**N-(Di)icosyl-substituted benzo[a]phenoxazinium chlorides: synthesis and evaluation as near-infrared membrane probes**

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Five benzo[a]phenoxazinium chlorides containing alkyl chains with twenty carbon atoms on 5- or 9-positions of the tetracyclic ring were efficiently synthesised and characterised by UV/Vis/NIR spectroscopy. The absorption and emission maxima in ethanol lie in the range 627-641 nm and 645-676 nm, respectively, with quantum yields varying from 0.14 to 0.38. Preliminary photophysical studies with these fluorochromophores in zwitterionic (2,3-bis(palmitoyl-oxy)propyl-2-(trimethylammonio)ethyl phosphate, DPPC) and cationic (N,N-dimethyl-N-octadecyloctadecan-1-aminium bromide, DODAB) vesicles were carried out. The results showed that the new benzo[a]phenoxazinium derivatives are able to detect the gel to liquid-crystalline lipid phase transition through variations, either in the H-aggregation extent or in an acid-base equilibrium.

**Introduction**

Cellular membranes, selective barriers separating inside and outside cellular volumes, are crucial to the interactions with the cell exterior by enabling the transfer of many important compounds for cell metabolism and for chemical and electrical signaling. [1-3] In all biomembranes, the lipid bilayer constitutes the backbone, in which various proteins and glycan-containing membrane anchors are embedded.

The understanding of the biological functions of cell membranes is directly connected with their fundamental physicochemical properties. Several parameters, such as membrane electrostatics, phase state, hydration, and dynamics of the constituting molecules, establish the membrane structure and control the binding and transport of molecular and ionic species. Furthermore, they determine the correct insertion, proper folding, and function of membrane proteins. [4]
Fluorescent probing is one of the most suitable methods for the monitoring of these parameters in situ and the environment-sensitive fluorophores are of extreme importance. These compounds provide information on the properties of the molecular environment through changes in their photophysical characteristics.\textsuperscript{[5-10]}

The variety of fluorescent markers used in studies of the biophysical properties of lipid membranes, includes 9-(dicyanovinyl) julolidine (DCVJ), 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), Prodan, 7-(dialkylamino)-coumarin, styryl, 3-hydroxychromone (3HC) and their derivatives, as well as Nile Red and Nile Blue; the last two are oxazine dyes.\textsuperscript{[11-20,8,4]}

The synthesis of novel fluorochromophores based on the oxazine core with substituents, which allow for their interaction with a variety of biological molecules, is important for labelling purposes.\textsuperscript{[21]} Most biological macromolecules and structures have hydrophobic and hydrophilic zones, hence the presence of a long alkyl chain in the fluorescence label allows it to easily bind to the hydrophobic parts of biomolecules or biomembranes, enabling the fluorophore to probe its environment.\textsuperscript{[22]}

When these types of probes are incorporated in biomembranes, their oxazine core can report on the local changes of the membrane properties, namely micropolarity, the hydration level, charge and microviscosity. These properties are important to understand the structure, dynamics and function of the biological membrane, as previously mentioned. The cationic nature of benzophenoxazinium fluorophores is expected to allow them to probe the charge density of the biomembrane.

Considering these facts, and as part of our current research interests in the synthesis and characterisation of fluorescence probes,\textsuperscript{[23-30]} this work describes the synthesis of five benzo[\textit{a}]phenoxazinium chlorides with different combinations of substituents at 5-, 9- and 10-positions of the polycyclic aromatic ring, as well as the study of their photophysical behaviour in homogeneous media and in zwitterionic/cationic vesicles, regarding the changes in the substitution positions.

**Results and Discussion**

**Synthesis**

Benzo[\textit{a}]phenoxazinium chlorides 1\textit{a-e} were synthesised by the condensation of 5-(alkylamino)-2-nitrosophenol hydrochlorides 2\textit{a-e} with N-alkynaphthalen-1-amines 3\textit{a} or 3\textit{b} in acid media (Scheme 1). The required nitrosophenol 2\textit{a-e} was obtained by nitrosation of the corresponding 3-alkylaminophenol derivative with sodium nitrite and hydrochloric acid, in water or a mixture of ethanol-water as the solvent.\textsuperscript{[23-31]} The 3-(icosylamino)phenol and 3-(diicosylamino)phenol, precursors of compounds 2\textit{d} and 2\textit{e}, as well as N-icosynaphthalen-1-amine 3\textit{a} or N-propynaphthalen-1-amine 3\textit{b}\textsuperscript{[28,29]} were obtained by alkylation of 3-aminophenol and naphthalen-1-amine with 1-bromoicosane or 1-bromopropane (in the case of compound 3\textit{b}), in ethanol, in moderate to good yields. Precursors of nitrosophenols 2\textit{a-c} were commercial reagents.
Condensation of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride 2a, 5-diethylamino-2-nitrosophenol hydrochloride 2b and 5-dimethylamino-2-nitrosophenol hydrochloride 2c, with N-icosynaphthalen-1-amine 3a in the presence of hydrochloric acid, gave the benzo[a]phenoxazinium chlorides 1a-c. Starting from 5-(icosylamino)-2-nitrosophenol hydrochloride 2d and 5-(diicosylamino)-2-nitrosophenol hydrochloride 2e, and using N-propynaphthalen-1-amine 3b, compounds 1d and 1e were obtained, respectively. After purification by column chromatography on silica gel, cationic dyes 1a–e were isolated as blue solid materials in good to excellent yields (Table 1), and were fully characterised by high resolution mass spectrometry, IR and NMR (¹H and ¹³C) spectroscopy.

Scheme 1. Synthesis of benzo[a]phenoxazinium chlorides 1a-e.
The IR spectra of these benzo[a]phenoxazinium dyes showed the expected bands due to stretching vibrations of the amine function (3450-3402 cm\(^{-1}\)), the C-H linkage of the methyl and methylenic groups (2956-2850 cm\(^{-1}\)), the C-C linkage of the aliphatic chains (1186-817 cm\(^{-1}\)), as well as a strong band of the C=N bond (1657-1590 cm\(^{-1}\)) as a result of the oxazine ring.

Table 1. Synthesis and UV/Vis/NIR data for compounds 1a-e in ethanol.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield (%)</th>
<th>(\varepsilon) (M(^{-1}) cm(^{-1}))</th>
<th>(\lambda_{abs}) (nm)</th>
<th>(\lambda_{em}) (nm)</th>
<th>(\Phi_F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>73</td>
<td>89888</td>
<td>627</td>
<td>645</td>
<td>0.38</td>
</tr>
<tr>
<td>1b</td>
<td>68</td>
<td>56930</td>
<td>637</td>
<td>671</td>
<td>0.23</td>
</tr>
<tr>
<td>1c</td>
<td>85</td>
<td>63961</td>
<td>631</td>
<td>670</td>
<td>0.14</td>
</tr>
<tr>
<td>1d</td>
<td>37</td>
<td>27413</td>
<td>627</td>
<td>647</td>
<td>0.36</td>
</tr>
<tr>
<td>1e</td>
<td>70</td>
<td>48553</td>
<td>641</td>
<td>676</td>
<td>0.14</td>
</tr>
</tbody>
</table>

The \(^1\)H NMR spectra showed signals from the methyl groups of the aliphatic chains in the form of triplets or a broad singlet (1e) (\(\delta\) 0.83-1.09 ppm); the methyl group directly linked to the aromatic ring as a singlet (\(\delta\) 2.40 ppm; 1a). In compound 1c, with dimethylamine at 9-position of the benzo[a]phenoxazinium ring, the methyl protons occurred as singlets (\(\delta\) 3.24 ppm). The methyl protons of the ethyl groups (R\(_1\) and/or R\(_2\)) appeared as a multiplet (1a) or a triplet (1b) (\(\delta\) 1.19-1.40 ppm). For all compounds, the methylenic groups closed to the nitrogen atoms (NHCH\(_2\)CH\(_2\)) in 5- or 9-positions of heterocycles appeared as broad singlets or a multiplet (1c) (\(\delta\) 1.71-1.95 ppm), and the protons directly linked to the nitrogen atoms (NHCH\(_2\)CH\(_2\)) appeared as broad singlets or a multiplet (1c) (\(\delta\) 3.27-3.81 ppm). Spectra also showed the expected aromatic protons, in particular, H-10 as doublets or a broad singlet (1d) (\(\delta\) 6.92-7.11 ppm), H-8 as singlets (1a and 1b), a meta doublet (1c) (\(J\) 2.7 Hz) or broad singlets (1d and 1e) (\(\delta\) 6.10-6.48 ppm), and H-11, which appeared as a singlet (1a), a multiplet (1e) or as doublets (\(\delta\) 7.37-7.90 ppm).

The \(^{13}\)C NMR spectra showed the signals corresponding to the aliphatic N-substituents in the heterocycle, namely the methyl groups (\(\delta\) 11.55-14.05 ppm), and the methyl group directly attached to the benzene ring (1a) (\(\delta\) 18.29 ppm). For compound 1c with dimethylamine at 9-position of the benzo[a]phenoxazinium ring, the methyl carbons appeared at \(\delta\) 41.10 ppm. For all compounds, carbons of methylenic groups closed to the nitrogen atom (NHCH\(_2\)CH\(_2\)) in 5- and 9-positions of heterocycles appeared between 21.98 and 30.77 ppm and the carbon atoms directly linked to nitrogen (NHCH\(_2\)CH\(_2\)) occurred between 38.67 and 52.02 ppm. Spectra also showed the expected aromatic signals such as, C-6 (\(\delta\) 92.29-93.44 ppm), C-8 (\(\delta\) 93.08-95.81 ppm) and C-11 (\(\delta\) 131.01-132.39 ppm).
Photophysical studies

Electronic absorption and emission spectra of 10⁻⁶ M solutions in degassed absolute ethanol were measured for all the synthesised benzo[a]phenoxazinium chlorides 1a-e. It was observed that the absorption maxima ($\lambda_{\text{abs}}$) for 1a-e lie in the range of 627-641 nm, with molar absorptivities ($\varepsilon$) between 27413 and 89888 M⁻¹ cm⁻¹. The emission maxima ($\lambda_{\text{em}}$) were found to be in the range of 645-676 nm, by exciting at 570 nm. It can also be seen that 1a, with one C₂₀ alkyl chain at 5-position and one methyl group at 10-position, has the same absorption and emission maxima as 1d, which has one C₂₀ alkyl chain at 9-position but without the methyl group at 10-position. The fluorescence quantum yields measured with Oxazine 1 as a standard (fluorescence quantum yield, $\Phi_F = 0.11$ in ethanol)[32] for 1a and 1d are similar (0.38 and 0.36). Hence it can be formulated that similar substitutions at 5- and 9-positions reveal similar fluorescence parameters, irrespective of the substitution at 10-position.

Similarly, dyes 1b, 1c and 1e have comparable values for absorption maxima, emission maxima and quantum yields. In contrast to 1a and 1d, which are monoalkyl substituted at 9-position, derivatives with a dialkyl substitution at 9-position showed a batochromic shift in absorption and emission maxima, the latter being superior (about 35 nm), but presenting lower fluorescence quantum yields (0.14 or 0.23). The above results suggested that mono-substitution at 9-position displayed better fluorescence quantum yields than the di-substitution, irrespective of the chain length and the substitution at 10-position.

As an initial photophysical study in biological model systems, compounds 1a, 1d and 1e were incorporated in zwitterionic (DPPC) and cationic (DODAB) vesicles (Figure 1). It was observed that, with the exception of compound 1d in DPPC, these compounds are able to detect the gel to liquid-crystalline lipid phase transition by simple absorption measurements as well as by steady state fluorescence emission (Figures 2-4).

Figure 1. Structures of DPPC and DODAB.

It was previously shown that, depending on the physicochemical environment, 5,9-disubstituted benzo[a]phenoxazinium dyes can form H-aggregates (absorption at ~50 nm to the blue) and are involved in acid base equilibria (absorption at ~100 nm to the blue).[23-30] Recently, we have also found that the main site of acid-base equilibria is the amine at 5-position.[29] An acid-base equilibrium was not observed in aqueous
solutions, probably due to the fact that the basic neutral form was H-bonded and showed a similar photophysical behaviour to the positive acid form.

In the DPPC vesicles, all the compounds seem to be well hydrated near the beginning of the membrane interface as the basic form is not observed. It can also be seen that the icosylamino substituent at the 9-position (compounds 1d and 1e) prevents the formation of H-aggregates, which are non-fluorescent. This aggregation process happens for compound 1a (Figure 2) when DPPC is present in the gel phase, but is lost when the DPPC membrane undergoes its phase transition. The temperature increases should reduce the fluorescence intensity as the rate of the competing non-radiative internal conversion process increases. However, an increase in the fluorescence intensity is observed for compound 1a, which is explained by the absence of non-fluorescent H-aggregates in the liquid crystalline membrane phase. The 9-dialkylated-amino derivative 1e (Figure 4) detects the lipid phase transition with a shift to the blue in both absorption and emission maxima. Considering that the hydration and fluidity of the interface increase in the liquid-crystalline phase,\[^{[18]}\] this can be interpreted by the relocation of the compound towards the hydrophobic interior of the membrane, as it has been previously shown\[^{[23]}\] that for these types of compounds a decrease in polarity results in a blue spectral shift. The presence of the double chain is important for this relocalization process since it is not observed for the single-chain equivalent derivative (compound 1d).

The behaviour of the benzo\([a]\)phenoxazinum chlorides 1a-e in positive DODAB vesicles is completely different. In this case, an acid-base equilibrium is clearly observed for compounds 1d and 1e (Figures 3 and 4), which can be interpreted by their deeper position in the interior of the membrane to get way of the positive charge of DODAB molecules. Although the basic form is dominant, the fluorescence spectra is typical of the acid form, since its fluorescence quantum yield is much higher.\[^{[23,29]}\] Upon the gel to liquid-crystalline phase transition, the proportion of the basic form nearly goes to unity and the fluorescence spectrum is now dominated by a low intensity band that corresponds to the basic form. This effect is greater for the compound with a single side-chain at the 9-position of the benzo\([a]\)phenoxazinum 1d. The absence of an acid-base equilibrium in compound 1a can easily be explained by the fact that, upon membrane insertion, the 5-amino position will be buried within the aliphatic chain, making deprotonation impossible. Furthermore, H-aggregation is observed for this compound, decreasing with the membrane phase transition, as observed for DPPC.
Figure 2. Absorption and fluorescence spectra of compound 1a in DODAB (left) and DPPC (right) vesicles below ($T = 25^\circ C$) and above ($T = 55^\circ C$) the gel to liquid-crystalline lipid phase transition.

Figure 3. Absorption and fluorescence spectra of compound 1d in DODAB (left) and DPPC (right) vesicles below ($T = 25^\circ C$) and above ($T = 55^\circ C$) the gel to liquid-crystalline lipid phase transition.
Figure 4. Absorption and fluorescence spectra of compound 1e in DODAB (left) and DPPC (right) vesicles below \((T = 25°C)\) and above \((T = 55°C)\) the gel to liquid-crystalline lipid phase transition.

Conclusions

In summary, 5,9-diaminobenzo[a]phenoxazinium dyes 1a–e, possessing (di)icosylamino side-chains at 5- or 9-positions of the polyaromatic system were efficiently synthesised. These dyes displayed strong absorption, and fluorescence emission in the near-infrared region with good fluorescence quantum yields. Results of photophysical behaviour in biomembranes showed that the absorption and fluorescence spectra depended on the charge of the biological membrane and on the lipid chains organization. As a result, the synthesised molecules were able to detect the gel to liquid-crystalline lipid phase transition by simple absorption and fluorescence measurements as well as to provide information on the charge of the membranes.

Experimental Section

General Information: 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 60F₂₅₄) and spots were visualised under UV light or with the naked eye. 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 or neat samples. 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 an internal reference at 25 °C. All chemical shifts are given in ppm using δH Me3Si = 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 ressonance and bidimensional heteronuclear HMBC and HMOC correlation techniques. Mass spectrometry analyses were performed at the “C.A.C.T.I. - Unidad de Espectrometria de Masas”, at the University of Vigo, Spain. UV-Visible absorption spectra (200 – 800 nm) were obtained using either a Shimadzu UV/2501PC or Shimadzu UV/3101PC spectrophotometers. Fluorescence spectra were collected using either FluoroMax-4 or FluoroMax-3 spectrofluorometers. All chemical reagents were used as received. Labelled vesicles were prepared by injection of an ethanolic solution containing the benzo[a]phenoxazinium chloride 1a-e and either DODAB or DPPC into a required amount of an aqueous pH 7 buffered solution. The injection was made above the gel to liquid crystalline phase transition and the DODAB/DPPC: dye molar ratio was kept at 500:1. The dye concentration was 2.0×10^-6 M. The resulting solutions were allowed to equilibrate for one day before absorption/fluorescence measurements were undertaken.

General method for the preparation of compounds 1a-e: To a cold solution (ice bath) of 5-(alkylamino)-2-nitrosophenol hydrochloride 2a-e in ethanol (2-3 mL), N-alkylated-naphthylamine 3a,b and concentrated hydrochloride acid (5.0×10^-2 mL) were added. The mixture was refluxed during 5 to 15 hours, and monitored by TLC (dichloromethane/methanol or chloroform/methanol). After evaporation of the solvent and column chromatography purification on silica gel, the required dye 1a-e was obtained as a blue material.

N-[5-(Icosylamino)-10-methyl-9H-benzo[a]phenoxazin-9-ylide-nejethanaminium chloride 1a. The product of the reaction of 2a (0.085 g, 4.73×10^-4 mol) with 3a (0.20 g, 4.73×10^-4 mol) (reflux time 5 h) was chromatographed with dichloromethane/n-hexane and dichloromethane/methanol, mixtures of increasing polarity, as the eluent, to give compound 1a (0.20 g, 73%). mp = 125.0-126.2 °C. TLC (dichloromethane/methanol, 95:5): Rf = 0.79. 1H NMR (CDCl3, 300 MHz): δ = 0.86 (t, J = 6.9 Hz, 3 H, NH(CH2)9CH3), 1.19-1.40 (2×m, 37 H, 17×CH2 and NHCH2CH3), 1.81 (br s, 2 H, NHCH2CH3), 2.40 (s, 3 H, CH3), 3.27 (br s, 2 H, NHCH2CH3), 3.47 (br s, 2 H, NHCH2CH3), 6.16 (s, 1 H, 8-H ), 6.22 (s, 1 H, 6-H), 6.71 (br s, 1 H, NH), 7.37 (s, 1 H, 11-H), 7.70-7.90 (br s, 2 H, 2-H and 3-H ), 8.73 (d, J = 7.2 Hz, 1 H, 1-H), 8.94 (br s, 1 H, 4-H), 10.34 (br s, 1-H, NH). 13C NMR (CDCl3, 75.4 MHz): δ = 13.84 (NCH2CH3), 14.05 (N(CH2)9CH3), 18.29 (CH3), 22.60 (CH3), 27.10 (NHCH2CH3), 28.60 (CH2), 29.27 (CH2), 29.30 (CH2), 29.51 (CH2), 29.55 (CH2), 29.57 (CH2), 29.62 (CH2), 30.87 (CH2), 31.84 (CH2), 38.67 (NHCH2CH3), 44.48 (NHCH2CH3), 92.29 (C-6), 93.08 (C-8), 123.44 (Ar-C), 123.98 (C-1), 125.10 (C-4), 127.00 (C-10), 129.67 (Ar-C), 129.93 (C-3), 130.55 (Ar-C), 131.01 (C-11), 131.54 (C-2), 133.38 (Ar-C), 146.76 (Ar-C), 150.57 (Ar-C), 154.10 (C-9), 156.80 (C-5). IR (KBr 1%, cm^-1): ν = 3450, 3240, 2956, 2922, 2852, 1642, 1591, 1562, 1544, 1520, 1500, 1451, 1436, 1348, 1343, 1315, 1295, 1260, 1234, 1185, 1163, 1130, 1054, 1086, 1010, 881, 817, 773, 734, 708, 666. HRMS: m/z (El): calcd for C39H58N5O [M]+: 584.45766; found: 584.45744.
N-Ethyl-N-[5-(icosylamino)-9H-benzo[a]phenoxazin-9-ylidene] ethanaminium chloride 1b. The product of the reaction of 2b (0.092 g, 4.73×10⁻⁴ mol), with 3a (0.20 g, 4.73×10⁻⁴ mol) (reflux time 9 h) was chromatographed with dichloromethane/n-hexane and dichloromethane/methanol, mixtures of increasing polarity, as the eluent, to give compound 1b (0.19 g, 68%). mp = 133.2-134.2 °C. TLC (dichloromethane/methanol, 9:1): R₉ = 0.51.¹¹H NMR (CDCl₃, 300 MHz): δ = 0.84 (t, J = 6.6 Hz, 3 H, NH(CH₂)₁₉CH₃), 1.10-1.30 (m, 32 H, 16×CH₂), 1.31 (t, J = 6.9 Hz, 3 H, N(CH₂CH₂)₂), 1.45 (br s, 2 H, CH₂), 1.87 (br s, 2 H, NHCH₂CH₃), 3.50-3.62 (m, 2 H, N(CH₂CH₂)₂), 3.77 (br s, 2 H, NHCH₂CH₃), 6.48 (s, 2 H, 8-H and 6-H), 6.92 (d, J = 9.0 Hz, 1 H, 10-H), 7.62 (d, J = 9.0 Hz, 1 H, 11-H), 7.65-7.75 (m, 2 H, 2-H and 3-H), 8.55-8.65 (m, 1 H, 1-H), 9.26 (br s, 1 H, 4-H).¹³C NMR (CDCl₃, 75.4 MHz): δ = 12.58 (N(CH₂CH₂)₂), 13.98 (NH(CH₂)₁₉CH₃), 22.54 (CH₃), 27.13 (CH₂), 29.01 (NHCH₂CH₃), 29.21 (CH₂), 29.30 (CH₂), 29.47 (3×CH₂), 29.51 (4×CH₂), 29.56 (4×CH₂), 31.77 (2×CH₃), 45.05 (NHCH₂CH₃), 45.80 (N(CH₂CH₂)₂), 93.20 (C-6), 95.54 (C-8), 113.57 (C-10), 128.81 (C-1), 124.28 (Ar-C), 126.22 (C-4), 128.19 (Ar-C), 130.28 (C-3), 130.50 (Ar-C), 131.60 (C-2), 132.25 (C-11), 135.25 (Ar-C), 147.14 (Ar-C), 151.15 (Ar-C), 152.71 (C-9), 158.29 (C-5). IR (KBr 1%, cm⁻¹): ν = 3440, 2955, 2921, 2851, 1640, 1588, 1548, 1494, 1461, 1436, 1384, 1328, 1276, 1257, 1166, 1123, 1075, 1013, 947, 865, 757, 700, 666. HRMS: m/z (EI): calcd for C₈₀H₆₀N₅O [M⁺]: 598.47259; found: 598.47309.

N-[5-(Icosylamino)-9H-benzo[a]phenoxazin-9-ylidene]-N-methylmethanaminium chloride 1c. The product of the reaction of 2c (0.072 g, 4.74×10⁻⁴ mol), with 3a (0.201 g, 4.74×10⁻⁴ mol) (reflux time 7 h) was chromatographed with dichloromethane/n-hexane and dichloromethane/methanol, mixtures of increasing polarity, as the eluent, to give compound 1c (0.23 g, 85%). mp = 171.2-173.7 °C. TLC (dichloromethane/methanol, 9.5:0.5): R₉ = 0.37.¹¹H NMR (CDCl₃, 300 MHz): δ = 0.83 (t, J = 6.6 Hz, 3 H, NH(CH₂)₁₉CH₃), 1.10-1.50 (2×m, 34 H, 17×CH₂), 1.76-1.90 (m, 2 H, NHCH₂CH₃), 3.24 (s, 6 H, N(CH₃)₂), 3.60-3.76 (m, 2 H, NHCH₂CH₃), 4.62 (br s, 1 H, NH), 6.31 (d, J = 2.7 Hz, 1 H, 10-H), 6.38 (s, 1 H, 6-H), 6.92 (dd, J = 9.0 and 2.4 Hz, 1 H, 10-H), 7.49 (d, J = 9.3 Hz, 1 H, 11-H), 7.56-7.70 (m, 2 H, 2-H and 3-H), 8.51 (d, J = 8.1 and 1.2 Hz, 1 H, 1-H), 8.95 (d, J = 7.8 Hz, 1 H, 4-H).¹³C NMR (CDCl₃, 75.4 MHz): δ = 13.95 (NH(CH₂)₁₉CH₃), 22.51 (CH₂), 27.07 (CH₂), 28.95 (CH₃), 29.18 (2×CH₂), 29.30 (CH₂), 29.48 (5×CH₂), 29.53 (5×CH₂), 30.77 (NHCH₂CH₃), 31.75 (CH₂), 41.10 (N(CH₃)₂), 44.77 (NHCH₂CH₃), 93.02 (C-6), 95.68 (C-8), 114.35 (C-10), 123.62 (Ar-C), 123.88 (C-1), 125.36 (C-4), 128.62 (Ar-C), 129.93 (Ar-C), 130.30 (C-3), 131.61 (C-11), 131.90 (C-2), 134.39 (Ar-C), 146.63 (Ar-C), 150.88 (Ar-C), 154.63 (C-9), 157.67 (C-5). IR (KBr 1%, cm⁻¹): ν = 3439, 2955, 2920, 2851, 1657, 1642, 1589, 1552, 1537, 1513, 1494, 1463, 1427, 1383, 1330, 1291, 1204, 1178, 1149, 1125, 1010, 905, 865, 850, 836, 773, 741, 715, 666. HRMS: m/z (EI): calcd for C₈₀H₆₀N₅O [M⁺]: 570.44321; found: 570.44179.

N-[5-(Propylamino)-9H-benzo[a]phenoxazin-9-ylidene]icosan-1-aminium chloride 1d. The product of the reaction of 2d (0.505 g, 1.13×10⁻³ mol) with 3b (0.148 g, 8.0×10⁻² mol) (reflux time 12 h) was chromatographed with chloroform and chloroform/methanol, mixtures of increasing polarity, as the eluent, to give compound 1d (0.185 g, 37%). mp = 87-89°C. TLC (chloroform/methanol, 9:1): R₉ = 0.55.¹¹H NMR
N-Icosyl-N-[5-(propylamino)-9H-benzo[a]phenoxazin-9-ylidene]icosan-1-aminium chloride 1e. The product of the reaction of 2e (0.500 g, 7.1×10⁻⁴ mol) with 3b (0.088 g, 4.8×10⁻⁴ mol) (reflux time 15 h) was chromatographed with chloroform and chloroform/methanol, mixtures of increasing polarity, as the eluent, to give compound 1e (0.303g, 70%). mp = 140-142°C. TLC (chloroform/methanol, 94:6): Rf = 0.26. ¹H NMR (CDCl₃, 400 MHz): δ = 0.87 (t, J = 7.2 Hz, 3 H, NH(CH₃)₂); 1.07 (t, J = 7.2 Hz, 3 H, NHCH₂CH₂CH₃), 1.10-1.45 (m, 34 H, NHCH₂CH₂(CH₂)₇CH₃), 1.73 (br s, 2 H, NHCH₂CH₂(CH₂)₇CH₃), 1.90 (br s, 2 H, NHCH₂CH₂CH₃), 3.03 (br s, 2 H, NHCH₂CH₂(CH₂)₇CH₃), 3.49 (br s, 2 H, NHCH₂CH₂CH₃), 6.10 (br s, 1 H, H, 8-H), 6.16 (br s, 1 H, 6-H), 7.11 (br s, 1 H, 10-H), 7.34 (d, J = 8.0 Hz, 1 H, 11-H), 7.74 (br s, 3 H, 3-H, 2-H, N-H), 8.32 (br s, 1 H, NH), 8.64 (d, J = 6.8 Hz, 1 H, 4-H), 8.85 (br s, 1 H, 1-H). ¹³C NMR (CDCl₃, 100.6 MHz): δ = 11.55 (NHCH₂CH₂CH₃), 14.05 (2×NH(CH₃)₉CH₃), 22.33 (5×CH₂), 22.62 (NHCH₂CH₂CH₃), 26.97 (5×CH₂), 27.40 (2×NCH₂CH₂(CH₂)₇CH₃), 29.29 (4×CH₂), 29.38 (4×CH₂), 29.53 (4×CH₂), 29.59 (4×CH₂), 29.64 (4×CH₂), 31.85 (4×CH₂), 46.51 (NHCH₂CH₂CH₃), 52.02 (2×NCH₂CH₂(CH₂)₇CH₃), 93.44 (C-6), 95.81 (C-8), 113.69 (C-10), 124.03 (C-4), 124.23 (Ar-C), 126.33 (C-1), 128.41 (2×Ar-C), 130.70 (C-3), 131.95 (C-2), 132.39 (C-11), 135.61 (Ar-C), 147.33 (Ar-C), 151.52 (Ar-C), 153.14 (C-9), 158.76 (C-5), IR (KBr 1%, cm⁻¹): ν = 3430, 2955, 2918, 2850, 1638, 1590, 1548, 1490, 1466, 1434, 1384, 1330, 1288, 1236, 1182, 1163, 1124, 1016, 1000, 721, 666. HRMS: m/z (FAB): calcd for C₉₀H₅₈N₃O [M⁺+1]: 584.45724; found: 584.45744.

General method for preparation of compounds 2d and 2e: To an ice-cold solution of the 3-((di)icosylamino)phenol in ethanol, concentrated hydrochloric acid was added and stirred until the reaction mixture became homogenous. The solution of sodium nitrite in water was then added drop-wise within an interval of 20 min. The resulting mixture was stirred for the time mentioned and monitored by TLC (dichloromethane/methanol, 95:5). After evaporation of the reaction, the 5-((di)icosylamino)-2-nitrosophenol hydrochloride 2d or 2e was obtained as a yellow solid and was used in the following step without any purification.
5-(Icosylamino)-2-nitrosophenol hydrochloride 2d. From the reaction of 3-(icosylamino)phenol (0.392 g, 1×10⁻³ mol) in ethanol (4 mL) and concentrated hydrochloric acid (0.2 mL) with sodium nitrite (0.076 g, 1.1×10⁻³ mol) in water (0.4 mL), compound 2d was obtained (0.418 g; 98%).

5-(Diicosylamino)-2-nitrosophenol hydrochloride 2e. From the reaction of 3-(diicosylamino)phenol (0.300 g, 4.5×10⁻⁴ mol) in ethanol (3 mL) and concentrated hydrochloric acid (0.2 mL) with sodium nitrite (0.040 g; 5.8×10⁻⁴ mol) in water (0.1 mL), compound 2e was obtained (0.290 g; 93%).

**Synthesis of 3-(icosylamino)phenol and 3-(diicosylamino)phenol:** To a solution of 3-aminophenol (1.0 g, 9.1×10⁻³ mol) in ethanol (5 mL), 1-bromoicosane (3.976 g, 1.08×10⁻² mol) was added and the reaction mixture was refluxed for 44 h. After purification by column chromatography on silica gel with chloroform and chloroform/methanol as the eluent, 3-(icosylamino)phenol was obtained as a white solid (2.466 g, 70%), mp = 81.7-83.7°C. TLC (dichloromethane): Rf = 0.14. ¹H NMR (CDCl₃, 400 MHz): δ = 0.89 (t, J = 6.8 Hz, 3H, N(CH₂)₂CH₃), 1.25-1.40 (m, 34 H, NCH₂CH₃), 1.56-1.65 (m, 2 H, NCH₂CH₂CH₃), 3.08 (t, J = 7.2 Hz, 2 H, NCH₂CH₂CH₂CH₃), 3.35 (t, J = 7.2 Hz, 1 H, NH), 4.60 (br s, 1 H, OH), 6.10 (t, J = 2.0 Hz, 1 H, 2-H), 6.16 (dd, J = 8.0 Hz, J = 2.0 Hz, 1 H, 4-H), 6.20 (dd, J = 8.4 and 2.0 Hz, 1 H, 6-H), 7.01 (t, J = 8.0 Hz, 1 H, 5-H). ¹³C NMR (CDCl₃, 100.6 MHz): δ = 14.11 (N(CH₂)₂CH₃), 22.68 (2×CH₂), 27.15 (NCH₂CH₂CH₂CH₃), 29.35 (2×CH₂), 29.44 (2×CH₂), 29.51 (2×CH₂), 29.59 (2×CH₂), 29.60 (2×CH₂), 29.65 (2×CH₂), 29.69 (2×CH₂), 31.92 (CH₂), 43.96 (NCH₂CH₂CH₂CH₃), 99.35 (C-2), 103.98 (C-4), 105.90 (C-6), 130.12 (C-5), 150.15 (C-3), 156.69 (C-1). IR (KBr 1%, cm⁻¹): ν = 3372, 3274, 2954, 2918, 2849, 1631, 1594, 1518, 1505, 1486, 1473, 1464, 1391, 1374, 1333, 1252, 1239, 1205, 1191, 1165, 994, 946, 853, 846, 753, 729, 720, 688, 666. HRMS: m/z (ESI): calcd for C₃₀H₄₈NO [M⁺+1]: 390.3718; found: 390.37240.

In addition to the 3-(icosylamino)phenol, 3-(diicosylamino)phenol was also obtained as a purple solid (1.582 g, 26%). Mp = 55.7-57.7 °C. Rf = 0.20 (dichloromethane). IR (KBr 1%, cm⁻¹): ν = 3496, 3383, 2918, 2850, 1618, 1578, 1503, 1467, 1399, 1373, 1297, 1282, 1270, 1257, 1240, 1224, 1209, 1193, 1181, 1170, 1147, 1110, 1090, 1033, 999, 829, 720, 755, 689, 666 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz): δ = 0.89 (t, J = 6.8 Hz, 6 H, 2×N(CH₂)₂CH₃), 1.10-1.50 (m, 68 H, 2×NCH₂CH₂CH₂CH₃), 1.58 (br s, 4 H, 2×NCH₂CH₂CH₂CH₂CH₃), 3.22 (t, J = 8.0 Hz, 4 H, 2×NCH₂CH₂CH₂CH₂CH₃), 4.56 (br s, 1 H, OH), 6.09 (dd, J = 8.0 and 2.4 Hz, 1 H, 4-H), 6.12 (t, J = 2.0 Hz, 1 H, 2-H), 6.23 (dd, J = 8.4 and 2.4 Hz, 1 H, 6-H), 7.04 (t, J = 8.0 Hz, 1 H, 5-H). ¹³C NMR (CDCl₃, 100.6 MHz): δ = 14.11 (2×N(CH₂)₂CH₃), 22.69 (4×CH₂), 27.19 (2×NCH₂CH₂CH₂CH₂CH₃), 27.27 (4×CH₂), 29.36 (4×CH₃), 29.56 (4×CH₂), 29.63 (4×CH₂), 29.71 (12×CH₂), 31.93 (2×CH₂), 51.11 (2×NCH₂CH₂CH₂CH₂CH₃), 98.49 (C-2), 102.00 (C-4), 104.65 (C-6), 130.01 (C-5), 149.82 (C-3), 156.66 (C-1). HRMS: m/z (ESI): calcd. for C₄₆H₆₈NO [M⁺+1] 670.68544; found: 670.68604.

N-Icosynaphthalen-1-amine 3a: To a solution of naphthalen-1-amine (1.01 g; 6.98×10⁻³ mol) in ethanol (2 mL), 1-bromoicosane (2.65 g, 7.33×10⁻³ mol) was added and the resulting mixture was refluxed for 17 h 30
min, and monitored by TLC (chloroform/n-hexane, 7:3). The solvent was evaporated and the crude mixture was purified by column chromatography on silica gel using chloroform/n-hexane, mixtures of increasing polarity, as the eluent, to give compound 3a as a white oily solid (2.66 g, 90%). Rf = 0.69 (chloroform/n-hexane, 7:3). IR (neat, cm⁻¹): 3397, 2954, 2917, 2849, 1620, 1583, 1523, 1466, 1409, 1380, 1283, 1253, 1140, 1120, 785, 768, 722, 666 cm⁻¹. ¹H NMR (CDCl₃, 300 MHz): δ = 0.90 (t, J = 6.9 Hz, 3 H, NH(CH₂)₉CH₃), 1.40-1.50 (m, 34 H, NHCH₂CH₂(CH₂)₁₇CH₃), 1.72-1.86 (m, 2 H, NHCH₂CH₂(CH₂)₁₇CH₃), 3.30 (t, J = 6.9 Hz, 2 H, NHCH₂CH₂(CH₂)₁₇CH₃), 6.73 (br s, 1 H, 4-H), 7.28 (d, J = 7.8 Hz, 1 H, 2-H), 7.37 (t, J = 7.8 Hz, 1 H, 3-H), 7.42-7.50 (m, 2 H, 6-H and 7-H), 7.78-7.82 (m, 1 H, 8-H), 7.84-7.90 (m, 1 H, 5-H). ¹³C NMR (CDCl₃, 75.4 MHz): δ = 14.11 (NH(CH₂)₉CH₃), 22.68 (2×CH₂), 26.63 (2×CH₂), 27.14 (CH₂), 27.94 (CH₂), 28.31 (NHCH₂CH₂(CH₂)₁₇CH₃), 29.22 (CH₂), 29.32 (CH₂), 29.35 (CH₂), 29.53 (CH₂), 29.56 (CH₂), 29.69 (CH₂), 30.91 (CH₂), 31.91 (CH₂), 43.19 (CH₂), 46.57 (CH₂), 48.82 (CH₂), 53.00 (NHCH₂CH₂(CH₂)₁₇CH₃), 117.85 (C-4), 120.33 (C-5), 122.21 (C-2), 124.14 (C-4a), 126.05 (C-7), 126.17 (C-6), 126.71 (C-3), 128.69 (C-8), 134.34 (C-8a), 138.03 (C-1). HRMS: m/z (EI): calcd. for C₃₀H₄₉N [M⁺] 423.3865; found 423.3866.

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