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# 2-Arylbenzothiazole, benzoxazole and benzimidazole derivatives as fluorogenic substrates for the detection of nitroreductase and aminopeptidase activity in clinically important bacteria

Marie Cellier [a], Olivier J. Fabrega [b], Elizabeth Fazackerley [b], Arthur L. James [b], Sylvain Orenga [a], John D. Perry [c], Vindhya L. Salwatura [b], Stephen P. Stanforth [b,*]

[a] Research & Development Microbiology, bioMérieux SA, 3 route de Port Michaud, 38 390 La-Balme-les-Grottes, France
[b] School of Life Sciences, Northumbria University, Newcastle upon Tyne NE1 8ST, UK
[c] Department of Microbiology, Freeman Hospital, Newcastle upon Tyne NE7 7DN, UK

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ABSTRACT

A series of 2-(2-nitrophenyl)benzothiazole **7**, 2-(2-nitrophenyl)benzoxazole **10** and 2-(2-nitrophenyl)benzimidazole **13** derivatives have been synthesised and assessed as indicators of nitroreductase activity across a range of clinically important Gram negative and Gram positive bacteria. The majority of Gram negative bacteria produced strongly fluorescent colonies with substrates **7** and **10** whereas fluorescence production in Gram positive bacteria was less widespread. The L-alanine **16** and **19** and β-alanine **21** and **23** derivatives have been prepared from 2-(2-aminophenyl)benzothiazole **14** and 2-(2-aminophenyl)benzoxazole **17**. These four compounds have been evaluated as indicators of aminopeptidase activity. The growth of Gram positive bacteria was generally inhibited by these substrates but fluorescent colonies were produced with the majority of Gram negative bacteria tested.



## 1. Introduction

Enzymatic substrates have emerged as important and powerful tools for the detection and identification of bacteria[1,2] and in this paper we describe the synthesis of a number of new fluorogenic nitroreductase and aminopeptidase substrates for this purpose. These substrates have subsequently been evaluated for their ability to detect a range of Gram negative and Gram positive bacteria of clinical importance.

Nitroreductase activity in bacteria can often be detected by observing the change in fluorescence when the nitro-group of a weakly fluorescent substrate **1** is reduced by a nitroreductase enzyme giving a strongly fluorescent amine (or hydroxylamine product) **2** (Scheme 1). Many bacteria can utilise aromatic nitrocompounds as substrates and hence the detection of nitroreductase activity by observing a substantial change in fluorescence intensity can offer a useful method for analysing biological and environmental samples for the presence of bacteria.[3] A series of several 7-nitrocoumarin derivatives has been prepared by one of us (ALJ) and assessed against 30 strains encompassing 24 distinct microbial genera, including a wide range of clinically important species.[4,5] Other coumarin-derived nitroreductase substrates have also been prepared by us.[6]

Aminopeptidase activity in bacteria has frequently been detected by observing the change in fluorescence when the amide bond in a weakly fluorescent substrate **3** is hydrolysed by an aminopeptidase producing a highly fluorescent amine product **2** (Scheme 1). The substrate **3** is designed so that the amide component is derived from an amino acid, for example, the L-alanine derivative **3** (R = Me). L-Alanine aminopeptidase activity has previously been used to differentiate between Gram negative and Gram positive bacteria because Gram negative organisms demonstrate the presence of this enzyme but in contrast, Gram positive bacteria do not. β-Alanyl aminopeptidase has been detected in *Pseudomonas* sp. and new chromogenic substrates have recently been described for identifying *Pseudomonas aeruginosa*, a common respiratory pathogen in cystic fibrosis patients.[7] Colormetric methods for detecting aminopeptidase activity have also been reported recently.[7,8]

Fluorogenic enzyme substrates derived from *O*-substituted 2-(2-hydroxyphenyl)benzothiazoles, 2-(2-hydroxyphenyl)benzoxazoles and 2-(2-hydroxyphenyl)benzimidazoles have been prepared for the detection of phosphatase and other enzymatic

* Corresponding author. Tel.: +44 191 227 4784; fax: + 44 191 227 3519.
*E-mail address:* steven.stanforth@northumbria.ac.uk (S.P. Stanforth).

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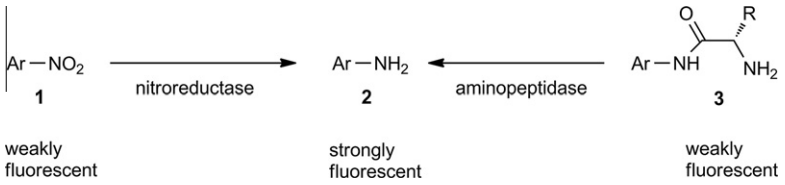



**Scheme 1.** Generation of fluorescence from nitroreductase and aminopeptidase activity.

activities (Fig. 1).[9,10] The core 2-(2-hydroxyphenyl)heterocycles are highly fluorescent in comparison to their *O*-substituted derivatives which has been attributed to excited-state intramolecular proton transfer (ESIPT). However, there was no indication of whether substrates based upon the corresponding 2-(2-aminophenyl)heterocyclic core, that is, structure **2** in Scheme 1 could also furnish useful fluorogenic enzyme substrates.

## 2. Synthesis of nitroreductase substrates

A selection of 2-(2-nitrophenyl)benzothiazole **7**, 2-(2-nitrophenyl)benzoxazole **10** and 2-(2-nitrophenyl)benzimidazole **13** derivatives have been prepared as potential nitroreductase substrates as part of this work (Scheme 2). The benzothiazole derivatives were prepared by reacting 2-aminothiophenol **4** with appropriately substituted 2-nitrobenzaldehydes **5**, yielding in most cases, the isolatable dihydrobenzothiazoles **6** which were subsequently dehydrogenated by treatment with *p*-chloroanil producing the required products **7**. Reaction of 2-aminophenol **8** with 2-nitrobenzaldehydes **5** afforded Schiff bases **9** which underwent cyclisation and dehydrogenation giving the required substrates **10** when treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). *N*-Methyl-1,2-diphenylamine **11** and aldehydes **5** in the presence of potassium peroxymonosulfate ('Oxone®') afforded the benzimidazoles **13** directly without isolation of the intermediate dihydrobenzimidazoles **12**.

## 3. Evaluation of nitroreductase substrates

The nitroreductase substrates **7**, **10** and **13** were evaluated against the clinically important bacteria listed in Table 1.[11] The benzothiazole substrates **7a–d** generally gave strongly blue

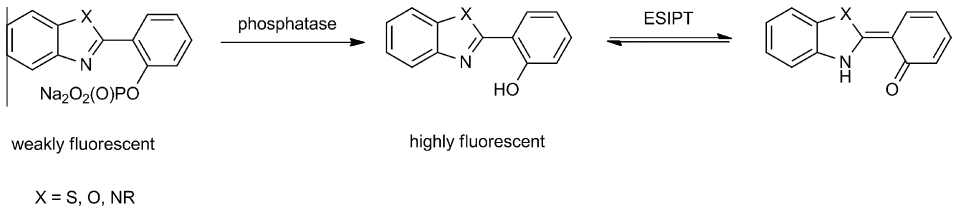
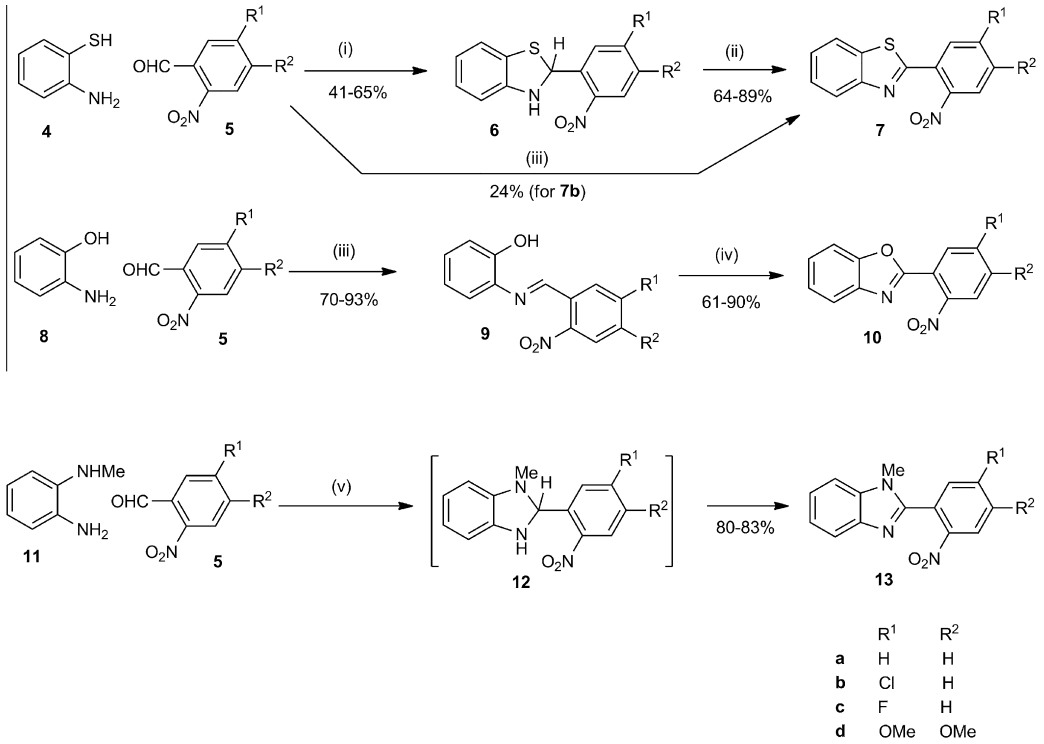



**Figure 1.** Fluorogenic substrates based upon the 2-(2-hydroxyphenyl)heterocyclic core system.




**Scheme 2.** Synthesis of nitroreductase substrates **7**, **10** and **13**. Reagents and conditions: (i) EtOH, rt or reflux (**6a**, **6c** and **6d**); (ii), 2,3,5,6-tetrachloro-1,4-benzoquinone (*p*-chloranil), $CH_2Cl_2$, reflux (**7a**, **7c** and **7d**); (iii), EtOH, reflux; (iv), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), $CH_2Cl_2$, rt; (v), 'Oxone®', DMF, rt.

**Table 1**
Fluorescent colonies produced by various organisms in the presence of the substrates **7**, **10** and **13**

| Substrate | Fluorescence[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **7a** | **7b** | **7c** | **7d** | **10a** | **10b** | **10c** | **10d** | **13a** | **13b** |
| *Gram negative organisms*[b] | | | | | | | | | | |
| *Escherichia coli* NCTC 10418 | ++ Blue | ++ Blue | ++ Blue | ++ Blue | ++ Purple | ++ Blue | ++ Blue[d] | + Blue | NF | ± Purple[d] |
| *Serratia marcescens* NCTC 10211 | ++ Blue | ++ Blue | ++ Blue | ++ Blue | ++ Purple | + Blue | ++ Blue[d] | + Blue | NF | ± Purple[d] |
| *Pseudomonas aeruginosa* NCTC 10662 | NF | ± Blue | NF | ++ Blue | ++ Purple | ± Blue | NF | NF | NF | ± Purple[d] |
| *Salmonella typhimurium* NCTC 74 | ++ Blue | ++ Blue | ++ Blue | ++ Blue | ++ Purple | + Blue | ++ Blue[d] | NF | NF | ± Purple[d] |
| *Morganella morganii* WS 462403 | ++ Blue[d] | + Blue[d] | ++ Blue | ++ Blue[d] | ++ Purple | ++ Blue | ++ Blue[d] | ± Blue | ± Blue | ± Purple[d] |
| *Enterobacter cloacae* NCTC 11936 | ++ Blue | + Blue | ++ Blue | ++ Blue | ++ Purple | + Blue | ++ Blue[d] | + Blue | NF | + Purple |
| *Providencia rettgeri* NCTC 7475 | ++ Blue | + Blue | ++ Blue | ++ Blue | ++ Purple | ++ Blue | ++ Blue[d] | + Blue | NF | ± Purple |
| *Gram positive organisms*[b,c] | | | | | | | | | | |
| *Staphylococcus epidermidis* NCTC 11047 | Trace blue | NF | NF | Trace blue | + Purple | NF | NF | NF | NF | NF |
| *Staphylococcus aureus* NCTC 6571 | ++ Blue | ++ Blue | ++ Blue | ++ Blue | ++ Purple | + Blue | ++ Blue[d] | + Blue | NF | + Purple |
| *Staphylococcus aureus* (MRSA) NCTC 11939 | ++ Blue | ++ Blue | ++ Blue | ++ Blue | ++ Purple | + Blue | ++ Blue[d] | + Blue | NF | + Purple |

[a] ++ strong fluorescence, + moderate fluorescence, ± weak fluorescence, NF no significant fluorescence. Substrate concentration = 100 mg $L^{-1}$.
[b] All Gram negative bacteria exhibited good growth in the presence of the substrate. Gram positive organisms generally exhibited moderate growth.
[c] The following Gram positive organisms did not give fluorescent colonies with any of the substrates: *Enterococcus faecalis* NCTC 775, *Enterococcus faecium* NCTC 7171, *Streptococcus pyogenes* NCTC 8306, *Listeria monocytogenes* NCTC 11994.
[d] There was noticeable diffusion of the fluorophore into the medium.

fluorescent colonies with the majority of the seven Gram negative organisms shown in Table 1. With the exception of *P. aeruginosa*, most of these Gram negative organisms produced blue or purple fluorescent colonies with the benzoxazole substrates **10a–c** but in contrast substrate **10d** only moderately or weakly fluorescent colonies were produced with the seven Gram negative bacteria. Figure 2 illustrates the fluorescent colonies produced when substrate **10c** was incubated with *Serratia marcescens*. The benzimidazole derivatives **13a** and **13b** were poor substrates in comparison with their benzothiazole and benzoxazole analogues. No significant fluorescence was observed for substrate **13a** and most Gram negative bacteria and substrate **13b** gave only weakly fluorescent purple colonies with Gram negative bacteria. Some undesirable diffusion of the fluorescence into the surrounding media was noted particularly with substrates **10c** and **13b**.

In contrast to the Gram negative bacteria, the Gram positive bacteria *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus pyogenes* and *Listeria monocytogenes* did not produce fluorescent colonies with any of the substrates and *Staphylococcus epidermidis* gave weakly fluorescent colonies with the benzothiazole substrates **7a** and **7d**. *Staphylococcus aureus* NCTC 6571 and *S. aureus* NCTC 11939 (MRSA) however gave strongly fluorescent colonies with all of the benzothiazole substrates **7a–d**. Similarly, the benzoxazole substrates **10a** and **10c** gave strongly fluorescent colonies with *S. aureus* NCTC 6571 and *S. aureus* NCTC 11939 (MRSA) whereas the other benzoxazole substrates produced inferior results with these two bacteria. Substrate **10a** however was superior to substrate **10c** for *P. aeruginosa* and *S. epidermidis* detection. The benzimidazole derivatives **13a** and **13b** were generally poor substrates for the Gram positive bacteria.

In order to confirm that enzyme activity was responsible for the production of fluorescence *Escherichia coli* BL21 was grown and the cell culture was subsequently sonicated and centrifuged. The resulting cell-free extract was incubated with the substrate **10c** overnight producing a fluorescent solution (Fig. 3). Additionally, when *E. coli* was grown in a broth in the presence of substrate **7a**, the corresponding amine **14** was the only product detected by HPLC. The identity of the amine **14** was confirmed by comparison with an authentic sample, thus confirming that the anticipated reduction of the nitro-group had taken place with this organism.

## 4. Synthesis of aminopeptidase substrates

In view of the superior results obtained with the benzothiazole and benzoxazole nitroreductase substrates described above, L-alanine and β-alanine aminopeptidase substrates were prepared from

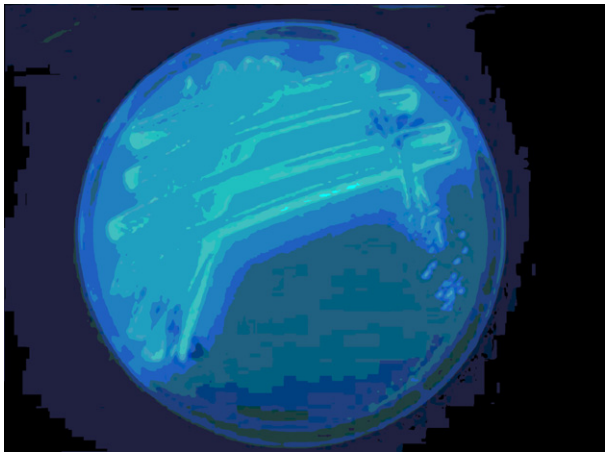


**Figure 2.** Fluorescent colonies produced from substrate **10c** and *Serratia marcescens* (NCTC 10211) viewed under UV light at 365 nm.

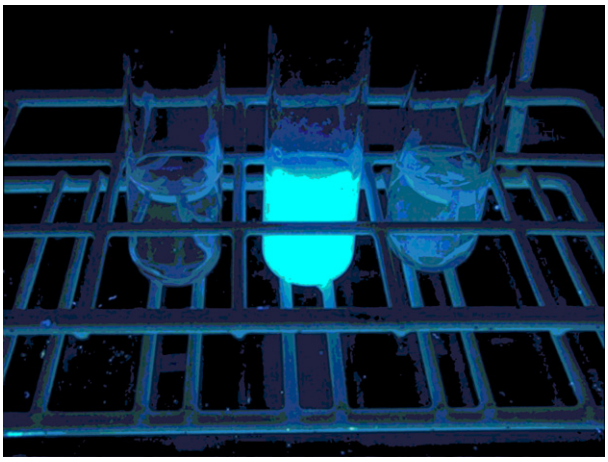
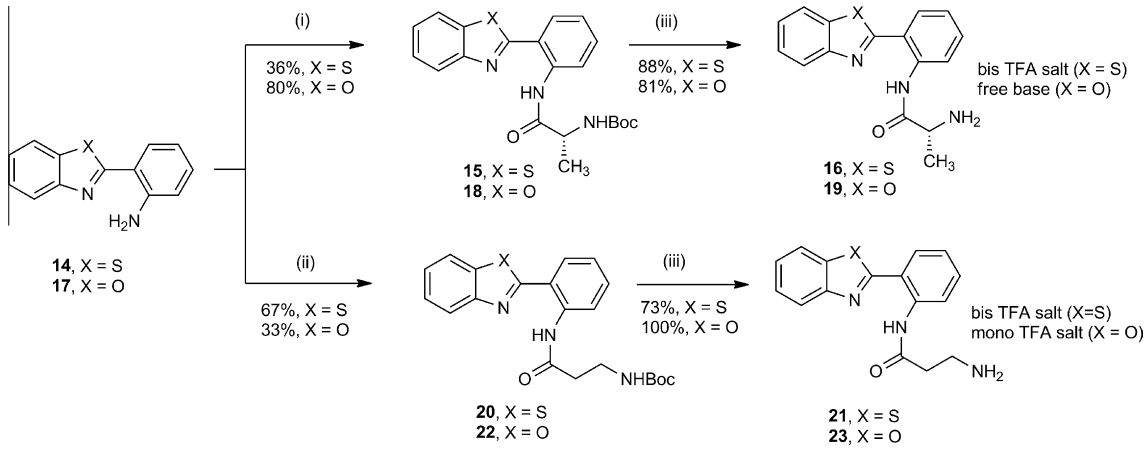


**Figure 3.** Incubation of substrate **10c** with a *Escherichia coli* BL21 cell-free extract in a Tris buffer solution (*p*H 7.4) viewed under UV light (365 nm). Left: buffer solution and substrate **10c**; Centre: buffer solution, substrate **10c** and cell-free extract; Right: buffer solution and cell-free extract.




**Scheme 3.** Synthesis of aminopeptidase substrates **16**, **19**, **21** and **23**. Reagents and conditions: (i) Boc-L-alanine, *N*-methylmorpholine (NMM), *iso*butylchloroformate (IBCF), −10 to −15 °C, then add **14** or **17**; (ii), Boc-β-alanine, NMM, IBCF, −10 to −15 °C, then add **14** or **17**; (iii), trifluoroacetic acid, rt.

**Table 2**
Fluorescent colonies produced by various bacteria in the presence of the substrates **16**, **19**, **21** and **23**

| Substrate | 16 | | 19 | | 21 | | 23 | |
|---|---|---|---|---|---|---|---|---|
| | Growth[a] | Fluorescence[b] | Growth[a] | Fluorescence[b] | Growth[a] | Fluorescence[b] | Growth[a] | Fluorescence[b] |
| *Escherichia coli* NCTC 10418 | ++ | + Blue | ++ | ++ Blue[c] | + | ++ Yellow | ++ | + Green |
| *Serratia marcescens* NCTC 10211 | ++ | + Blue | ++ | ++ Blue[c] | + | ++ Yellow | ++ | + Green |
| *Pseudomonas aeruginosa* NCTC 10662 | ++ | + Yellow | ++ | ++ Blue[c] | ++ | ++ Yellow | ++ | + Green |
| *Salmonella typhimurium* NCTC 74 | NG | — | ++ | ++ Blue[c] | NG | — | + | — |
| *Morganella morganii* WS 462403 | ++ | + Blue | ++ | ++ Blue[c] | ++ | ++ Yellow | ++ | + Green |
| *Enterobacter cloacae* NCTC 11936 | ++ | + Blue | ++ | ++ Blue[c] | NG | — | ++ | + Green |
| *Providencia rettgeri* NCTC 7475 | ++ | + Blue | ++ | ++ Blue[c] | NG | — | ± | ± Green |

[a] Growth in absence of substrate: ++ strong growth, + moderate growth, ± poor growth, NG no growth. Substrate concentration = 100 mg $L^{-1}$.
[b] ++ strong fluorescence, + moderate fluorescence, ± weak fluorescence, NF no significant fluorescence.
[c] There was noticeable diffusion of the fluorophore into the medium.

these two heterocyclic cores as shown in Scheme 3. Reduction of the nitro-derivative **7a** using tin(II) chloride gave the amine **14** which was condensed with Boc-L-alanine using the mixed anhydride method giving compound **15**. Removal of the Boc group by treatment with trifluoroacetic acid afforded the required substrate **16** as its TFA salt. Similarly, the nitro-derivative **10a** afforded the substrate **19** via the amine **17** and Boc-protected intermediate **18**. By replacing Boc-L-alanine with Boc-β-alanine, amines **14** and **17** gave the corresponding Boc-protected derivatives **20** and **22** from which the β-alanine aminopeptidase substrates **21** and **23** were obtained.

## 5. Evaluation of aminopeptidase substrates

The four substrates **16**, **19**, **21** and **23** were evaluated for aminopeptidase activity[12] against the seven Gram negative and seven Gram positive bacteria listed in Table 1. The results for the Gram negative bacteria are only shown in Table 2 because all of these substrates were generally inhibitory to the Gram positive organisms and only occasionally weakly fluorescent colonies were produced (data not shown). These substrates would therefore have potential in the differentiation between Gram negative and Gram positive bacteria. With the exception of *Salmonella typhimurium*, the L-alanine aminopeptidase benzothiazole substrate **16** gave moderately fluorescent colonies with Gram negative bacteria. In contrast, benzoxazole substrate **19** gave strongly blue fluorescent colonies but diffusion of the fluorophore into the surrounding medium was observed. The β-alanine aminopeptidase substrate **21** gave strongly yellow fluorescent colonies with *E. coli, S. marcescens, P. aeruginosa* and *Morganella morganii* but this substrate inhibited the growth of the other Gram negative bacteria. It is interesting to note the contrast in fluorescent colours between substrates **16** and **21** that possess the same benzothiazole core structure. The L-alanyl substrate **16** generally produced blue fluorescent colonies following hydrolysis whereas in contrast the β-alanyl substrate **21** gave yellow fluorescent colonies. The origin of this effect has not yet been established but may be a consequence of either the localisation of the fluorophore in different regions of the organism or the result of further metabolic transformation of the initially formed fluorophore. Compound **23** was not a particularly effective substrate producing only moderately or weakly fluorescent colonies with most Gram negative bacteria. We have previously demonstrated that aminopeptidase activity is linked to enzyme activity.[8]

## 6. Conclusions

Nitroreductase activity was efficiently detected by the benzoxazole substrate **10a** across all of the Gram negative bacteria and *S. aureus* strains examined. In general, the full range of benzothiazole substrates **7**, enabled detection of most Gram negative bacteria but the benzimidazole substrates **13a** and **13b** were ineffective. The benzoxazole substrate **19** was effective for determining L-alanyl aminopeptidase activity although there was diffusion of the fluorophore into the surrounding medium. The benzothiazole substrate **21** allowed detection of β-alanyl aminopeptidase activity in cases where microorganism growth was not inhibited by this substrate.

## 7. Experimental

### 7.1. Synthetic work

$^1$H NMR spectra (270 MHz) and $^{13}$C NMR spectra (68 MHz) were recorded on a Jeol EX270 instrument. High resolution mass spectrometry (HRMS) (electrospray) was performed by the EPSRC mass spectrometry service. Infra-red spectra were obtained via a diamond anvil sample cell using a Perkin Elmer 1000 spectrometer. Melting points are reported uncorrected as determined on a Stuart SMP 1 melting point apparatus. Thin layer chromatography was performed on Merck plastic foil plates pre-coated with silica gel 60 $F_{254}$. Merck silica gel 60 was used for column chromatography.

#### 7.1.1. General procedure for the preparation of the dihydrobenzothiazoles 6a–d and benzothiazoles 7a–d

A mixture of 2-aminothiophenol **4** (1 equiv) and the appropriate nitrobenzaldehyde derivative **5** (1 equiv) in EtOH was heated either at reflux for 2–3 h or stirred at room temperature. After cooling to room temperature (if necessary), the solution was evaporated and the residue was partitioned between water and ethyl acetate. The organic layer was separated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were dried ($MgSO_4$) and evaporated giving the crude product **6**. To a solution of compound **6** (1 equiv) in $CH_2Cl_2$ at room temperature was added *p*-chloranil (1 equiv) and the resulting mixture was stirred overnight. The mixture was washed with a dilute aqueous NaOH solution, the organic phase was separated, dried ($MgSO_4$) and evaporated. The crude product **7** thus obtained was recrystallised from ethanol.

**7.1.1.1. 2-(2-Nitrophenyl)-2,3-dihydrobenzothiazole 6a and 2-(2-nitrophenyl)benzothiazole 7a.** Compound **6a**[13] was prepared from 2-nitrobenzaldehyde (1.00 g, 6.6 mmol), and 2-aminothiophenol (0.83 g, 6.6 mmol) in EtOH (50 mL) under nitrogen at room temperature for 1 h. Red needles (1.01 g, 59%); $\nu_{max}$ cm$^{-1}$ 3387, 1587, 1518, 1344, 1326, 1271, 1058; $\delta_H$ (270 MHz; $CDCl_3$) 4.57 (1H, broad s, >N*H*), 6.75–6.82 (3H, m, 2 × Ar-*H* and >C*H*-Ar), 6.93–7.06 (2H, m, Ar-*H*), 7.45 (1H, dd, *J* = 1.5 and 7.8 Hz, Ar-*H*), 7.63 (1H, ddd, *J* = 0.6, 0.6 and 7.7 Hz, Ar-*H*), 7.95–8.05 (2H, m, Ar-*H*). Compound **7a** was prepared from compound **6a** (1.01 g, 3.91 mmol) and *p*-chloroanil (0.96 g, 3.91 mmol) in $CH_2Cl_2$ (75 mL). Yellow solid (0.78 g, 77%), mp 117 °C (lit.[14] 122–123 °C); $\nu_{max}$ cm$^{-1}$1531, 1362, 1305, 1232; $\delta_H$ (270 MHz; $CDCl_3$) 7.40 (1H, ddd, *J* = 1.3, 7.6 and 7.6 Hz, Ar-*H*), 7.48 (1H, ddd, *J* = 1.3, 7.6 and 7.6 Hz, Ar-*H*), 7.54–7.68 (2H, m, Ar-*H*); 7.74 (1H, dd, *J* = 1.6 and 7.6 Hz, Ar-*H*), 7.84–7.91 (2H, m, Ar-*H*), 8.02 (1H, dd, *J* = 1.3 and 8.2 Hz, Ar-*H*).

**7.1.1.2. 2-(3-Chloro-6-nitrophenyl)benzothiazole 7b.** Compound **7b** was prepared directly from 2-aminothiophenol **4** (1.35 g, 0.011 mol) and 3-chloro-6-nitrobenzaldehyde (2.0 g, 0.011 mol) in EtOH (20 mL) at reflux. The intermediate dihydro-derivative **6b** was not isolated and was oxidised to compound **7b** under the reaction conditions. Yellow-brown solid (0.66 g, 24%), mp 126–128 °C; (found: MH$^+$, 290.9992. Calcd for $C_{13}H_8{}^{35}ClN_2O_2S$: MH, 290.9990). $\nu_{max}$ cm$^{-1}$ 2962, 1526, 1340, 1259, 753; $\delta_H$ (270 MHz; $CDCl_3$) 7.36–7.48 (2H, m, Ar-*H*), 7.52 (1H, dd, *J* = 8.7 and 2.2 Hz, Ar-*H*), 7.70 (1H, d, *J* = 2.2 Hz, Ar-*H*), 7.83 (1H, d, *J* = 8.7 Hz, Ar-*H*), 7.87 (1H, dd, *J* = 7.7 and 1.5 Hz, Ar-*H*), 8.01 (1H, dd, *J* = 7.7 and 1.5 Hz, Ar-*H*); $\delta_C$ (68 MHz; $CDCl_3$) 121.8, 124.2, 126.1, 126.3, 126.9, 130.0, 130.9, 131.9, 135.9, 138.9, 147.2, 153.4, 161.0.

**7.1.1.3. 2-(3-Fluoro-6-nitrophenyl)-2,3-dihydrobenzothiazole 6c and 2-(3-fluoro-6-nitrophenyl)benzothiazole 7c.** Compound **6c** was prepared from 2-aminothiophenol **4** (5.00 g, 0.04 mol) and 3-fluoro-6-nitrobenzaldehyde (6.75 g, 0.04 mol) in EtOH (200 mL) at reflux. Vivid red needles (7.23 g, 65%), mp 156–158 °C (EtOH); $\nu_{max}$ cm$^{-1}$: 3376, 1584, 1516, 1342, 1326, 1257, 1214, 1046; $\delta_H$ (270 MHz; $CDCl_3$) 4.56 (1H, broad s, >N*H*), 6.82–6.91 (3H, m, 3 × Ar-*H* or 2 × Ar-*H* and >C*H*-Ar), 6.99–7.14 (3H, m, 3 × Ar-*H* or 2 × Ar-*H* and >C*H*-Ar), 7.69 (1H, dd, *J* = 9.7 and 2.7 Hz, Ar-*H*), 8.18 (1H, dd, *J* = 9.2 and 5.2 Hz, Ar-*H*); $\delta_C$ (68 MHz; $CDCl_3$) 64.1, 112.2, 115.7 (d, *J* = 25.8 Hz), 115.8 (d, *J* = 23.1 Hz), 122.0, 122.4, 125.8, 127.3, 128.5 (d, *J* = 10.2 Hz), 141.8, 143.8 (d, *J* = 7.5 Hz), 145.5, 165.8 (d, *J* = 258.4 Hz). Compound **7c** was prepared from compound **6c** (2.23 g, 8.07 mmol) and *p*-chloranil (1.98 g, 8.07 mmol) in $CH_2Cl_2$ (125 mL). Fluffy yellow solid (1.42 g, 64%), mp 114–116 °C (EtOH); (found: MH$^+$, 275.0283. Calcd for $C_{13}H_8FN_2O_2S$: MH, 275.0285); $\nu_{max}$ cm$^{-1}$ 3082, 1510, 1348, 1313, 1269, 1093, 1223; $\delta_H$ (270 MHz; $CDCl_3$) 7.33 (1H, ddd, *J* = 9.0, 7.2 and 2.7 Hz, Ar-*H*), 7.45–7.59 (3H, m, Ar-*H*), 7.96 (1H, ddd, *J* = 7.8, 1.5 and 1.0 Hz, Ar-*H*), 8.03 (1H, dd, *J* = 9.0 and 5.0 Hz, Ar-*H*), 8.10 (1H, ddd, *J* = 7.8, 1.5 and 1.0 Hz, Ar-*H*); $\delta_C$ (68 MHz; $CDCl_3$) 117.8 (d, *J* = 23.8 Hz), 119.2 (d, *J* = 25.2 Hz), 121.8, 124.2, 126.3, 126.9, 127.4 (d, *J* = 9.5 Hz), 131.2 (d, *J* = 8.8 Hz), 135.9, 153.4, 161.1, 163.9 (d, *J* = 257.9 Hz).

**7.1.1.4. 2-(3,4-Dimethoxy-6-nitrophenyl)-2,3-dihydrobenzothiazole 6d and 2-(3,4-dimethoxy-6-nitrophenyl)benzothiazole 7d.** Compound **6d** was prepared from 2-aminothiophenol **4** (1.88 g, 15 mmol) and 6-nitroveratraldehyde (3.17 g, 15 mmol) in EtOH (50 mL) at reflux for 2 h. After cooling at room temperature, the solution was partially concentrated giving compound **6d** as a yellow solid (1.98 g, 41%); $\delta_H$ (270 MHz; $CDCl_3$) 3.82 (3H, s, –O*CH*$_3$), 3.88 (3H, s, –O*CH*$_3$), 4.51 (1H, br d, *J* = 5.0 Hz, >N*H*), 6.72–6.78 (2H, m, Ar-*H*), 6.88–7.02 (2H, m, Ar-*H*), 6.92 (1H, d, *J* = 5.0 Hz, >C*H*–), 7.47 (1H, s, Ar-*H*), 7.59 (1H, s, Ar-*H*). Compound **7d** was prepared from compound **6d** (1.59 g, 5 mmol) and *p*-chloranil (1.23 g, 5 mmol) in $CH_2Cl_2$ (80 mL). Yellow solid (1.40 g, 89%), mp 153–154 °C; (found: MH$^+$, 317.0584. Calcd for $C_{15}H_{13}N_2O_4S$: MH, 317.0596); $\nu_{max}$ cm$^{-1}$ 3008, 2937, 2845, 1496, 1330, 1272, 1230, 1088; $\delta_H$ (270 MHz; $CDCl_3$) 3.94 (3H, s, –O*CH*$_3$), 3.96 (3H, s, –O*CH*$_3$), 7.07 (1H, s, Ar-*H*), 7.35–7.42 (1H, m, Ar-*H*), 7.44–7.51 (1H, m, Ar-*H*), 7.56 (1H, s, Ar-*H*), 7.84–7.89 (1H, m, Ar-*H*), 8.00–8.05 (1H, m, Ar-*H*); $\delta_C$ (68 MHz; $CDCl_3$) 56.7, 56.8, 108.0, 113.3, 121.7, 123.0, 123.7, 125.8, 126.5, 136.1, 141.5, 150.1, 152.4, 153.2, 163.6.

#### 7.1.2. General procedure for the preparation of the Schiff bases 9a–d and benzoxazoles 10a–d

A mixture of 2-hydroxyaniline **8** (1 equiv) and the appropriate 2-nitrobenzaldehyde derivative **5** (1 equiv) in ethanol (30 mL) was heated at reflux for 3–4 h. After cooling to room temperature the resulting precipitate **9a–d** was collected and dried. To a stirred solution of the Schiff's base **9a–d** (1 equiv) in $CH_2Cl_2$ (30 mL) at room temperature, DDQ (1 equiv) was added over a 1 min. The reaction mixture was stirred at room temperature for 1–2 days, filtered and evaporated giving the crude product **10a–d**.

**7.1.2.1. 2-[(2-Nitrobenzylidene)amino]phenol 9a and 2-(2-nitrophenyl)benzoxazole 10a.** Compound **9a** was synthesised from 2-hydroxyaniline **8** (4.03 g, 0.037 mol) and 2-nitrobenzaldehyde (3.73 g, 0.02 mol). Yellow needles (6.36 g, 70%), mp 105–107 °C (lit.[15] mp 104 °C); $\delta_H$ (270 MHz; $CDCl_3$) 6.91 (1H, td, *J* = 7.4 and 1.5 Hz, Ar-*H*), 7.01 (1H, dd, *J* = 9.4 and 1.2 Hz, Ar-*H*), 7.15 (1H, s, –O*H*), 7.23 (1H, t, *J* = 8.7 Hz, Ar-*H*), 7.33 (1H, dd, *J* = 7.9 and 1.5 Hz, Ar-*H*), 7.61 (1H, td, *J* = 8.9 and 1.5 Hz, Ar-*H*), 7.72 (1H, t, *J* = 8.4 Hz, Ar-*H*), 8.03 (1H, dd, *J* = 7.9 and 1.5 Hz, Ar-*H*), 8.24 (1H, dd, *J* = 7.7 and 1.7 Hz, Ar-*H*), 9.13 (1H, s, =C*H*–); $\delta_C$ (68 MHz; $CDCl_3$) 115.5, 116.5, 120.5, 124.8, 129.6, 130.6, 131.5, 131.5, 133.5, 134.8, 149.4, 152.1, 152.9. Compound **10a** was prepared from compound **9a** (6.36 g, 0.026 mol) and DDQ

(5.90 g, 0.026 mol). Pale orange solid (4.41 g, 69%), mp 104–106 °C (lit.[15] mp 104–106 °C); $\delta_H$ (270 MHz; $CDCl_3$) 7.36–7.45 (2H, m, Ar-*H*), 7.54–7.62 (1H, m, Ar-*H*), 7.66–7.79 (2H, m, Ar-*H*), 7.79–7.84 (1H, m, Ar-*H*), 7.89 (1H, dd, *J* = 7.7 and 1.7 Hz, Ar-*H*), 8.14 (1H, dd, *J* = 7.4 and 1.7 Hz, Ar-*H*); $\delta_C$ (68 MHz; $CDCl_3$) 108.7, 110.3, 116.3, 116.8, 119.4, 124.4, 124.8, 128.8, 132.5, 141.9, 147.9, 149.3, 163.2.

**7.1.2.2. 2-[(3-Chloro-6-nitrobenzylidene)amino]phenol 9b and 2-(3-chloro-6-nitrophenyl)benzoxazole 10b.** Compound **9b** was synthesised from 2-hydroxyaniline **8** (2.18 g, 0.02 mol) and 5-chloro-2-nitrobenzaldehyde (5.59 g, 0.037 mol). Orange needles (4.17 g, 80%), mp 144–146 °C (EtOH); (found: $MH^+$, 277.0373. Calcd for $C_{13}H_{10}{}^{35}ClN_2O_3$: MH, 277.0374); $\nu_{max}$ $cm^{-1}$ 3442, 1558, 1376, 1521, 1461, 1341, 1301, 1176, 1143, 756; $\delta_H$ (270 MHz; $CDCl_3$) 6.95 (1H, t, *J* = 8.2 Hz, Ar-*H*), 7.05 (1H, dd, *J* = 8.2 and 1.2 Hz, Ar-*H*), 7.10 (1H, broad s, –O*H*), 7.28 (1H, t, *J* = 8.2 Hz, Ar-*H*), 7.33 (1H, dd, *J* = 8.2 and 1.5 Hz, Ar-*H*), 7.60 (1H, dd, *J* = 8.7 and 2.5 Hz, Ar-*H*), 8.05 (1H, d, *J* = 8.7 Hz, Ar-*H*), 8.23 (1H, d, *J* = 2.5 Hz, Ar-*H*), 9.27 (1H, s, =C*H*–); $\delta_C$ (68 MHz; $CDCl_3$) 115.8, 116.6, 120.5, 126.5, 129.3, 130.8, 131.3, 132.3, 134.4, 140.4, 147.4, 150.8, 153.0. Compound **10b** was prepared from compound **9b** (1.38 g, 0.005 mol) and DDQ (1.14 g, 0.005 mol). Greenish brown powder (0.97 g, 70%), mp 130–132 °C (EtOH); (found: $MH^+$, 274.0141. Calcd for $C_{13}H_8Cl^{35}N_2O_3$: MH, 274.0140); $\nu_{max}$ $cm^{-1}$ 1616, 1572, 1534, 1461, 1371, 1236, 1182, 1153, 743; $\delta_H$ (270 MHz; $CDCl_3$) 7.40–7.45 (2H, m, Ar-*H*), 7.56–7.61 (1H, m, Ar-*H*), 7.65 (1H, dd, *J* = 8.7 and 2.2 Hz, Ar-*H*), 7.80–7.85 (1H, m, Ar-*H*), 7.87 (1H, d, *J* = 8.7 Hz, Ar-*H*), 8.16 (1H, d, *J* = 2.2 Hz, Ar-*H*); $\delta_C$ (68 MHz; $CDCl_3$) 111.1, 120.9, 123.3, 125.3, 125.7, 126.5, 131.4, 131.8, 138.8, 141.4, 147.3, 151.6, 157.5.

**7.1.2.3. 2-[(3-Fluoro-6-nitrobenzylidene)amino]phenol 9c and 2-(3-fluoro-6-nitrophenyl)benzoxazole 10c.** Compound **9c** was synthesised from 2-hydroxyaniline **4** (10.91 g, 0.100 mol) and 3-fluoro-6-nitrobenzaldehyde (16.91 g, 0.100 mol). Orange solid, (24.09 g, 93%) mp 167–168 °C; $\delta_H$ (270 MHz; $CDCl_3$) 6.92–6.99 (1H, m, Ar-*H*), 7.03–7.08 (1H, m, Ar-*H*), 7.09 (1H, broad s, –O*H*), 7.27–7.35 (2H, m, Ar-*H*), 7.35–7.40 (1H, m, Ar-*H*), 7.97 (1H, dd, *J* = 9.2 and 2.7 Hz, Ar-*H*), 8.18 (1H, dd, *J* = 9.2 and 5.0 Hz, Ar-*H*), 9.23 (1H, d, *J* = 2.0 Hz, =C*H*–); $\delta_C$ (68 MHz; $CDCl_3$) 115.8, 116.1 (d, *J* = 25.2 Hz), 116.6, 118.4 (d, *J* = 22.4 Hz), 120.6, 128.0 (d, *J* = 9.5 Hz), 130.8, 134.0 (d, *J* = 8.8 Hz), 145.4, 150.9, 153.0, 165.0 (d, *J* = 255.0 Hz). The compound was used for the next step without further characterisation. Compound **10c** was synthesised from compound **9c** (24.09 g, 0.093 mol) and DDQ (21.04 g, 0.093 mol). Beige solid (14.49 g, 61%), mp 112–114 °C; (found: $MH^+$, 259.0513. Calcd for $C_{13}H_8FN_2O_3$: MH, 259.0513); $\nu_{max}$ $cm^{-1}$ 3084, 1589, 1527, 1357, 1298, 1219, 1056; $\delta_H$ (270 MHz; $CDCl_3$) 7.34–7.47 (3H, m, Ar-*H*), 7.57–7.62 (1H, m, Ar-*H*), 7.80–7.87 (1H, m, Ar-*H*), 7.84 (1H, dd, *J* = 8.2 and 2.9 Hz, Ar-*H*), 7.97 (1H, dd, *J* = 8.2 and 4.7 Hz, Ar-*H*); $\delta_C$ (68 MHz; $CDCl_3$) 111.1, 118.7 (d, *J* = 21.8 Hz), 118.7 (d, *J* = 26.5 Hz), 121.0, 124.4 (d, *J* = 9.9 Hz), 125.3, 126.5, 127.0 (d, *J* = 9.4 Hz), 141.4, 145.4, 151.1, 157.7, 157.7, 164.0 (d, *J* = 257.0 Hz).

**7.1.2.4. 2-[(3,4-Dimethoxy-6-nitrobenzylidene)amino]phenol 9d and 2-(3,4-dimethoxy-6-nitrophenyl)benzoxazole 10d.** Compound **9d** was synthesised from 2-hydroxyaniline **8** (3.27 g, 0.03 mol) and 6-nitroveratraldehyde (6.33 g, 0.03 mol). Orange needles (8.20 g, 90%), mp 154–156 °C (EtOH); (found: $MH^+$, 303.0973. Calcd for $C_{15}H_{15}N_2O_5$: MH, 303.0975); $\nu_{max}$ $cm^{-1}$ 3416, 2838, 1589, 1566, 1461, 1384, 1344, 1317, 1181, 1149; $\delta_H$ (270 MHz; $CDCl_3$) 4.01 (3H, s, –OC$H_3$), 4.05 (3H, s, –OC$H_3$), 6.92 (1H, t, *J* = 7.9 Hz, Ar-*H*), 7.01 (1H, dd, *J* = 7.9 and 1.5 Hz, Ar-*H*), 7.12 (1H, broad s, –O*H*), 7.22 (1H, t, *J* = 7.9 Hz, Ar-*H*), 7.32 (1H, dd, *J* = 7.9 and 1.5 Hz, Ar-*H*), 7.62 (1H, s, Ar-*H*), 7.69 (1H, s, Ar-*H*), 9.25 (1H, s, =C*H*–); $\delta_C$ (68 MHz; $CDCl_3$) 56.7, 56.8, 107.6, 109.6, 115.4, 116.7, 120.5, 125.5, 129.9, 135.3, 142.8, 151.0, 152.5, 153.1, 153.2. Compound **10d** was synthesised from compound **9d** (1.52 g, 0.005 mol) and DDQ (1.13 g, 0.005 mol). Orange-brown powder (1.35 g, 90%), mp 148–150 °C (EtOH); (found: $MH^+$, 301.0819. Calcd for $C_{15}H_{13}N_2O_5$: MH, 301.0819); $\nu_{max}$ $cm^{-1}$ 1519, 1582, 1448, 1378, 1189, 1177; $\delta_H$ (270 MHz; $CDCl_3$) 4.00 (3H, s, –OC$H_3$), 4.01 (3H, s, –OC$H_3$), 7.37–7.41 (2H, m, Ar-*H*), 7.43 (1H, s, Ar-*H*), 7.52 (1H, s, Ar-*H*), 7.53–7.57 (1H, m, Ar-*H*), 7.78–7.82 (1H, m, Ar-*H*); $\delta_C$ (68 MHz; $CDCl_3$) 56.1, 56.2, 107.8, 111.0, 112.9, 115.8, 120.4, 124.9, 125.8, 141.5, 142.4, 150.9, 151.1, 152.2, 159.8.

**7.1.3. Preparation of the benzimidazoles 13a and 13b**

**7.1.3.1. 1-Methyl-2-(2-nitrophenyl)benzimidazole 13a.** Compound **13a** (80%) was prepared using a similar procedure to that described below for compound **13b**, mp 134–136 °C (EtOH) (lit.[14] 135–137 °C); $\delta_H$ (270 MHz; $CDCl_3$) 3.65 (3H, s, –$NCH_3$), 7.30–7.50 (3H, m, Ar-*H*), 7.56–7.90 (4H, m, Ar-*H*), 8.35 (1H, dd, *J* = 8.0 and 2.0 Hz, Ar-*H*); $\delta_C$ (68 MHz; $CDCl_3$) 30.7, 109.8, 120.2, 122.6, 123.3, 124.9, 126.1, 131.2, 133.0, 133.7, 135.8, 143.0, 148.8, 149.9.

**7.1.3.2. 1-Methyl-2-(3-chloro-6-nitrophenyl)benzimidazole 13b.** To a stirred solution of *N*-methyl-1,2-phenylenediamine **11** (0.5 g, 0.004 mol) and 5-chloro-2-nitrobenzaldehyde (0.76 g, 0.004 mol) in DMF (25 mL) and $H_2O$ (1 mL) at room temperature, Oxone® (1.47 g, 0.0024 mol) was added in portions over 15 min. The mixture was stirred overnight at room temperature. Water (10 mL) was added and the reaction mixture was extracted twice with $CH_2Cl_2$ (20 mL). The combined $CH_2Cl_2$ layers were washed several times with water, dried ($MgSO_4$) and evaporated giving compound **13b** (0.94 g, 83%) as yellow-brown needles, mp 148–150 °C (EtOH) (lit.[14] 144–145 °C); $\delta_H$ (270 MHz; $CDCl_3$) 3.65 (3H, s, –C$H_3$), 7.30–7.45 (2H, m, Ar-*H*), 7.70 (2H, m, Ar-*H*), 7.85 (1H, m, Ar-*H*), 7.80 (1H, m, Ar-*H*), 8.20 (1H, d, *J* = 9.4 Hz, Ar-*H*); $\delta_C$ (68 MHz; $CDCl_3$) 30.8, 109.8, 120.3, 122.8, 123.7, 126.4, 127.9, 131.2, 133.2, 135.7, 140.4, 142.9, 147.1, 148.5.

**7.1.4. General procedure for the preparation of *t*-Boc-L-alanyl derivatives 15, 18, 20 and 22**

2-(2-Aminophenyl)benzothiazole **14** was prepared by $SnCl_2.2H_2O$ reduction of compound **7a**.[16] 2-(2-Aminophenyl)benzoxazole **17**[17] was prepared by a similar procedure. *t*-Boc-L-alanine or *t*-Boc-β-alanine (1.33 equiv) was dissolved in dry THF (10 mL). The solution was cooled down to −15 °C and *N*-methylmorpholine (NMM) (1.66 equiv) was added drop-wise over 1 min, followed by *iso*butyl chloroformate (IBCF) (1.33 equiv). The mixture was stirred for 5–10 min and then a solution of either 2-(2-aminophenyl)benzothiazole **14** (1 equiv) or 2-(2-aminophenyl)benzoxazole **17** (1 equiv) dissolved in minimum amount of dry THF (*ca* 5 mL) was added drop-wise over 2 min at −15 °C. The reaction mixture was stirred in an ice-bath for 2–3 h, allowed to warm to room temperature and stirred at room temperature overnight. The reaction mixture was filtered and the resulting filtrate was reduced in volume and poured into a cooled aqueous solution of sodium carbonate. The resulting precipitate was collected and recrystallised from either methanol or ethanol.

**7.1.4.1. 2-[(*t*-Boc-L-alanylamino)phenyl]benzothiazole 15.** Compound **15** was synthesised from *t*-Boc-L-alanine (1.51 g, 8 mmol), NMM (1.01 g, 10 mmol), IBCF (1.09 g, 8 mmol) and 2-(2-aminophenyl)benzothiazole **14** (1.36 g, 6 mmol). Yellow solid (0.87 g, 36%), mp 202–204 °C (MeOH); (found: $MNa^+$, 420.1347. Calcd for $C_{21}H_{23}NaN_3O_3S$: MNa, 420.1352); $\nu_{max}$ $cm^{-1}$ 3336, 1707, 1681, 1514, 1250, 1158, 1062; $\delta_H$ (270 MHz; $CDCl_3$) 1.43 (9H, s, 3 × –C$H_3$), 1.58 (3H, d, *J* = 7.4 Hz, –C$H_3$), 4.51 (1H, quintet,

$J$ = 7.4 Hz, >C*H*–), 5.33 (1H, d, $J$ = 5.9 Hz, >N*H*), 7.19 (1H, td, $J$ = 7.7 and 1.2 Hz, Ar-*H*), 7.41–7.56 (3H, m, Ar-*H*), 7.88 (1H, dd, $J$ = 7.9 and 1.5 Hz, Ar-*H*), 7.93 (1H, broad d, $J$ = 7.7 Hz, Ar-*H*), 8.14 (1H, broad d, $J$ = 7.4 Hz, Ar-*H*), 8.83 (1H, broad d, $J$ = 8.4 Hz, Ar-*H*) (one >NH was not located); $\delta_C$ (68 MHz; $CDCl_3$) 19.5, 28.4, 52.1, 80.3, 119.5, 121.0, 121.5, 123.1, 123.5, 125.9, 126.6, 129.9, 132.0, 133.3, 137.7, 152.9, 155.4, 168.7, 172.1.

**7.1.4.2. 2-[(*t*-Boc-L-alanylamino)phenyl]benzoxazole 18.** Compound **18** was synthesised from *t*-Boc-L-alanine (5.03 g, 0.0266 mol), NMM (3.36 g, 0.033 mol), IBCF (3.63 g, 0.0266 mol) and 2-(2-aminophenyl)benzoxazole **17** (4.20 g, 0.02 mol). Cream crystals (8.00 g, 80%), mp 152–154 °C (EtOH); (found: $MH^+$, 382.1760. Calcd for $C_{21}H_{24}N_3O_4$: MH, 382.1761); $\nu_{max}$ $cm^{-1}$ 3365, 3210, 3105, 1675, 1624, 1585, 1556, 1496, 1183, 1165; $\delta_H$ (270 MHz; $CDCl_3$) 1.44 (9H, s, 3 × –C*H*$_3$), 1.59 (3H, d, $J$ = 7.30 Hz, –C*H*$_3$), 4.54 (1H, quintet, $J$ = 7.3 Hz, >C*H*–), 5.39 (1H, d, $J$ = 7.3 Hz, >N*H*), 7.23 (1H, t, $J$ = 7.1 Hz, Ar-*H*), 7.40 (1H, t, $J$ = 1.5 Hz, Ar-*H*), 7.54 (1H, m, Ar-*H*), 7.61 (2H, m, Ar-*H*), 7.81 (1H, t, $J$ = 6.4 Hz, Ar-*H*), 8.24 (1H, dd, $J$ = 7.9 and 1.7 Hz, Ar-*H*), 8.84 (1H, d, $J$ = 8.7 Hz, Ar-*H*), 12.35 (1H, s broad, >N*H*); $\delta_C$ (68 MHz; $CDCl_3$) 19.6, 28.4, 52.0, 80.0, 110.5, 113.5, 120.0, 120.5, 123.3, 124.9, 125.8, 128.4, 132.9, 138.8, 141.0, 149.3, 155.5, 162.0, 172.4.

**7.1.4.3. 2-[(*t*-Boc-β-alanylamino)phenyl]benzothiazole 20.** Compound **20** was synthesised from *t*-Boc-β-alanine (1.51 g, 8 mmol), NMM (1.01 g, 10 mmol), IBCF (1.09 g, 8 mmol) and compound **14** (1.36 g, 6 mmol). Yellow-green solid (1.60 g, 67%), mp 114–116 °C (MeOH); (found: $MNa^+$, 420.1357. Calcd for $C_{21}H_{23}NaN_3O_3S$: MNa, 420.1352); $\nu_{max}$ $cm^{-1}$ 3365, 1706, 1679, 1624, 1247, 1162, 974; $\delta_H$ (270 MHz; $CDCl_3$) 1.40 (9H, s, 3 × –C*H*$_3$), 2.79 (2H, t, $J$ = 6.0 Hz, >C*H*$_2$), 3.60 (2H, q, $J$ = 6.0 Hz, >C*H*$_2$), 5.27 (1H, broad s, >N*H*), 7.17 (1H, td, $J$ = 7.7 and 1.2 Hz, Ar-*H*), 7.41–7.57 (3H, m, Ar-*H*), 7.86 (1H, dd, $J$ = 7.9 and 1.5 Hz, Ar-*H*), 7.92 (1H, dd, $J$ = 7.9 and 1.0 Hz, Ar-*H*), 8.05 (1H, d, $J$ = 7.9 Hz, Ar-*H*), 8.77 (1H, dd, $J$ = 8.4 and 1.2 Hz, Ar-*H*) (one >NH was not located); $\delta_C$ (68 MHz; $CDCl_3$) 28.5, 36.6, 38.2, 79.3, 119.1, 120.8, 121.5, 123.0, 123.4, 126.0, 126.8, 129.9, 132.0, 133.2, 137.8, 152.8, 156.0, 168.7, 170.9.

**7.1.4.4. 2-[(*t*-Boc-β-alanylamino)phenyl]benzoxazole 22.** Compound **22** was synthesised from *t*-Boc-β-alanine (0.75 g, 4 mmol), NMM (0.50 g, 5 mmol), IBCF (0.54 g, 4 mmol) and compound **17** (0.63 g, 3 mmol). Cream crystals (0.40 g, 33%), mp 143–145 °C (MeOH); (found: $MH^+$, 382.1760. Calcd for $C_{21}H_{24}N_3O_4$: MH, 382.1761); $\nu_{max}$ $cm^{-1}$ 3365, 1679, 1619, 1244, 1168, 1040; $\delta_H$ (270 MHz; $CDCl_3$) 1.42 (9H, s, 3 × –C*H*3), 2.84 (2H, t, $J$ = 5.9 Hz, >C*H*$_2$), 3.60 (2H, q, $J$ = 5.9 Hz, >C*H*$_2$), 5.27 (1H, broad s, >N*H*), 7.19–7.25 (1H, m, Ar-*H*), 7.38–7.45 (2H, m, Ar-*H*), 7.51–7.58 (1H, m, Ar-*H*), 7.60–7.66 (1H, m, Ar-*H*), 7.76–7.83 (1H, m, Ar-*H*), 8.22–8.27 (1H, m, Ar-*H*), 8.77–8.82 (1H, m, Ar-*H*), 12.01 (1H, broad s, >N*H*).

### 7.1.5. General procedure for the preparation of 2-[(2-alanylamino)phenyl]benzothiazoles 2-[(2-alanylamino)phenyl]benzoxazoles 16, 19, 21 and 23

A solution of the *t*-Boc protected derivative **15**, **20** or **22** in TFA was stirred at room temperature overnight and then evaporated yielding the products **16**, **21** and **23**, respectively, as their TFA salts.[18] Compound **18** was deprotected using HCl/EtOAc as detailed below.

**7.1.5.1. 2-[(2-L-alanylamino)phenyl]benzothiazole 16 bis TFA salt.** Compound **16** was prepared as its bis TFA salt from compound **15** (0.30 g, 0.755 mmol) and TFA (7 mL). Yellow-green solid (0.35 g, 88%), mp 150–154 °C; (found: $M^+$, 298.1008. Calcd for $C_{16}H_{16}N_3OS$: $M^+$, 298.1009); $\nu_{max}$ $cm^{-1}$: 2940, 1653, 1596, 1704 , 1141; $\delta_H$ (270 MHz; DMSO-$d_6$) 1.63 (3H, d, $J$ = 7.2 Hz, –C*H*$_3$), 4.30–4.40 (1H, m, >C*H*–), 7.34–7.41 (1H, m, Ar-*H*), 7.49–7.57 (1H, m, Ar-*H*), 7.58–7.66 (2H, m, Ar-*H*), 8.00–8.05 (1H, m, Ar-*H*), 8.11–8.16 (1H, m, Ar-*H*), 8.18–8.23 (1H, m, Ar-*H*), 8.29–8.37 (1H, m, Ar-*H*), 8.33 (3H, broad s, –N$H_3^+$), 11.97 (1H, broad s, >N*H*); $\delta_C$ (68 MHz; DMSO-$d_6$) 17.1, 50.0, 122.2, 122.8, 123.3 (2C), 125.7, 126.7, 127.5, 130.9, 132.5, 134.0, 136.4, 152.9, 167.6, 169.2.

**7.1.5.2. 2-[(2-L-alanylamino)phenyl]benzoxazole 19.** Compound **18** (6.10 g, 0.016 mol) was dissolved in a minimum amount of dry ethyl acetate. The resulting solution was added drop-wise to dry ethyl acetate that had been saturated with HCl (15 mL). The mixture was stirred overnight and saturated aqueous $Na_2CO_3$ solution (20 mL) and $CH_2Cl_2$ (30 mL) was added. After stirring for 3 h organic layer was separated, washed with water, dried ($MgSO_4$) and evaporated giving compound **19** (3.59 g, 81%) as a cream powder, mp 222–224 °C (EtOH–$H_2O$); (found: $MH^+$, 282.1233. Calcd for $C_{16}H_{16}N_3O_2$: MH, 282.1237); $\nu_{max}$ $cm^{-1}$ 3414, 3352, 3093, 2921, 1667, 1619, 1525, 1581, 1437, 1245, 1194, 1139; $\delta_H$ (270 MHz; $CDCl_3$) 1.53 (3H, d, $J$ = 6.9 Hz, –C*H*$_3$), 1.74 (2H, broad s, –N*H*$_2$), 3.76 (1H, q, $J$ = 6.9 Hz, >C*H*–), 7.20 (1H, m, Ar-*H*), 7.38 (2H, m, Ar-*H*), 7.52 (1H, m, Ar-*H*), 7.60 (1H, m, Ar-*H*), 7.73 (1H, m, Ar-*H*), 8.21 (1H, dd, $J$ = 8.2 and 1.5 Hz, Ar-*H*), 8.84 (1H, dd, $J$ = 8.7 and 1.5 Hz, Ar-*H*) (one >N*H* was not located); $\delta_C$ (68 MHz; $CDCl_3$) 21.9, 52.7, 110.7, 113.6, 119.7, 120.7, 123.2, 124.9, 125.7, 128.5, 132.8, 138.9, 141.1, 149.4, 162.0, 175.9.

**7.1.5.3. 2-[(2-β-alanylamino)phenyl]benzothiazole 21 bis TFA salt.** Compound **21** was prepared as its bis TFA salt from compound **20** (0.25 g, 0.629 mmol) and TFA (7 mL). Yellow-green solid (0.24 g, 73%), mp 170–174 °C; (found: $M^+$, 298.1008. Calcd for $C_{16}H_{16}N_3OS$: $M^+$, 298.1009); $\delta_H$ (270 MHz; DMSO-$d_6$) 2.91 (2H, t, $J$ = 6.7 Hz, >C*H*$_2$), 3.19 (2H, sextet, $J$ = 6.7 Hz, >C*H*$_2$), 7.30–7.38 (1H, m, Ar-*H*), 7.51–7.66 (3H, m, Ar-*H*), 7.88 (3H, broad s, –N$H_3^+$), 8.03–8.08 (1H, m, Ar-*H*), 8.12–8.17 (1H, m, Ar-*H*), 8.19–8.24 (1H, m, Ar-*H*), 8.38–8.45 (1H, m, Ar-*H*), 11.94 (1H, br s, >N*H*); $\delta_C$ (68 MHz; DMSO-$d_6$) 35.0, 35.5, 121.4, 122.6, 122.8, 123.3, 125.1, 126.7, 127.4, 130.6, 132.5, 133.9, 137.3, 152.8, 167.8, 169.5.

**7.1.5.4. 2-[(2-β-alanylamino)phenyl]benzoxazole 23 TFA salt.** Compound **23** was prepared as its TFA salt from compound **22** (0.17 g, 0.45 mmol) and TFA (5 mL). Beige solid (0.19 g, 100%), mp 188–191 °C; (found: $M^+$, 282.1236. Calcd for $C_{16}H_{16}N_3O_2$: $M^+$, 282.1237); $\nu_{max}$ $cm^{-1}$ 3146, 1679, 1623, 1594, 1116, 1180, 1040; $\delta_H$ (270 MHz; DMSO-$d_6$) 2.93 (2H, t, $J$ = 6.7 Hz, >C*H*$_2$), 3.19 (2H, sextet, $J$ = 6.7 Hz, >C*H*$_2$), 7.33–7.39 (1H, m, Ar-*H*), 7.46–7.56 (2H, m, Ar-*H*), 7.62–7.69 (1H, m, Ar-*H*), 7.84 (3H, broad s, –N$H_3^+$), 7.85–7.89 (2H, m, Ar-*H*), 8.21–8.26 (1H, m, Ar-*H*), 8.50–8.55 (1H, m, Ar-*H*), 11.52 (1H, broad s, >N*H*); $\delta_C$ (68 MHz; DMSO-$d_6$) 35.0, 35.5, 111.7, 114.4, 120.2, 121.5, 124.5, 125.8, 126.8, 129.3, 133.4, 138.4, 140.9, 149.5, 161.9, 169.3.

## 7.2. Microbiological work

### 7.2.1. Agar plate preparation

Each substrate (10 mg) was dissolved in 1-methyl-2-pyrrolidone (0.2 mL) and added to molten Columbia agar (100 mL) (Oxoid, Basingstoke) at 50 °C to a final concentration of 100 mg/L. Agar plates were then prepared and dried. Bacterial strains (obtained from the National Collection of Type Cultures, London, UK) were sub-cultured onto Columbia agar. Colonies of each strain were sampled using a loop and suspended in 0.85 % sterile physiological saline to generate a suspension equivalent to $10^8$ colony forming units per mL using a densitometer. Each agar plate was then inoculated with 10 μL of this suspension and spread to obtain single colonies. Plates were incubated at 37 °C in air for 24 h.

### 7.2.2. Cell-free extract preparation

*E. coli* BL21 was grown in a nutrient broth for 24 h, centrifuged to form a pellet which was subsequently suspended in the minimum amount of Tris *p*H 7.4 buffer. The mixture was then sonicated and centrifuged again. The supernatant liquid was separated and used directly.

### 7.2.3. HPLC method for determining the reduction product of substrate 7a

Substrate **7a** (100 mg) was dissolved in 1-methyl-2-pyrrolidone (200 μL) and added to BD Difco™ nutrient broth (100 mL). This was inoculated with *E. coli* and put in an agitated incubator at 37 °C for 48 h. The mixture was the centrifuged at 20,000 rpm for 10 min and the aqueous phase removed. The aqueous phase was then extracted with $CH_2Cl_2$ (3 × 75 mL), the solvent was evaporated under reduced pressure and the residue obtained was dissolved in acetonitrile (10 mL). HPLC analysis was conducted using a Thermo Finnigan Surveyor system with a Waters symmetry C18, 5 μm, 15 cm × 0.46 cm column [mobile phase: 40% acetonitrile and 60% water (both containing 1% acetic acid)] at a flow rate of 1.0 mL/min and a detection wavelength of 254 nm. See Supplementary data for HPLC traces.

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## Supplementary data

Supplementary data (HPLC traces) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.043.

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