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Near-Infrared fluorophores based on *N*-(di)icosyl-substituted benzo[*a*]phenoxazinium chlorides as biomembrane probes

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Abstract: Five benzo[*a*]phenoxazinium dyes containing alkyl chains with twenty carbon atoms on 5- or 9-positions of the tetracyclic ring were efficiently synthesised and characterised by UV/Visible and fluorescence 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 in zwitterionic (2,3-bis(palmitoyloxy)propyl 2-(trimethylammonio)ethyl phosphate, DPPC) and cationic (*N*,*N*-dimethyl-*N*-octadecyloctadecan-1-aminium bromide, DODAB) vesicles are reported showing that these molecules are able to detect the gel to liquid-crystalline lipid phase transition through variations either in H-aggregation extent or in an acid-base equilibrium.

Keywords: Benzo[*a*]phenoxazinium dyes; Biomembrane probes; Near-infrared fluorescent labels

1. Introduction

The synthesis of novel fluorochromophores based on the oxazine core with substituents, which allow for their interaction with a variety of biological molecules, would be important for labelling purposes.[1] Most of biological macromolecules and structures have hydrophobic and hydrophilic zones, and hence the presence of a long hydrocarbon chain in the fluorescence label, which allow it to easily bind to the hydrophobic parts of biomolecules or biomembranes enabling the fluorophore to probe its environment.[2]

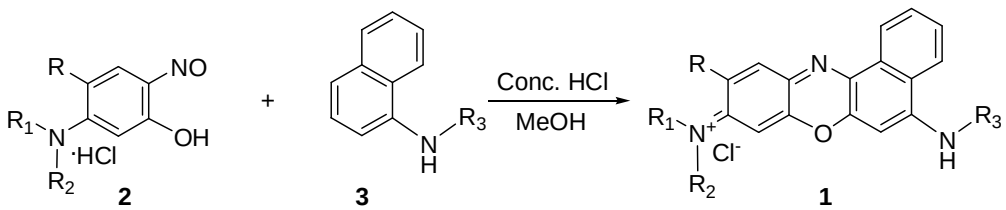
Considering these facts and as part of our current research interests in the synthesis and characterisation of fluorescence probes,[3] this work describes the synthesis of five benzo[*a*]phenoxazinium chlorides with different combinations of substituents at 5-, 9- and 10-positions of the polycyclic aromatic ring, as well as the study of the variation in photophysical

behaviour, in homogeneous media and in zwitterionic/cationic vesicles, regarding the changes in the substitution positions.

2. Results and Discussion

Benzo[*a*]phenoxazinium chlorides **1a-e** were synthesised by the condensation of 5-(alkylamino)-2-nitrosophenol hydrochlorides **2a-e** with *N*-alkylnaphthalen-1-amines **3a** or **3b** in acid media (Scheme). The required nitrosophenol **2a-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.[4] The 3-(icosylamino)phenol and 3-(diicosylamino)phenol, precursors of compounds **2d** and **2e**, as well as *N*-icosylnaphthalen-1-amine **3a** or *N*-propylnaphthalen-1-amine **3b**[5] were obtained by alkylation of 3-aminophenol and naphthalen-1-amine with 1-bromoicosane or 1-bromopropane (in the case of compound **3b**), in ethanol, in good to moderate yields. Precursors of nitrosophenols **2a-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*-icosylnaphthalen-1-amine **3a** in the presence of hydrochloric acid, produced 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*-propylnaphthalen-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), and were fully characterised by the usual analytical techniques.



a R = CH_3, R_1 = H, R_2 = Et
b R = H, R_1 = R_2 = Et
c R = H, R_1 = R_2 = Me
d R = R_1 = H, R_2 = $(CH_2)_{19}CH_3$
e R = H, R_1 = R_2 = $(CH_2)_{19}CH_3$

a R = $(CH_2)_{19}CH_3$
b R = $(CH_2)_2CH_3$

a R = CH_3, R_1 = H, R_2 = Et, R_3 = $(CH_2)_{19}CH_3$
b R = H, R_1 = R_2 = Et, R_3 = $(CH_2)_{19}CH_3$
c R = H, R_1 = R_2 = Me, R_3 = $(CH_2)_{19}CH_3$
d R = H, R_1 = H, R_2 = $(CH_2)_{19}CH_3$, R_3 = $(CH_2)_2CH_3$
e R = H, R_1 = R_2 = $(CH_2)_{19}CH_3$, R_3 = $(CH_2)_2CH_3$

Scheme. Synthesis of benzo[*a*]phenoxazinium chlorides **1a-e**.

Table. Synthesis, UV/Visible and fluorescence data for compounds **1a-e** in ethanol.

Compound	Yield (%)	ε ($M^{-1}cm^{-1}$)	λ_{abs} (nm)	λ_{em} (nm)	Φ_F
1a	73	89888	627	645	0.38
1b	68	56946	637	671	0.23
1c	85	63961	631	670	0.14
1d	25	27413	627	647	0.36
1e	30	48553	641	676	0.14

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 (λ_{abs}) for **1a-e** lie in the range 627-641 nm, with molar absorptivities (ε) between 27413 and 89888 $M^{-1}cm^{-1}$. The emission maxima (λ_{em}) were found to be in the range 645-676 nm, by exciting at 570 nm. It can also be seen that **1a** with one C_{20} alkyl chain at 5-position and one methyl group at 10-position have exactly the same absorption and emission maxima as **1d**, which has one C_{20} alkyl chain at 9-position but without the methyl group at 10-position. Hence it can be formulated that similar substitutions at 5- and 9-positions reveal similar photophysical parameters irrespective of the substitution at 10-position. The fluorescence quantum yields measured with Oxazine 1 as a standard (fluorescence quantum yield, Φ_F = 0.11 in ethanol)[6] for **1a** and **1d** are same as expected 0.38 and 0.36.

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, these derivatives with 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 substitution at 10-position.

As a preliminary photophysical study in biological model systems, compounds **1a**, **1d** and **1e** were incorporated in zwitterionic (DPPC) and cationic (DODAB) vesicles (Figures 1-3). 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 (Figure 2).

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).[3,5] Recently,

we have also found that the main site of acid-base equilibria is the amine at 5-position.[5b] 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.[5b]

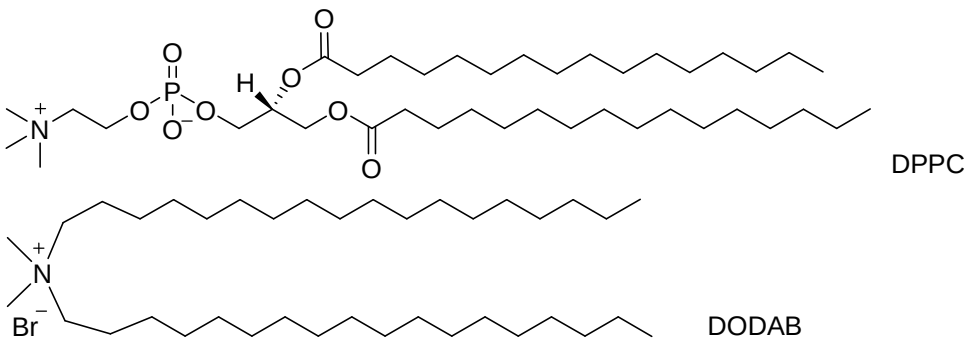


Figure 1. Structures of DPPC and DODAB.

In the DPPC vesicles, all the compounds seem to be very hydrated near the beginning of the membrane interface. 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 happens for compound **1a** when the DPPC is present in the gel ordered phase, but are lost when the DPPC membrane undergoes its phase transition. The 9-dialkylated-amino derivative **1e** detects the lipid phase transition with a 10 nm shift to the blue. As the hydration and fluidity of the interface increases in the liquid-crystalline phase,[7] this can be interpreted by the relocation of the compound towards the hydrophobic interior of the membrane.

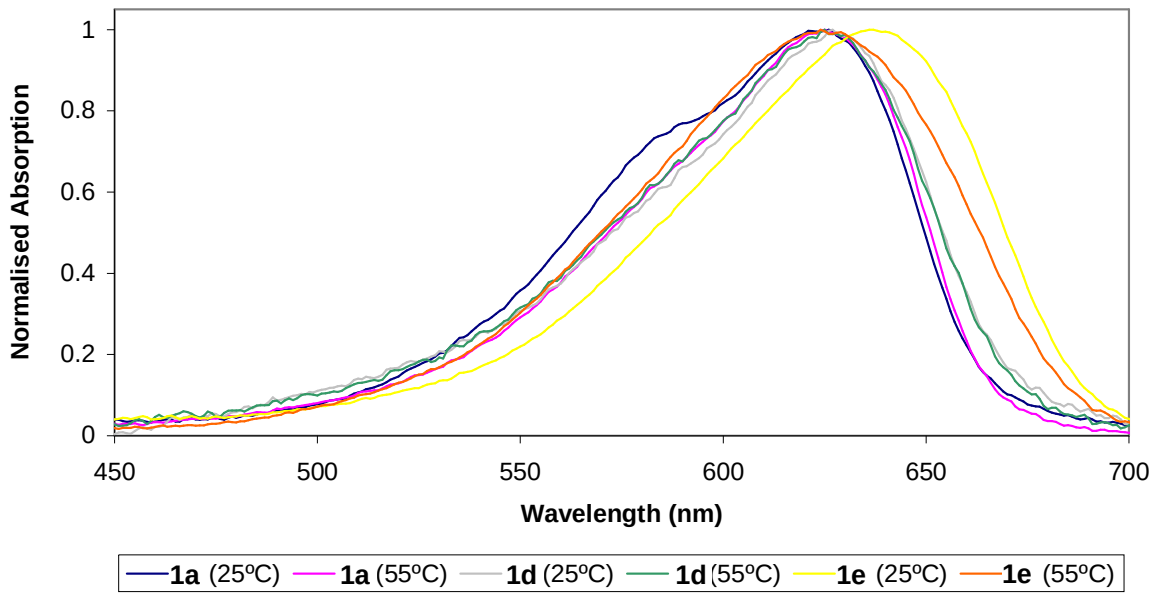


Figure 2. Normalised absorption spectra of compounds **1a**, **1d** and **1e** in DPPC vesicles below (T = 25ºC) and above (T = 55ºC) the gel to liquid-crystalline lipid phase transition.

The behaviour of the benzo[*a*]phenoxazinum chlorides **1a-e** in positive DODAB vesicles is completely different (Figure 3). In this case, an acid-base equilibrium is clearly observed for compounds **1d** and **1e**, which can be interpreted by their closer position in the interior of the membrane to get way of the positive charge of DODAB molecules. Upon the gel to liquid-crystalline phase transition, the proportion of basic form increases. 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 hydrocarbon chain making deprotonation impossible. Furthermore, for this compound H-aggregation is observed, decreasing again with the membrane phase transition.

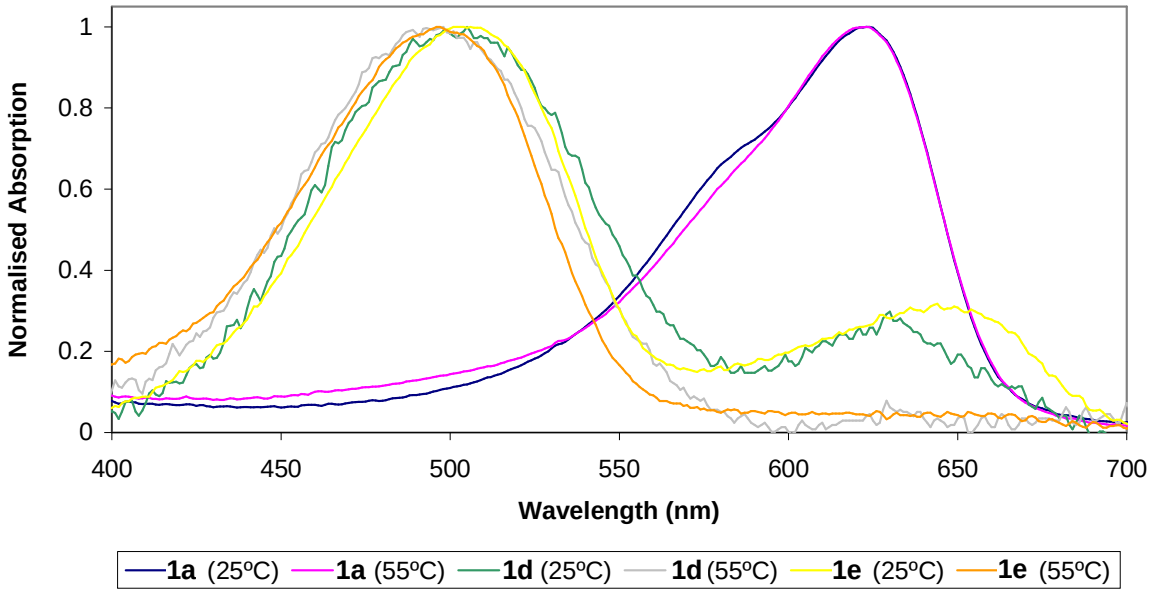


Figure 3. Normalised absorption spectra of compounds **1a**, **1d** and **1e** in DODAB vesicles below (T = 25ºC) and above (T = 55ºC) the gel to liquid-crystalline lipid phase transition.

3. Conclusion

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. Preliminary results of photophysical behaviour in biomembranes showed that the absorption 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 measurements as well as to provide information on the charge of the membranes.

4. Experimental

4.1. Typical procedure for the synthesis of 1a-e (described for **1e**): To an ice cold solution of 5-(diicosylamino)-2-nitrosophenol hydrochloride **2e** (0.500 g, 7.1×10^{-4} mol) in methanol (3 mL), concentrated hydrochloric acid (0.04 mL) was added followed by *N*-propylnaphthalen-1-amine **3b** (0.88 g, 4.8×10^{-4} mol). The reaction mixture was refluxed for 15h and monitored by TLC (chloroform/methanol, 9.4:0.6). After evaporation of the methanol and purification by column chromatography on silica gel with chloroform and chloroform/methanol, mixtures of increasing polarity, as the eluent, *N*-icosyl-*N*-[5-(propylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene]icosan-1-aminium chloride **1e** was obtained as a blue solid (0.303g, 30%). Mp = 140-142°C. R_f = 0.26 (chloroform/methanol: 94:6). FTIR (KBr): ν_{max} 3430, 2955, 2918, 2850, 1638, 1590, 1548, 1490, 1466, 1434, 1384, 1330, 1288, 1236, 1182, 1163, 1124, 1016, 1000, 721, 666 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.87 (6 H, t *J* 7.2 Hz, 2×N$(CH_2)_{19}$*CH_3*), 1.09 (3 H, br s, NHCH$_2$CH$_2$*CH_3*), 1.20-1.50 (68 H, m, 2×NCH$_2$CH$_2$*$(CH_2)_{17}$*CH$_3$), 1.71 (4 H, br s, 2×NCH$_2$*CH_2*$(CH_2)_{17}$CH$_3$), 1.95 (2 H, br s, NHCH$_2$*CH_2*CH$_3$), 3.49 (4 H, br s, 2×N*CH_2*CH$_2$$(CH_2)_{17}CH_3$), 3.81 (2 H, br s, NH*$CH_2$*CH$_2CH_3$), 6.55 (1 H, br s, 8-H), 6.61 (1 H, br s, 6-H), 6.95 (1 H, d *J* 8.4 Hz, 10-H), 7.70-7.90 (3 H, m, 11-H, 3-H and 2-H), 8.73 (1 H, br s, 4-H), 9.30 (1 H, br s, 1-H), 11.68 (1 H, br s, NH) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 11.55 (NHCH$_2$CH$_2$*CH_3*), 14.05 (2×N$(CH_2)_{19}$*CH_3*), 22.33 (5×CH$_2$), 22.62 (NHCH$_2$*CH_2*CH$_3$), 26.97 (5×CH$_2$), 27.40 (2×NCH$_2$*CH_2*$(CH_2)_{17}$CH$_3$), 29.29 (4×CH$_2$), 29.38 (4×CH$_2$), 29.53 (4×CH$_2$), 29.59 (4×CH$_2$), 29.64 (4×CH$_2$), 31.85 (4×CH$_2$), 46.51 (NH*CH_2*CH$_2$CH$_3$), 52.02 (2×N*CH_2*CH$_2$$(CH_2)_{17}CH_3$), 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) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS: m/z (FAB): calcd. for $C_{59}H_{98}N_3O$ [M^+] 864.76994; found 864.77044.

4.2. Typical procedure for the synthesis of compounds 2a-e (described for **2e**): To an ice-cold solution of the 3-(diicosylamino)phenol (0.300 g; 4.5×10^{-4} mol) in ethanol (3 mL), concentrated hydrochloric acid (0.2 mL) was added and stirred until the reaction mixture became homogenous. The solution of sodium nitrite (0.040 g; 5.8×10^{-4} mol) in water (0.1 mL) was then added drop-wise within an interval of 20 min. The resulting mixture was stirred overnight and monitored by TLC (dichloromethane/methanol, 95:5). After evaporation of the reaction, 5-(diicosylamino)-2-nitrosophenol hydrochloride **2e** was obtained as a yellow solid (0.290 g; 93%) and was used in the following step without any purification.

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 44h. After purification by column chromatography on silica gel with chloroform and chloroform/methanol, as the eluent, 3-(diicosylamino)phenol was obtained as a purple solid (1.582 g, 26%). Mp = 55.7-57.7 °C. R_f = 0.20 (dichloromethane). FTIR (KBr): ν_{max} 3496, 3383, 2918, 2850, 1618, 1578, 1503, 1467, 1373, 1399, 1297, 1282, 1270, 1257, 1240, 1224, 1209, 1193, 1181, 1170, 1147, 1110, 1090, 1033, 999, 829, 720, 755, 689, 666 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.89 (6 H, t *J* 6.8 Hz, 2×N$(CH_2)_{19}$*CH_3*), 1.10-1.50 (68 H, m, 2×NCH_2CH_2*$(CH_2)_{17}$*CH_3), 1.58 (4 H, br s, 2×NCH_2*CH_2*$(CH_2)_{17}CH_3$), 3.22 (4 H, t *J* 8.0 Hz, 2×N*CH_2*$CH_2(CH_2)_{17}CH_3$), 4.56 (1 H, br s, OH), 6.09 (1 H, dd *J* 8.0 and 2.4 Hz, 4-H), 6.12 (1 H, t *J* 2.0 Hz, 2-H), 6.23 (1 H, dd *J* 8.4 and 2.4 Hz, 6-H), 7.04 (1 H, t *J* 8.0 Hz, 5-H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 14.11 (2×N$(CH_2)_{19}$*CH_3*), 22.69 (2×CH_2), 27.19 (2×NCH_2*CH_2*$(CH_2)_{17}CH_3$), 27.27 (2×CH_2), 29.36 (2×CH_2), 29.56 (2×CH_2), 29.63 (2×CH_2), 29.71 (6×CH_2), 31.93 (2×CH_2), 51.11 (2×N*CH_2*$CH_2(CH_2)_{17}CH_3$), 98.49 (C-2), 102.00 (C-4), 104.65 (C-6), 130.01 (C-5), 149.82 (C-3), 156.66 (C-1) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS: m/z (EI): calcd. for $C_{46}H_{88}NO$ [M^+] 670.68544; found 670.68604.

In addition to the 3-(diicosylamino)phenol, 3-(icosylamino)phenol was also obtained as a white solid (2.466 g, 69%) and spectroscopic data confirmed its structure.

4.3. Typical procedure for the synthesis of 3a,b (described for **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 17h30min, and monitored by TLC (chloroform/*n*-hexane, 7:3). The solvent was evaporated and the crude mixture was purified by colunm chromatography on silica gel using chloroform/*n*-hexane, mixtures of increasing polarity, as the eluent. *N*-icosylnaphthalen-1-amine **3a** was obtained as a white oily solid (2.66 g, 90%). R_f = 0.69 (chloroform/*n*-hexane, 7:3). FTIR (neat): ν_{max} 3397, 2954, 2917, 2849, 1620, 1583, 1523, 1466, 1409, 1380, 1283, 1253, 1140, 1120, 785, 768, 722, 666 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.90 (3 H, t *J* 6.9 Hz, NH$(CH_2)_{19}$*CH_3*), 1.40-1.50 (34 H, m, NHCH_2CH_2*$(CH_2)_{17}$*CH_3), 1.72-1.86 (2 H, m, NHCH_2*CH_2*$(CH_2)_{17}CH_3$), 3.30 (2 H, t *J* 6.9 Hz, NH*CH_2*$CH_2(CH_2)_{17}CH_3$), 6.73 (1 H, br s, 4-H), 7.28 (1 H, d *J* 7.8 Hz, 2-H), 7.37 (1 H, t *J* 7.8 Hz, 3-H), 7.42-7.50 (2 H, m, 6-H and 7-H), 7.78-7.82 (1 H, m, 8-H), 7.84-7.90 (1 H, m, 5-H) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ 14.11 (NH$(CH_2)_{19}$*CH_3*), 22.68 (2×CH_2), 26.63

(2×CH_2), 27.14 (CH_2), 27.94 (CH_2), 28.31 (NHCH$_2$*CH$_2$*(CH_2)$_{17}$CH$_3$), 29.22 (CH_2), 29.32 (CH_2), 29.35 (CH_2), 29.53 (CH_2), 29.56 (CH_2), 29.69 (CH_2), 30.91 (CH_2), 31.91 (CH_2), 43.19 (CH_2), 46.57 (CH_2), 48.82 (CH_2), 53.00 (NH*CH$_2$*CH$_2$(CH_2)$_{17}$CH$_3$), 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) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS: m/z (EI): calcd. for $C_{30}H_{49}N$ [M^+] 423.3865; found 423.3866.

Acknowledgements

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