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Synthesis and Testing of Chromogenic Phenoxazinone Substrates for β-Alanyl Aminopeptidase

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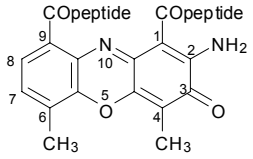

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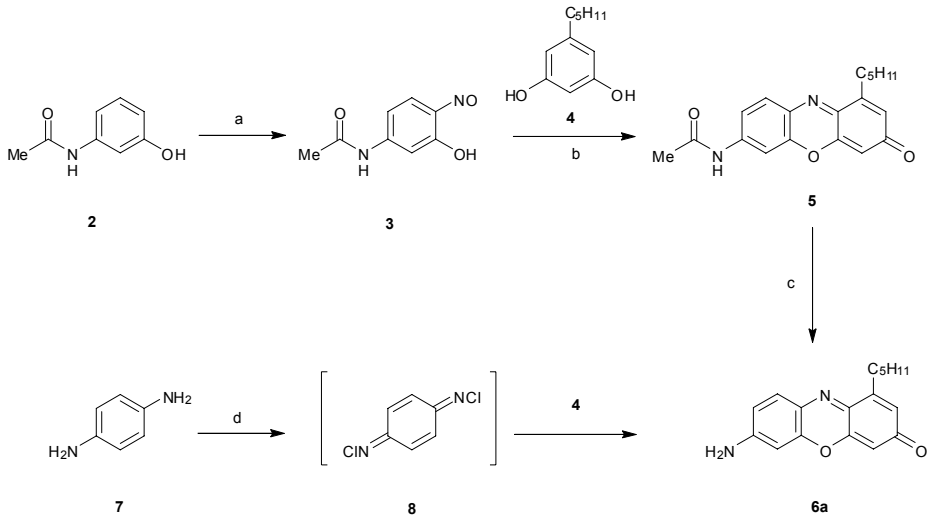

2 **3** **5** **7** **8** **6a**

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Scheme 1 *Reagents and conditions*: [a] 'HNO_2'; [b] ^{n}BuOH, concn. H_2SO_4; [c] 85% H_2SO_4, ethanol; [d] MeOH, $CO(NH_2)_2$, Cl_2, then **4**, reflux.

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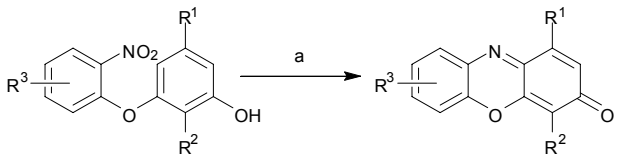
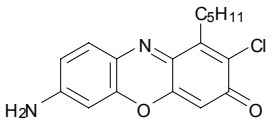


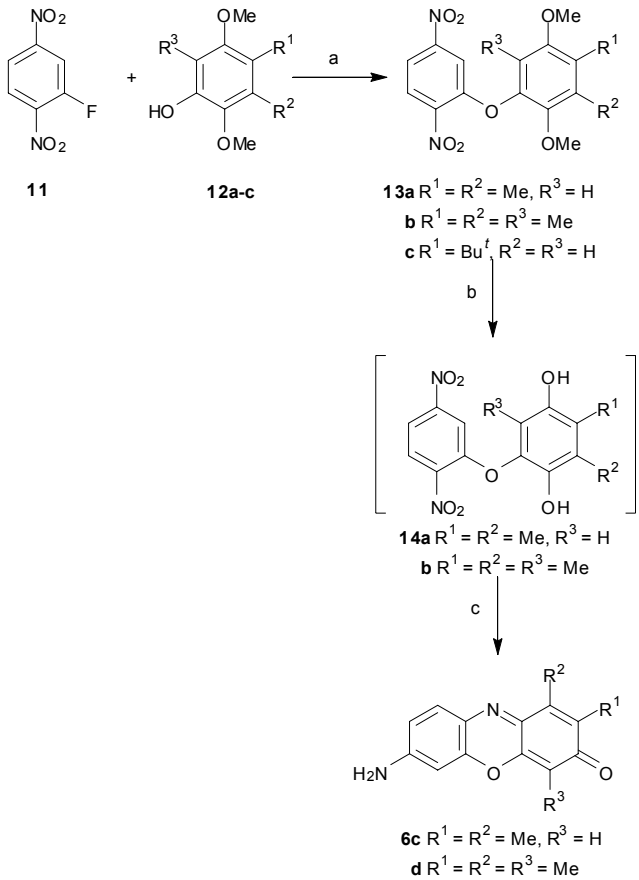
9 **10**

R^1, R^2, R^3 = H, Cl, Me, OH, or CO_2Me

Scheme 2 *Reagents and conditions:* [a] Zn, NH_4Cl, H_2O, DME, 40 °C



6b



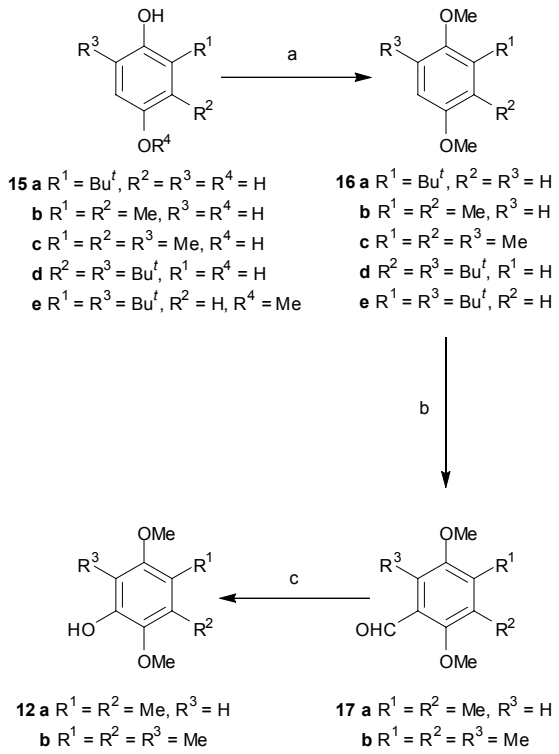
11 **12a-c**

13a R^1 = R^2 = Me, R^3 = H
b R^1 = R^2 = R^3 = Me
c R^1 = Bu^t, R^2 = R^3 = H

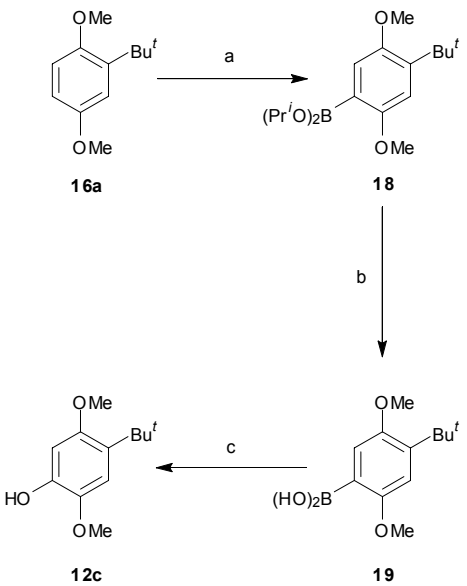
14a R^1 = R^2 = Me, R^3 = H
b R^1 = R^2 = R^3 = Me

6c R^1 = R^2 = Me, R^3 = H
d R^1 = R^2 = R^3 = Me

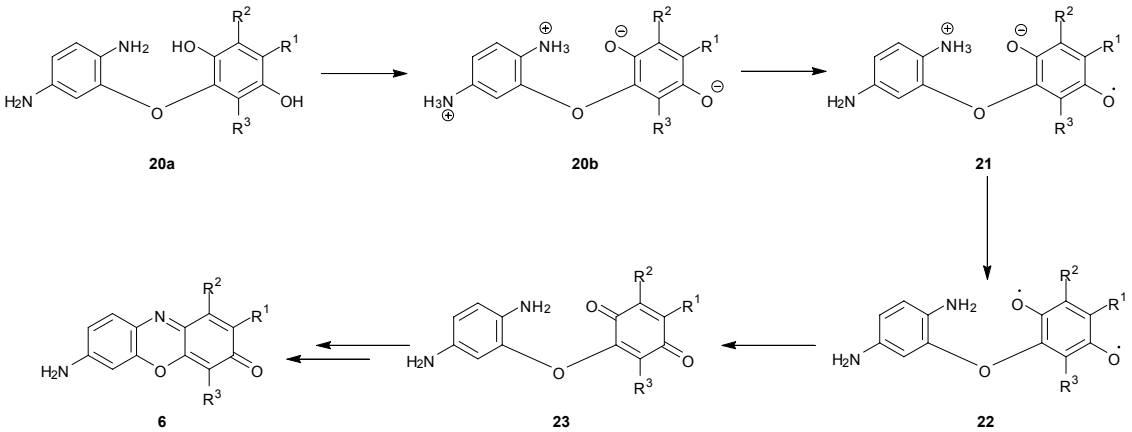
Scheme 3 *Reagents and conditions*: [a] NaH, DMF, 40 °C; [b] BBr_3, DCM, -78 °C; [c] 5 % Pd-on-C, MeOH then air / silica.



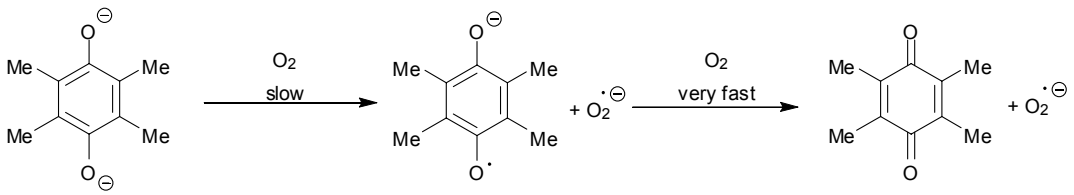
Scheme 4 *Reagents and conditions*: [a] NaH, MeI, DMF, 40°C, 1 h; [b] hexamine, TFA, reflux; [c] i, MMPP, MeOH; ii, NaOH then HCl.



Scheme 5 *Reagents and conditions*: [a] ^{n}BuLi, THF, -78°C then $B(OPr^i)_3$; [b] 10 % NH_4Cl; [c] 27 % H_2O_2, THF.



Scheme 6



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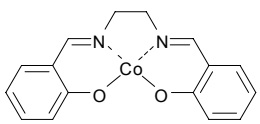
Scheme 7



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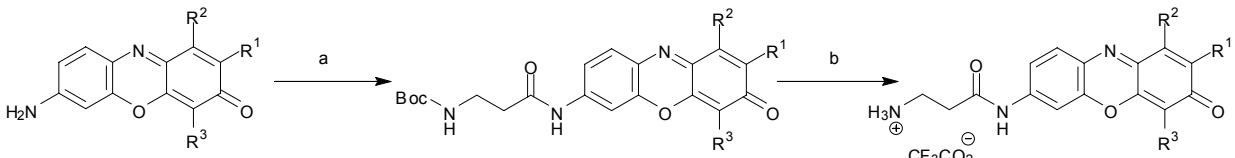
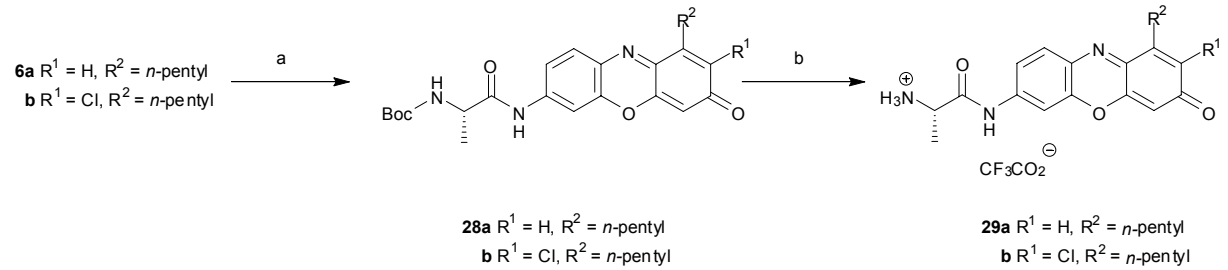


6a R^1 = H, R^2 = *n*-pentyl
b R^1 = Cl, R^2 = *n*-pentyl
c R^1 = R^2 = Me, R^3 = H
d R^1 = R^2 = R^3 = Me

26a R^1 = H, R^2 = *n*-pentyl
b R^1 = Cl, R^2 = *n*-pentyl
c R^1 = R^2 = Me, R^3 = H
d R^1 = R^2 = R^3 = Me

27a R^1 = H, R^2 = *n*-pentyl
b R^1 = Cl, R^2 = *n*-pentyl
c R^1 = R^2 = Me, R^3 = H
d R^1 = R^2 = R^3 = Me

Scheme 8 *Reagents and conditions*: [a] 5% Pd-on-C, DMF, *N*-^{t}Boc-β-alanine, HOBt, DIC, DCM; [b] TFA.



Scheme 9 *Reagents and conditions*: [a] 5% Pd-on-C, DMF, *N*-^{t}Boc-L-alanine, HOBt, DIC, DCM; [b] TFA.

Experimental

General

Melting points were determined on a Gallenkamp apparatus and are uncorrected. Elemental analyses were performed on an Exeter Analytical CE-440 Elemental Analyzer. IR spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrophotometer. ^{1}H NMR spectra were acquired on a Bruker AVANCE 300 at 300 MHz or AVANCE 500 at 500 MHz. Coupling constants are given in Hz and all chemical shifts are relative to the chemical shift of the residual non-deuterated solvent. ^{13}C NMR spectra were obtained on the Bruker AVANCE 300 at 75 MHz. Low resolution electrospray mass spectra were obtained on a Bruker Esquire 3000+ and high resolution spectra on a Bruker APEX II FT mass spectrometer. Thin layer chromatography was performed on Merck silica gel 60F_{254}. All solvents were purified according to standard procedures. Diethyl ether and tetrahydrofuran were freshly distilled over sodium wire with a trace of benzophenone. Fisons silica gel 60 (35-70 micron) was used for wet flash chromatography. The samples were applied in liquid form or were pre-adsorbed onto silica 60 (35-70 micron).

General procedure for the preparation of dimethoxybenzenes 16a-e[17]

In a dry 2-necked round bottom flask equipped with a condenser, a magnetic stirring bar and a calcium chloride guard tube, the hydroquinone 12 (1 equiv.) was dissolved in dry DMF (50 ml) and NaH (2.2 equiv., 60% dispersion in oil) was added in small portions. After the base had been added and the evolution of H_2 had ceased, methyl iodide (4 equiv.) was added dropwise over 15-20 min.. When the addition was finished, the reaction mixture was stirred at 40 °C for 2 hours. Brine (200 ml) was added to the flask and the resulting mixture was extracted with diethyl ether (3 × 50 ml). The combined organic layers were washed with water (2 × 50 ml) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was subjected to column chromatography.

2-*tert*-Butyl-1,4-dimethoxybenzene 16a

Prepared from 2-*tert*-butylhydroquinone **12a** (2.90 g, 17.5 mmol) and purified by column chromatography using petroleum ether (60-80 °C) : diethyl ether (95:5) as eluent; yellow oil (3.35 g, 99%) (lit.[17] bp 240 °C / 50 mm Hg) (Found: M^+, 194.1301. Calc. for $C_{12}H_{18}O_2$: M, 194.1316); δ_H (300 MHz; $CDCl_3$) 1.40 (9H, s, C(CH_3)$_3$), 3.80 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 6.72 (1H, dd, J = 8.8 Hz and J=3.1 Hz, H-5), 6.84 (1H, d, J = 8.8 Hz, H-6), 6.93 (1H, d, J = 3.1 Hz, H-3); δ_C (75 MHz; $CDCl_3$) 30.1 (CH_3, C(CH_3)$_3$), 35.3 (quat., C(CH_3)$_3$), 56.0 (CH_3, OCH_3), 56.0 (CH_3, OCH_3), 110.3 (CH, C-5), 112.8 (CH, C-6), 114.7 (CH, C-3), 140.3 (quat., C-2), 153.4 (quat., C-1 or C-4), 153.7 (quat., C-4 or C-1).

1,4-Dimethoxy-2,3-dimethylbenzene 16b

Prepared from 2,3-dimethylhydroquinone **12b** (1.96 g, 14.2 mmol) and purified by column chromatography using petroleum ether (60-80 °C) : diethyl ether (95:5) as eluent; white solid (2.27 g, 80%); mp 75-76 °C (lit.[21] mp 73-74 °C); δ_H (300 MHz; $CDCl_3$) 2.09 (6H, s, 2 × CH_3), 3.70 (6H, s, 2 × OCH_3), 6.58 (2H, s, 2 × ArH); δ_C (75 MHz; $CDCl_3$) 12.4 (CH_3, 2 × ArCH_3), 56.5 (CH_3, 2 × OCH_3), 108.4 (2 × CH), 127.1 (2 × quat.), 152.4 (2 × quat.).

1,4-Dimethoxy-2,3,5-trimethylbenzene 16c

Prepared from 2,3,5-trimethylhydroquinone **12c** (2.175 g, 14.3 mmol) and purified by column chromatography using petroleum ether (60-80 °C) : diethyl ether (95:5) as eluent; colourless oil (2.367 g, 92%) (lit.[22] mp 37-38 °C) (Found: MH^+, 181.1225. Calc. for $C_{11}H_{17}O_2$: MH, 181.1223); δ_H (300 MHz; $CDCl_3$) 2.17 (3H, s, CH_3), 2.23 (3H, s, CH_3), 2.33 (3H, s, CH_3), 3.70 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 6.58 (1H, s, H-6); δ_C (75 MHz; $CDCl_3$) 12.2 (CH_3), 13.0 (CH_3), 16.7 (CH_3), 56.2 (OCH_3), 60.5 (OCH_3), 110.8 (CH, C-6), 124.2 (quat., C-5), 128.1 (quat., C-2 or C-3), 131.0 (quat., C-3 or C-2), 151.0 (quat., C-4), 153.9 (quat., C-1).

2,5-Di-*tert*-butyl-1,4-dimethoxybenzene 16d

Prepared from 2,5-di-*tert*-butylhydroquinone **12d** (2.79 g, 12.55 mmol) and purified by column chromatography using petroleum ether (60-80 °C) : diethyl ether (95:5) as eluent; white solid (2.67 g, 85%), mp 99-100 °C (lit.[23] mp 101-102 °C); δ_H (300 MHz; $CDCl_3$) 1.40 (18H, s, C(CH_3)$_3$), 3.84 (6H, s, 2 × OCH_3), 6.86 (2H, s, 2 × ArH); δ_C (75 MHz; $CDCl_3$) 30.2 (CH, C(CH_3)$_3$), 35.0 (quat., C(CH_3)$_3$), 56.3 (2 × OCH_3), 112.1 (2 × CH), 136.8 (2 × quat.), 152.4 (2 × quat.).

2,6-Di-*tert*-butyl-1,4-dimethoxybenzene 16e

Prepared from 3,5-di-*tert*-4-hydroxyanisole **12e** (2.06 g, 8.72 mmol) and purified using petroleum ether (60-80 °C) : diethyl ether (98:2) as eluent; colourless oil (1.79 g, 82%); (Found: C, 77.05; H, 10.5. $C_{16}H_{26}O_2$ requires C, 76.75; H, 10.5 %); δ_H (300 MHz; $CDCl_3$) 1.35 (18H, s, C(CH_3)$_3$), 3.59 (3H, s, OCH_3), 3.70 (3H, s, OCH_3), 6.75 (2H, s, 2 × ArH); δ_C (75 MHz; $CDCl_3$) 32.4 (CH, C(CH_3)$_3$), 36.4 (quat., C(CH_3)$_3$), 55.7 (OCH_3), 64.6 (OCH_3), 112.2 (2 × CH), 144.9 (2 × quat.), 153.8 (2 × quat.), 154.7 (2 × quat.).

Formylation of dimethoxybenzenes *via* the Duff reaction

The dimethoxybenzene (1 equiv.) was dissolved in TFA (20 ml) and hexamine (1.05 equiv.) was added to the resulting solution. The reaction mixture was refluxed under dry conditions for 2 hours. The TFA was evaporated under reduced pressure, the residue was dissolved in ether (100 ml) and the organic solution was washed with water (3 × 50 ml) and then dried over $MgSO_4$. The solvent was evaporated and the residue subjected to column chromatography, eluting with petroleum ether (60-80 °C) : diethyl ether (80:20).

2,5-Dimethoxy-3,4-dimethylbenzaldehyde 17a

Prepared from 1,4-dimethoxy-2,3-dimethylbenzene **16b** (2.270 g, 13.7 mmol). 2,5-Dimethoxy-3,4-dimethylbenzaldehyde **17a** was isolated as a white solid (1.18 g, 44%); mp 61-62 °C (lit.[24] mp 67.5-68.5 °C) (Found: MH^+, 195.1019. Calc. for $C_{11}H_{15}O_3$: MH, 195.1016); ν_{max} (KBr)/cm^{-1} 1685 (C=O), 1595 (C=C); δ_H (300 MHz; $CDCl_3$) 2.26 (3H, s, CH_3), 2.29 (3H, s, CH_3), 3.83 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 7.16 (1H, s, H-6), 10.38 (1H, s, CHO); δ_C (75 MHz; $CDCl_3$) 12.5 (CH_3), 13.3 (CH_3), 56.1 (OCH_3), 64.2 (OCH_3), 105.4 (CH, C-6), 127.0 (quat., C-1), 132.3 (quat., C-3), 135.8 (quat., C-4), 154.7 (quat., C-2), 156.9 (quat., C-5), 190.4 (CHO).

2,5-Dimethoxy-3,4,6-trimethylbenzaldehyde 17b

Prepared from 1,4-dimethoxy-2,3,5-trimethylbenzene **16c** (2.274 g, 12.6 mmol). 2,5-Dimethoxy-3,4,6-trimethylbenzaldehyde **17b** was isolated as a yellow solid (1.21 g, 46%); mp 65-66 °C (lit.[25] mp 80 °C) (Found: MH^+, 209.1176. Calc. for $C_{12}H_{16}O_3$: MH, 209.1172); ν_{max} (KBr)/cm^{-1} 1685 (C=O), 1586 (C=C), 1255 (C-O); δ_H (500 MHz; $CDCl_3$) 2.23 (3H, s, CH_3), 2.31 (3H, s, CH_3), 2.53 (3H, s, CH_3), 3.70 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 10.47 (1H, s, CHO); δ_C (125 MHz; $CDCl_3$) 12.5 (CH_3), 13.3 (CH_3), 14.2 (CH_3), 60.7 (OCH_3), 63.8 (OCH_3), 126.0 (quat., C-1), 129.7 (quat., C-3), 132.1 (quat., C-6), 140.2 (quat., C-4), 153.6 (quat., C-2), 160.0 (quat., C-5), 194.9 (CHO).

2,5-Dimethoxy-3,4-dimethylphenol 12a

Prepared from 3,4-dimethyl-2,5-dimethoxybenzaldehyde **17a** (1.126 g, 5.8 mmol). Using light petroleum (60-80 °C) : diethyl ether (60:40) as eluent, 2,5-dimethoxy-3,4-dimethylphenol **12a** was isolated as a yellow solid (0.239 g, 23%), mp 69-71 °C (lit.[21] mp 70-71 °C); (Found: C, 65.9; H, 7.7. $C_{10}H_{14}O_3$ requires C, 65.9; H, 7.7 %); ν_{max} (film)/cm^{-1} 3263 (OH), 1598 (C=C), 1261 (C-O); δ_H (300 MHz; $CDCl_3$) 2.09 (3H, s, CH_3), 2.22 (3H, s, CH_3), 3.74 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 4.74 (1H, br s, OH), 6.44 (1H, s, H-6); δ_C (75 MHz; $CDCl_3$) 11.7 (CH_3), 13.0 (CH_3), 56.1 (CH, OCH_3), 61.4 (CH, OCH_3), 97.0 (CH, C-6), 117.4 (quat., C-4), 130.6 (quat., C-3), 139.5 (quat., C-2), 147.1 (quat., C-1), 154.7 (quat., C-5); *m/z* 183 (MH^+).

2,5-Dimethoxy-3,4,6-trimethylphenol 12b

Prepared from 3,4,6-trimethyl-2,5-dimethoxybenzaldehyde **17b** (1.205 g, 5.8 mmol). Using light petroleum (60-80 °C) : diethyl ether (75:25) as eluent, 2,5-dimethoxy-3,4,6-trimethylphenol **12a** was isolated as a white solid (0.856 g, 75%) mp 105-106 °C, (Found: C, 67.4; H, 8.2. $C_{11}H_{16}O_3$ requires C, 67.3; H, 8.2 %); ν_{max} (KBr)/cm^{-1} 3401 (OH), 1265 (C-O); δ_H (300 MHz; $CDCl_3$) 2.18 (3H, s, CH_3), 2.23 (6H, s, 2 × CH_3), 3.71 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 5.71 (1H, br s, OH); δ_C (75 MHz; $CDCl_3$) 9.1 (CH_3), 12.0 (CH_3), 12.5 (CH_3), 60.2 (OCH_3), 60.9 (OCH_3), 115.0 (quat.), 121.0 (quat.), 126.8 (quat.), 141.7 (quat.), 145.3 (quat.), 153.4 (quat.).

Preparation of 4-*tert*-Butyl-2,5-dimethoxyphenol 12c

In a flame-dried flask, 2-*tert*-butyl-1,4 dimethoxybenzene 16a (2.27 g, 11.7 mmol) was dissolved in dry THF (30 ml), the resulting solution was cooled to –78 °C and a solution of BuLi (2.82M in hexanes, 5.0 ml, 14.0 mmol) was added dropwise. After the addition was complete, the reaction mixture was allowed to warm to room temperature, stirred for 15 min. at this temperature and cooled again to –78 °C. Triisopropyl borate (3.2 ml, 0.01402 mol) was introduced dropwise and the reaction mixture was left to stir at room temperature. After 12 hours the reaction mixture was quenched with 10% NH_4Cl solution (50 ml) and water (50 ml) and extracted with diethyl ether (3 × 50ml). The combined organic layers were washed with brine (50 ml) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure, the residue was dissolved in THF (10 ml) and aqueous H_2O_2 (27% w/v, 5 ml) was added. The emulsion was stirred at room temperature for 30 min.. Water (100 ml) was added and the mixture was extracted with diethyl ether (3 × 50 ml), and dried over $MgSO_4$. The solvent was removed under vacuum and the residue was purified by column chromatography using light petroleum (60-80 °C) : ethyl acetate (80:20) as eluent to give the title compound **12c** as a white solid (1.010 g, 41%), mp 46-47 °C (lit.[26] mp 54-55 °C); ν_{max}(film)/cm^{-1} 3542, 3433 (OH), 1600 (C=C); δ_H (300 MHz; $CDCl_3$) 1.22 (9H, s, $C(CH_3)_3$), 3.61 (3H, s, OCH_3), 3.71 (3H, s, OCH_3), 6.43 (1H, s, H-6), 6.70 (1H, s, H-3); δ_C (75 MHz; $CDCl_3$) 30.6 (CH_3, $C(CH_3)_3$), 34.9 (quat., $C(CH_3)_3$), 56.1 (OCH_3), 57.3 (OCH_3), 100.9 (CH, C-6), 111.2 (CH, C-3), 130.1 (quat., C-4), 139.9 (quat.), 144.6 (quat.), 153.6 (quat.).

General preparation of biaryl ethers 13a-c

The appropriate phenol (1 equiv.) was dissolved in dry DMF (10 ml) and NaH (60% dispersion in oil; 1.1 equiv.) was added in small portions. After the evolution of gas was complete, the resulting solution of the sodium phenolate was stirred at room temperature for 15 min.. A solution of 2,5-dinitrofluorobenzene 18 (1 equiv.) in dry THF (5 ml) was then added dropwise to the flask and the reaction mixture was stirred for 2 hours. Finally, the contents of the flask were poured into water (50 ml), extracted with ether (3 × 20 ml) and the combined organic layers were dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue was subjected to column chromatography on silica.

1-(2',5'-Dinitrophenoxy)-3,4-dimethyl-2,5-dimethoxybenzene 13a

Prepared from 2,5-dinitrofluorobenzene **11** (0.293 g, 1.6 mmol) and 2,5-dimethoxy-3,4-dimethylphenol **12a** (0.287 g, 1.6 mmol). Purified using light petroleum (60-80 °C) : ethyl acetate (85:15) as eluent to give 1-(2′,5′-dinitrophenoxy)-3,4-dimethyl-2,5-dimethoxybenzene 13a as an orange solid (0.415 g, 76 %); mp 135-136 °C; (Found: MH^+, 349.1030. Calc. for $C_{16}H_{17}O_7N_2$: M, 349.1017); ν_{max} (KBr)/cm^{-1} 1551, 1346 (NO_2), 1246 (C-O); δ_H (300 MHz; $CDCl_3$) 2.10 (3H, s, CH_3), 2.17 (3H, s, CH_3), 3.58 (3H, s, OCH_3), 3.71 (3H, s, OCH_3), 6.49 (1H, s, H-6), 7.52 (1H, d, J = 2.2 Hz, H-6'), 7.84 (1H, dd, J = 8.8 Hz and 2.2 Hz, H-4'), 7.93 (1H, d, J = 8.8 Hz, H-3'); δ_C (75 MHz; $CDCl_3$) 12.4 (CH_3), 13.0 (CH_3), 56.3 (OCH_3), 61.7 (OCH_3), 102.6 (CH, C-6), 112.8 (CH, C-6'), 116.7 (CH, C-4'), 125.5 (quat.), 126.4 (CH, C-3'), 133.7 (quat.), 143.1 (quat.), 143.6 (quat.), 150.8 (quat.), 152.6 (quat.), 154.8 (quat.).

1-(2',5'-Dinitrophenoxy)-3,4,6-trimethyl-2,5-dimethoxybenzene 13b

Prepared from 2,5-dinitrofluorobenzene **11** (0.812 g, 4.362 mmol) and 2,5-dimethoxy-3,4,6-trimethylphenol **12b** (0.856 g, 4.362 mmol). Purified using light petroleum (60-80 °C) : diethyl ether (75:25) as eluent and recrystallised from ethanol to give 1-(2',5'-dinitrophenoxy)-3,4,6-trimethyl-2,5-dimethoxybenzene 13b as a yellow solid (1.412 g, 89 %); mp 136-137 ºC (Found: C, 56.35; H, 5.0; N, 7.6. $C_{17}H_{18}N_2O_7$ requires C, 56.35; H, 5.0; N, 7.7 %); ν_{max} (KBr)/cm^{-1} 1544, 1348 (NO_2), 1249 (C-O); δ_H (300 MHz; $CDCl_3$) 2.15 (3H, s, CH_3), 2.21 (3H, s, CH_3), 2.26 (3H, s, CH_3), 3.66 (3H, s, OCH_3), 3.71 (3H, s, OCH_3), 7.46 (1H, d, J = 2.2 Hz, H-6'), 7.93 (1H, dd, J= 8.9 and 2.2 Hz, H-4'), 8.03 (1H, d, J = 8.9 Hz, H-3'); δ_C (75 MHz; $CDCl_3$) 12.4 (CH_3), 12.8 (CH_3), 13.2 (CH_3), 60.8 (OCH_3), 61.5 (OCH_3), 111.8 (CH, C-6'), 116.6 (CH, C-4'), 122.6 (quat.), 126.5 (CH, C-3'), 130.0 (quat.), 130.5 (quat.), 142.5 (quat.), 142.8 (quat.), 146.6 (quat., C-2), 150.9 (quat.), 152.1 (quat.), 153.9 (quat., C-5).

1-(2',5'-Dinitrophenoxy)-4-*tert*-butyl-2,5-dimethoxybenzene 13c

Prepared from 2-fluoro-1,4-dinitrobenzene **11** (0.829 g, 4.457 mmol) and 4-*tert*-butyl-2,5-dimethoxyphenol **12c** (0.937 g, 4.457 mmol). Purified using light petroleum (60-80 °C):diethyl ether (80:20) to give ether 13c as a yellow solid (1.435 g, 86 %), mp 132-133 ºC; (Found: C, 57.4; H, 5.35; N, 7.25. $C_{17}H_{18}N_2O_7$ requires C, 57.4; H, 5.4; N, 7.4 %); ν_{max}(KBr)/cm^{-1} 1545, 1349 (NO_2), 1245 (C-O); δ_H (300 MHz; $CDCl_3$) 1.33 (9H, s, $C(CH_3)_3$), 3.58 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 6.66 (1H, s, H-3), 6.94 (1H, s, H-6), 7.56 (1H, d, J = 2.2 Hz, H-6'), 7.84 (1H, dd, J = 8.9 and 2.2 Hz, H-4'), 7.93 (1H, d, J = 8.9 Hz, H-3'); δ_C (75 MHz; $CDCl_3$) 30.0 (CH, $C(CH_3)_3$), 35.5 (quat., $C(CH_3)_3$), 56.2 (OCH_3), 57.4 (OCH_3), 106.9 (CH, C-3), 113.1 (CH, C-6'), 114.2 (CH, C-6), 116.7 (CH, C-4'), 126.5 (CH, C-3'), 138.3 (quat., C-4), 140.0 (quat., C-1), 143.2 (quat., C-5'), 144.2 (quat.), 150.7 (quat.), 152.6 (quat.), 153.7 (quat.).

General procedure for preparation of 7-aminophenoxazin-3-ones 6c,d

The dihydroxydiaryl ether (1.3 mmol) was dissolved in methanol (5 ml) and 5% Pd/C (10 % w/w) was added to the solution. The reaction mixture was stirred at room temperature in the hydrogenator under a hydrogen atmosphere for 4 hours. Sufficient silica to adsorb the residue for the subsequent column chromatography was added to the flask, and the mixture was stirred vigorously for a further 4 hours in air. When the oxidation was complete, the solvent was removed and the residue was subjected to column chromatography on silica eluting with light petroleum (60-80 °C) : ethyl acetate (50:50 to 0:100) then with ethyl acetate : methanol (90:10).

7-Amino-1,2-dimethylphenoxazin-3-one 6c

Prepared from 1-(2',5'-dinitrophenoxy)-3,4-dimethyl-2,5-dimethoxybenzene **13a** (0.266 g, 0.76 mmol) in a one-pot reaction. 7-Amino-1,2-dimethylphenoxazin-3-one **6c** was obtained as a brown-red solid (0.125 g, 68 %); mp > 270 ºC; ν_{max} (KBr)/cm^{-1} 3315, 3201 (NH_2), 1600 (C=O), 1545 (C=C); δ_H (300 MHz; DMSO-d_6) 2.02 (3H, s, CH_3), 2.33 (3H, s, CH_3), 6.10 (1H, s, H-4), 6.47 (1H, d, J = 2.3 Hz, H-6), 6.67 (1H, dd, J = 8.8 and Hz, H-8), 6.73 (2H, s, NH_2), 7.48 (1H, d, J = 8.8 Hz, H-9); δ_C (75 MHz; DMSO-d_6) 13.3 (CH_3), 13.5 (CH_3), 98.0 (CH, C-6), 104.4 (CH, C-4), 113.8 (CH, C-8), 125.9 (quat., C-7), 132.6 (CH, C-9), 136.7 (quat.), 138.1 (quat.), 141.0 (quat.), 147.0 (quat., C-9a), 150.3 (quat., C-4a), 155.3 (quat., C-5a), 184.4 (quat., C-3).

7-Amino-1,2,4-trimethylphenoxazin-3-one 6d

1-(2′,5′-Dinitrophenoxy)-3,4,6-trimethyl-2,5-dihydroxybenz-ene **14b** was prepared from 1-(2′,5′-dinitrophenoxy)-3,4,6-trimethyl-2,5-dimethoxybenzene **13b** (0.626 g, 1.73 mmol), using light petroleum (60-80 °C) : diethyl ether (70:30) as eluent, and isolated as an orange solid (0.435 g, 75 %); mp 180-181 ºC; (Found: MH$^+$, 255.1129. Calc. for $C_{15}H_{15}O_2N_2$: MH, 255.1128); ν_{max} (KBr)/cm^{-1} 3490 (NH_2 and OH), 1606 (C=O), 1591 (C=C); δ_H (300 MHz; DMSO-d_6) 1.98 (3H, s, CH_3), 2.11 (3H, s, CH_3), 2.14 (3H, s, CH_3), 7.23 (1H, d, J = 2.3 Hz, H-6'), 7.94 (1H, br s, OH), 7.99 (1H, dd, J = 8.9 and 2.3 Hz, H-4'), 8.28 (1H, d, J = 8.9 Hz, H-3'), 8.38 (1H, br s, OH); δ_C (75 MHz; DMSO-d_6) 10.6 (CH_3), 13.3 (CH_3), 13.7 (CH_3), 111.0 (CH), 116.4 (quat.), 117.4 (quat.), 124.1 (quat.), 124.2 (quat.), 127.7 (CH), 137.6 (quat.), 140.7 (quat.), 143.3 (quat.), 146.9 (quat.), 150.9 (quat.), 152.0 (quat.).

1-(2',5'-Dinitrophenoxy)-3,4,6-trimethyl-2,5-dihydroxybenz-ene **14b** (0.435 g, 1.30 mmol) was then treated as described above to give 7-amino-1,2,4-trimethylphenoxazin-3-one **6d** as a brown-red solid (0.237 g, 72 %); mp > 270 ºC; ν_{max} (KBr)/cm^{-1} 3320, 3211 (NH_2), 1610 (C=C); δ_H (300 MHz; DMSO-d_6) 1.96 (3H, s, CH_3), 2.04 (3H, s, CH_3), 2.31 (3H, s, CH_3), 6.49 (1H, d, J = 2.3 Hz, H-6), 6.60 (2H, br s, NH_2), 6.63 (1H, dd, J = 8.7 and 2.3 Hz, H-8), 7.44 (1H, d, J = 8.7 Hz, H-9); δ_C (75 MHz; DMSO-d_6) 8.5 (CH_3), 13.3 (CH_3), 13.6 (CH_3), 98.2 (CH, C-6), 111.9 (quat., C-4), 113.3 (CH, C-8), 125.4 (quat., C-7), 132.3 (CH, C-9), 136.0 (quat.), 137.1 (quat.), 141.3 (quat.), 146.4 (quat., C-9a), 147.2 (quat., C-4a), 154.8 (quat., C-5a), 184.1 (quat., C-3).

7-*N*-(*N*-^{t}Boc-β-alanyl)amino-1-pentylphenoxazin-3-one 26a and 7-*N*-(*N*-^{t}Boc-β-alanyl)amino-2-chloro-1-pentylphenoxazin-3-one 26b

Acetic acid (30 %, 200 ml) was added dropwise, with stirring, to a solution in which sodium borohydride (4 - 6 g) and sodium hydroxide (0.2 g) were dissolved in water (200 ml). The hydrogen gas produced was passed into a three-necked flask in which a mixture of 7-amino-1-pentylphenoxazin-3-one **6a** and 7-amino-2-chloro-1-pentylphenoxazin-3-one **6b**[14] (0.564 g) was dissolved in dry DMF (15 ml), and the solution was diluted with dry THF (15 ml). 5 % Pd/C (0.2 g) was added and hydrogen gas was bubbled slowly through the solution for 1 hour after the reduction appeared to be complete, as evidenced by the replacement of the purple colour of the solution by a weak grey-green colour. In a separate flask, *N*-^{t}Boc-β-alanine (0.756 g, 4.0 mmol) and *N*-methylmorpholine (0.408 g, 4.0 mmol) were dissolved in dry THF (10 ml), the solution was cooled to -20 °C and isobutyl chloroformate (0.56 ml, 4.0 mmol) was added with stirring. The mixture was stirred at -20 °C for a further 30 min., after which time the mixture was introduced into the reduced resorufamine solution at -10 °C with the continued passage of hydrogen gas. After 15 min., hydrogen was no longer admitted, the system was sealed and the reaction mixture was stirred overnight at room temperature. The reaction mixture was filtered and solvent was evaporated under reduced pressure, the residual solid was dissolved in DCM (50 ml), filtered, and the DCM solution washed with $NaHCO_3$ (5 %, 2 × 50 ml) and water (50 ml). The organic phase was dried ($MgSO_4$), filtered and concentrated to afford a residue consisting of two products, which was purified by column chromatography on silica, eluting with petrol / ethyl acetate (6:4), to give 7-*N*-(*N*-^{t}Boc-β-alanyl)amino-2-chloro-1-pentylphenoxazin-3-one **26b** (as the first spot) as an orange solid (0.12 g) mp 226-227 ºC; (Found: MH$^+$,

488.1942. Calc. for $C_{25}H_{31}O_5N_3Cl$: MH, 488.1945); ν_{max} (KBr) / cm^{-1} 3388 (NH), 3269 (NH), 1699 (C=O), 1603 (C=O), 1577 (C=C); δ_H (300 MHz, DMSO-d_6) 0.89 (3H, t, J = 6.8 Hz, 5′-CH_3), 1.38 (13H, m, 3′-CH_2, 4′-CH_2, C(CH_3)), 1.59 (2H, m, 2′-CH_2), 2.56 (2H, t, J = 6.8 Hz, $NHCH_2CH_2CO$), 3.00 (2H, m, 1′-CH_2), 3.25 (2H, q, J = 6.8 Hz, $NHCH_2CH_2CO$), 6.40 (1H, s, H-4), 6.93 (1H, d, J = 4.95 Hz, NH), 7.52 (1H, d, J = 8.4 Hz, H-8), 7.79 (1H, d, J = 8.2 Hz, H-9), 7.93 (1H, s, H-6), 10.60 (1H, br, ArNH); δ_C (75.5 MHz, DMSO-d_6) 14.6 (CH_3, C-5′), 22.6 (CH_2, C-4′), 28.4 (CH_2, C-2′), 28.5 (CH_2, C-1′), 29.1 (3 × CH_3), 32.0 (CH_2, C-3′), 37.1 (CH_2), 37.9 (CH_2), 78.55 (quat.), 105.3 (CH, C-4), 108.2 (CH, C-6), 117.5 (CH, C-8), 129.6 (quat., C-7), 131.9 (CH, C-9), 136.7 (quat., C-2), 142.9 (quat., C-1), 143.65 (quat., C-9a), 144.5 (quat., C-5a), 145.1 (quat., C-10a), 149.6 (quat., C-4a), 156.4 (C=O), 171.4 (C=O), 187.8 (C=O, C-3).

7-*N*-(*N*-^{t}Boc-β-alanyl)amino-1-pentylphenoxazin-3-one **26a** (second spot) was obtained as an orange solid (0.11 g) mp 212.5-214.0 °C; (Found: MH^+, 454.2330. Calc. for $C_{25}H_{32}O_5N_3$: MH, 454.2334); ν_{max} (KBr) / cm^{-1} 3379 (NH), 3265 (NH), 1703 (C=O), 1647 (C=O), 1612 (C=O), 1591 (C=C); δ_H (300 MHz, CD_3OD) 0.96 (3H, t, J = 7.0 Hz, 5′-CH_3), 1.29 - 1.44 (15H, m, 2′-CH_2, 3′-CH_2, 4′-CH_2, C(CH_3)$_3$), 2.63 (2H, t, J = 6.6 Hz, $NHCH_2CH_2CO$), 2.91 (2H, t, J = 8.0 Hz, 1′-CH_2), 3.43 (2H, t, J = 6.7 Hz, $NHCH_2CH_2CO$), 6.26 (1H, d, J = 2.1 Hz, H-4), 6.68 (1H, d, J = 2.1 Hz, H-2), 7.50 (1H, dd, J = 8.7 and 2.3 Hz, H-8), 7.81 (1H, d, J = 8.8 Hz, H-9), 8.01 (1H, d, J = 2.1 Hz, H-6); δ_C (75.5 MHz, CD_3OD) 14.4 (CH_3, C-5′), 22.85 (CH_2, C-4′), 28.8 (C(CH_3)$_3$), 29.1 (CH_2, C-2′), 30.0 (CH_2, C-1′), 32.0 (CH_2, C-3′), 37.1 (CH_2), 37.9 (CH_2), 78.55 (quat.), 106.25 (CH, C-4), 106.4 (CH, C-6), 116.7 (CH, C-8), 129.75 (quat., C-7), 131.3 (CH, C-9), 131.75 (CH, C-2), 142.4 (quat., C-1), 144.9 (quat., C-9a), 146.8 (quat., C-5a), 147.65 (quat., C-10a), 150.4 (quat., C-4a), 157.3 (carbamate C=O), 172.25 (amide C=O), 184.7 (C=O, C-3).

7-N-(N-^{t}Boc-L-alanyl)amino-1-pentylphenoxazin-3-one 28a and 7-*N*-(*N*-^{t}Boc-L-alanyl)aminophenoxazin-2-chloro-1-pentyl-3-one 28b

Prepared using the same method as for the β-Ala derivatives **26a** and 26b, from a mixture of 7-amino-1-pentylphenoxazin-3-one **6a** and 7-amino-2-chloro-1-pentylphenoxazin-3-one **6b**[14] (0.564 g) and *N*-^{t}Boc-L-alanine (0.756 g, 4.0 mmol), *N*-methylmorpholine (0.408 g, 4.0 mmol) and isobutyl chloroformate (0.56 ml, 4.0 mmol). Column chromatography on silica, eluting with petrol / ethyl acetate (7:3) gave 7-*N*-(*N*-^{t}Boc-L-alanyl)amino-2-chloro-1-pentylphenoxazin-3-one **28b** (as the first spot) as an orange solid (0.12 g) (Found: MH^+, 488.1944. Calc. for $C_{25}H_{31}O_5N_3Cl$: MH, 488.1945); mp 231.0-232.5 °C; $[\alpha]^{20}_D$ -250° (c 0.10, $CHCl_3$); ν_{max} (KBr)/cm^{-1} 3442 (NH), 1701 (C=O), 1645 (C=O), 1604 (C=O), 1253 (C-O); δ_H (300 MHz, $CDCl_3$) 0.90 (3H, t, J = 6.6 Hz, 5′-CH_3), 1.20 (4H, m, 3′-CH_2, 4′-CH_2), 1.40 - 1.48 (14H, m, 2′-CH_2, C(CH_3)$_3$, ala-CH_3), 2.90 (2H, m, 1′-CH_2), 4.36 (1H, m, CH_α), 5.15 (1H, d, J = 6.7 Hz, ala-NH), 6.24 (1H, s, H-4), 7.04 (1H, d, J = 7.6 Hz, H-8), 7.53 (1H, d, J = 8.4 Hz, H-9), 7.77 (1H, s, H-6), 9.54 (1H, br s, ArNH); δ_C (75.5 MHz, $CDCl_3$) 14.4 (5′-CH_3), 17.6 (ala-CH_3), 22.7 (4′-CH_2), 28.3 (2′-CH_2), 28.5 (1′-CH_2), 28.8 (C(CH_3)$_3$), 32.2 (3′-CH_2), 55.15 (CH), 81.7 (quat.), 105.8 (CH, C-4), 106.2 (CH, C-6), 117.0 (CH, C-8), 129.8 (quat., C-7), 131.4 (CH, C-9), 137.8 (quat., C-2), 142.9 (quat., C-1), 143.8 (quat., C-9a), 144.5 (quat., C-5a), 145.1 (quat., C-10a), 149.6 (quat., C-4a), 157.5 (carbamate C=O), 172.35 (amide C=O), 185.85 (C=O, C-3).

7-*N*-(*N*-^{t}Boc-L-alanyl)amino-1pentylphenoxazin-3-one **28a** (second spot) as a brown solid (0.10 g) mp 209-211 °C; (Found: MH^+, 454.2332. Calc. for $C_{25}H_{32}O_5N_3$: MH, 454.2334); $[\alpha]^{20}_D$ -273 ° (c 0.11, $CHCl_3$); ν_{max} (KBr)/cm^{-1} 3437 (NH), 1714 (C=O), 1647 (C=O), 1614 (C=O), 1591 (C=C), 1252 (C-O); δ_H (300 MHz, $CDCl_3$) 0.73 (3H, t, J = 6.6 Hz, 5′-CH_3), 1.18 (4H, m, 3′-CH_2, 4′-CH_2), 1.27 - 1.42 (14H, m, 2′-CH_2, C(CH_3)$_3$, ala-CH_3), 2.58 (2H, m, 1′-CH_2), 4.24 (1H, m, CH_α), 5.05 (1H, d, J = 6.95 Hz, ala-NH), 5.95 (1H, s, H-4), 6.38 (1H, s, H-2), 6.93 (1H, d, J = 7.3 Hz, H-8), 7.39 (1H, d, J = 8.6 Hz, H-9), 7.67 (1H, s, H-6), 9.35 (1H, br s, ArNH); δ_C (75.5 MHz, $CDCl_3$) 14.4 (5′-CH_3), 17.6 (ala-CH_3), 22.8 (4′-CH_2), 28.8 (C(CH_3)$_3$), 29.0 (2′-CH_2), 29.95 (1′-CH_2), 32.0 (3′-CH_2), 55.5 (CH), 81.6 (quat.), 106.2 (CH, C-4), 106.4 (CH, C-6), 116.6 (CH, C-8), 129.7 (quat., C-7), 131.2 (CH, C-9), 131.7 (CH, C-2), 142.5 (quat., C-1), 144.9 (quat., C-9a), 146.8 (quat., C-5a), 147.6 (quat., C-10a), 150.4 (quat., C-4a), 157.1 (carbamate C=O), 172.2 (amide C=O), 186.7 (C=O, C-3).

General procedure for the peptide coupling of 7-aminophenoxazin-3-ones 6c,d

The 7-aminophenoxazin-3-one **6c,d** (0.4 mmol) was dissolved in dry DMF (5 ml) and 5% Pd/C (0.010 g) was added to the solution. The flask was placed in a hydrogenator at room temperature and an atmosphere of hydrogen was maintained while the reaction mixture was stirred for 1 hour. The completion of the reduction was indicated by the replacement of the deep purple colour of the solution by a greyish-green colour. In a separate flask, *N*-^{t}Boc-β-alanine (0.089g, 0.47 mmol), HOBt (0.072 g, 0.47 mmol), and DIC (0.07 ml, 0.47 mmol) were dissolved in dry DCM (5 ml) and the resulting mixture was stirred at room temperature for 1 hour. After this period, the contents of the second flask were introduced into the first flask (which contained the reduced form of 7-aminophenoxazin-3-one) *via* syringe, under an inert atmosphere. The mixture was stirred for a further 20 hours at room temperature then filtered through celite and the solvent evaporated under reduced pressure. The residue was redissolved in ethyl acetate (20 ml), the organic layer was washed with 1M HCl (20 ml), 10% Na_2CO_3 (20 ml) and water (20 ml). The organic solution was dried over $MgSO_4$, filtered and evaporated under reduced pressure to give a residue, which was purified by column chromatography using light petroleum (60-80 °C) : ethyl acetate (30:70) as eluent.

7-*N*-(*N*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2-dimethylphenoxazin-3-one 26c

Prepared from 7-amino-1,2-dimethylphenoxazin-3-one **6c** (0.110 g, 0.4578 mmol). 7-*N*-(*N*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2-dimethylphenoxazin-3-one **26c** was obtained as a brown-red solid (0.104 g, 55%); mp 222-223 °C (decomp.); (Found: MH^+, 412.1871. Calc. for $C_{22}H_{26}O_5N_3$: MH, 412.1867); ν_{max} (KBr)/cm^{-1} 3341, 3272 (NH), 1705, 1689 (C=O), 1616 (C=C), 1253 (C-O); δ_H (300 MHz; DMSO-d_6) 1.38 (9H, s, C(CH_3)$_3$), 2.06 (3H, s, CH_3), 2.36 (3H, s, CH_3), 2.53-2.55 (2H, m, H-2′), 3.22-3.28 (2H, m, H-3′), 6.22 (1H, s, H-4), 6.88 (1H, br s, NH), 7.48 (1H, dd, J = 8.7 and 2.0 Hz, H-8), 7.75 (1H, d, J = 8.7 Hz, H-9), 7.87 (1H, d, J = 2.0 Hz, H-6), 10.47 (1H, s, ArNH); δ_C (75 MHz; DMSO-d_6) 13.4 (CH_3), 13.5 (CH_3), 29.1 (CH_3, C(CH_3)), 37.3 (CH_2, C-3'), 37.9 (CH_2, C-2'), 78.5 (quat., C(CH_3)$_3$), 103.8, (CH), 105.5 (CH, C-4), 117.0 (CH, C-8), 129.4 (quat., C-7), 131.3 (CH, C-9), 138.4 (quat.), 139.0 (quat.), 143.7 (quat.), 144.9 (quat.), 147.0 (quat., C-4a), 150.0 (quat.), 156.3 (quat., carbamate C=O), 171.2 (quat., amide C=O), 185.1 (quat., C-3).

7-*N*-(*N*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2,4-trimethylphenoxazin-3-one 26d

Prepared from 7-amino-1,2,4-trimethylphenoxazin-3-one **6d** (0.100 g, 0.39 mmol). 7-*N*-(*N*-^{t}Butoxycarbonyl-β-alanyl)amino-1,2,4-trimethylphenoxazin-3-one **26d** was obtained as an orange solid (0.113 g, 68 %); mp 215-216 °C; (Found: MH^+, 426.2025. Calc. for $C_{23}H_{28}O_5N_3$: MH, 426.2023); ν_{max} (KBr)/cm^{-1} 3341 (NH), 1704 (C=O), 1686 (C=O), 1616 (C=C), 1250 (C-O); δ_H (500 MHz; DMSO-d_6) 1.39 (9H, s, $C(CH_3)_3$), 1.98 (3H, s, CH_3), 2.05 (3H, s, CH_3), 2.31 (3H, s, CH_3), 2.55 (2H, t, J = 7.0 Hz, H-2′), 3.24-3.28 (2H, m, H-3′), 6.92 (1H, t, J = 5.2 Hz, NH), 7.37 (1H, dd, J = 8.7 and 2.1 Hz, H-8), 7.68 (1H, d, J = 8.7 Hz, H-9), 7.91 (1H, d, J = 2.1 Hz, H-6), 10.43 (1H, s, ArNH); δ_C (125 MHz; DMSO-d_6) 8.5 (CH_3), 13.3 (CH_3), 13.7 (CH_3), 29.1 (CH_3, $C(CH_3)_3$), 37.2 (CH_2, C-3'), 37.9 (CH_2, C-2'), 78.5 (quat., $C(CH_3)_3$), 105.6 (CH, C-6), 113.1 (quat., C-4), 116.6 (CH, C-8), 129.0 (quat., C-7), 131.0 (CH, C-9), 137.4 (quat.), 138.3 (quat.), 143.3 (quat., C-9a), 145.2 (quat., C-5a), 146.0 (quat., C-4a), 146.8 (quat., C-4a), 156.2 (quat., carbamate C=O), 171.1 (quat., amide C=O), 184.7 (quat., C-3).

Deprotection of *N*-^{t}butoxycarbonyl group

The corresponding *N*-^{t}butoxycarbonyl protected compound **26** (0.2 mmol) was dissolved in dry DCM (3 ml) and TFA (1 ml) or neat TFA (2 ml) was added to the solution. The reaction mixture was stirred at room temperature until completion of reaction (as indicated by TLC). The solvent and excess of TFA were evaporated under reduced pressure and the residue was purified by column chromatography on silica, using a gradient eluent starting with light petroleum (60-80 °C) : ethyl acetate (50:50 to 0:100) and, finally, ethyl acetate : methanol (90:10).

7-*N*-(β-Alanyl)amino-1-pentylphenoxazin-3-one trifluoroacetate salt 27a

Prepared from 7-*N*-(*N*-^{t}Boc-β-Alanyl)amino-1-pentylphenoxazin-3-one **26a** (80 mg, 0.18 mmol) and TFA (2 ml). After work-up, 7-*N*-(β-Alanyl)amino-1-pentylphenoxazin -3-one trifluoroacetate salt **27a** was obtained as a brown solid (80 mg, 97 %) mp 215-216 °C; (Found: M^+, 354.1798. Calc. for $C_{20}H_{24}O_3N_3$: M, 354.1812); ν_{max} (KBr)/cm^{-1} 3448, 3259 (NH), 1678 (C=O), 1647 (C=O), 1612 (C=O), 1250 (C-O); δ_H (300 MHz, DMSO-d_6) 0.89 (3H, t, J = 6.9 Hz, 5′-CH_3), 1.16 (4H, m, 3′-CH_2, 4′-CH_2), 1.62 (2H, m, 2′-CH_2), 2.77 (4H, m, 1′-CH_2,CH_2), 3.13 (2H, m, CH_2), 6.19 (1H, d, J = 1.4 Hz, H-4), 6.59 (1H, d, J = 1.4 Hz, H-2), 7.50 (1H, d, J = 8.6 Hz, H-8), 7.8 (1H, d, J = 8.7 Hz, H-9), 7.9 (4H, br, H-6, NH_3^+), 10.82 (1H, br, ArNH); δ_C (75.5 MHz, DMSO-d_6) 14.7 (CH_3, C-5′), 22.7 (CH_2, C-4′), 29.1 (CH_2, C-2′), 29.8 (CH_2, C-1′), 31.8 (CH_2, C-3′), 34.5 (CH_2), 35.5 (CH_2), 105.6 (4-CH), 106.0 (CH, C-6), 117.1 (CH, C-8), 122.6 (CH, C-9), 129.4 (quat., C-7), 131.7 (CH, C-2), 143.8 (quat., C-1), 145.2 (quat., C-9a), 146.7 (quat., C-5a), 147.4 (quat., C-10a), 150.85 (quat., C-4a), 170.2 (C=O), 185.9 (C=O, C-3).

7-*N*-(β-Alanyl)amino-2-chloro-1-pentylphenoxazin-3-one trifluoroacetate salt 27b

Prepared from 7-*N*-(*N*-^{t}Boc-β-Alanyl)amino-2-chloro-1-pentylphenoxazin-3-one **26b** (80 mg, 0.18 mmol) and TFA (2 ml). After work-up, 7-*N*-(β-Alanyl)amino-2-chloro-1-pentylphenoxazin-3-one trifluoroacetate salt **27b** was obtained as a brown solid (80 mg, 97 %) mp 220-221 °C; (Found: M^+, 388.1422. Calc. for $C_{20}H_{23}O_3N_3Cl$: M, 388.1422); ν_{max} (KBr) / cm^{-1} 3454, 3265 (NH), 1678 (C=O), 1601 (C=O), 1577 (C=C), 1252 (C-O); δ_H (300 MHz, DMSO-d_6) 0.89 (3H, m, 5′-CH_3), 1.38 (4H, m, 3′-CH_2, 4′-CH_2), 1.59 (2H, m, 2′-CH_2), 2.81 (2H, m, CH_2), 3.00 (1′-CH_2), 3.14 (2H, m, CH_2), 6.42 (1H, s, H-4), 7.55 (1H, d, J = 7.9 Hz, H-8), 7.82 (1H, d, J = 8.4 Hz, H-9), 7.93 (4H, br, H-6, NH_3^+), 10.85 (1H, br, ArNH); δ_C (75.5 MHz, DMSO-d_6) 14.6 (CH_3, C-5′), 22.6 (CH_2, C-4′), 28.4 (CH_2, C-2′), 29.8 (CH_2, C-1′), 32.0 (CH_2, C-3′), 34.6 (CH_2), 35.5 (CH_2), 105.4 (CH, C-4), 105.5 (CH, C-6), 117.5 (CH, C-8), 129.7 (quat., C-7), 132.0 (CH, C-9), 136.8 (quat., C-2), 143.7 (quat., C-1), 144.4 (quat., C-9a), 144.85 (quat., C-5a), 145.1 (quat., C-10a), 150.8 (quat., C-4a), 169.8 (C=O), 177.8 (C=O, C-3).

7-*N*-(β-Alanyl)amino-1,2-dimethylphenoxazin-3-one trifluoroacetate salt 27c

Prepared from 7-*N*-(*N*-^{t}Boc-β-Alanyl)amino-1,2-dimethylphenoxazin-3-one **26c** (0.047 g, 0.1138 mmol) dry DCM (3 ml) and TFA (1 ml). 7-*N*-(β-Alanyl)amino-1,2-dimethylphenoxazin-3-one trifluoroacetate salt **27c** was isolated as a red solid (0.046 g, 95%) mp 191-192 °C; (Found: M^+, 312.1338. Calc. for $C_{17}H_{18}O_3N_3$: M, 312.1343); ν_{max} (KBr)/cm^{-1} 3274, 3192, 3111 (NH), 1676 (C=O), 1592 (C=C), 1254 (C-O); δ_H (300 MHz, CD_3OD) 2.01 (3H, s, CH_3), 2.29 (3H, s, CH_3), 2.78 (2H, t, J = 6.2 Hz, H-2′), 3.20-3.22 (2H, m, H-3′), 6.03 (1H, s, H-4), 7.27 (1H, dd, J = 8.7 and 2.2 Hz, H-8), 7.55 (1H, d, J = 8.7 Hz, H-9), 7.80 (1H, d, J = 2.2 Hz, H-6); δ_C (125 MHz; CD_3OD) 11.8 (CH_3), 12.0 (CH_3), 33.1 (CH_2), 35.6 (CH_2), 104.8 (CH, C-4), 105.8 (CH, C-6), 116.6 (CH, C-8), 129.8 (quat., C-7), 130.8 (CH, C-9), 139.0 (quat.), 139.3 (quat.), 142.8 (quat.), 144.7 (quat.), 146.6 (quat., C-4a) 150.0 (quat.), 169.9 (quat., amide C=O), 186.4 (quat., C-3).

7-*N*-(β-Alanyl)amino-1,2,4-trimethylphenoxazin-3-one trifluoroacetate salt 27d

Prepared from 7-*N*-(*N*-^{t}Boc-β-Alanyl)amino-1,2,4-trimethylphenoxazin-3-one **26d** (0.081 g, 0.1897 mmol). 7-*N*-(β-Alanyl)amino-1,2,4-trimethylphenoxazin-3-one trifluoro-acetate salt **27d** was isolated as a red solid (0.080 g, 96%) mp 217-219 °C (decomp.); (Found: M^+, 326.1506. Calc. for $C_{18}H_{20}O_3N_3$: M, 326.1499); ν_{max} (KBr)/cm^{-1} 3328, 3108 (NH), 1701 (C=O), 1686 (C=O), 1578 (C=C), 1207 (C-O); δ_H (300 MHz; DMSO-d_6) 1.96 (3H, s, CH_3), 2.04 (3H, s, CH_3), 2.30 (3H, s, CH_3), 2.76-2.81 (2H, m, H-2′), 3.11-3.16 (2H, m, H-3′), 7.38 (1H, dd, J = 8.7 and 2.2 Hz, H-8), 7.69 (1H, d, J = 8.7 Hz, H-9), 7.86 (3H, br s, -N$^{\oplus}$H$_3$), 7.88 (1H, d, J = 2.2 Hz, H-6), 10.69 (1H, s, ArNH); δ_C (75 MHz; DMSO-d_6) 7.6 (CH_3), 12.4 (CH_3), 12.8 (CH_3), 33.5 (CH_2), 34.7 (CH_2), 104.9 (CH, C-6), 112.3 (quat., C-4), 115.7 (CH, C-8), 128.3 (quat., C-7), 130.2 (CH, C-9), 136.5 (quat.), 137.5 (quat.), 142.0 (quat.), 144.3 (quat.), 145.1 (quat., C-4a), 146.2 (quat., C-10a), 169.1 (quat., amide C=O), 183.9 (quat., C-3).

7-*N*-(L-Alanyl)amino1-pentylphenoxazin-3-one trifluoroacetate salt 29a

Prepared from 7-*N*-(*N*-^{t}Boc-L-Alanyl)amino-1-pentlyphenoxazin-3-one **28a** (80 mg, 0.18 mmol) and TFA (2 cm^3). After work-up, 7-*N*-(L-Alanyl)amino-1-pentylphenoxazin-3-one trifluoroacetate salt **29a** was obtained as a brown solid (80 mg, 97 %) mp 168-170 °C; (Found: C, 56.5; H, 5.15; N, 9.0. $C_{22}H_{24}O_5N_3F_3$ requires C, 56.5; H, 5.2; N, 9.0 %) (Found: M^+, 354.1809. Calc. for $C_{20}H_{24}O_3N_3$: M, 354.1812); $[\alpha]^{20}_D$ + 145 ° (c 0.06, MeOH); ν_{max} (KBr) / cm^{-1} 3452 (NH), 3276 (NH), 1682 (C=O), 1645 (C=O), 1585 (C=C), 1250 (C-O); δ_H (300 MHz, CD_3OD) 0.84 (3H, t, J = 6.9 Hz, 5′-CH_3), 1.31 (4H, m, 3′-CH_2,

4′-CH_2), 1.55 (3H, d, J = 7.05 Hz, ala-CH_3), 1.58 (2H, m, 2′-CH_2), 2.76 (2H, m, 1′-CH_2), 4.05 (1H, m, CH_α), 6.09 (1H, d, J = 2.1 Hz, H-4), 6.53 (1H, d, J = 2.1 Hz, H-3), 7.41 (1H, dd, J = 8.7 and 2.2 Hz, H-8), 7.68 (1H, d, J = 8.7 Hz, H-9), 7.83 (1H, d, J = 2.2 Hz, H-6); δ_C (75.5 MHz, CD_3OD) 13.2 (5′-*C*H_3), 16.4 (ala-CH_3), 22.35 (4′-*C*H_2), 29.1 (2′-*C*H_2), 29.7 (1′-*C*H_2), 31.7 (3′-*C*H_2), 50.2 (CH), 105.4 (CH, C-4), 106.4 (CH, C-6), 117.2 (CH, C-8), 130.2 (quat., C-7), 131.1 (CH, C-9), 131.3 (CH, C-2), 142.75 (quat., C-1), 145.1 (quat., C-9a), 146.7 (quat., C-5a), 148.5 (quat., C-10a), 151.05 (quat., C-4a), 168.9 (C=O), 187.3 (C=O, C-3).

7-*N*-(L-Alanyl)amino-2-chloro-1-pentylphenoxazin-3-one trifluoroacetate salt 29b

Prepared from 7-*N*-(*N*-^{t}Boc-L-Alanyl)amino-2-chloro-1-pentylphenoxazin-3-one **28b** (0.10 g, 0.21 mmol) and TFA (2 cm^3). After work-up, 7-*N*-(L-Alanyl)amino-2-chloro-1-pentyl phenoxazin-3-one trifluoroacetate salt **29b** was obtained as a brown solid (0.095 g, 92 %) mp > 290 °C; (HRMS Found: M^+, 388.1422. Calc. for $C_{20}H_{23}O_3N_3Cl$: M, 388.1422), $[\alpha]^{20}_D$ + 100 ° (c 0.07, MeOH); ν_{max} (KBr)/cm^{-1} 3452 (NH), 3276 (NH), 1682 (C=O), 1645 (C=O), 1583 (C=C), 1250 (C-O); δ_H (300 MHz, CD_3OD) 0.85 (3H, t, J = 6.8 Hz, 5′-C*H*$_3$), 1.31 (4H, m, 3′-C*H*$_2$, 4′-C*H*$_2$), 1.47 (2H, m, 2′-C*H*$_2$), 1.56 (3H, d, J = 6.8 Hz, ala-CH_3), 2.98 (2H, m, 1′-C*H*$_2$), 4.05 (1H, q, J = 6.9 Hz, CH_α), 6.17 (1H, s, H-4), 7.36 (1H, d, J = 7.6 Hz, H-8), 7.64 (1H, d, J = 8.7 Hz, H-9), 7.89 (1H, s, H-6); δ_C (75.5 MHz, CD_3OD) 13.3 (5′-*C*H_3), 16.4 (ala-CH_3), 22.4 (4′-*C*H_2), 28.0 (2′-*C*H_2), 28.2 (1′-*C*H_2), 32.0 (3′-*C*H_2), 50.2 (CH), 104.85 (CH, C-4), 106.0 (CH, C-6), 117.3 (CH, C-8), 130.1 (quat., C-7), 131.4 (CH, C-9), 136.8 (quat., C-2), 143.3 (quat., C-1), 144.0 (quat., C-9a), 144.8 (quat., C-5a), 144.9 (quat., C-10a), 150.2 (quat., C-4a), 169.0 (C=O), 178.8 (C=O, C-3).

Columbia agar solution preparation

Gram-positive and Gram-negative bacteria were cultured on Columbia agar. 1 Litre of Columbia agar was prepared as follows; Columbia agar (41 g) was dissolved by boiling in distilled water (1 l). The solution was then autoclaved at 116 °C for 10 min. and left to cool at 50 °C.

Media preparation

The substrates to be tested were initially dissolved in DMSO or distilled water to give solutions of 10 mg/ml. The substrate solutions were incorporated into Columbia agar solution (200 ml) and added to sterile plates to give final concentrations of 50 mg/l. Columbia agar alone was used as a growth control. Solidified plates were surface dried in a warm air cabinet for 5 min..

Bacterial suspension preparation

Bacterial strains were obtained from the National Collection of Type Cultures (NCTC), Colindale, U.K., the American Type Culture Collection (ATCC), Cockeysville, U.S.A., or were isolated from clinical samples (wild strains) at the Microbiology Department of the Freeman Hospital, Newcastle-upon-Tyne, U.K..

McFarland tubes were labelled with numbers corresponding to the bacterial code on the plates. Sterile distilled water (2 ml) was added to each tube. Each bacterium was inoculated into the tube using a sterile loop. A densitometer was used to adjust the turbidity to 0.5 McFarland units (1.5×10^8 organisms/ml).

Multipoint inoculation

Each bacterial suspension (200 µl) was pipetted into the corresponding tubes of a multipoint inoculator. Each set of plates received 1 µl of bacterial suspension, giving 1.5×10^5 organisms per spot on each inoculation. Twenty strains were inoculated per plate and the plates were incubated for 24 and 48 hours at 30 °C, and 24 and 48 hours at 37 °C.

Activity determination

The activity of the test substrates was determined by the development of red, pink, purple or orange colonies after incubation. The control plate was first taken for each substrate tested and examined for growth and colour. Each test plate was then compared to the control and the presence of red, pink, purple or orange colour was considered as positive evidence for the hydrolysis of the substrate by alanyl aminopeptidase; no colour or a pale yellow was considered as negative.

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