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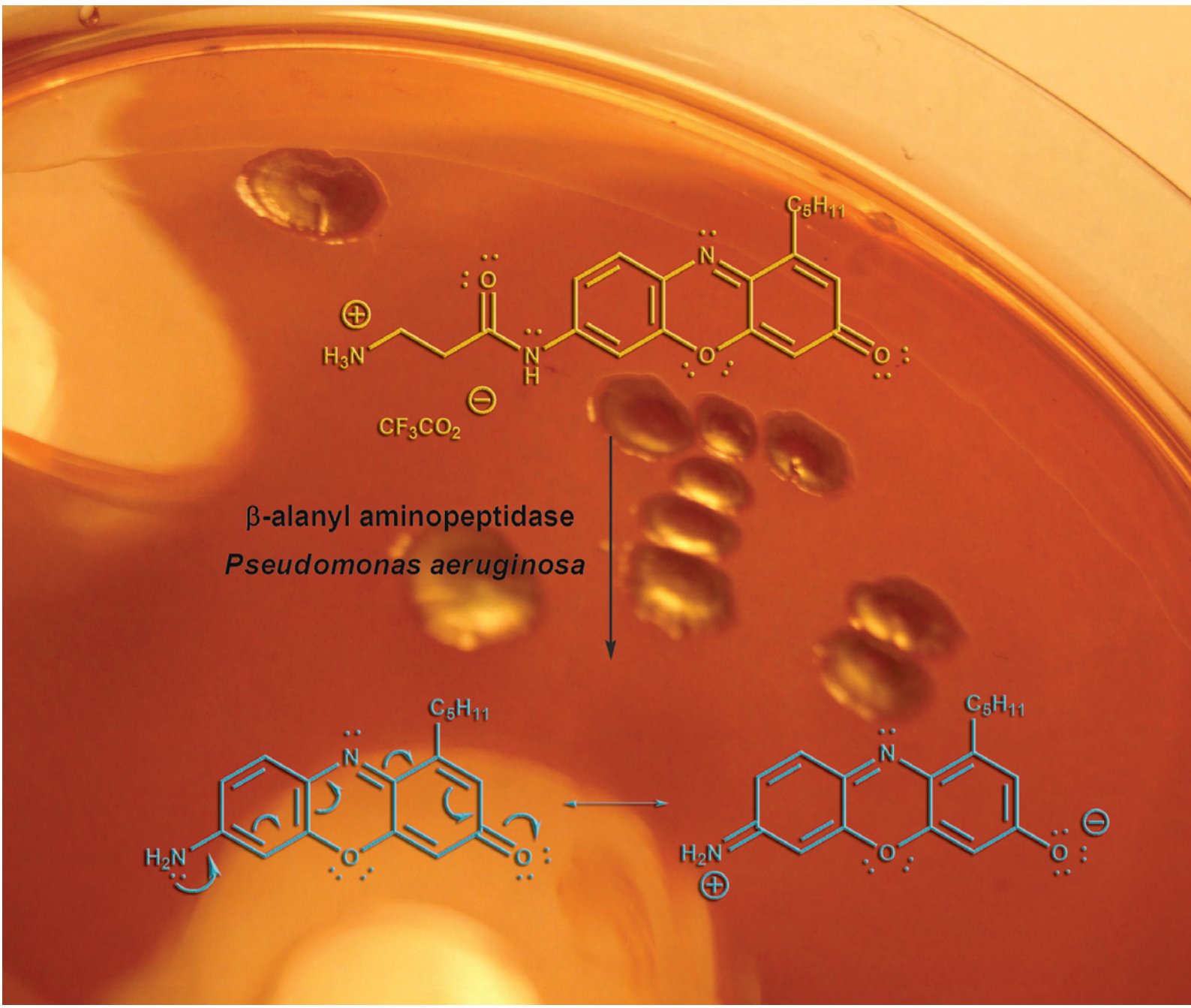

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Synthesis and testing of chromogenic phenoxazinone substrates for β-alanyl aminopeptidase†

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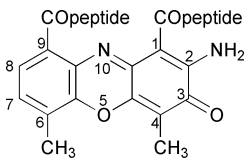
Novel 7-*N*-(β-alanyl)aminophenoxazin-3-one salts **27a–d** have been synthesized and tested as chromogenic substrates for β-alanyl aminopeptidase, which is present in *Pseudomonas aeruginosa*, the most common respiratory pathogen in patients with cystic fibrosis. The biological results show that 7-*N*-(β-alanyl)amino-1-pentylphenoxazin-3-one trifluoroacetate salt **27a** is a chromogenic substrate for this bacterium, with a low degree of diffusion in nutrient media for growing bacterial cultures and a bright red colour, making it easily distinguishable from the agar background.

Techniques for the detection and differentiation of different species of bacteria often utilize the action of bacterial enzymes on chromogenic or fluorogenic substrates[1]—liberating coloured or fluorescent products, respectively. Such substrates are of particular interest as they allow the rapid and accurate detection of bacteria, since these tests may be performed on the primary isolation media, thus avoiding time-consuming isolation procedures prior to the bacterial identification step.

We wish to report here the preparation and testing of chromogenic phenoxazinone substrates for β-alanyl aminopeptidase. β-Alanyl aminopeptidase has been detected in *Pseudomonas* sp.,[2] while alanyl aminopeptidase is a periplasmic aminopeptidase present in significant quantities in *Pseudomonas aeruginosa*,[3] the most common respiratory pathogen in patients with cystic fibrosis.[4] *P. aeruginosa* is also responsible for 28% of the cases of bacteremia in patients receiving organ transplants[5] and causes urinary tract infections and a variety of systemic infections in patients with severe burns,[6] and in cancer and AIDS patients who are immunosuppressed.[7,8] It has been shown that in such patients as few as 10–100 cells of *Pseudomonas aeruginosa* can lead to gut colonization by this Gram-negative aerobic bacterium,[9] which is naturally resistant to many antibiotics.[10,11] Although classical microbiological techniques for the detection and identification of *P. aeruginosa* are satisfactory in many situations, methods for the rapid detection of this bacterium are still under investigation. For example, *P. aeruginosa* identification using a disk of phenanthroline and 9-chloro-9-[4-(dimethylamino)]-9,10-dihydro-10-phenylacridine hydrochloride (PC disk) has been reported and compared to a monoclonal antibody detection method.[12]

Phenoxazinones are the chromophores in a number of synthetic dyestuffs and natural coloured matters and the phenoxazinone chromophore has been found in a number of mould metabolites and other natural products. The actinomycins[13] are a group of antibiotics produced by *Streptomyces* species, which are very toxic but some of which show chemotherapeutic activity in certain neoplastic diseases. The actinomycins contain a 2-amino-4,6-dimethylphenoxazin-3(3*H*)one-1,9-dicarboxylic acid nucleus **1** bound *via* peptide links at the carboxyl groups to pentapeptide side chains. Compounds with different amino acid sequences in the peptide chains have been isolated, *e.g.* actinomycin A, and synthesised, as have their analogues.[13]



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Results and discussion

7-Amino-1-pentylphenoxazin-3-one (1-pentylresorufamine [PRF]) **6a** can be prepared by nitrosation of 3-acetamidophenol **2**, condensation of the resulting 2-nitroso-5-acetamidophenol **3** with olivetol **4** and acidic hydrolysis of amide **5** using 85% H_2SO_4, Scheme 1 (steps a–c),[14] or by the reaction of 1,4-dichlorobenzoquinonediimine **8**, generated *in situ* by the oxidative chlorination of *p*-phenylenediamine **7**,[15] with olivetol **4**,[14] Scheme 1 (step d). The overall yield and purity of the resorufamines **6** prepared *via* these routes is poor, so that these methods are not suitable for multi-gram syntheses or the preparation of a range of derivatives, and an alternative route to these phenoxazinones is thus required. In particular, the route from the diimine leads to the production of chlorinated derivatives, *e.g.* the 2-chloro-1-pentyl derivative **6b**, which are difficult to separate from the desired 1-pentylresorufamine **6a**.

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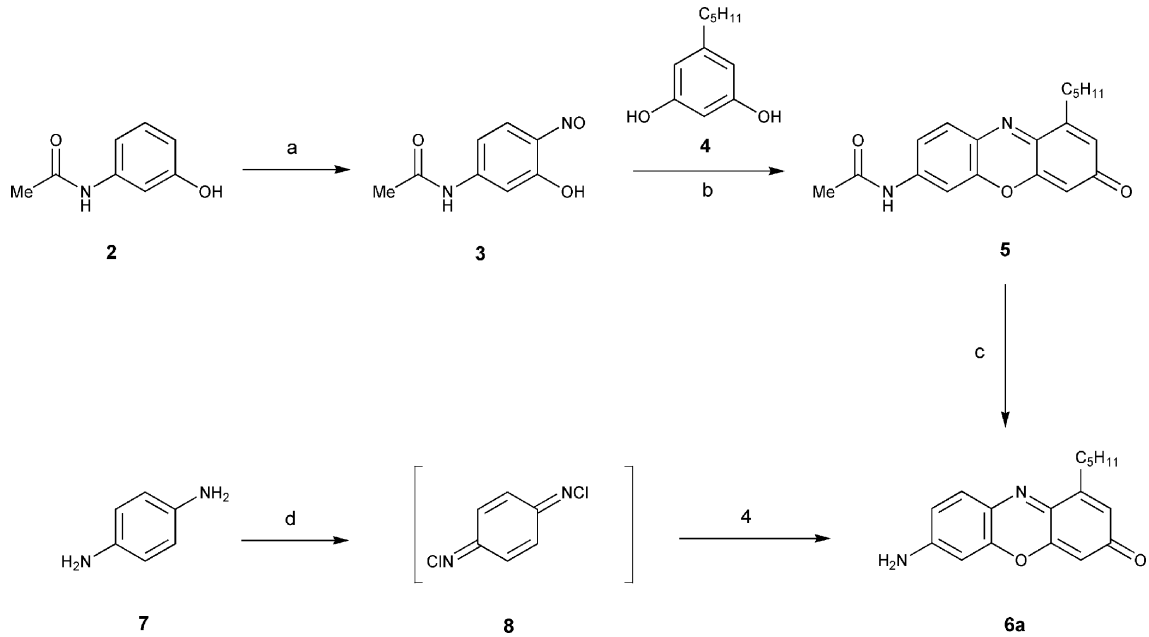
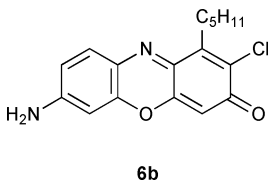
† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b716978g

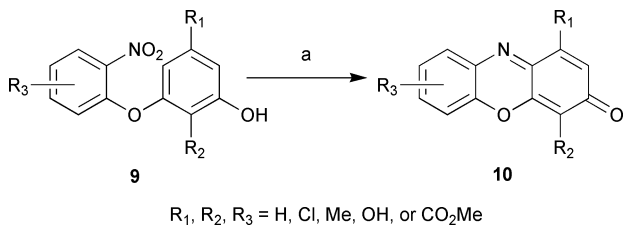


Scheme 1 *Reagents and conditions*: [a] 'HNO_2'; [b] ^{n}BuOH, conc. H_2SO_4; [c] 85% H_2SO_4, ethanol; [d] MeOH, $CO(NH_2)_2$, Cl_2, then **4**, reflux.



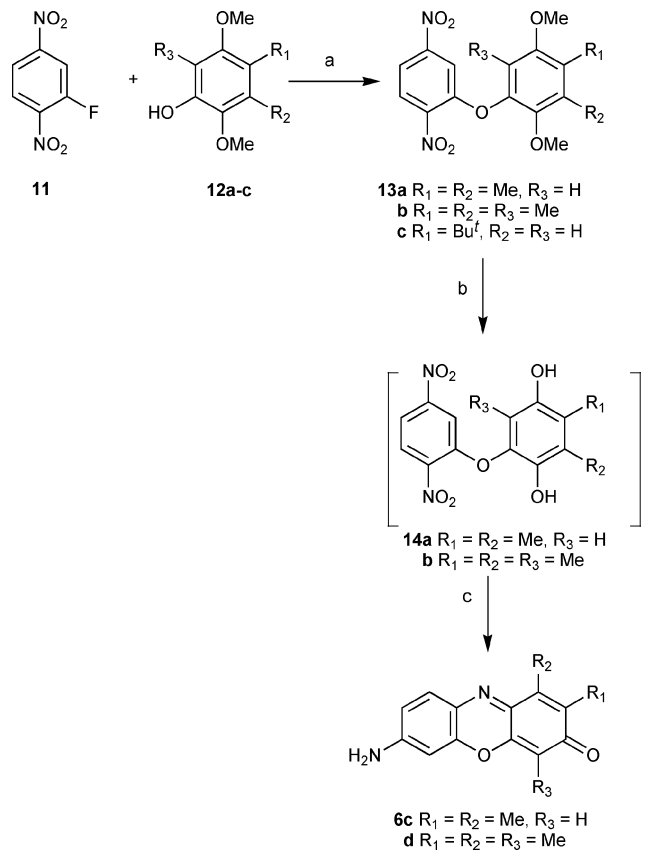
6b

Similar heterocyclic systems **10** have previously been synthesised by Bird and Latif[16] *via* the reductive cyclisation of 3-hydroxy-2′-nitrodiphenyl ethers **9**, Scheme 2, but the preparation of resorufamines by this method was unsuccessful. An alternative route was therefore investigated, involving the reduction of a dinitrodihydroxydiaryl ether **14**, which can be prepared *via* the coupling of 2,5-dinitrofluorobenzene **11** with suitably substituted phenols **12**, Scheme 3, prepared *via* a sequence of reactions involving; methylation of a hydroquinone, formylation and Baeyer–Villiger oxidation.



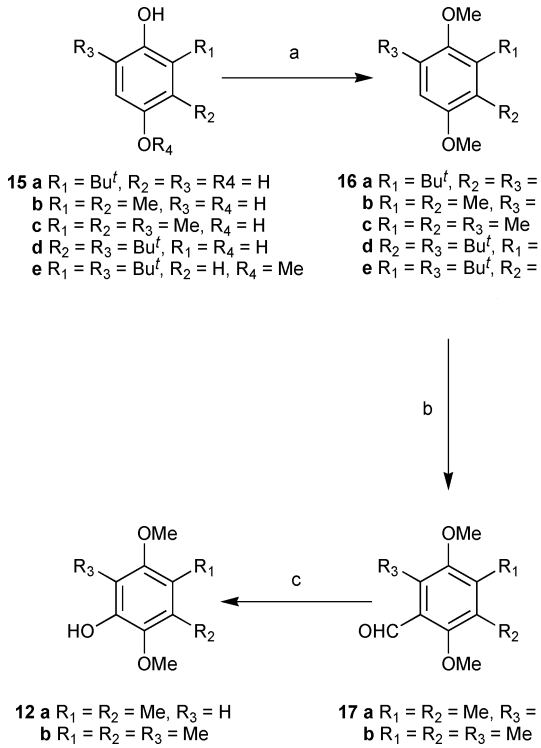
Scheme 2 *Reagents and conditions:* [a] Zn, NH_4Cl, H_2O, DME, 40 °C.

Alkylation of hydroquinones **15a–d** and monomethyl ether **15e** with methyl iodide was carried out using an adaptation of the method of Michman *et al.*[17] and the yields of the corresponding dimethyl ethers **16a–e** were excellent (82–99%), Scheme 4. The introduction of the hydroxyl group onto the aromatic ring, to form the phenols **12**, was then accomplished *via* the Baeyer–Villiger oxidation of the corresponding benzaldehydes **17**, which were prepared using the Duff reaction,[18] with



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.

hexamine (hexamethylenetetramine) as the formylating agent, in refluxing trifluoroacetic acid. No product was isolated from the attempted formylation of the *tert*-butyl substituted hydroquinone ethers **16a**,**d**,**e**, Scheme 3, presumably due to the cumulative

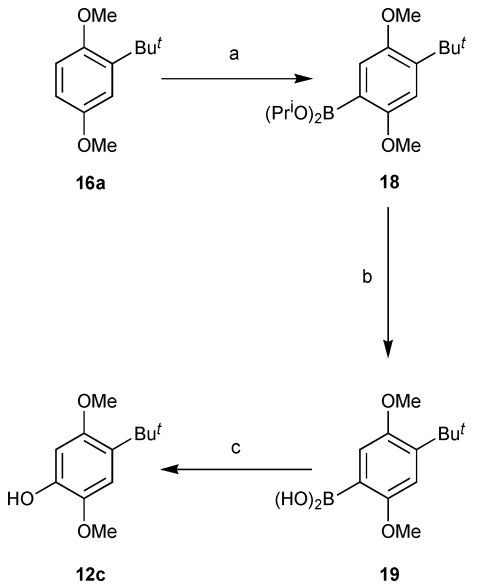


Scheme 4 *Reagents and conditions*: [a] NaH, MeI, DMF, 40 °C, 1 h; [b] hexamine, TFA, reflux; [c] i, MMPP, MeOH; ii, NaOH then HCl.

steric hindrance of the bulky *tert*-butyl and methoxy groups. Baeyer–Villiger oxidation of aldehydes **17a**,**b** with magnesium monoperoxyphthalate (MMPP), followed by the hydrolysis of the formate esters, gave the corresponding phenols **12a**,**b**, Scheme 4.

A similar steric effect was observed during *ortho*-lithiation of ether **16a** with *n*-BuLi in THF at −78 °C. The anion initially formed reacts with triisopropyl borate to give the corresponding arylboronic ester **18**, hydrolysis of which, under acidic conditions, afforded boronic acid **19**, which was oxidised with 27% H_2O_2 to form phenol **12c** in a total yield of 41%, Scheme 5.

The next step in the preparation of the diaryl ethers is the nucleophilic aromatic substitution of the fluorine in 2,5-

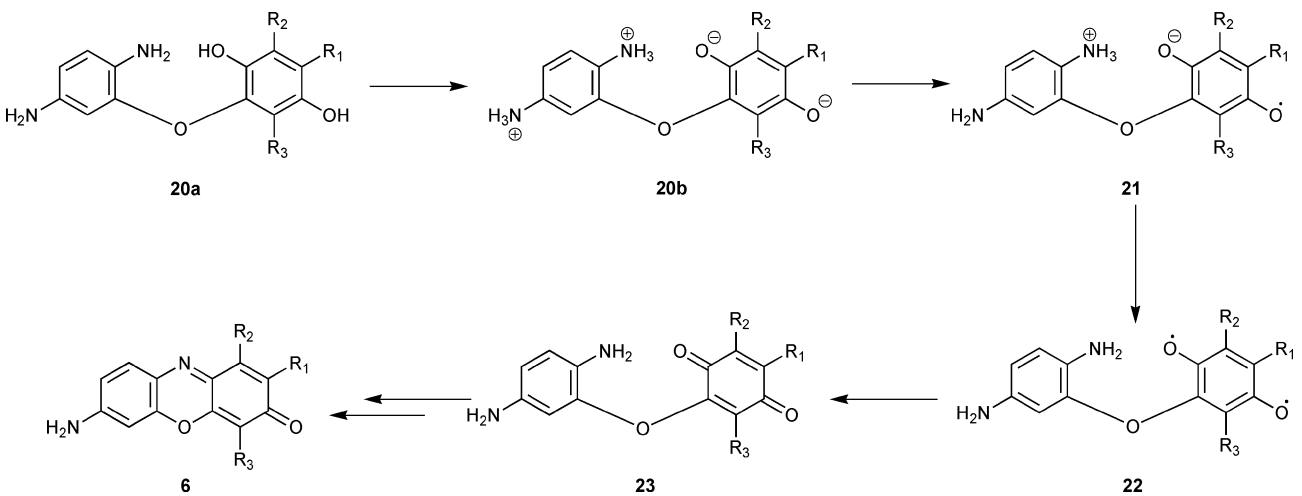


Scheme 5 *Reagents and conditions*: [a] ^{n}BuLi, THF, −78 °C then $B(OPr^i)_3$; [b] 10% NH_4Cl; [c] 27% H_2O_2, THF.

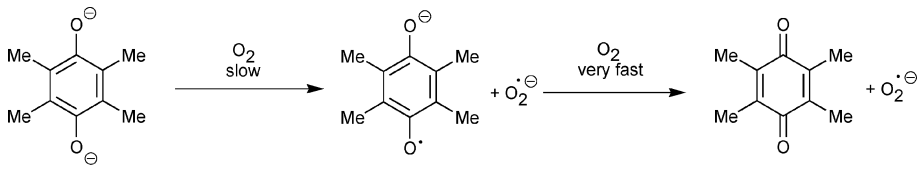
dinitrofluorobenzene **11** and this proceeded smoothly to give the ethers **13a–c** in good yields, as shown in Scheme 3. Cleavage of the methoxyaryl ethers **13** was carried out under standard conditions, with BBr_3 in DCM at −78 °C, and the resulting hydroquinones **14a**,**b** were subjected to further transformation without purification, Scheme 3.

The final stage in the preparation of aminophenoxazinones **6c** and **6d**, involved the reduction of the nitro groups in ethers **14a**,**b** with palladium-on-carbon under hydrogen (1 atm.), followed by aerial oxidation and subsequent cyclization on work-up/chromatography on silica.

The reduction of the nitro groups in the ethers **14** leads to the formation of the diamine **20a**, which can exist as a zwitterion **20b**. This zwitterion undergoes oxidation by molecular oxygen,[19] involving the autoxidation of the dianion, Scheme 6, in a process similar to the autoxidation of duroquinone dianion **24**, Scheme 7. By analogy, this process would be expected to be autocatalytic,



Scheme 6



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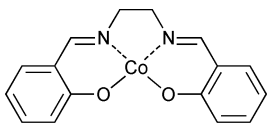
Scheme 7

to be accelerated by the quinone, and to proceed at a rate which is independent of oxygen pressure. The addition of an oxygen-transfer catalyst, salcomine **25**, did not influence the rate, thus confirming that this is an autocatalytic oxidation. According to this mechanism, the semiquinone **21** formed in the first step undergoes attack by a hydroxyperoxide radical, resulting in the formation of a biradical **22**. Such biradicals readily self-terminate through the formation of a quinone **23**. In a slightly acidic medium, in this case due to the presence of the silica, this quinone undergoes intramolecular cyclization to the resorufamine **6**, Scheme 6.



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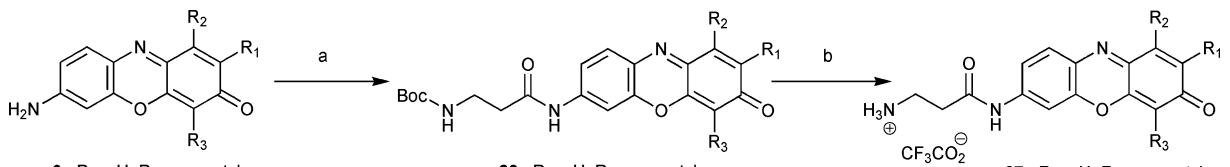
For the biological tests, the aminophenoxazinones **6a–d** were coupled to Boc-β-Ala-OH using *N*,*N*′-diisopropylcarbodiimide as the coupling reagent, affording the protected monopeptides **26**, Scheme 8. The Boc group was then removed under standard conditions (TFA in DCM) to give the corresponding trifluoroacetate salts **27**. It should be noted that the nucleophilicity of the amino group in phenoxazinones **6** is very low and coupling by this method is impossible without reduction prior to the coupling, Scheme 8. In a similar manner, 7-*N*-(L-Ala)aminophenoxazinones **29a**,**b** were prepared by the coupling of the aminophenaxozinones **6a–d** with Boc-L-Ala-OH to give the carbamates **28a**,**b**, Scheme 9, followed by deprotection.

Compounds **27a–d** are weakly coloured as the amino lone-pair is no longer available for conjugation through the system to the carbonyl group at the 3-position. These compounds were tested for their activity to act as a substrate for the enzyme, β-alanyl aminopeptidase, which is present in significant amounts in *Pseudomonas aeruginosa.* β-Alanyl aminopeptidase cleaves these chromatogenic substances to release the coloured 7-aminophenoxazinones **6** and one of the advantages of this test is the simple visual interpretation of the results, which can be carried out without the use of any specialized instrumentation.

7-Amino-1-pentyl-3*H*-phenoxazin-3-one (1-pentylresorufamine [PRF]) **6a**, which is produced by the β-alanyl aminopeptidase hydrolysis of the 7-*N*-(β-alanyl)amino derivative **27a** (β-Ala-PRF), was shown to be a very good chromophore, having a low degree of diffusion in nutrient media for growing bacterial cultures and a bright red colour, making it easily distinguishable from a pale yellow agar background. Fig. 1 shows the results obtained after 24 hours incubation using 7-*N*-(β-alanyl)amino-1-pentylphenoxazin-3-one (β-Ala-PRF) **27a**, 7-*N*-(β-alanyl)amino-1,2-dimethyl-(β-Ala-diMRF) **27c** and 7-*N*-(β-alanyl)amino-1,2,4-trimethyl-3*H*-phenoxazin-3-one (β-Ala-triMRF) **27d** trifluoroacetate salts at a concentration of 50 mg l^{-1} in the presence of *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Escherichia coli.* The selectivity of these substrates for *P. aeruginosa* is clearly shown by the absence of the red colour in the case of *Escherichia coli*, Fig. 1, and *Pseudomonas fluorescens*, Fig. 2, which do not have β-alanyl aminopeptidase and so cannot release the chromogen. *Burkholderia cepacia* strains may exhibit β-alanyl aminopeptidase

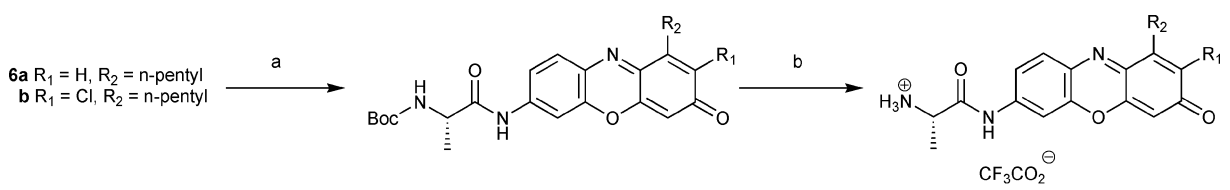


6a R_1 = H, R_2 = n-pentyl
b R_1 = Cl, R_2 = n-pentyl
c R_1 = R_2 = Me, R_3 = H
d R_1 = R_2 = R_3 = Me

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*-ᵗBoc-β-alanine, HOBt, DIC, DCM; [b] TFA.



6a R_1 = H, R_2 = n-pentyl
b R_1 = Cl, R_2 = n-pentyl

28a R_1 = H, R_2 = n-pentyl
b R_1 = Cl, R_2 = n-pentyl

29a R_1 = H, R_2 = n-pentyl
b R_1 = Cl, R_2 = n-pentyl

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

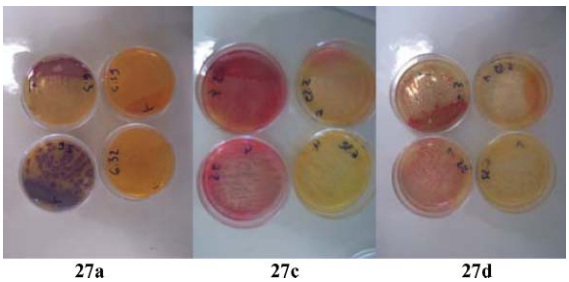


Fig. 1 Incubation of 7-*N*-(β-alanyl)amino-1-pentylphenoxazin-3-one (β-Ala-PRF) **27a**, 7-*N*-(β-alanyl)amino-1,2-dimethyl-(β-Ala-diMRF) **27c** and 7-*N*-(β-alanyl)amino-1,2,4-trimethyl-3*H*-phenoxazin-3-one (β-Ala-triMRF) **27d** trifluoroacetate salts, at a concentration of 50 mg l^{-1}, for 24 hours, with *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Escherichia coli.* In each photograph, the *Pseudomonas aeruginosa* plates are top left and bottom left, *Burkholderia cepacia* top right, and *Escherichia coli* bottom right.

activity (see data in Table 1) and the presence of a weak red colouration indicates some enzymatic activity for this strain. In contrast, the L-Ala derivative **29a** is hydrolyzed by all the Gram-negative bacteria tested, Table 2, including *Escherichia coli* (NCTC 10418), thus suggesting that β-alanyl aminopeptidase is responsible for the specific hydrolysis of the β-Ala analogue **27a**.

Another requirement of a test substrate is that it should localise to the bacterial cells, aiding the visualisation of colonies. Fig. 1 also highlights the importance of lipophilic substituents on the

Table 1 Assessment of β-Ala-pentylresorufamines **27a**,**b** (50 mg l^{-1}) as substrates for non-fermenting bacteria (% of strains with positive response due to β-alanyl aminopeptidase activity)

Species	No. of strains	**27a**	**27b**
Burkholderia cepacia	34	47	26
Burkholderia gladioli	6	0	83
Other *Burkholderia* sp.	9	33	44
Pseudomonas aeruginosa	74	99	73
Other *Pseudomonas* sp.	17	41	24
Pandorea sp.	8	25	25
Ralstonia picketti	4	50	50
Other *Ralstonia* sp.	16	19	13
Sphingobacterium spiritivorum	1	0	0
Stenotrophomonas maltophilia	2	0	0
Acinetobacter sp.	7	0	14
Brevindumonas sp.	2	0	0
Chryseobacterium meningosepticum	1	0	100
Moraxella sp.	3	67	33
Oligella sp.	1	100	0

Table 2 Detection of L-alanyl aminopeptidase activity *via* the hydrolysis of L-Ala-pentylresorufamine **29a** (50 mg l^{-1}). Positive results are indicated by a purple colour, and + and − signify the growth or inhibition of growth of the bacteria, respectively

Species	Growth	Colour
Escherichia coli NCTC 10418	+	Purple
Klebsiella pneumoniae NCTC 10896	+	Purple
Salmonella hadar	+	Purple
Proteus mirabilis NCTC 10975	+	Purple
Providencia rettgeri NCTC 7475	+	Purple
Pseudomonas aeruginosa NCTC 10662	+	Purple
Staphylococcus aureus NCTC 6571	−	No colour
Listeria monocytogenes NCTC 11994	−	No colour
Streptococcus pyogenes NCTC 8306	−	No colour

chromogen for good localisation. The dimethyl substitution in **6c** (generated by the hydrolysis of **27c**) is not sufficient to localise this chromogen and the colour is seen to be diffused across the medium. The trimethyl substituted 7-aminophenoxazin-3-one **6d** (from **27d**) is visibly localised, although not as efficiently as the pentyl-substituted derivative (β-Ala-PRF) **6a**. The increased lipophilicity of the substituted chromogens aids in the retention of the colour in the cell wall of the bacterium and also results in an increase in the hydrophobic properties, which prevents the spread of chromogen into the medium. The same is true with an increase in length of the alkyl chain (as in the case of β-Ala-PRF **6a**), Fig. 1.

The data in Table 1 shows that β-Ala-PRF **27a** can be used to distinguish between *P. aeruginosa* and other non-fermenting bacteria, since β-alanyl aminopeptidase activity is present in all *P. aeruginosa* strains but not in *Burkholderia gladioli*, *Acinetobacter* sp., *Stenotrophomonas maltophilia*, *Brevindumonas* sp. or *Chryseobacterium meningosepticum.* The detection of 99% of strains using the pentyl derivative **27a** as a test substrate for the detection of *P. aeruginosa* is vitally important as a recent study of 1225 isolates from cystic fibrosis patients from 31 centres in the U.K. showed that, although cross-infection by transmissable strains does occur, at least 72% of patients harboured strains with unique genotypes.[20]

It has therefore been established that compounds **27a**,**c**,**d** are sensitive, effective and specific for the detection of *Pseudomonas aeruginosa*, but in the case of **27c**, spreading of the colour of the chromogen **6c** from the colonies into the medium prevents it from being used as a diagnostic tool on agar plates (it could, however, be used in solution-based tests). In addition, 7-*N*-(β-alanyl)amino-1-pentylphenoxazin-3-one (β-Ala-PRF) **27a**, like the L-Ala derivative **29a**, inhibited the growth of Gram-positive bacteria, *e.g. Staphylococcus aureus*, *Enterococcus faecalis*,

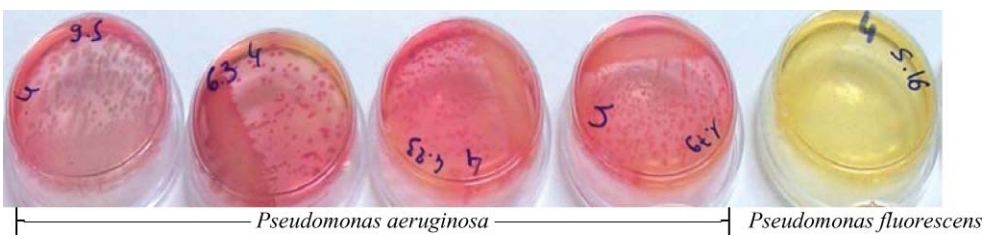


Fig. 2 Incubation of 7-*N*-(β-alanyl)amino-1,2-dimethylpentylphenoxazin-3-one (β-Ala-diMRF) **27c** trifluoroacetate salt, at a concentration of 50 mg l^{-1}, for 18 hours, with *Pseudomonas aeruginosa* and *Pseudomonas fluorescens.* From left, dishes 1–4 *Pseudomonas aeruginosa* and dish 5 *Pseudomonas fluorescens*.

Table 3 Impact of 7-*N*-(β-alanyl)amino-1-pentylphenoxazin-3-one **27a** (50 mg l^{-1}) in Columbia agar medium on growth and colour of colonies of different strains of bacteria after 24 hours of incubation. Positive results are indicated by a purple colour, and + and − signify the growth or inhibition of growth of the bacteria, respectively

Species	Growth	Colour
Escherichia coli	+	No colour
Klebsiella pneumoniae	+	No colour
Proteus mirabilis	+	No colour
Pseudomonas aeruginosa	+	Red
Pseudomonas fluorescens	+	No colour
Burkholderia cepacia	+	Red
Staphylococcus aureus	−	No colour
Streptococcus pyogenes	−	No colour
Enterococcus faecalis	−	No colour
Listeria monocytogenes	−	No colour
Candida albicans	±	No colour

Listeria monocytogenes, and *Streptococcus pyogenes*, Table 3, when incorporated into Columbia agar at 50 mg l^{-1}, and this suppression of the growth of Gram-positive bacteria in the presence of Gram-negative bacteria is a useful additional property when developing a selective means for the detection of *Pseudomonas aeruginosa.*

These β-Ala-resorufamines **27** represent some of the first chromogenic substrates which can be used directly in culture media to differentiate bacterial colonies based upon the detection of β-alanyl aminopeptidase activity.

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). Experimental and spectroscopic data are given for one example of each synthetic method; data for the other compounds included in this work are reported in the ESI accompanying this paper.†

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 **15** (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 **15a** (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 **15b** (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.).

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.[22] 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.[23] mp 80 °C) (found: MH^+, 209.1176. Calc. for $C_{12}H_{17}O_3$: 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 (OCH_3), 61.4 (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 2,5-dimethoxy-3,4,6-trimethylbenzaldehyde **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 **12b** 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 (2.82 M 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 × 50 ml). 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.[24] 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 **11** (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 $C_{16}H_{17}N_2O_7$: MH, 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.).

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 a 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 °C; 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-dihydroxybenzene **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 °C; (found: MH$^+$, 255.1129. Calc. for $C_{15}H_{15}N_2O_2$: 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, O*H*), 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-dihydroxybenzene **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 °C; 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, N*H*$_2$), 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 °C; (found: MH$^+$, 488.1942. Calc. for $C_{25}H_{31}ClN_3O_5$ 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(*C*H$_3$)), 1.59 (2H, m, 2′-CH_2), 2.56 (2H, t, J = 6.8 Hz, NHCH$_2$CH$_2$CO), 3.00 (2H, m, 1′-CH_2), 3.25 (2H, q, J = 6.8 Hz, NHCH$_2$CH$_2$CO), 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 $C_{25}H_{32}N_3O_5$: 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′-C*H*$_3$), 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$_2$CH$_2$CO), 2.91 (2H, t, J = 8.0 Hz, 1′-CH_2), 3.43 (2H, t, J = 6.7 Hz, NHCH$_2$CH$_2$CO), 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).

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 (0.089 g, 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 1 M 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 $C_{22}H_{26}N_3O_5$: 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 $C_{20}H_{24}N_3O_3$: 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 $C_{20}H_{23}ClN_3O_3$: 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 $C_{17}H_{18}N_3O_3$: 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 trifluoroacetate salt **27d** was isolated as a red solid (0.080 g, 96%) mp 217–219 °C (decomp.); (found: M^+, 326.1506. Calc. for $C_{18}H_{20}N_3O_3$: 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, NH_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)amino-1-pentylphenoxazin-3-one trifluoroacetate salt 29a

Prepared from 7-*N*-(*N*-^{t}Boc-L-alanyl)amino-1-pentylphenoxazin-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. $C_{22}H_{24}F_3N_3O_5$ requires C, 56.5; H, 5.2; N, 9.0%) (found: M^+, 354.1809. Calc. for $C_{20}H_{24}N_3O_3$: M, 354.1812); $[\alpha]_D^{20}$ +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′-CH_3), 16.4 (ala-CH_3), 22.35 (4′-CH_2), 29.1 (2′-CH_2), 29.7 (1′-CH_2), 31.7 (3′-CH_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-pentylphenoxazin-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 $C_{20}H_{23}ClN_3O_3$: M, 388.1422), $[\alpha]_D^{20}$ +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′-CH_3), 1.31 (4H, m, 3′-CH_2, 4′-CH_2), 1.47 (2H, m, 2′-CH_2), 1.56 (3H, d, $J = 6.8$ Hz, ala-CH_3), 2.98 (2H, m, 1′-CH_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′-CH_3), 16.4 (ala-CH_3), 22.4 (4′-CH_2), 28.0 (2′-CH_2), 28.2 (1′-CH_2), 32.0 (3′-CH_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^{-1}. The substrate solutions were incorporated into Columbia agar solution (200 ml) and added to sterile plates to give final concentrations of 50 mg l^{-1}. 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, USA, 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 per 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.

References

1 A. Carricajo, S. Boiste, J. Thore, G. Aubert, Y. Gille and A. M. Freydiere, *Eur. J. Clin. Microbiol. Infect. Dis.*, 1999, **18**, 796; F. Fenollar and D. Raoult, *Eur. J. Clin. Microbiol. Infect. Dis.*, 2000, **19**, 33.
2 H. Komeda and Y. Asano, *FEBS J.*, 2005, **272**, 3075.
3 B. Fricke and H. Aurich, *J. Basic Microbiol.*, 1993, **33**, 291.
4 M. Laurans, A. Arion, M. Fines-Guyon, A. Regeasse, J. Brouard, R. Leclercq and J. H. Duhamel, *Arch. Pediatr.*, 2006, **13**, S22.
5 R. G. Brooks and J. S. Remington, Transplant-related infections, in *Hospital infections*, ed. J. V. Bennett and P. S. Brachman, Little, Brown and Co., Boston, 2nd edn, 1986, pp. 581–618.
6 J. C. Valdez, M. C. Peral, M. Rachid, M. Santana and G. Perdigon, *Clin. Microbiol. Infect.*, 2005, **11**, 472.
7 I. Gustafsson and J. L. Martinez, *Rev. Med. Microbiol.*, 2005, **16**, 155.
8 V. Nagappan and P. Kazanjian, *HIV Clinical Trials*, 2005, **6**, 213.
9 D. von der Waaij, *J. Antimicrob. Chemother.*, 1982, **10**, 263.
10 H. Nikaido, *Science*, 1994, **264**, 382.
11 P. Richard, *J. Infect. Dis.*, 1994, **170**, 377.
12 S. Fujita, A. Tonohata, T. Matsuoka, N. Okado and T. Hashimoto, *J. Clin. Microbiol.*, 1992, **30**, 2728.
13 H. Hollstein, *Chem. Rev.*, 1974, **74**, 625; H. Brockmann, *Angew. Chem.*, 1960, **72**, 939.
14 R. J. Anderson, P. W. Groundwater, A. James, D. Monget and A. V. Zaytsev, PCT Patent WO 2006/030119 A1.
15 R. Willstätter and E. Mayer, *Ber. Dtsch. Chem. Ges.*, 1904, 1498.
16 C. W. Bird and M. Latif, *Tetrahedron*, 1980, **36**, 1813; C. W. Bird and M. Latif, *Tetrahedron*, 1980, **36**, 529.
17 M. Michman, M. Oron and H. J. Schaefer, *Collect. Czech. Chem. Commun.*, 2000, **65**, 924.
18 L. N. Ferguson, *Chem. Rev.*, 1946, **38**, 230.
19 T. H. James and A. Weissberger, *J. Am. Chem. Soc.*, 1938, **60**, 98.
20 F. W. Scott and T. L. Pitt, *J. Med. Microbiol.*, 2004, **53**, 609.
21 B. M. Trost, H. C. Shen, L. Dong, J.-P. Surivet and C. Sylvain, *J. Am. Chem. Soc.*, 2004, **126**, 11966.
22 T. Smith, *J. Am. Chem. Soc.*, 1944, **66**, 1523.
23 L. Giraud and A. Giraud, *Synthesis*, 1998, 1153.
24 C. J. K. Adderley and F. R. Hewgill, *J. Chem. Soc. C*, 1968, 1438.