln *A vs* irradiation time for the photolysis of caged peptides **4a-d** at the various wavelengths of irradiation in MeOH/HEPES buffer (80:20) and ACN/HEPES (80:20) solution.

Conclusions

Two photosensitive groups based on coumarin and pyrene were used for the first time in the preparation of ester and carbamate cages of Ala-Ala-Pro-Val (AAPV), a bioactive tetrapeptide that inhibits human neutrophil elastase (HNE), an enzyme involved in chronic inflammatory disease of the skin. Caged AAPV was subjected to photolysis at selected wavelengths (254, 300, and 350 nm), to assess the feasibility and influence of the photosensitive group and the type of linkage in the controlled photorelease of the active molecule, which was monitored by HPLC-UV.

The obtained data confirmed the applicability of the tested photoactive groups for the release of AAPV, especially for the derivative bearing the carbamate-linked pyrenylmethyl group, which displayed the shortest irradiation times for the release at the various wavelengths of irradiation (*ca*. 4 minutes at 254 nm, 8 minutes at 300 nm and 46 minutes at 350 nm).

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Conflict of interest

The authors declare that they have no conflict of interest.

Research involving human participants and/or animals

This article does not contain any studies with human participants or animals performed by any of the authors.

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CAPTIONS

Table 1. UV/vis absorption data for caged peptides **4a-d** in MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) solutions.

Table 2. UV/vis absorption and fluorescence data for caged peptide Boc-AAPV-OMcm **4c** in different organic solvents and aqueous mixtures.

Table 3. Irradiation times (t_{irr} in min), photochemical quantum yields (Φ_{phot}, $\times 10^{-3}$) and rate contants (k, in min^{-1}) for the photolysis of caged peptides **4a-d** by irradiation at 254, 300, and 350 nm in MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) solutions.

[a] A = MeOH/HEPES (80:20); B = ACN/HEPES (80:20). [b] Not determined.

Scheme 1. Synthesis of AAPV derivatives **2a-d** by MW-SPPS on a 2-chlorotrityl chloride resin. Reaction conditions: *i)* 20% piperidine in DMF, rt; *ii)* Boc_2O, aq. 1M NaOH 1,4-dioxane, rt ; *iii)* $SOCl_2$, MeOH, rt.

Scheme 2. Synthesis of caged AAPV derivatives **4a-d**. Reaction conditions: *i)* KF, DMF, rt; *ii)* 20% piperidine in DMF, rt; *iii)* CDI, DMF, rt.

Scheme 3. Photolysis of caged peptides **4a-d** by irradiation at 254, 300, and 350 nm in MeOH/HEPES buffer (80:20) and ACN/HEPES buffer (80:20) solutions.

Figure 1. Normalised fluorescence spectra of caged peptide **4c** in different solvents (C = 7.6×10^{-6} M).

Figure 2. Plot of ln *A vs* irradiation time for the photolysis of caged peptides **4a-d** at the various wavelengths of irradiation in MeOH/HEPES buffer (80:20) and ACN/HEPES (80:20) solution.

TABLES

Table 1.

Compound	UV/vis absorption			
	MeOH/HEPES (80:20)		ACN/HEPES (80:20)	
	λ_{max} (nm)	log ε	λ_{max} (nm)	log ε
H-AAPV-OMcm **4a**	325	3.89	323	3.84
H-AAPV-OPym **4b**	342	4.39	342	4.43
Boc-AAPV-OMcm **4c**	325	4.29	320	4.07
Poc-AAPV-OMe **4d**	342	4.38	341	4.30

Table 2.

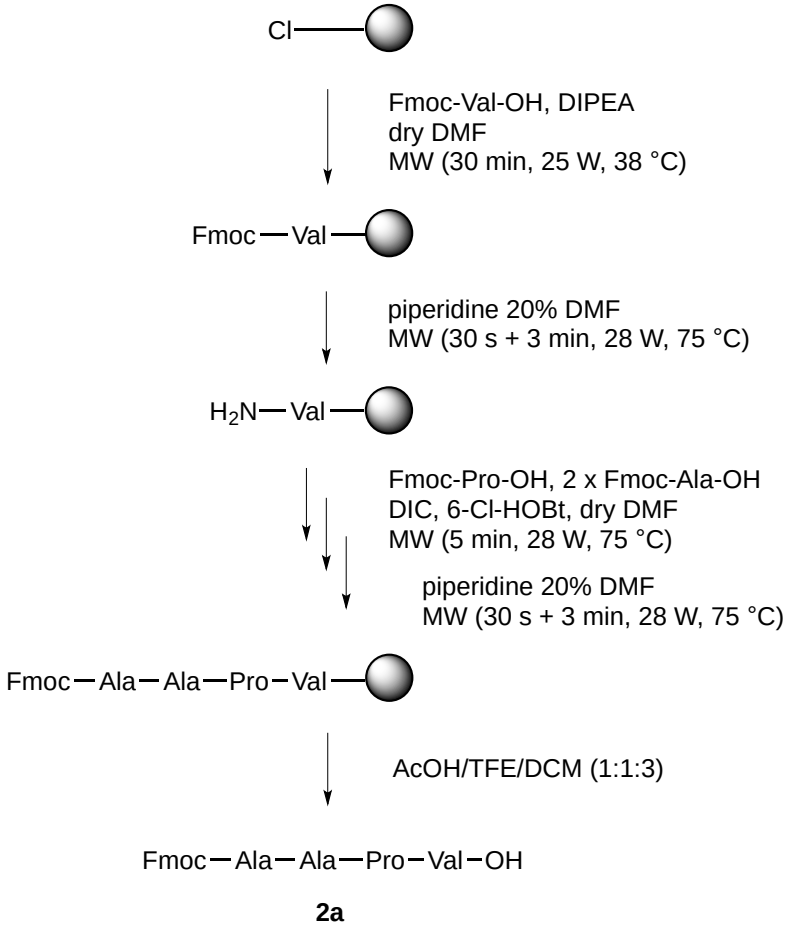
Solvent	UV/vis absorption		Fluorescence		
	λ_{max} (nm)	log ε	λ_{em} (nm)	Φ_F	Stokes' shift (nm)
EtOH	325	4.05	394	0.34	69
MeOH	319	4.11	399	0.58	80
ACN	319	4.24	393	0.12	74
MeOH/HEPES (80:20)	325	4.29	400	0.76	75
ACN/HEPES (80:20)	320	3.54	397	0.56	77

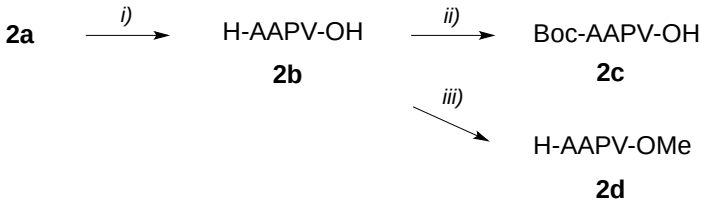
Table 3.

Cpd.	Solvent [a]	254 nm			300 nm			350 nm		
		t_{irr}	Φ_{phot}	k	t_{irr}	Φ_{phot}	k	t_{irr}	Φ_{phot}	k
4a	A	40	0.335	0.083	41	0.019	0.114	352	0.018	0.009
	B	46	0.400	0.106	55	0.148	0.081	1108	0.007	0.006
4b	A	18	0.162	0.113	32	0.075	0.129	310	0.006	0.006
	B	81	0.056	0.068	53	0.042	0.115	389	0.004	0.003
4c	A	57	0.087	0.076	347	0.012	0.025	2080	0.001	0.002
	B	74	0.125	0.040	163	0.038	0.017	2169	0.002	0.001
4d	A	4.4	1.050	0.806	8.3	0.274	0.404	46	0.045	0.073
	B	[b]	[b]	[b]	[b]	[b]	[b]	162	0.013	0.018

SCHEMES

Scheme 1.





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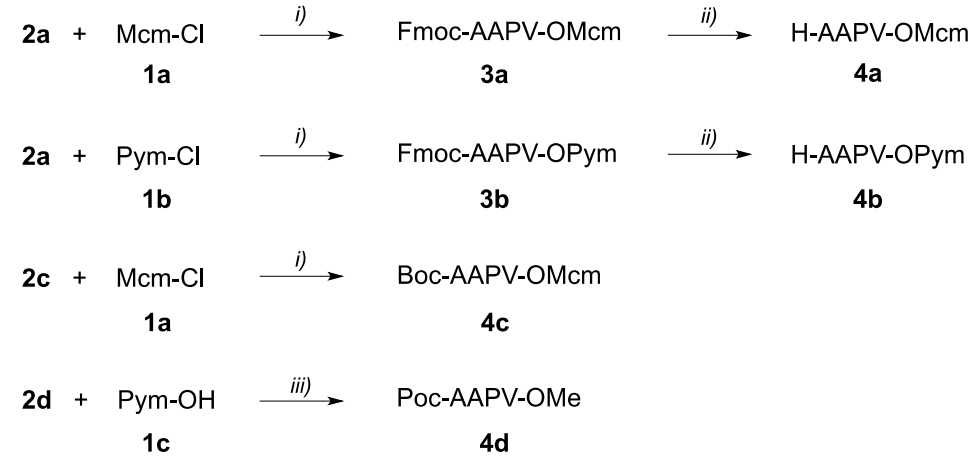

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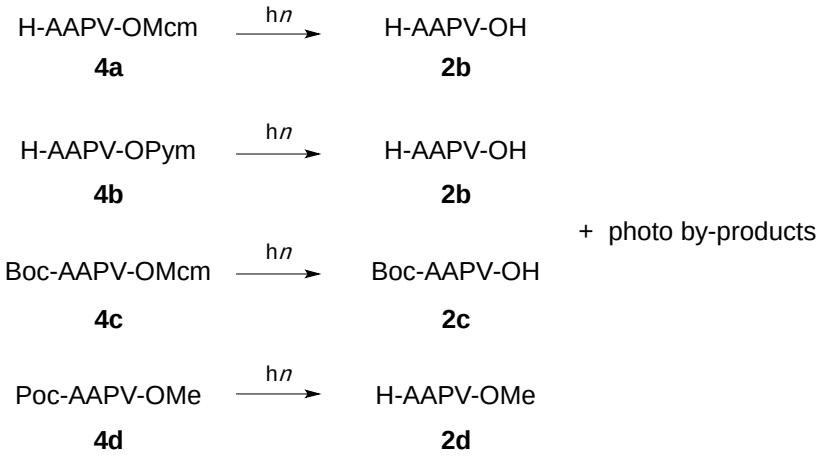


FIGURES

Figure 1.

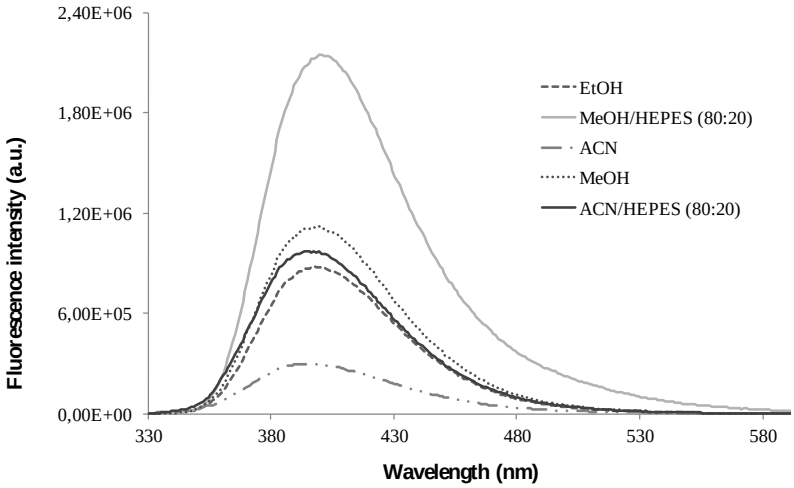
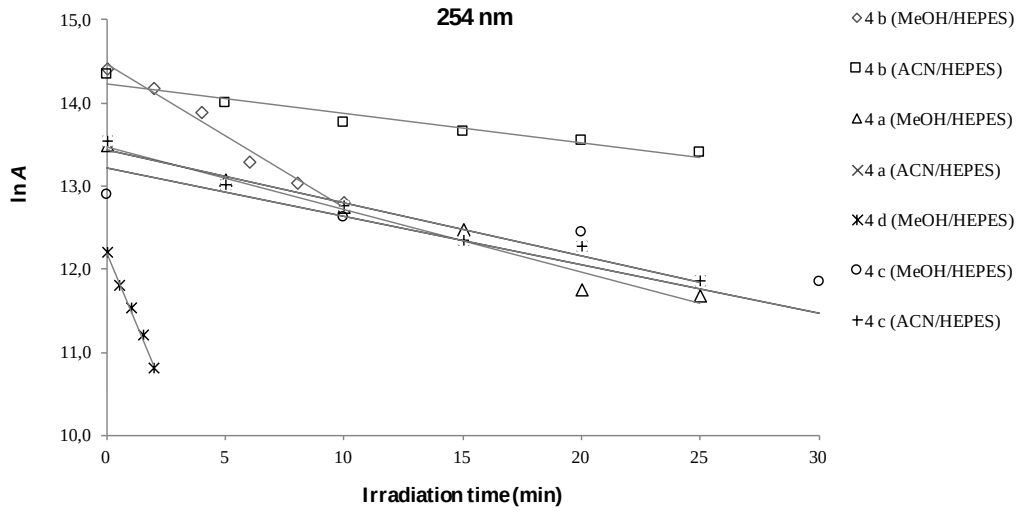
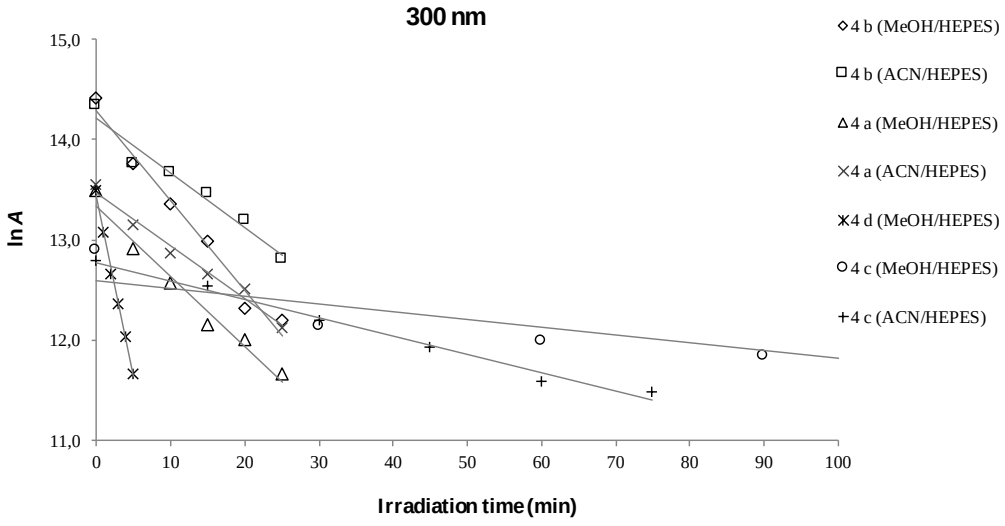


Figure 2.


254 nm
ln A
Irradiation time (min)
4 b (MeOH/HEPES)
4 b (ACN/HEPES)
4 a (MeOH/HEPES)
4 a (ACN/HEPES)
4 d (MeOH/HEPES)
4 c (MeOH/HEPES)
4 c (ACN/HEPES)


300 nm
ln A
Irradiation time (min)
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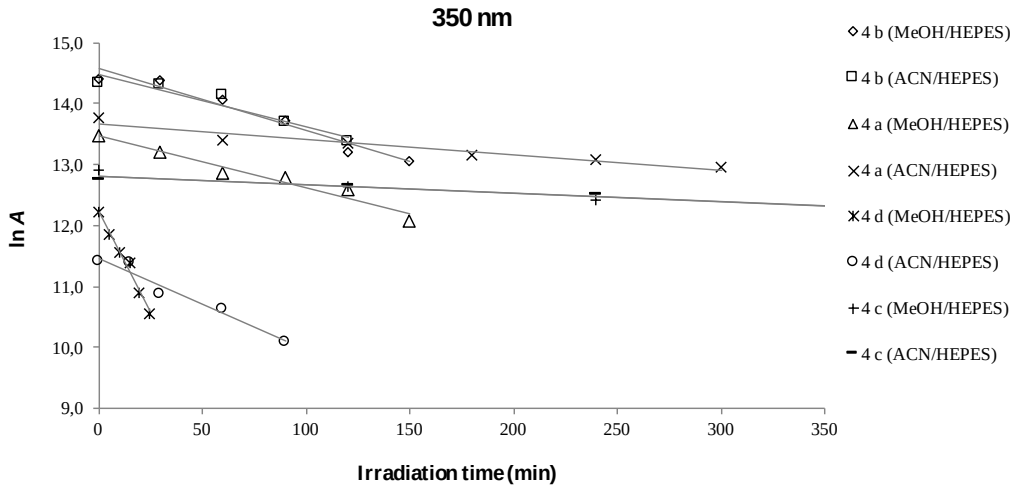
4 b (ACN/HEPES)
4 a (MeOH/HEPES)
4 a (ACN/HEPES)
4 d (MeOH/HEPES)
4 c (MeOH/HEPES)
4 c (ACN/HEPES)


350 nm
ln A
Irradiation time (min)
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4 b (ACN/HEPES)
4 a (MeOH/HEPES)
4 a (ACN/HEPES)
4 d (MeOH/HEPES)
4 d (ACN/HEPES)
4 c (MeOH/HEPES)
4 c (ACN/HEPES)