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# Synthesis, characterisation and antimicrobial activity of new benzo[*a*]phenoxazine based fluorophores

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**Abstract**—*N*-[5-(3-Aminopropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride (Bze-$NH_2$) was prepared and used as a precursor in the synthesis of new polycyclic cationic dyes. In addition to the potentiality of Bze-$NH_2$ as a non-covalent fluorescent probe, the presence of a free amino group in its structure prompted us to study the application of this functionalised heterocycle in the covalent labelling of glycine and valine amino acids, as models of biomolecules. All compounds obtained showed strong absorbance and high emission at long-wavelengths ($\lambda_{em}$ >640 nm). Furthermore, all benzo[*a*]phenoxazine derivatives synthesised were evaluated as antifungal agents against *Saccharomyces cerevisiae*, considering the commercial Nile Blue A (NB) as a lead compound. The results revealed that they exhibited good activity, which was usually superior to NB, the most effective compound displaying a minimum inhibitory concentration (MIC) value of 15 μM.


During the last years, interesting research studies have been dedicated to the design and synthesis of novel organic fluorophores for life science applications.[1] Despite the huge number of available dyes, new fluorophoric systems are still required for biomedical applications, as well as emerging and more challenging biotechnology such as genetic analysis, DNA sequencing, in vivo imaging and proteomics.[2,3] Long-wavelength fluorophores suitable for non-covalent and covalent labelling of analytes are of special interest for biological purposes.

Among these fluorescent markers are the cationic polycyclic benzo[*a*]phenoxazine dyes reported in various applications, such as for monitoring hydrophobic surfaces in proteins, like lipid stains in membranes, for studying the interaction between the probe and DNA and for tagging natural α-amino acids.[4–8] In fact, the presence of a functional group in benzo[*a*]phenoxazinium salts, such as a carboxyl, amine or hydroxyl group, which is an essential requirement for covalent labelling of molecules, also confer them the possibility of other chemical modifications, in addition to its intrinsic non-covalent character.

Furthermore, oxazine heterocycles, such as phenoxazine and benzo[*a*]phenoxazine derivatives also play an important role as antiproliferative agents with potential application both as antitumour and as antimicrobial drugs.[9–12] Previous studies undertaken by our research group showed that benzo[*a*]phenoxazine compounds had interesting potentialities as antifungal agents.[13] In addition, compounds with enhanced activity may potentially arise by binding with different biologically active chemical structures, thus producing synergistic effects. Besides representing the oldest and largest class of organic and industrially synthesised dyes, azo compounds may also display biological activity, exhibiting inhibition of the vesicular uptake of L-glutamate,[14] antiviral action in the inhibition of HIV entry into the host cell[15] and antibacterial activity.[16]

Anthraquinone derivatives play an interesting role in different fields, namely, as potent inhibitors of hepatic glucose-6-phosphate translocase, an important target for the treatment of type II diabetes,[17] and as an antibacterial agent against *Helicobacter pylori*.[18] Pyrene compounds are known carcinogenics, able to bind DNA and inhibit cell growth.[19–21]

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On the other hand, the sulfonamide group (–$SO_2NH$–) occurs in various biologically active compounds, which include antimicrobial drugs, saluretics, carbonic anhydrase and γ-secretase inhibitors, insulin-releasing sulfonamides, antithyroid agents and antitumour drugs.[22–25] Sulfonamides represents one of the most widely used antibacterial agents in the world, mainly due to their low cost, low toxicity and excellent activity against common bacterial diseases.

Considering these facts and our recent research work related to the synthesis, characterisation and applications of fluorescent heterocycles,[26,27,13] we decided to prepare a new amino functionalised benzo[*a*]phenoxazinium chloride, to investigate its use either as a precursor in the synthesis of new potential non-covalent cationic probes and as a covalent label of biomolecules, using glycine and valine as representative models as well as to evaluate the antifungal activity of all compounds synthesised against *Saccharomyces cerevisiae*.

*N*-[5-(3-Aminopropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride, Bze-$NH_2$ (**1**) was prepared by the reaction of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride (**2**), with $N^1$-(naphthalen-1-yl)propane-1,3-diamine (**3**) in an acidic medium (Scheme 1).[28] The required nitrosophenol **2** was synthesised using the usual procedure involving the treatment of 3-ethylamino-4-methylphenol with sodium nitrite in an acid solution.[29] Diamine **3** was prepared by alkylation of 1-naphthylamine with 3-bromopropylamine, in ethanol at reflux.[30]

Non-covalent labelling that results from strong hydrophobic and/or ionic interactions between the marker and the biomolecule of interest is of extreme importance. Benzo[*a*]phenoxazinium chloride **1** has a cationic structure that allows for its potential use as a non-covalent marker. However, the presence of a functional group also enables the use of this heterocycle as a precursor in the synthesis of other cationic dyes. Thus, the amine function of compound **1** was bonded to the carboxylic group, or the sulfonyl chloride of chromophoric or fluorogenic reagents, through an amide or sulfonamide linkage, respectively. Reaction between Bze-$NH_2$ (**1**) and 3-{[4-(diethylamino)phenyl]diazenyl}-benzoic acid (**4a**), anthraquinone-2-carboxylic acid (**4b**) or 1-pyrenecarboxylic acid (**4c**), using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) under standard conditions, produced the polycyclic benzo[*a*]phenoxazine derivatives **5a**–**c**.[31] Benzo[*a*]phenoxazinium chloride **1** was also reacted with *p*-toluenosulfonylchloride Tos-Cl (**4d**), resulting in the corresponding sulfonamide **5d** (Scheme 2).[32]

After purification by extraction with solvents (compound **1**) or silica gel chromatography, precursor **1** and cationic dyes **5a**–**d** were obtained in yields ranging from 11% to 98% (Table 1) and were characterised by high resolution mass spectrometry, IR, $^1H$ and $^{13}C$ NMR spectroscopy.

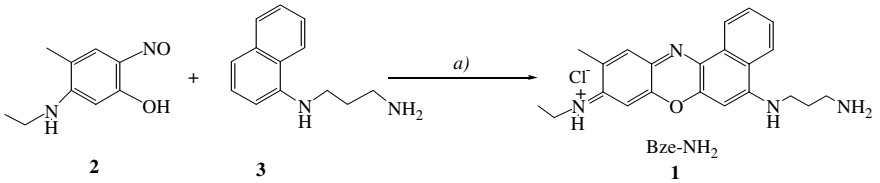
The IR spectra showed bands, a result of the stretching vibrations of the carbonyl groups of the amide type at about 1642 $cm^{-1}$ (compounds **5a**–**c**) and the sulfonamide function at 1316 and 1184 $cm^{-1}$ (compound **5d**). $^1H$ NMR spectra showed the aliphatic signals of the alkyl groups of the N-substituents of the benzo[*a*]phenoxazine nucleus from 1.41 to 3.87 ppm as well as its aromatic protons (e.g., H-8, singlet at 6.66–6.83 ppm; H-6, singlet 6.89–6.95 ppm, H-11, singlet 7.62–7.65 ppm). In addition, all signals of the other unities appeared. The following protons should be highlighted: those of the $N(CH_2CH_3)_2$ at 1.37 ($CH_3$, triplet) and 3.40–3.60 ($CH_2$, multiplet) for dye **5a**; the aromatic protons at 7.72–7.84 and 8.18–8.50 for **5b**, at 7.80–7.98 and 8.04–8.34 for **5c**; the $CH_3$ at 2.38 (singlet) in the case of **5d**.

The confirmation of the presence of the newly formed amide linkages was also supported by $^{13}C$ NMR spectra, which showed signals of the carbonyl group at about $\delta$ 170 ppm for compounds **5a**–**c**.

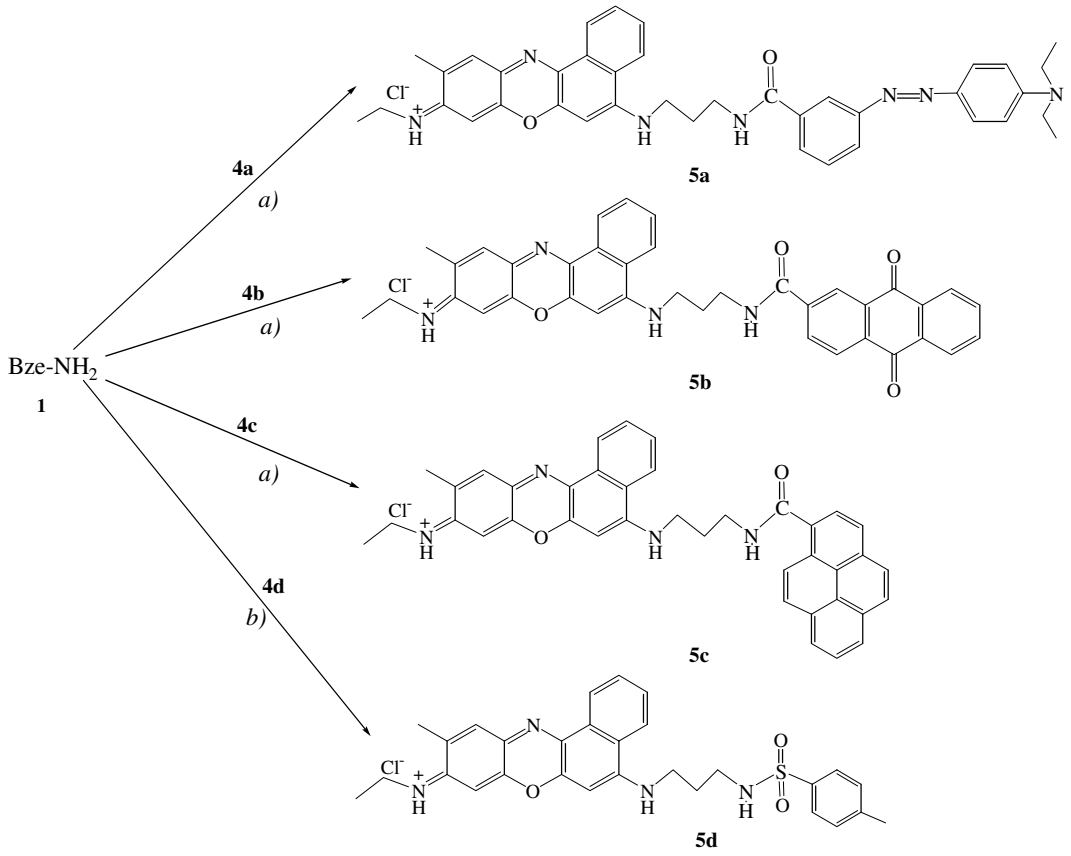
In order to investigate the possibility of using the functionalised benzo[*a*]phenoxazininium chloride **1** as a fluorescent covalent label of α-amino acids, *N*-*tert*-butyloxycarbonyl-L-glycine, Boc-Gly-OH (**6a**) and *N*-*tert*-butyloxycarbonyl-L-valine, Boc-Val-OH (**6b**) were chosen as models. Derivatisation at the C-*terminus* of **6a**,**b** with heterocycle **1** was carried out with *N*,*N*′-dicyclohexylcarbodiimide, assisted by 1-hydroxybenzotriazole under standard conditions (Scheme 3).[33]

After silica gel chromatography, the corresponding fluorescent conjugates **7a**,**b** were obtained as blue solid materials in good yields (47% **7a**, 54% **7b**) (Table 1) and their structure was confirmed by the usual analytic techniques.

The IR spectra showed bands due to the stretching vibrations of the amine function at 3400 $cm^{-1}$ and the carbonyl groups of the amide type from 1650 to 1659 $cm^{-1}$. $^1H$ NMR spectra presented aliphatic signals




**Scheme 1.** Synthesis of benzo[*a*]phenoxazinium chloride **1**. Reagents and conditions: (a) $H^+$, ethanol, reflux.




**4a** 3-{[4-(diethylamino) phenyl]diazenyl} benzoic acid
**4b** anthraquinone-2-carboxylic acid
**4c** 1-pyrenecarboxylic acid
**4d** *p*-toluenosulfonylchloride

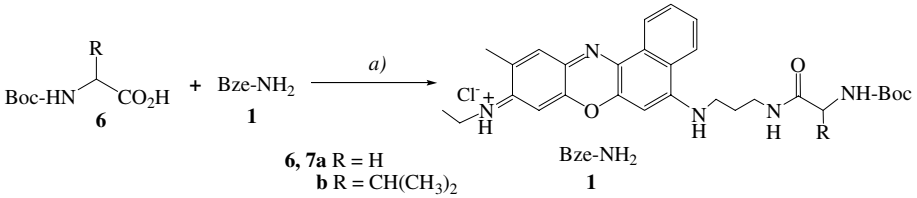
**Scheme 2.** Synthesis of cationic polycyclic dyes **5a**–**d**. Reagents and conditions: (a) DCC/HOBt, rt; (b) 1 M $NaOH/H_2O$, 0 °C and rt.

**Table 1.** Yields, UV–vis and fluorescence data for compounds **1**, **5a**–**d** and **7a**,**b**, in ethanol

| Compd | Yield [%] | Vis | Fluorescence | | |
|---|---|---|---|---|---|
| | | $\lambda_{abs}$ [nm] ($\varepsilon$) | $\lambda_{exc}$ [nm] | $\lambda_{em}$ [nm] | $\Phi_F$ |
| **1** | 98 | 620 (20,282) | 580 | 644 | 0.33 |
| **5a** | 11 | 631 (49,900) | 590 | 643 | 0.14 |
| **5b** | 27 | 631 (36,300) | 590 | 644 | 0.22 |
| **5c** | 30 | 631 (18,950) | 590 | 642 | 0.22 |
| **5d** | 47 | 632 (32,250) | 590 | 642 | 0.23 |
| **7a** | 47 | 630 (45,000) | 590 | 643 | 0.37 |
| **7b** | 54 | 630 (39,500) | 600 | 643 | 0.47 |

of the alkyl groups of the N-substituents of the benzo[*a*]phenoxazine nucleus from 1.37 to 3.90 ppm, as well as their aromatic protons (e.g., for H-8, singlet at 6.42–6.93 ppm; H-6, singlet 7.11–7.54 ppm, H-11, singlet or multiplet (**7b**) 7.20–7.76). In addition to the mentioned signals, spectra of compounds **7a**,**b** also showed the protons of the amino acid residues, such as a singlet for the methyl groups of the Boc unity ($\delta$ 1.37 ppm, **7a** and 1.45 ppm, **7b**), a multiplet for the α-CH in valine derivative ($\delta$ 4.20–4.40 ppm), and for the glycine $CH_2$ ($\delta$ 4.35–4.40 ppm). A broad doublet for α-NH at about 5.14 ppm. In $^{13}C$ NMR, signals of the carbonyl function were found at $\delta$ 171.24 (**7a**) and 173.0 (**7b**) ppm (amide type), as well as at about $\delta$ 156 ppm (urethane of the Boc protecting group).

Electronic absorption spectra of $10^{-6}$ M solutions of fluorophore **1**, cationic dyes **5a**–**d**, and the amino acid conjugates **7a**,**b** in degassed absolute ethanol were measured. Summarised data of this study are presented in Table 1. Comparison of the wavelengths of maximum




**Scheme 3.** Synthesis of fluorescent conjugates **7a**,**b**. Reagents and conditions: (a) DCC/HOBt, DMF, rt.

absorption of compounds **5a**–**d** ($\lambda_{abs}$) with their precursor **1**, revealed a slight bathochromic shift (about 11 nm). Molar absorptivity ($\varepsilon$) was also increased in all derivatives (except for **5c**), in relation to benzo[*a*]phenoxazine **1**, the highest value being that of dye **5a**, which was about 2.50 times greater than precursor **1**. The same tendency was observed for conjugates **7a**,**b** in relation to compound **1**; the red shift was that of 10 nm and the best molar absorptivity for tagging glycine (**7a**).

Evaluation of the fluorescent properties of compounds **1**, **5a**–**d** and **7a**,**b**, in ethanol, using Oxazine 1 as a standard ($\Phi_F = 0.11$ in ethanol[34]) was also performed (Table 1). All compounds exhibited good fluorescence with wavelengths of maximum emission ($\lambda_{em}$) at about 643 nm and quantum yields ranging from 0.14 to 0.47, the highest values being for compounds **7a**,**b** ($\Phi_F$ 0.37 **7a**, 0.47 **7b**), which seem to be related to the presence of the amino acid residues. Figures 1 and 2 show the comparison between normalised absorbance and the fluorescence spectra of compounds **1**, **5c** and **1**, **5b**–**d**, in ethanol, respectively.

In order to evaluate the biological activity of the new benzo[*a*]phenoxazine derivatives **1**, **5a**–**d** and **7a**,**b**, as antifungal agents, minimum inhibitory concentrations (MIC) were determined for the different compounds, taking the commercial analogue Nile Blue A (NB) (Scheme 4) as the lead compound and using a broth microdilution method for antifungal susceptibility testing (Table 2).[35]

Regarding the behaviour of cationic dyes **5a**–**d** and their precursor **1** when compared to Nile Blue A against *S. cerevisiae*, there was an increase in the inhibitory activity for all synthesised fluorophores (except for compound **5b**—its MIC value was equal to that of NB, 60 μM). The linkage of precursor **1** to different groups, all belonging to classes of compounds associated to negative effects on biological systems, resulted in increase in activity for the azo compound, no change for pyrene and sulfonamide and decreased activity for the anthraquinone group. Comparison of the activity of compound **1**, both free as well as linked to the amino acid residues **7a**,**b** showed that the presence of valine did not affect the MIC value (30 μM), whereas the more polar glycine moiety suppressed its activity.

In summary, it was possible to conclude that in addition to its potentiality for non-covalent interaction with analytes, the presence of a functional group on the [5-(3-aminopropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride (**1**), affords the possibility of its use as a versatile reagent either as precursor in the synthesis of new long-wavelength fluorophores suitable for non-covalent labelling and as a covalent label of biomolecules, namely, amino acids. Furthermore, another interesting application was related to their potential use as antimicrobials, namely, antifungal agents. Considering the MIC values of the new benzo[*a*]phenoxazine derivatives, it was possible to conclude that they are independent from the $(CH_2)_3N$ moiety of the benzo[*a*]phenoxazine derivative and that the introduction of azo dye component bond to this amino group resulted in the enhancement of the inhibitory activity.

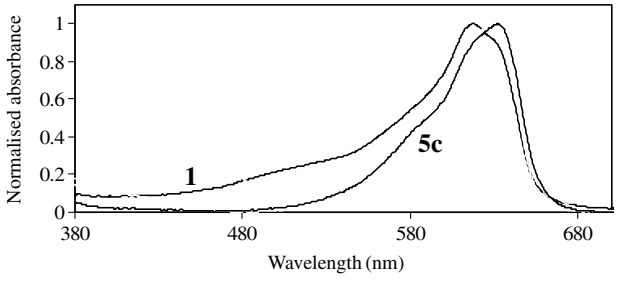



**Figure 1.** Normalised absorbance spectra of compounds **1** and **5c**, in ethanol.

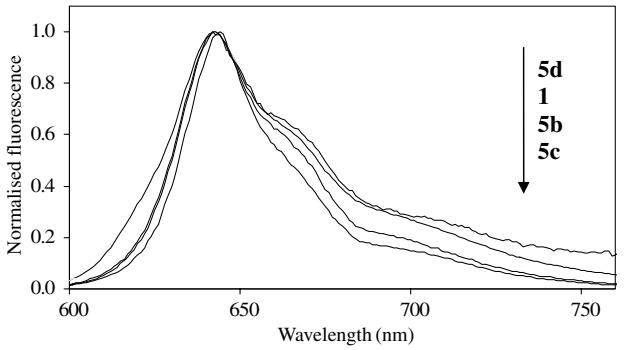
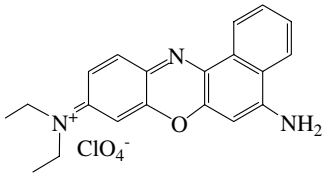



**Figure 2.** Normalised fluorescence spectra of compounds **1** and **5b**–**d**, in ethanol.




**Scheme 4.** Chemical structure of Nile Blue A.

**Table 2.** Activity against *Saccharomyces cerevisiae* W303-1B of compounds **1**, **5a**–**d** and **7a**,**b**

| Compound | MIC[a] |
|---|---|
| Nile Blue A | 60 |
| **1** | 30 |
| **5a** | 15 |
| **5b** | 60 |
| **5c** | 30 |
| **5d** | 30 |
| **7a** | >120 |
| **7b** | 30 |

[a] Minimal inhibitory concentration of growth (μM).

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## References and notes


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28. Synthesis of *N*-[5-(3-aminopropylamino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene]ethanaminium chloride (**1**): To a cold solution (ice bath) of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride **2** (0.323 g; $1.80 \times 10^{-3}$ mol) in ethanol (6 mL), $N^1$-(naphthalen-1-yl)propane-1,3-diamine (**3**) (0.360 g; $1.80 \times 10^{-3}$ mol) and concentrated hydrochloride acid ($5.0 \times 10^{-2}$ mL) were added. The mixture was refluxed for 1 h and 25 min, and monitored by TLC (silica: dichloromethane/methanol, 1:1). After successive washes with dichloromethane/*n*-hexane (4:1 and 3:2), dye **1** was obtained as a blue material (0.638 g, 98%). Mp above 300 °C. $R_f$ 0.53 (silica: chloroform/methanol, 6:1). FTIR (Nujol): $\nu_{max}$ 3400, 2954, 2854, 1644, 1556, 1504, 1463, 1455, 1376, 1157 $cm^{-1}$. $^1H$ NMR ($CD_3OD$, 300 MHz): $\delta$ 1.42 (3H, br s, $NHCH_2CH_3$), 2.20–2.33 (2H, m, $NHCH_2CH_2CH_2$), 2.40 (3H, s, $CH_3$), 3.21 (2H, br s, $NHCH_2CH_2CH_2$), 3.50–3.70 (2H, m, $NHCH_2CH_2CH_2$), 3.90 (2H, br s, $NHCH_2CH_3$), 6.93 (1H, s, 8-H), 7.11 (1H, s, 6-H), 7.76 (1H, s, 11-H), 7.86 (1H, br s, 2-H), 7.96 (1H, br s, 3-H), 8.46 (1H, br s, 1-H), 8.98 (1H, br s, 4-H) ppm. $^{13}C$ NMR ($CD_3OD$, 75.4 MHz): $\delta$ 14.14 ($NHCH_2CH_3$), 17.22 ($CH_3$), 27.63 ($NHCH_2CH_2CH_2$), 38.48 ($NHCH_2CH_2CH_2$), 39.82 ($NHCH_2CH_3$), 42.41 ($NHCH_2CH_2CH_2$), 94.05 (C-6), 94.52 (C-8), 123.93 (C-10), 124.70 (C-4), 125.44 (C-1), 129.49 (Ar-C), 130.75 (C-2), 132.62 (Ar-C), 132.73 (C-3), 133.03 (C-11 and Ar-C), 134.12 (Ar-C), 149.78 (Ar-C), 152.98 (Ar-C), 157.23 (C-9), 158.46 (C-5) ppm. HRMS (FAB): calcd for $C_{22}H_{25}N_4O$ [$M^+$] 361.2028; found 361.2040.
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30. Synthesis of $N^1$-(naphthalen-1-yl)propane-1,3-diamine (**3**): To a solution of 1-naphthylamine (1.0 g; $6.98 \times 10^{-3}$ mol) in ethanol (3 mL), 3-bromopropylamine hydrobromide (1.61 mL, $7.33 \times 10^{-3}$ mol) was added and the resulting mixture was refluxed for 13 h and 30 min, and monitored by TLC (silica: dichloromethane/methanol, 5:1). The solvent was removed under reduced pressure and the crude mixture was purified by silica gel chromatography (dichloromethane/methanol 5.2:0.8). Compound **3** was obtained as an oil (1.05 g, 75%). $R_f$ 0.52 (silica: dichloromethane/methanol, 5:1). FTIR (Nujol): $\nu_{max}$ 3401, 2953, 2923, 2852, 1583, 1530, 1503, 1483, 1463, 1435, 1409, 1377, 1339, 1318, 1285, 1236, 1144, 1130, 1077 $cm^{-1}$. $^1H$ NMR ($CD_3OD$, 300 MHz): $\delta$ 2.10–2.20 (2H, m, $NHCH_2CH_2CH_2$), 3.16 (2H, t, $J$ 6.6 Hz, $NHCH_2CH_2CH_2$), 3.46 (2H, t, $J$ 6.6 Hz, $NHCH_2CH_2CH_2$), 6.66 (1H, d, $J$ 7.8 Hz, 4-H), 7.19 (1H, d, $J$ 8.1 Hz, 2-H), 7.32 (1H, t, $J$ 7.8 Hz, 3-H), 7.40–7.60 (2H, m, 6-H and 7-H), 7.71–7.80 (1H, m, 8-H), 8.06–8.14 (1H, m, 5-H) ppm. $^{13}C$ NMR ($CD_3OD$, 75.4 MHz): $\delta$ 27.54 ($NHCH_2CH_2CH_2$), 39.09 ($NHCH_2CH_2CH_2$), 41.59 ($NHCH_2CH_2CH_2$), 105.08 (C-4), 117.96 (C-2), 122.04 (C-5), 125.03 (C-4a), 125.39 (C-7), 126.57 (C-6), 127.53 (C-3), 129.16 (C-8), 135.74 (C-8a), 144.66 (C-1) ppm. HRMS (EI): calcd for $C_{13}H_{16}N_2$ [$M^+$] 200.1313; found 200.1310.
31. Typical procedure for the synthesis of compounds **5a**–**c** (described for **5a**): 3-{[4-(diethylamino)phenyl]diazenyl}-benzoic acid (**4a**) (0.156 g; $5.54 \times 10^{-4}$ mol) was reacted with benzo[*a*]phenoxazinium chloride **5a** (0.200 g; $5.54 \times 10^{-4}$ mol) in DMF (2 mL) by a standard DCC/HOBt coupling. After silica gel chromatography (chloroform/methanol, 5.7:0.3), *N*-{5-[3-(3-(4-diethylamino)-phenyldiazenylbenzamido)propyl-amino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride **5a** was obtained as a greenish solid (0.038 g, 11%). $R_f$ 0.45 (silica: chloroform/methanol, 6:1). FTIR (KBr): $\nu_{max}$ 3426, 2930, 2855, 2738, 2677, 1642, 1590, 1563, 1544,

1520, 1495, 1455, 1435, 1398, 1313, 1293, 1257, 1185, 1139, 1031 $cm^{-1}$. $^1H$ NMR ($CD_3OD$, 300 MHz): $\delta$ 1.26 (3H, t, $J$ 7.2 Hz, $NHCH_2CH_3$), 1.37 (6H, t, $J$ 7.2 Hz, $N(CH_2CH_3)_2$), 2.25 (2H, br s, $NHCH_2CH_2CH_2$), 2.28 (3H, s, $CH_3$), 3.40–3.60 (6H, m, $N(CH_2CH_3)_2$ and $NHCH_2CH_2CH_2$), 3.66 (2H, br s, $NHCH_2CH_3$), 3.87 (2H, br s, $NHCH_2CH_2CH_2$), 6.66 (1H, s, 8-H), 6.77 (2H, d, $J$ 9.0 Hz, 2 × Ar-H *ortho* $N(CH_2CH_3)_2$), 6.95 (1H, s, 6-H), 7.51 (1H, d, $J$ 8.1 Hz, 4′-H), 7.62 (1H, s, 11-H), 7.73 (2H, d, $J$ 9.0 Hz, 2 × Ar-H *meta* $N(CH_2CH_3)_2$), 7.81 (2H, t, $J$ 7.2 Hz, 5′-H and 6′-H), 7.84–7.94 (2H, m, 2-H and 3-H), 8.15 (1H, s, 2′-H), 8.36 (1H, d, $J$ 8.4 Hz, 1-H), 8.88 (1H, d, $J$ 8.1 Hz, 4-H) ppm. The assignments were supported by the spin decoupling-double resonance. $^{13}C$ NMR ($CD_3OD$, 75.4 MHz): $\delta$ 12.98 ($N(CH_2CH_3)_2$), 14.17 ($NHCH_2CH_3$), 17.68 ($CH_3$), 29.20 ($NHCH_2CH_2CH_2$), 30.77 ($NHCH_2CH_2CH_2$), 38.36 ($N(CH_2CH_3)_2$), 39.69 ($NHCH_2CH_3$), 43.05 ($NHCH_2CH_2CH_2$), 93.96 (C-6), 94.43 (C-8), 112.08 (2 × Ar-C *ortho* $N(CH_2CH_3)_2$), 121.29 (C-2′), 123.67 (C-1), 124.80 (Ar-C), 125.55 (C-4), 126.48 (C-4′), 126.59 (2 × Ar-C *meta* $N(CH_2CH_3)_2$), 128.55 (C-5′), 128.70 (C-10), 130.33 (C-2), 130.75 (C-6′), 132.02 (1 × Ar-C), 132.58 (1 × Ar-C), 132.65 (C-3), 132.84 (C-11), 136.15 (C-1′ and 1 × Ar-C), 145.50 (C-1″), 149.32 (1 × Ar-C), 152.07 (C-3′), 152.89 (C-4″), 154.44 (1 × Ar-C), 156.65 (C-9), 158.56 (C-5), 170.82 (CONH) ppm. The assignments were supported by the bidimensional heteronuclear HMBC correlation technique. HRMS: $m/z$ (FAB): calcd for $C_{39}H_{42}N_7O_2$ [$M^+$] 640.3391; found 640.3391.

32. Synthesis of *N*-{10-methyl-5-[3-(4-methylphenylsulfonamido)propylamino]-9*H*-benzo[*a*]phenoxazin-9-ylidene}-ethanaminium chloride (**5d**): To a vigorously stirred cold solution of benzo[*a*]phenoxazine chloride **1** (0.170 g; $4.71 \times 10^{-4}$ mol) in distilled water (3.7 mL) and aqueous solution of 1 M NaOH (4.90 mL), *p*-toluenosulfonyl chloride, Tos-Cl (**4d**) (0.126 g; $6.60 \times 10^{-4}$ mol) was added. The reactional mixture was stirred for 2 h at low temperature and small quantities of 1 M NaOH were added to maintain the alkalinity of the mixture at about pH 9.0. After no additional base was required, stirring was continued at room temperature for another 3 h. The mixture was extracted with chloroform (3 × 20 mL) and after evaporation, the residue obtained was purified by silica gel chromatography (chloroform/methanol, 5.7:0.3). Compound **5d** was obtained as a blue solid (0.114 g; 47%). Mp above 300 °C. TLC (silica: chloroform/methanol, 5.5:0.5): $R_f$ 0.30. FTIR (KBr): $\nu_{max}$ 3451, 2953, 2925, 2854, 1643, 1592, 1563, 1546, 1521, 1455, 1384, 1316, 1259, 1184, 1160, 1139, 1091, 1010 $cm^{-1}$.$^1H$ NMR ($CD_3OD$, 300 MHz): $\delta$ 1.41 (3H, t, $J$ 7.2 Hz, $NHCH_2CH_3$), 2.0–2.10 (2H, m, $NHCH_2CH_2CH_2$), 2.35 (3H, s, $CH_3$), 2.38 (3H, s, $CH_3$ Tos), 3.07 (2H, t, $J$ 5.7 Hz, $NHCH_2CH_2CH_2$), 3.54 (2H, q, $J$ 7.2 Hz, $NHCH_2CH_3$), 3.72–3.82 (2H, m, $NHCH_2CH_2CH_2$), 6.83 (1H, s, 8-H), 6.89 (1H, s, 6-H), 7.34 (2H, d, $J$ 7.8 Hz, 2 × Ar-H *meta* $SO_2$), 7.65 (1H, s, 11-H), 7.75 (2H, d, $J$ 8.4 Hz, 2 × Ar-H *ortho* $SO_2$), 7.81 (1H, t, $J$ 7.5 Hz, 2-H), 7.91 (1H, t, $J$ 7.5 Hz, 3-H), 8.28 (1H, d, $J$ 7.8 Hz, 1-H), 8.86 (1H, d, $J$ 7.8 Hz, 4-H) ppm. The assignments were supported by the spin decoupling-double resonance technique. $^{13}C$ NMR ($CD_3OD$, 75.4 MHz): $\delta$ 14.16 ($NHCH_2CH_3$), 17.67 ($CH_3$), 21.41 ($CH_3$ Tos), 29.52 ($NHCH_2CH_2CH_2$), 39.76 ($NHCH_2CH_3$), 41.52 ($NHCH_2CH_2CH_2$), 49.93 ($NHCH_2CH_2CH_2$), 94.07 (C-6), 94.55 (C-8), 124.81 (1 × Ar-C), 125.54 (C-4), 126.96 (C-1), 128.08 (2 × Ar-C *meta* $SO_2$), 128.92 (C-10), 129.78 (C-2), 130.78 (2 × Ar-C *ortho* $SO_2$), 132.30 (1 × Ar-C), 132.54 (1 × Ar-C), 132.69 (C-11), 132.89 (C-3), 134.41 (1 × Ar-C), 138.50 (C-4′), 144.80 (C′-1), 149.48 (1 × Ar-C), 152.86 (1 × Ar-C), 156.84 (C-9), 158.57 (C-5) ppm. The assignments were supported by the bidimensional heteronuclear HMQC correlation technique. HRMS: $m/z$ (FAB): calcd for $C_{29}H_{31}N_4O_3S$ [$M^+$] 515.2117; found 515.2128.

33. Typical procedure for the synthesis of compounds **7a**,**b** (described for **7b**): *N*-*tert*-butyloxycarbonyl-valine (0.090 g, $4.16 \times 10^{-4}$ mol) was reacted with benzo[*a*]phenoxazinium chloride **1** in DMF (2 mL) by a standard DCC/HOBt coupling. After silica gel chromatography (dichloromethane/methanol, 5.5:0.5), *N*-{5-[3-(2-*tert*-butoxycarbonylamino-3-methylbutanamido)propylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride **7b** was obtained as a blue solid (0.084 g, 54%). Mp 129.0–131.4 °C. $R_f$ 0.33 (silica: dichloromethane/methanol, 5.4:0.6). FTIR (Nujol): $\nu_{max}$ 3400, 2954, 2924, 2854, 1651, 1590, 1463, 1456, 1377, 1311, 1159 $cm^{-1}$. $^1H$ NMR ($CDCl_3$, 300 MHz): $\delta$ 0.86–1.0 (9H, m, γ-$CH_3$ Val and $NHCH_2CH_3$), 1.45 (9H, s, $C(CH_3)_3$), 2.02 (2H, br s, $NHCH_2CH_2CH_2$), 2.20 (1H, br s, β-CH Val), 2.36 (3H, s, $CH_3$), 2.50 (2H, br s, $NHCH_2CH_2CH_2$), 3.40 (2H, br s, $NHCH_2CH_3$), 3.80 (2H, br s, $NHCH_2CH_2CH_2$), 4.20–4.40 (1H, m, α-CH Val), 5.14 (1H, d, $J$ 8.4 Hz, α-NH Val), 5.87 (1H, br s, NH), 6.42 (1H, s, 8-H), 6.54 (1H, s, 6-H), 7.50 (1H, s, 11-H), 7.75–7.90 (2H, m, 2-H, NH), 8.33 (1H, br s, 3-H), 8.75–8.85 (1H, m, 1-H), 9.05 (1H, br s, 4-H) ppm. $^{13}C$ NMR ($CDCl_3$, 75.4 MHz): $\delta$ 14.05 ($NHCH_2CH_3$), 17.49 ($CH_3$), 17.58 (γ-$CH_3$ Val), 19.09 (γ-$CH_3$ Val), 28.06 ($NHCH_2CH_2CH_2$), 28.30 ($C(CH_3)_3$), 31.27 (β-CH Val), 35.67 ($NHCH_2CH_2CH_2$), 38.84 ($NHCH_2CH_3$), 41.10 ($NHCH_2CH_2CH_2$), 60.14 (α-CH Val), 79.89 ($C(CH_3)_3$), 92.82 (C-6), 93.57 (C-8), 123.94 (Ar-C), 124.34 (C-4), 125.32 (C-1), 125.78 (Ar-C), 129.52 (C-10), 130.70 (C-3), 131.50 (C-11), 132.06 (C-2), 134.89 (Ar-C), 151.28 (2 × Ar-C), 153.69 (Ar-C), 155.88 (C-9, $COC(CH_3)_3$), 158.0 (C-5), 173.0 (CONH) ppm. The assignments were supported by the bidimensional heteronuclear HMQC correlation technique. HRMS (FAB): calcd for $C_{32}H_{42}N_5O_4$ [$M^+$] 560.3237; found 560.3245.

34. Sens, R.; Drexhage, K. H. *J. Lumin.* **1981**, *24*, 709–712.

35. Antifungal activity tests: Minimum inhibitory concentrations of growth (MIC) for the different compounds were determined using a broth microdilution method for antifungal susceptibility testing of yeasts (NCCLS M27-A). The yeast strain *S. cerevisiae* W303-1B (*MATa*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*) was used. Cells were incubated at 30 °C in RPMI 1640 medium, buffered to pH 7.0 with 0.165 M morpholenepropanesulfonic acid (MOPS) buffer (Sigma) and supplemented with the required amino acids. Initial cell concentration was $0.5 \times 10^3$ cells/mL. MIC values were determined visually after 48 h of incubation, until the lowest concentration of the resulting drug revealed no detectable growth. Stock solutions of the compounds were prepared in DMSO and a final dilution was carried out in an RPMI 1640 medium (Sigma, St. Louis, MO).