Synthesis of functionalised fluorescent dyes and their coupling to amines and amino acids

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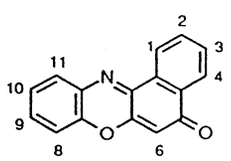
A series of novel functionalised benzophenoxazinones, analogues of Nile Red, is prepared and their fluorescence properties evaluated. The ring system is prepared by reaction of 5-diethylamino-2-nitrosophenol with 1,6-dihydroxynaphthalene followed by alkylation of the 2-hydroxy group with 6-bromohexanoic acid derivatives. Subsequent ester cleavage under a variety of conditions gives the acid 6. Replacement of the 9-diethylamino group with the *N*-ethyl-(3-sulfonylpropyl)amino group is carried out to increase water solubility and the resulting dye 14 has similar fluorescence properties. The acid 6 is coupled to a range of amino compounds.

Introduction

The use of fluorescent dyes to label biologically important molecules such as DNA, proteins, drugs *etc*. has grown markedly in recent years,[1] particularly as a result of advances in instrumentation. Fluorescence spectroscopy is therefore established as an extremely sensitive technique. Although there are many fluorescent dyes that are commercially available, few operate in or near the far visible–near infra-red region (600–1000 nm), a highly desirable operating region where there is minimum interference from absorption scattering and from fluorescence of biological molecules and reduced risk of photodecomposition.

A successful fluorescent label must have a number of characteristics: high quantum yields and molar absorptivities, excitation maxima accessible to simple light sources such as laser diodes, a large Stokes shift, chemical and photochemical stability and a reactive functional group such as a carboxylic or sulfonic acid to allow coupling to biomolecules by covalent bond formation. With these factors in mind, we have investigated new functionalised dyes based on the benzophenoxazinone structure **1**,[2] and we now report our results in detail.



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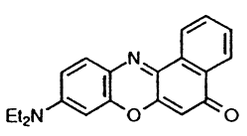

2 (Nile Red)

Results and discussion

Our initial targets were the 1- and 2-hydroxy derivatives **3** and **4** of Nile Red **2**. These were prepared by reaction of 5-diethylamino-2-nitrosophenol hydrochloride with 1,5- or 1,6-dihydroxynaphthalene in dimethylformamide (DMF),[3] and gave the benzophenoxazinones **3** and **4** in 70 and 65% yield respectively (Scheme 1).

Although the 1-hydroxy derivative **3** was only weakly fluorescent, the 2-hydroxybenzophenoxazinone **4** was strongly fluorescent and had a large Stokes shift of 81 nm ($\lambda_{excitation}$ 551 nm; $\lambda_{emission}$ 632 nm) and therefore formed the basis for further functionalisation. A carboxylic acid was chosen as the reactive functional group for eventual coupling to biomolecules and this was incorporated with a carbon chain spacer by the reaction of the phenol **4** with ethyl 6-bromohexanoate. The resulting ethyl ester **5** proved unstable to aqueous alkaline conditions and therefore the ester was hydrolysed enzymically using pig liver esterase (PLE) in a phosphate buffer and gave the corresponding acid **6** in 62% yield. Although the desired acid was obtained, the enzymic hydrolysis was slow and therefore an alternative was sought. The corresponding benzyl ester **7** was readily prepared and subsequently cleaved to the acid **6** using boron trifluoride–methyl sulfide complex[4] in 74% yield (Scheme 2). Initially in the work-up of this reaction, methanol was used to redissolve the material and in the presence of the residual boron trifluoride reesterification to the methyl ester **8** occurred. The methyl ester was cleaved in excellent yield using potassium trimethylsilanolate.[5] Subsequently it was discovered that the benzyl ester **7** could also be hydrolysed to the acid **6** using aqueous potassium carbonate. All the ester and acid derivatives **5–8** retained the strong fluorescence of the parent 2-hydroxy compound **4**.

Although the desired functional fluorescent dye **6** had been readily obtained, its absorption wavelength (551 nm) was somewhat below the ideal far visible–near infra-red range (600–1000 nm). Additionally the dye **6** had limited water solubility. In an attempt to address the problem of absorption wavelength, the dicyanomethylidene derivative **9** was prepared. Replacement of a carbonyl group by cyano- or dicyanomethylidene has been shown to result in a significant bathochromic shift in other dye systems.[1c] Condensation of the ester **5** with malononitrile in the presence of titanium(IV) chloride and pyridine[6] gave the blue dicyanomethylidene dye **9** in 69% yield (Scheme 3). As expected a large shift in absorption wavelength from 550 to 643 nm was observed, but unfortunately the material was no longer fluorescent. Attempts to prepare the monocyanomethylidene derivative using either diisopropyl cyanomethylphosphonate[7] or trimethylsilylacetonitrile[8] in Wadsworth–Emmons or Peterson reactions respectively were unsuccessful. Likewise, an attempt to prepare the corresponding *N*-cyanoimine[9] was also unsuccessful.

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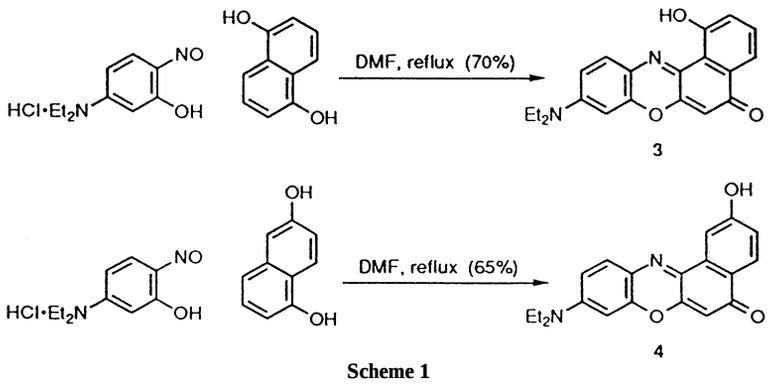
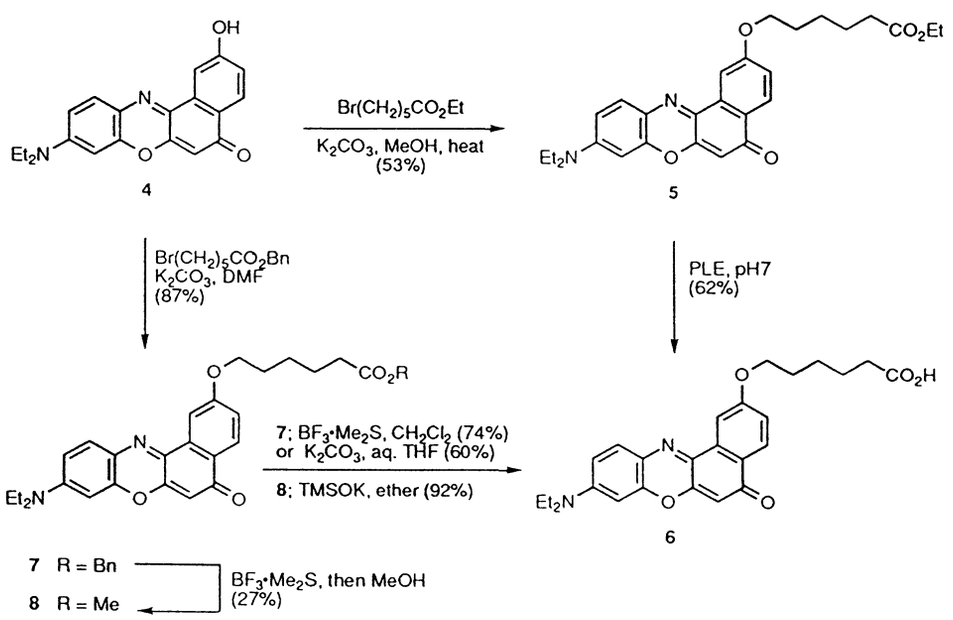
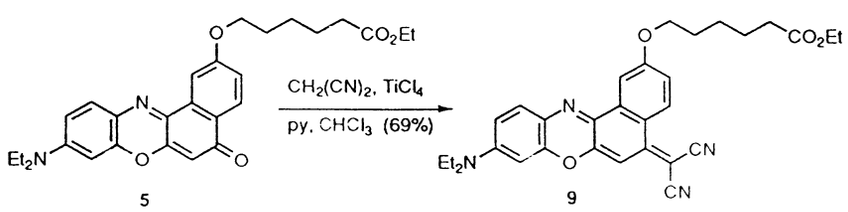


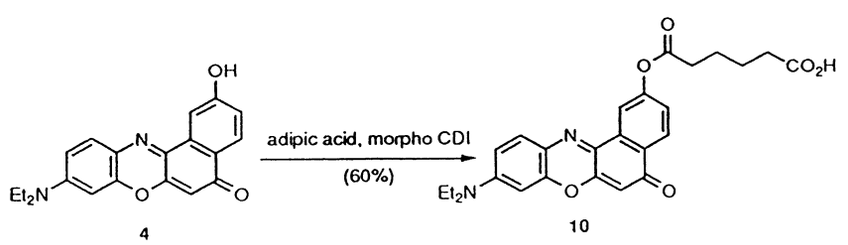
Scheme 1



Scheme 2



Scheme 3

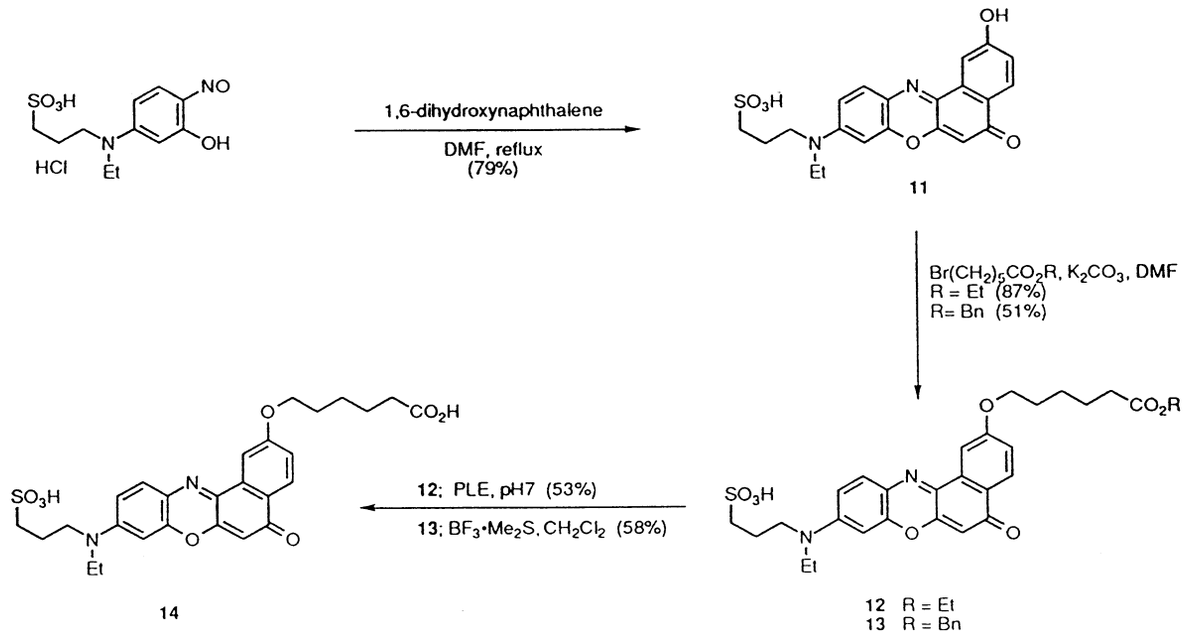


Scheme 4

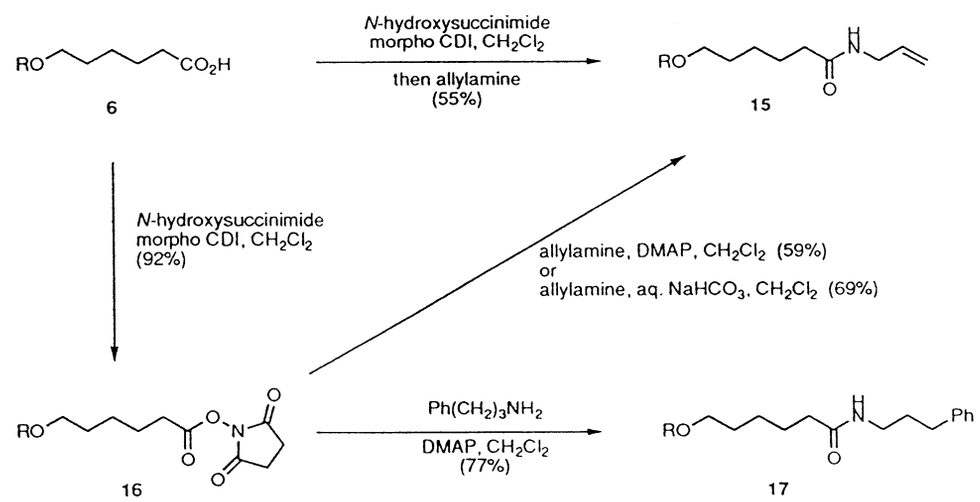
Initial attempts to improve the water solubility of the benzophenoxazinone dyes involved the preparation of the simple adipate derivative **10** by a 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (morpho CDI) coupling of the phenol **4** with adipic acid (Scheme 4).

However, a more successful approach involved the replacement of the 9-diethylamino substituent by a *N*-ethyl-(3-sulfonylpropyl)amino group. The ring system was readily prepared by condensation of 3-(*N*-ethyl-3-hydroxy-4-nitrosoanilino)propanesulfonic acid with 1,6-dihydroxynaphthalene in boiling DMF to give **11** in 79% yield. Subsequent functionalisation with ethyl or benzyl 6-bromohexanoate gave the corresponding esters **12** and **13**, cleavage of which as before gave the desired acid **14** (Scheme 5). The optical properties of these compounds were very similar to the corresponding 9-diethylamino derivatives, *i.e.* absorption at *ca.* 550 nm and a strong fluorescence with a large Stokes shift (*ca.* 80 nm).

In order to allow labelling of proteins and other biomol-



Scheme 5

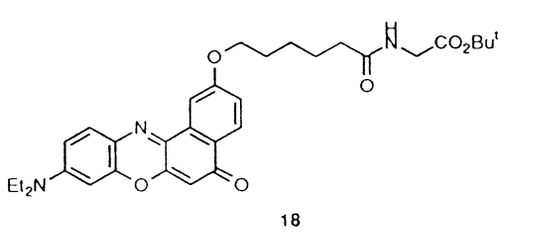


R = 9-diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yl

Scheme 6

ecules, the dye must undergo efficient covalent bond formation with functional groups such as the amino group. Therefore a series of model coupling reactions were carried out with the dye **6**. The most effective coupling method proved to be the *N*-hydroxysuccinimide active ester. In the first instance, this was carried out *in situ* by carbodiimide coupling the dye **6** and *N*-hydroxysuccinimide followed by quenching with the amine. With allylamine as the amine component, the amide **15** was obtained in 55% yield (Scheme 6).

Subsequently it proved possible to isolate the active ester **16** in 92% yield; the active ester **16** was a shelf stable dark red solid, ideal for coupling to a variety of amino compounds. Coupling with allylamine either in dichloromethane or in a 2-phase water–dichloromethane system gave **15** in 59 and 69% yield respectively; reaction with 3-phenylpropylamine gave the amide **17** in 77% yield (Scheme 6). Finally the dye was coupled to aminoacid derivatives to give the amides **18** and **19** from glycine *tert*-butyl ester and tryptophan methyl ester respectively.



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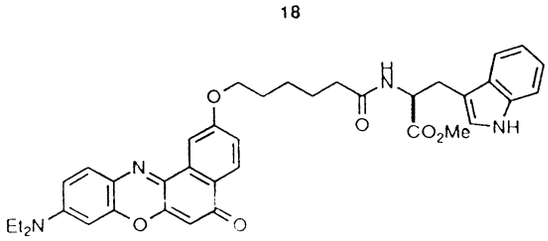

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Fluorescence properties

The fluorescence properties of the novel functionalised benzophenoxazinones are summarised in Table 1. They were also compared with those of the parent dye, Nile Red **2**. Modification at the 2- and 9-positions results in a 20–30% decrease in fluorescence intensity but otherwise the optical properties are little changed. Relative fluorescence was determined from the

Table 1 Fluorescence properties of benzophenoxazinones **3–8**, **10–17** and **19** in methanol

Dye	Excitation peak/nm	Emission peak/nm
3	565	636
4	551	632
5	551	632
6	551	632
7	556	622
8	550	618
10	559	622
11	545	618
12	554	621
13	551	621
14	549	619
15	554	619
16	546	618
17	554	621
19	549	620

emission curve at the peak excitation wavelength using the 'area' function of the fluorimeter.

However, the new dyes exhibit better time dependent fluorescence than Nile Red. For example in pH 7.1 buffer, the fluorescence intensity of Nile Red decreases by a factor of 33 after 1 h compared with the fluorescence in methanol. This was associated with a large light scatter peak at the excitation wavelength, presumably due to aggregation of the fluorophore. On the other hand, the novel dye **14** showed a fluorescence in water which was only decreased 3.6 fold compared to its fluorescence in methanol. The intensity appeared not to vary with time. Further detailed studies on the fluorescence properties of the novel benzophenoxazinone dyes are in progress.

Experimental

Commercially available solvents and reagents were used throughout without further purification, except for those detailed below which were purified as described. *N*,*N*-Dimethylformamide (DMF) was dried by stirring over calcium hydride for 15 h, decanted and distilled under reduced pressure before storage over 4 Å molecular sieves under nitrogen. Methanol was distilled from magnesium turnings and iodine. Analytical thin layer chromatography was carried out using aluminium-backed plates coated with Merck Kieselgel 60 GF_{254}. Plates were visualised under UV light (at 254 and/or 360 nm). Flash chromatography was carried out using Merck Kieselgel 60 H silica or Matrex silica 60. Pressure was applied at the column head with hand bellows. Samples were applied pre-adsorbed on silica. Light petroleum refers to the fraction with bp 40–60 °C.

All compounds were chromatographically homogeneous, although they could not be obtained microanalytically pure. IR spectra were recorded in the range 4000–600 cm^{-1} using a Nicolet FT-205 spectometer, with internal calibration. Spectra were recorded as KBr discs. UV–visible spectra were obtained using a Shimadzu UV-160 spectrophotometer. Fluorescence spectra were recorded as dilute solutions in methanol on a Perkin-Elmer LS50B luminescence spectrometer using optiglass fluorescence cells. ^{1}H and ^{13}C NMR spectra were recorded using Bruker AC-250, Bruker DPX-400 and Bruker WH-400 (former SERC NMR Spectroscopy Centre, Warwick) instruments; *J* values were recorded in Hz. High and low-resolution mass spectra were recorded on a Kratos MS80 instrument or on VG Analytical ZAB-E instrument (EPSRC Mass Spectrometry Service, Swansea); some compounds did not give satisfactory high resolution mass spectra under EI conditions.

9-Diethylamino-1-hydroxy-5*H*-benzo[*a*]phenoxazin-5-one 3

5-Diethylamino-2-nitrosophenol hydrochloride (0.68 g, 2.96 mmol) and 1,5-dihydroxynaphthalene (0.43 g, 2.69 mmol) were heated under reflux in DMF (40 ml) for 4 h. The DMF was removed under reduced pressure. The crude mixture was purified by flash chromatography (ethyl acetate–isopropanol, 100–50%) to yield a dark green crystalline solid (0.62 g, 70%), mp 251–252 °C (methanol) (Found: M^+, 334.1317. $C_{20}H_{18}N_2O_3$ requires *M*, 334.1317); ν_{max}(KBr)/cm^{-1} 3400, 2970, 2910, 1588 and 1579; λ_{max}(MeOH)/nm 564 (ε 15 322), 419 (1503), 306 (4092), 261 (13 778) and 222 (14 446); δ_H(400 MHz; $CDCl_3$–[$^2H_6$]DMSO) 13.14 (1 H, br s, OH), 7.73 (1 H, d, *J* 7.6, 2-H), 7.44 (1 H, t, *J* 8.0, 3-H), 7.40 (1 H, d, *J* 9.0, 11-H), 7.16 (1 H, d, *J* 8.1, 4-H), 6.57 (1 H, dd, *J* 9.0 and 2.7, 10-H), 6.40 (1 H, d, *J* 2.6, 8-H), 6.29 (1 H, s, 6-H), 3.38 [4 H, q, *J* 7.1, $N(CH_2CH_3)_2$] and 1.18 [6 H, t, *J* 7.1, $N(CH_2CH_3)_2$]; δ_C(100.6 MHz; $CDCl_3$–[$^2H_6$]DMSO) 183.14 (CO), 159.12, 150.42, 146.51, 142.02, 131.95, 131.36, 128.94, 121.26, 120.28, 117.27, 114.10, 109.55, 106.70, 96.40, 77.10, 44.82 [$N(CH_2CH_3)_2$] and 12.26 [$N(CH_2CH_3)_2$]; *m/z* (EI) 335 (MH^+, 23%), 334 (M^+, 74), 320 (23), 319 (100), 291 (22) and 290 (32).

9-Diethylamino-2-hydroxy-5*H*-benzo[*a*]phenoxazin-5-one 4

5-Diethylamino-2-nitrosophenol hydrochloride (1.14 g, 4.96 mmol) and 1,6-dihydroxynaphthalene (0.19 g, 4.94 mmol) were heated under reflux in DMF (100 ml) for 4 h. The DMF was removed under reduced pressure. The crude mixture was purified by flash chromatography (ethyl acetate–isopropanol, 100–50%) to yield a dark green solid (1.1 g, 65%), mp >300 °C (Found: M^+, 334.1317. $C_{20}H_{18}N_2O_3$ requires *M*, 334.1317); ν_{max}(KBr)/cm^{-1} 3375, 2964, 1645, 1590 and 1561; λ_{max}(MeOH)/nm 547 (ε 30 366), 326 (5762), 265 (27 555) and 210 (23 380); δ_H(400 MHz; [$^2H_6$]DMSO) 10.42 (1 H, br s, OH), 7.96 (1 H, d, *J* 8.6, 4-H), 7.87 (1 H, d, *J* 2.4, 1-H), 7.55 (1 H, d, *J* 9.0, 11-H), 7.08 (1 H, dd, *J* 8.6 and 2.5, 3-H), 6.77 (1 H, dd, *J* 9.0 and 2.5, 10-H), 6.61 (1 H, d, *J* 2.5, 8-H), 6.13 (1 H, s, 6-H), 3.48 [4 H, q, *J* 7.0, $N(CH_2CH_3)_2$] and 1.15 [6 H, t, *J* 7.0, $N(CH_2CH_3)_2$]; δ_C(100.6 MHz; [$^2H_6$]DMSO) 181.64 (CO), 160.69, 151.66, 150.74, 146.47, 138.77, 133.82, 130.88 (CH), 127.52 (CH), 123.92, 118.42 (CH), 109.96 (CH), 108.18 (CH), 104.15 (CH), 96.09 (CH), 44.48 (CH_2CH_3) and 12.52 (CH_2CH_3); *m/z* (EI) 335 (MH^+, 20%), 334 (M^+, 63), 320 (25), 319 (100) and 291 (26).

Ethyl 6-(9-diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy)hexanoate 5

A mixture of the naphthol **4** (0.27 g, 0.81 mmol), potassium carbonate (0.34 g, 2.46 mmol) and ethyl 6-bromohexanoate (0.28 g, 1.26 mmol) in methanol (100 ml) was heated under reflux for 2 days. Excess of potassium carbonate was filtered off and the filtrate concentrated. The crude residue was evaporated and purified by column chromatography (diethyl ether) to yield a red crystalline solid (0.21 g, 53%), mp 158–160 °C (ethanol) (Found: M^+, 476.2311. $C_{28}H_{32}N_2O_5$ requires *M*, 476.2311); ν_{max}(KBr)/cm^{-1} 2927, 1735, 1628, 1600 and 1582; λ_{max}(MeOH)/nm 552 (ε 44 064), 267 (40 664) and 208 (38 216); δ_H(250 MHz; $CDCl_3$) 8.21 (1 H, d, *J* 8.7, 4-H), 8.04 (1 H, d, *J* 2.6, 1-H), 7.60 (1 H, d, *J* 9.1, 11-H), 7.15 (1 H, dd, *J* 8.7 and 2.5, 3-H), 6.65 (1 H, dd, *J* 9.1 and 2.7, 10-H), 6.45 (1 H, d, *J* 2.7, 8-H), 6.29 (1 H, s, 6-H), 4.15 (4 H, m, $CO_2CH_2CH_3$ and $ArOCH_2$), 3.47 [4 H, q, *J* 7.1, $N(CH_2CH_3)_2$], 2.36 (2 H, t, *J* 7.2, CH_2CO_2Et), 1.89 (2 H, m), 1.75 (2 H, m), 1.57 (2 H, m) and 1.26 [9 H, t, *J* 7.1, $N(CH_2CH_3)_2$ and $CO_2CH_2CH_3$]; δ_C(62.9 MHz; $CDCl_3$) 183.14 (CO), 173.86 (CO_2Et), 161.63, 151.92, 150.60, 146.69, 140.00, 133.95, 130.94 (CH), 127.57 (CH), 125.46, 124.57, 118.14, 109.39 (CH), 106.42 (CH), 105.13 (CH), 96.15 (CH), 67.95 (CH_2), 60.20 (CH_2), 44.97 (CH_2), 34.18 (CH_2), 28.84 (CH_2), 25.61 (CH_2), 24.65 (CH_2), 14.19 (CH_3) and 12.55 (CH_3); *m/z* (EI) 477 (MH^+, 15%), 476 (M^+, 67), 461 (73), 431 (13), 291 (15) and 233 (17).

6-(9-Diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy)-hexanoic acid 6

The ester **5** (0.055 g, 0.11 mmol) was suspended in ammonium

dihydrogen phosphate (0.1 M, 5 ml, pH 7) and porcine liver esterase (0.6 ml, 1500 units) added. The mixture was stirred at 37 °C for 7 days. The water was removed under reduced pressure. The residue was dissolved in methanol and the enzyme extract filtered off through Celite. The crude mixture was purified by reverse phase chromatography (methanol) to yield a dark red crystalline solid (0.032 g, 62%), mp 167–169 °C (methanol) (Found: MH^+, 449.2076. $C_{26}H_{28}N_2O_5$ + H requires *M* + H, 449.2076); ν_{max}(KBr)/cm^{-1} 3435, 2953, 1653, 1638, 1619 and 1593; λ_{max}(MeOH)/nm 550 (ε 30 423), 325 (5824), 267 (28 753) and 210 (20 759); δ_H(250 MHz; [$^2H_4$]MeOH) 8.02 (1 H, d, *J* 8.8, 4-H), 7.92 (1 H, d, *J* 2.4, 1-H), 7.52 (1 H, d, *J* 9.2, 11-H), 7.10 (1 H, dd, *J* 8.8 and 2.5, 3-H), 6.76 (1 H, dd, *J* 9.2 and 2.5, 10-H), 6.51 (1 H, d, *J* 2.5, 8-H), 6.14 (1 H, s, 6-H), 4.09 (2 H, t, *J* 7.7, ArOCH_2), 3.49 [4 H, q, *J* 7.1, N(CH_2CH$_3$)$_2$], 2.33 (2 H, t, *J* 7.1, CH_2CO$_2$H), 1.86 (2 H, m), 1.69 (2 H, m), 1.56 (2 H, m) and 1.24 [6 H, t, *J* 7.1, N(CH$_2$CH_3)$_2$]; δ_C(100.6 MHz; [$^2H_6$]DMSO) 181.49 (CO), 174.77 (CO$_2$H), 161.34, 151.78, 150.88, 146.54, 138.33, 133.64, 131.05 (CH), 127.30 (CH), 124.91, 124.00, 117.98 (CH), 110.12 (CH), 106.25 (CH), 104.14 (CH), 96.04 (CH), 67.95 (CH$_2$), 44.54 (CH$_2$), 34.01 (CH$_2$), 28.50 (CH$_2$), 25.26 (CH$_2$), 24.51 (CH$_2$) and 12.57 (CH$_3$); *m/z* (FAB) 449 (MH^+, 54%), 330 (42), 308 (73), 290 (56) and 165 (65).

Benzyl 6-(9-diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy)hexanoate 7

A mixture of the naphthol **4** (0.1 g, 0.3 mmol), potassium carbonate (0.12 g, 0.9 mmol) and benzyl 6-bromohexanoate (0.11 g, 0.38 mmol) in DMF (50 ml) was heated under reflux for 4 h. Residual potassium carbonate was filtered off and the filtrate concentrated under reduced pressure. The crude residue was purified by column chromatography (light petroleum–diethyl ether, 100–50%, 10% gradient) to yield a green crystalline solid (0.14 g, 87%), mp 146–148 °C (ethyl acetate) (Found: M^+, 538.2470. $C_{33}H_{34}N_2O_5$ requires *M*, 538.2467); ν_{max}(KBr)/cm^{-1} 2937, 2865, 1734 and 1595; λ_{max}(MeOH)/nm 551 (ε 27 169), 267 (28 144) and 210 (30 531); δ_H(250 MHz; $CDCl_3$) 8.20 (1 H, d, *J* 8.7, 4-H), 8.02 (1 H, d, *J* 2.5, 1-H), 7.59 (1 H, d, *J* 9.1, 11-H), 7.35 (5 H, br s, Ph), 7.13 (1 H, dd, *J* 8.7 and 2.6, 3-H), 6.63 (1 H, dd, *J* 9.1 and 2.6, 10-H), 6.44 (1 H, d, *J* 2.6, 8-H), 6.28 (1 H, s, 6-H), 5.13 (2 H, s, CH_2Ph), 4.14 (2 H, t, *J* 6.2, ArOCH_2), 3.45 [4 H, q, *J* 7.0, N(CH_2CH$_3$)$_2$], 2.43 (2 H, t, *J* 7.3, CH_2CO$_2$Bn), 1.85 (2 H, m), 1.76 (2 H, m), 1.58 (2 H, m) and 1.25 [6 H, t, *J* 7.0, N(CH$_2$CH_3)$_2$]; δ_C(100.6 MHz; $CDCl_3$) 184.14 (CO), 173.34 (*C*O$_2$Bn), 161.60, 151.90, 150.59, 146.67, 139.76, 133.95, 130.95 (CH), 128.50 (CH), 128.14 (CH), 127.56 (CH), 125.44, 124.57, 118.13 (CH), 109.40 (CH), 106.39 (CH), 105.10 (CH), 96.12 (CH), 67.93 (CH$_2$), 66.09 (CH$_2$), 44.97 [N(*C*H$_2$CH$_3$)$_2$], 34.14 (CH$_2$), 28.82 (CH$_2$), 25.59 (CH$_2$), 24.64 (CH$_2$) and 12.57 [N(CH$_2$*C*H$_3$)$_2$]; *m/z* (CI) 539 (MH^+, 46%), 335 (22), 165 (22) and 132 (100).

Method for benzyl ester hydrolysis using boron trifluoride–methyl sulfide complex

The benzyl ester **7** (0.152 g, 0.28 mmol) was dissolved in dichloromethane (30 ml) and boron trifluoride–methyl sulfide complex (0.6 ml, 5.66 mmol) was added to it dropwise. The mixture was allowed to stir at room temperature for 7 days. The solvent was removed under reduced pressure and the residue extracted with ethyl acetate and washed with hydrochloric acid (0.5 M) and ammonium chloride solution. The organic layer was dried ($MgSO_4$) and concentrated. The residue was purified by reverse phase column chromatography (acetonitrile–methanol, 10% gradient) to yield the acid **6** as a purple solid (0.094 g, 74%).

Method for benzyl ester hydrolysis using potassium carbonate/THF–H_2O

To the benzyl ester **7** (0.117 g, 0.218 mmol) in a mixture of THF–H_2O (5:3, 20 ml) was added potassium carbonate (0.06 g, 0.43 mmol). The mixture was heated under reflux for 2 days. The THF was removed under reduced pressure and the residue extracted with ethyl acetate and washed with aqueous ammonium chloride. The organic layer was dried ($MgSO_4$) and concentrated. The residue was purified by reverse phase column chromatography (acetonitrile–methanol, 10% gradient) to yield the acid **6** as a purple solid (0.058 g, 60%).

Methyl 6-(9-diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy)hexanoate 8

Transesterified from benzyl ester **7** (27%), mp 152–154 °C; ν_{max}(KBr)/cm^{-1} 2929, 2868, 1739, 1620, 1601 and 1581; λ_{max}(MeOH)/nm 553 (ε 27 800), 265 (27 433) and 212 (21 459); δ_H(400 MHz; $CDCl_3$) 8.19 (1 H, d, *J* 8.7, 4-H), 8.01 (1 H, d, *J* 2.6, 1-H), 7.57 (1 H, d, *J* 9.0, 11-H), 7.13 (1 H, dd, *J* 8.7 and 2.6, 3-H), 6.62 (1 H, dd, *J* 9.1 and 2.7, 10-H), 6.42 (1 H, d, *J* 2.7, 8-H), 6.26 (1 H, s, 6-H), 4.16 (2 H, t, *J* 6.4, ArOCH_2), 3.68 (3 H, s, CO$_2$Me), 3.44 [4 H, q, *J* 7.1, N(CH_2CH$_3$)$_2$], 2.38 (2 H, t, *J* 7.4, CH_2CO$_2$Me), 1.88 (2 H, m), 1.74 (2 H, m), 1.58 (2 H, m) and 1.25 [6 H, t, *J* 7.1, N(CH$_2$CH_3)$_2$]; δ_C(100.6 MHz; $CDCl_3$) 183.51 (CO), 174.30 (CO), 152.30, 150.98, 147.09, 140.33, 134.33, 131.321, 128.83 (CH), 125.88 (CH), 124.95, 122.15, 117.61 (CH), 109.74 (CH), 106.88 (CH), 105.56 (CH), 96.58 (CH), 68.38 (CH$_2$), 51.78 (CO$_2$Me), 44.98 (CH$_2$), 34.26 (CH$_2$), 29.18 (CH$_2$), 25.97 (CH$_2$), 24.98 (N*C*H$_2$CH$_3$) and 12.90 (NCH$_2$*C*H$_3$); *m/z* (ES) 463 (MH^+, 28%), 234 (14), 194 (28), 179 (37), 98 (67) and 84 (100).

Method for methyl ester hydrolysis

The methyl ester **8** (0.028 g, 0.06 mmol) and potassium trimethylsilanolate (0.23 g, 0.18 mmol) were stirred in diethyl ether (10 ml) for 2 days and the reaction was followed by TLC. The ether was evaporated off and the resulting salt was purified by column chromatography (ethyl acetate–isopropanol, 100–50%, 10% gradient) to yield the acid **6** as a dark red crystalline solid (0.025 g, 92%).

Ethyl 6-(9-diethylamino-5-dicyanomethylidene-5*H*-benzo[*a*]-phenoxazin-2-yloxy)hexanoate 9

Titanium tetrachloride (0.032 ml, 0.17 mmol) was added under nitrogen to a stirred solution of ester (0.069 g, 0.14 mmol) and malononitrile (0.191 g, 2.9 mmol) in a mixture of dry chloroform (10 ml) and pyridine (0.6 ml). The resulting suspension was heated under reflux overnight. Water (50 ml) was added and the mixture was extracted with dichloromethane, dried (Na_2SO_4) and concentrated. The crude product was purified by flash chromatography (ethyl acetate) to yield a green crystalline solid (0.053 g, 69%), mp 114–116 °C (ethyl acetate) (Found: M^+, 524.2420. $C_{31}H_{32}N_4O_4$ requires *M*, 524.2423); ν_{max}(KBr)/cm^{-1} 2900, 2200, 1637, 1607 and 1583; λ_{max}(MeOH)/nm 643 (ε 13 606), 489 (1607), 450 (1624), 379 (4227), 296 (7109) and 220 (16 227); δ_H(250 MHz; $CDCl_3$) 8.82 (1 H, d, *J* 9.2, 4-H), 8.00 (1 H, d, *J* 2.8, 1-H), 7.52 (1 H, d, *J* 9.2, 11-H), 7.09 (1 H, dd, *J* 9.2 and 2.8, 3-H), 6.83 (1 H, s, 6-H), 6.74 (1 H, dd, *J* 9.2 and 2.7, 10-H), 6.36 (1 H, d, *J* 2.7, 8-H), 4.13 (4 H, m, CO$_2$CH_2CH$_3$ and ArOCH_2), 3.51 [4 H, q, *J* 7.2, N(CH_2CH$_3$)$_2$], 2.38 (2 H, t, *J* 7.2, CH_2CO$_2$Et), 1.90 (2 H, m), 1.73 (2 H, m), 1.61 (2 H, m) and 1.30 [9 H, m, N(CH$_2$CH_3)$_2$ and CO$_2$CH$_2$CH_3]; δ_C(62.9 MHz; $CDCl_3$) 173.80 (CO), 160.97, 153.49, 151.79, 147.02, 146.81, 138.22, 133.10, 131.51 (CH), 127.30 (CH), 127.02, 120.65, 118.45, 118.19 (CH), 117.28, 111.42 (CH), 106.65 (CH), 103.32 (CH), 95.73 (CH), 68.09 (CH$_2$), 60.23 (CH$_2$), 45.34 (CH$_2$), 34.17 (CH$_2$), 28.82 (CH$_2$), 25.58 (CH$_2$), 24.69 (CH$_2$), 14.21 (CH$_3$) and 12.66 (CH$_3$); *m/z* (EI) 525 (MH^+, 22%), 525 (M^+, 100), 509 (54), 479 (10), 338 (18), 97 (16) and 69 (70).

9-Diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yl (5-carboxy)-pentanoate 10

A mixture of the naphthol **4** (0.096 g, 0.28 mmol), 1-cyclohexyl-

3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (0.18 g, 0.42 mmol), adipic acid (0.046 g, 0.31 mmol) and a catalytic quantity of 4-dimethylaminopyridine were stirred overnight in dichloromethane (30 ml). The crude mixture was washed with water, dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography (ethyl acetate) to yield the acid **10** as a dark red amorphous solid (0.08 g, 60%), mp 85–87 °C; ν_{max}(film)/cm^{-1} 3347, 3055, 2985, 2930, 1757, 1639 and 1586; λ_{max}(MeOH)/nm 558 (ε 22 222), 267 (28 090) and 208 (17 094); δ_H(250 MHz; $CDCl_3$) 8.32 (2 H, 4-H and 1-H), 7.56 (1 H, d, *J* 9.1, 11-H), 7.34 (1 H, dd, *J* 8.7 and 2.3, 3-H), 6.62 (1 H, dd, *J* 9.2 and 2.7, 10-H), 6.44 (1 H, d, *J* 2.7, 8-H), 6.40 (1 H, s, 6-H), 3.47 [4 H, q, *J* 7.1, N(CH_2CH_3)$_2$], 2.68 (2 H, t, *J* 7.0, $COCH_2$), 2.49 (2 H, t, *J* 6.9, $COCH_2$) and 1.26 [6 H, t, *J* 7.1, N(CH_2CH_3)$_2$]; δ_C(100.6 MHz; $CDCl_3$), 182.91 (CO), 171.41 (CO), 153.18, 152.41, 151.05, 146.94, 139.05, 133.72, 131.30 (CH), 129.46, 127.66 (CH), 124.99, 123.48 (CH), 116.36 (CH), 109.93 (CH), 105.50 (CH), 96.39 (CH), 45.14 (CH_2CH_3), 34.05 (CH_2), 29.70 (CH_2), 24.24 (CH_2), 24.14 (CH_2) and 12.62 (CH_2CH_3); *m/z* (CI) 463 (MH^+, 84%), 335 (100), 146 (13) and 74 (18).

9-*N*-Ethyl-(3-sulfonylpropyl)amino-2-hydroxy-5*H*-benzo[*a*]-phenoxazin-5-one 11

3-(*N*-Ethyl-3-hydroxy-4-nitrosoanilino)propanesulfonic acid hydrochloride (0.53 g, 1.6 mmol) and 1,6-dihydroxynaphthalene (0.26 g, 1.6 mmol) were heated under reflux in DMF (40 ml) for 4 h. The DMF was removed under reduced pressure. The crude mixture was purified by flash chromatography (ethyl acetate–methanol, 100–50%, 10% gradient) to yield a dark green crystalline solid (0.54 g, 79%), mp 147–149 °C (methanol); ν_{max}(KBr)/cm^{-1} 3421, 2970, 1639, 1617, 1592 and 1560; λ_{max}(MeOH)/nm 547 (ε 28 969), 326 (5541), 264 (26 761) and 212 (21 805); δ_H(400 MHz; [$^2H_4$]MeOH) 7.92 (1 H, d, *J* 8.6, 4-H), 7.76 (1 H, d, *J* 2.4, 1-H), 7.35 (1 H, d, *J* 9.1, 11-H), 6.98 (1 H, dd, *J* 8.6 and 2.5, 3-H), 6.68 (1 H, dd, *J* 9.1 and 2.5, 10-H), 6.41 (1 H, d, *J* 2.5, 8-H), 6.03 (1 H, s, 6-H), 3.53 (2 H, t, *J* 7.7, $HO_3SCH_2CH_2CH_2N$), 3.46 (2 H, q, *J* 7.0, CH_3CH_2N), 2.91 (2 H, t, *J* 7.2, HO_3SCH_2), 2.11 (2 H, m, $HO_3SCH_2CH_2CH_2N$), 1.22 (3 H, t, *J* 7.0, CH_3CH_2N); δ_C(100 MHz; [$^2H_4$]MeOH) 182.91 (CO), 160.09, 151.65, 150.80, 145.82, 137.56, 133.52, 130.15 (CH), 126.49 (CH), 124.24, 123.14, 117.02 (CH), 109.73 (CH), 107.60 (CH), 102.64 (CH), 95.28 (CH), 48.47 (CH_2), 47.67 (CH_2), 44.40 (CH_2), 22.21 (CH_2) and 10.69 (CH_3); *m/z* (ES) 428 (M^-, 23%), 427 ($M - H^-$, 100).

Ethyl 6-(9-*N*-ethyl-(3-sulfonylpropyl)amino-5-oxo-5*H*-benzo[*a*]-phenoxazin-2-yloxy)hexanoate 12

A mixture of the naphthol **11** (0.34 g, 0.86 mmol), potassium carbonate (0.56 g, 4.06 mmol) and ethyl 6-bromohexanoate (0.95 g, 4.26 mmol) in DMF (25 ml) was heated under reflux overnight. Excess of potassium carbonate was filtered off and the filtrate concentrated. The crude residue was purified by column chromatography (ethyl acetate–methanol, 100–50%, 10% gradient) to yield a red crystalline solid (0.4 g, 87%), mp 150–152 °C (methanol–ethyl acetate); ν_{max}(KBr)/cm^{-1} 3442, 2928, 2869, 1733, 1619 and 1596; λ_{max}(MeOH)/nm 552 (ε 20 732), 326 (3963), 266 (19 539) and 212 (14 886); δ_H(400 MHz; [$^2H_4$]MeOH) 7.93 (1 H, d, *J* 8.6, 4-H), 7.81 (1 H, d, *J* 2.5, 1-H), 7.44 (1 H, d, *J* 9.1, 11-H), 7.00 (1 H, dd, *J* 8.7 and 2.5, 3-H), 6.76 (1 H, dd, *J* 9.3 and 2.5, 10-H), 6.50 (1 H, d, *J* 2.5, 8-H), 6.05 (1 H, s, 6-H), 4.04 (4 H, m, $ArOCH_2$ and $CO_2CH_2CH_3$), 3.50 (4 H, m, $HO_3SCH_2CH_2CH_2N$ and NCH_2CH_3), 2.82 (2 H, t, *J* 7.2, $HO_3SCH_2CH_2CH_2N$), 2.29 (2 H, t, *J* 7.3, CH_2CO_2Et), 2.03 (2 H, m, $HO_3SCH_2CH_2CH_2N$), 1.77 (2 H, m), 1.62 (2 H, m), 1.47 (2 H, m) and 1.16 (6 H, m, NCH_2CH_3 and $CO_2CH_2CH_3$); δ_C(100.6 MHz; [$^2H_4$]MeOH) 184.18 (CO), 174.70 (CO_2Et), 162.54, 153.12, 152.29, 147.35, 138.87, 134.64, 131.58 (CH), 127.60 (CH), 125.55, 125.35, 118.41 (CH), 111.20 (CH), 106.65 (CH), 96.67 (CH), 68.50 (CH_2), 60.66 (CH_2), 49.26 (CH_2), 45.72 (CH_2), 34.26 (CH_2), 29.14 (CH_2), 25.03 (CH_2), 23.44 (CH_2), 13.78 (CH_3) and 11.94 (CH_3); *m/z* (ES) 570 (M^-, 39%) and 569 ($M - H^-$, 100).

6-(9-*N*-Ethyl-(3-sulfonylpropyl)amino-5-oxo-5*H*-benzo[*a*]-phenoxazin-2-yloxy)hexanoic acid 14

The ester **12** (0.3 g, 0.55 mmol) was dissolved in ammonium dihydrogen phosphate (0.1 M, 50 ml, pH 7) and porcine liver esterase (6.3 ml, 20 000 units) added. The mixture was stirred at 37 °C for 14 days. The water was removed under reduced pressure. The residue was dissolved in methanol and the enzyme extract filtered off through Celite. The crude mixture was purified by reverse phase chromatography preadsorbed on silica (methanol) to yield a dark red solid (0.15 g, 53%), mp 222–225 °C; ν_{max}(KBr)/cm^{-1} 3441, 3064, 2934, 1732, 1640, 1619 and 1594; λ_{max}(MeOH)/nm 557 (ε 28 648), 531 (31 100), 325 (6233), 261 (28 726) and 216 (21 551); δ_H(250 MHz; [$^2H_4$]MeOH) 7.74 (1 H, d, *J* 8.8, 4-H), 7.49 (1 H, d, *J* 2.3, 1-H), 7.22 (1 H, d, *J* 9.1, 11-H), 6.82 (1 H, dd, *J* 8.7 and 2.5, 3-H), 6.65 (1 H, dd, *J* 9.1 and 2.3, 10-H), 6.31 (1 H, d, *J* 2.3, 8-H), 5.86 (1 H, s, 6-H), 3.89 (2 H, t, *J* 6.2, $ArOCH_2$), 3.50 (4 H, m, $HO_3SCH_2CH_2CH_2N$ and NCH_2CH_3), 7.10 (2 H, t, *J* 7.1, HO_3SCH_2), 2.37 (2 H, t, *J* 7.2, CH_2CO_2H), 2.10 (2 H, m, $HO_3SCH_2CH_2CH_2N$), 1.77 (4 H, m), 1.50 (2 H, m) and 1.22 (3 H, t, *J* 7.0, NCH_2CH_3); δ_C(100.6 MHz; [$^2H_4$]MeOH) 185.21 (CO), 177.96 (CO_2H), 163.71, 154.21, 153.57, 139.93, 135.77, 132.84 (CH), 128.77 (CH), 126.89, 126.47, 119.65 (CH), 112.52 (CH), 107.79 (CH), 105.32 (CH), 97.92 (CH), 69.78 (CH_2), 51.10 (CH_2), 49.07 (CH_2), 47.03 (CH_2), 35.41 (CH_2), 30.49 (CH_2), 27.24 (CH_2), 26.39 (CH_2), 24.79 (CH_2) and 13.28 (NCH_2CH_3); *m/z* (ES) 542 (M^+, 22%), 255 (14) and 177 (100).

Benzyl 6-(9-*N*-ethyl-(3-sulfonylpropyl)amino-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy)hexanoate 13

A mixture of the naphthol **11** (0.54 g, 1.26 mmol), potassium carbonate (0.52 g, 3.76 mmol) and benzyl 6-bromohexanoate (0.47 g, 1.65 mmol) in DMF (25 ml) was heated under reflux overnight. Excess of potassium carbonate was filtered off and the filtrate concentrated. The crude residue was purified by column chromatography (ethyl acetate–methanol, 100–50%, 10% gradient) to yield a red crystalline solid (0.4 g, 51%), mp 156–158 °C (methanol); ν_{max}(KBr)/cm^{-1} 3448, 2938, 1735, 1640, 1619 and 1595; λ_{max}(MeOH)/nm 551 (ε 7973), 326 (1546), 267 (7535) and 209 (7496); δ_H(400 MHz; [$^2H_4$]MeOH) 7.98 (1 H, d, *J* 8.7, 4-H), 7.83 (1 H, d, *J* 2.4, 1-H), 7.48 (1 H, d, *J* 9.1, 11-H), 7.30 (5 H, m, Ph), 7.03 (1 H, dd, *J* 8.8 and 2.5, 3-H), 6.80 (1 H, dd, *J* 9.1 and 2.6, 10-H), 6.54 (1 H, d, *J* 2.6, 8-H), 6.10 (1 H, s, 6-H), 5.12 (2 H, s, CH_2Ph), 4.03 (2 H, t, *J* 6.4, $ArOCH_2$), 3.54 (2 H, t, *J* 7.7, $HO_3SCH_2CH_2CH_2N$), 3.51 (2 H, q, *J* 7.1, NCH_2CH_3), 2.91 (2 H, t, *J* 7.2, HO_3SCH_2), 2.44 (2 H, t, *J* 7.3, CH_2OBn), 2.14 (2 H, m, $HO_3SCH_2CH_2CH_2N$), 1.80 (2 H, m), 1.74 (2 H, m), 1.52 (2 H, m) and 1.25 (3 H, t, *J* 7.1, NCH_2CH_3); δ_C(100.6 MHz; [$^2H_4$]MeOH) 184.97 (CO), 175.19 (CO_2Bn), 163.28, 153.85, 153.08, 148.11, 139.66, 137.73, 135.39, 132.37, 129.58 (CH), 129.23 (CH), 128.40 (CH), 126.35, 119.20 (CH), 111.98 (CH), 107.47 (CH), 97.48 (CH), 69.28 (CH_2), 67.23 (CH_2), 46.54 (CH_2), 35.07 (CH_2), 26.65 (CH_2), 25.90 (CH_2), 25.87 (CH_2), 24.30 (CH_2) and 12.80 (CH_3); *m/z* (ES) 633 (MH^+, 17%), 632 (M^+, 43), 281 (30), 255 (49), 177 (60), 130 (68) and 100 (100).

Method for benzyl ester hydrolysis using boron trifluoride–methyl sulfide

The benzyl ester **13** (0.088 g, 0.16 mmol) was dissolved in dichloromethane (30 ml) and boron trifluoride–methyl sulfide complex (0.34 ml, 3.2 mmol) was added to it dropwise. The mixture was allowed to stir at room temperature for 14 days. The solvent was removed under reduced pressure. The residue was purified by column chromatography (ethyl acetate–methanol, 100–50%, 10% gradient) to yield the acid **14** as a purple solid (0.049 g, 58%).

_N_-Allyl 6-(9-diethylamino-5-oxo-5_H_-benzo[_a_]phenoxazin-2-yloxy)hexanamide 15

The acid **6** (0.022 g, 0.048 mmol), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (0.031 g, 0.073 mmol), *N*-hydroxysuccinimide (0.016 g, 0.014 mmol) and a catalytic quantity of 4-dimethylaminopyridine were stirred overnight in dichloromethane (15 ml). Allylamine (0.014 g, 0.24 mmol) was added dropwise and the mixture allowed to stir for a further 2 h. The crude mixture was washed with water, dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography (ethyl acetate) to yield the allyl amide **15** as a dark red amorphous solid (0.012 g, 55%), mp 185–187 °C (Found: M^+, 487.2471. $C_{29}H_{33}N_3O_4$ requires *M*, 487.2471); ν_{max}(KBr)/cm^{-1} 3500, 3298, 2925, 1641, 1622, 1605 and 1581; λ_{max}(MeOH)/nm 552 (ε 26 298), 326 (4773), 267 (24 277) and 210 (19 577); δ_H(400 MHz; $CDCl_3$) 8.20 (1 H, d, *J* 8.7, 4-H), 8.02 (1 H, d, *J* 2.6, 1-H), 7.59 (1 H, d, *J* 9.1, 11-H), 7.14 (1 H, dd, *J* 8.7 and 2.6, 3-H), 6.65 (1 H, dd, *J* 9.1 and 2.7, 10-H), 6.44 (1 H, d, *J* 2.7, 8-H), 6.28 (1 H, s, 6-H), 5.83 (1 H, ddt, *J* 17.1, 10.2 and 5.7, $NCH_2CH{=}CH_2$), 5.63 (1 H, br s, NH), 5.19 (1 H, dq, *J* 17.1 and 1.6, *trans* $CH{=}CH_2$), 5.13 (1 H, dq, *J* 10.2 and 1.4, *cis* $CH{=}CH_2$), 4.18 (2 H, t, *J* 6.3, $ArOCH_2$), 3.90 (2 H, tt, *J* 5.7 and 1.5, $NCH_2CH{=}CH_2$), 3.46 [4 H, q, *J* 7.1, $N(CH_2CH_3)_2$], 2.27 (2 H, t, *J* 7.5, CH_2CONH), 1.87 (2 H, m), 1.76 (2 H, m), 1.58 (2 H, m) and 1.26 [6 H, t, *J* 7.1, $N(CH_2CH_3)_2$]; δ_C(100.6 MHz; $CDCl_3$), 183.29 (CO), 172.62 (CONH), 161.71, 152.03, 150.68, 146.82, 139.95, 134.27 (CH), 134.02, 131.03 (CH), 127.69 (CH), 125.52, 124.65, 118.24 (CH), 116.41 (CH_2), 109.46 (CH), 106.48 (CH), 105.22 (CH), 96.23 (CH), 68.00 (CH_2), 45.04 (CH_2), 41.89 (CH_2), 36.62 (CH_2), 29.67 (CH_2), 28.92 (CH_2), 25.79 (CH_2), 25.42 (CH_2) and 12.59 (CH_3); *m/z* (EI) 448 (MH^+, 30%), 487 (M^+, 100), 472 (35), 347 (23), 291 (18), 84 (25) and 69 (42).

Succinyl 6-(9-diethylamino-5-oxo-5_H_-benzo[_a_]phenoxazin-2-yloxy)hexanoate 16

The acid **6** (0.12 g, 0.26 mmol), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (0.34 g, 0.8 mmol), *N*-hydroxysuccinimide (0.09 g, 0.8 mmol) and a catalytic quantity of 4-dimethylaminopyridine were stirred overnight in dichloromethane (25 ml). The solvent was removed under reduced pressure and the residue purified by column chromatography (diethyl ether) to yield the active ester **16** as a dark red solid (0.13 g, 92%), mp 166–168 °C (not recrystallised) (Found: M^+, 545.2160. $C_{30}H_{31}N_3O_7$ requires *M*, 545.2162); ν_{max}(KBr)/cm^{-1} 3400, 1737, 1623, 1617 and 1589; λ_{max}(MeOH/nm) 552 (ε 30 929), 325 (5359), 268 (27 886) and 210 (34 608); δ_H(250 MHz; $CDCl_3$) 8.20 (1 H, d, *J* 8.7, 4-H), 8.02 (1 H, d, *J* 2.5, 1-H), 7.58 (1 H, d, *J* 9.2, 11-H), 7.15 (1 H, dd, *J* 8.7 and 2.5, 3-H), 6.63 (1 H, dd, *J* 9.2 and 2.7, 10-H), 6.43 (1 H, d, *J* 2.7, 8-H), 6.28 (1 H, s, 6-H), 4.18 (2 H, t, *J* 6.2, $ArOCH_2$), 3.45 [4 H, q, *J* 7.1, $N(CH_2CH_3)_2$], 2.84 (4 H, s, $COCH_2CH_2CO$), 2.68 [2 H, t, *J* 7.2, $CH_2CO_2N(COCH_2)_2$], 1.88 (4 H, m), 1.68 (2 H, m) and 1.25 [6 H, t, *J* 7.1, $N(CH_2CH_3)_2$]; δ_C(100.6 MHz; $CDCl_3$) 169.46 (CO), 168.85 (CO), 162.09 (CO), 152.42, 151.10, 147.22, 140.50, 134.45 (CH), 131.44 (CH), 128.11, 126.03, 125.09, 118.68 (CH), 109.86 (CH), 107.02 (CH), 105.68 (CH), 96.72 (CH), 68.28 (CH_2), 45.42 (CH_2), 31.30 (CH_2), 29.12 (CH_2), 25.98 (CH_2), 25.77 (CH_2), 24.78 (CH_2) and 13.00 [$N(CH_2CH_3)_2$]; *m/z* (EI) 546 (MH^+, 26%), 545 (M^+, 85), 530 (40), 430 (62), 415 (88), 262 (52) and 233 (100).

Reaction of active ester with allylamine

(i) To a stirred solution of the active ester **16** (0.039 g, 0.07 mmol) and a catalytic quantity of 4-dimethylaminopyridine in dichloromethane was added dropwise allylamine (0.026 ml, 0.35 mmol). The mixture was stirred overnight. The solvent was removed under reduced pressure and the residue purified by column chromatography (ethyl acetate) to yield the allyl amide **15** as a dark red solid (0.02 g, 59%).

(ii) To a stirred solution of the active ester **16** (0.06 g, 0.11 mmol) in dichloromethane (15 ml) was added aqueous sodium hydrogen carbonate (0.1 M, 8 ml) and aqueous sodium carbonate (0.1 M, 8 ml) containing allylamine (0.04 ml, 0.54 mmol) and tetrabutylammonium hydrogen sulfate (0.037 g, 0.11 mmol). The mixture was stirred for 2 h and followed by TLC. The layers were separated and the dichloromethane layer was dried ($MgSO_4$) and concentrated to yield the allylamide **15** as a dark red solid (0.037 g, 69%).

_N_-(3-Phenylpropyl)-6-(9-diethylamino-5-oxo-5_H_-benzo[_a_]-phenoxazin-2-yloxy)hexanamide 17

A solution of the active ester **16** (0.029 g, 0.05 mmol), 3-phenylpropylamine (0.045 g, 0.26 mmol) and a catalytic quantity of 4-dimethylaminopyridine in dichloromethane (15 ml) was stirred overnight. The solvent was removed under reduced pressure and the residue purified by column chromatography (ethyl acetate) to yield the amide **17** as a dark red crystalline solid (0.023 g, 77%), mp 79–81 °C (ethyl acetate) (Found: M^+, 565.2940. $C_{35}H_{39}N_3O_4$ requires *M*, 565.2940); ν_{max}(KBr)/cm^{-1} 3400, 3319, 2927, 1642, 1620 and 1603; λ_{max}(MeOH)/nm 554 (ε 54 401), 326 (10 008), 268 (50 527) and 209 (70 544); δ_H(250 MHz; $CDCl_3$) 8.20 (1 H, d, *J* 8.8, 4-H), 8.03 (1 H, d, *J* 2.6, 1-H), 7.40 (5 H, m, Ph), 7.14 (1 H, dd, *J* 8.8 and 2.6, 3-H), 6.65 (1 H, dd, *J* 9.1 and 2.7, 10-H), 6.46 (1 H, d, *J* 2.7, 8-H), 6.30 (1 H, s, 6-H), 5.30 (1 H, br s, NH), 4.17 (2 H, t, *J* 6.3, $ArOCH_2$), 3.47 [4 H, q, *J* 7.1, $N(CH_2CH_3)_2$], 3.31 (2 H, m), 2.66 (2 H, m), 2.19 (2 H, t, *J* 7.2, CH_2CON), 1.87 (4 H, m), 1.73 (2 H, m), 1.55 (2 H, m) and 1.27 [6 H, t, *J* 7.1, $N(CH_2CH_3)_2$]; δ_C(100.6 MHz; $CDCl_3$) 183.66 (CO), 173.18 (CONH), 165.05, 162.15, 161.54, 151.14, 147.22, 141.84, 140.40, 134.46, 131.44 (CH), 128.71 (CH), 128.09 (CH), 126.45 (CH), 125.10, 118.62 (CH), 109.90 (CH), 107.02 (CH), 105.61 (CH), 96.70 (CH), 68.44 (CH_2), 45.43 [$N(CH_2CH_3)_2$], 39.61 (CH_2), 37.05 (CH_2), 33.56 (CH_2), 31.49 (CH_2), 29.31 (CH_2), 26.17 (CH_2), 25.81 (CH_2) and 12.99 (CH_3); *m/z* (EI) 566 (MH^+, 13%), 565 (33), 117 (64) and 91 (100).

_N_-(_tert_-Butoxycarbonyl)methyl-6-(9-diethylamino-5-oxo-5_H_-benzo[_a_]phenoxazin-2-yloxy)hexanamide 18

A mixture of the acid **6** (10 mg, 22 μmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (ETC) (14 mg, 45 μmol) and *N*-hydroxysulfosuccinimide sodium salt (15 mg, 67 μmol) in DMF (1 ml) was stirred at room temperature. After 2.5 h a further portion of ETC (50 mg, 168 μmol) was added. The mixture was stirred at room temperature overnight and a solution of *tert*-butyl glycine hydrochloride (19 mg, 112 μmol) in pH 9.2 buffer ($NaHCO_3$–Na_2CO_3) (1.5 ml) was added dropwise. After 3 h the mixture was concentrated *in vacuo* and purified by flash column chromatography (C18, methanol–water, 50–80% gradient) to afford the title compound **18** as a dark red solid (11 mg, 88%), δ_H(270 MHz; $CDCl_3$) 8.21 (1 H, d, *J* 8.8, 4-H), 8.03 (1 H, d, *J* 2.5, 1-H), 7.61 (1 H, d, *J* 9.1, 11-H), 7.15 (1 H, dd, *J* 8.6 and 2.6, 3-H), 6.65 (1 H, dd, *J* 9.3 and 2.7, 10-H), 6.45 (1 H, d, *J* 2.8, 8-H), 6.29 (1 H, s, 6-H), 6.00 (1 H, br t, *J* 5.2, NH), 4.17 (2 H, t, *J* 6.3, $ArOCH_2$), 3.95 (2 H, d, *J* 5.2, $NHCH_2$), 3.47 [4 H, q, *J* 7.1, $N(CH_2CH_3)_2$], 2.30 (2 H, d, *J* 7.4, CH_2CON), 1.95–1.68 (4 H, m), 1.68–1.50 (2 H, m), 1.47 (9 H, s, Bu^t) and 1.26 [6 H, t, *J* 7, $N(CH_2CH_3)_2$].

_N_-[2-(Indol-3-yl)-1-methoxycarbonyl]ethyl-6-(9-diethylamino-5-oxo-5_H_-benzo[_a_]phenoxazin-2-yloxy)hexanamide 19

A solution of the active ester **16** (0.035 g, 0.06 mmol), L-tryptophan methyl ester hydrochloride (0.049 g, 0.19 mmol) and pyridine (0.015 g, 0.19 mmol) in dichloromethane (20 ml) was stirred overnight. The solvent was removed under reduced pressure and the residue purified by column chromatography

(ethyl acetate) to yield the tryptophan amide **19** as a dark red solid (0.027 g, 65%), mp 75–77 °C, ν_{max}(KBr)/cm^{-1} 3421, 3300, 2930, 1750, 1735, 1654, 1617, 1594 and 1572; λ_{max}(MeOH)/nm 551 (ε 38 033), 326 (7308), 268 (42 555) and 220 (70 697); δ_H(250 MHz; $CDCl_3$) 8.54 (1 H, br s, NH), 8.18 (1 H, d, *J* 8.7, 4-H), 8.00 (1 H, *J* 2.5, 1-H), 7.58 (1 H, d, *J* 9.1, 11-H), 7.56 (1 H, m), 7.36 (1 H, m), 7.16 (4 H, m), 6.61 (1 H, dd, *J* 9.1 and 2.7, 10-H), 6.43 (1 H, d, *J* 2.6, 8-H), 6.28 (1 H, s, 6-H), 6.05 (1 H, d, *J* 7.8, CONH), 4.97 (1 H, td, *J* 7.8 and 5.6, HNC*H*CO_2Me), 4.04 (2 H, t, *J* 6.4, ArCH_2), 3.71 (3 H, s, OMe), 3.45 [4 H, q, *J* 7.1, N(C*H*$_2$CH$_3$)$_2$], 3.32 [2 H, t, *J* 5.6, CH(CO_2Me)C*H*$_2$], 2.22 (2 H, t, *J* 7.4, CH_2CON), 1.81 (2 H, m), 1.72 (2 H, m), 1.63 (2 H, m) and 1.25 [6 H, t, *J* 7.1, N(CH$_2$C*H*$_3$)$_2$]; δ_C(100.6 MHz; $CDCl_3$) 183.67 (CO), 172.93 (CONH), 162.15 (CO_2Me), 152.46, 151.16, 147.22, 140.35, 136.59, 134.41, 131.38 (CH), 128.12 (CH), 125.92, 125.92, 125.10, 123.18 (CH), 122.60 (CH), 120.01 (CH), 119.88, 119.12, 118.86 (CH), 118.70 (CH), 111.79 (CH), 110.40, 109.97 (CH), 106.94 (CH), 105.61 (CH), 96.70 (CH), 68.42 (CH_2), 53.28 (*C*HCO$_2$CH$_3$), 52.70 (CO_2Me), 45.43 (N*C*H$_2$CH$_3$), 36.78 (CH_2), 29.23 (CH_2), 28.00 (CH_2), 25.92 (CH_2), 25.52 (CH_2) and 13.00 [N(CH$_2$*C*H$_3$)$_2$]; *m/z* (ES) 649 (MH^+, 68%), 489 (9), 348 (23) and 263 (100).

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