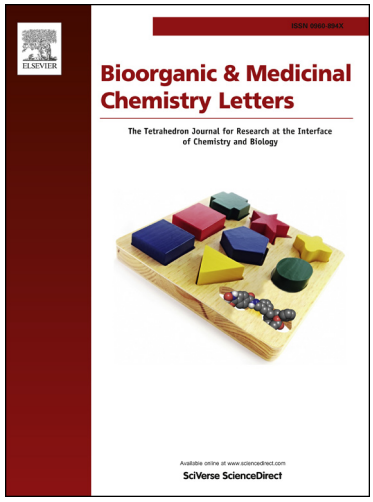

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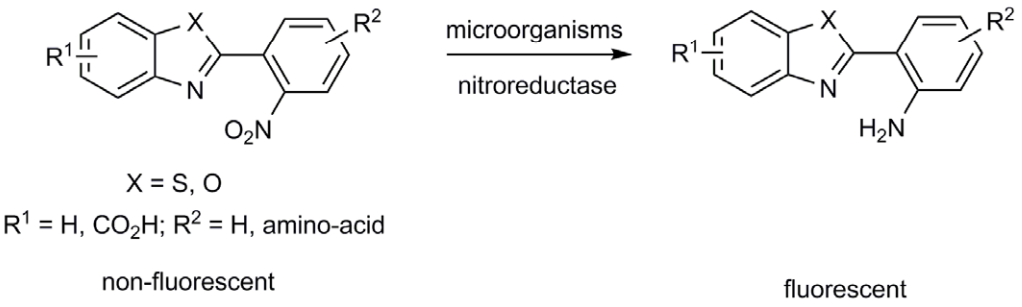

2-(Nitroaryl)benzothiazole and benzoxazole derivatives as fluorogenic substrates for the detection of nitroreductase activity in clinically important microorganisms

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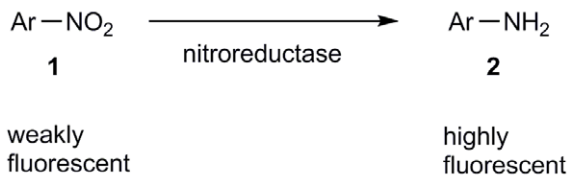
Abstract

A series of carboxy-substituted 2-(nitroaryl)benzothiazole derivatives and carboxy-substituted 2-(nitroaryl)benzoxazole derivatives were prepared and evaluated as potential nitroreductase substrates for the purpose of detecting clinically important microorganisms. Several of the substrates produced highly fluorescent colonies with the majority of a panel of 10 Gram-negative bacteria and also with two of a panel of 8 Gram-positive bacteria.

Keywords Nitroreductase, fluorescence, enzyme substrates, microorganism detection, bacterial detection

Methods that enable the detection, enumeration and identification of harmful bacteria are important in many sectors of the economy including the health-care sector (*e.g.* hospitals), the food industry (*e.g.* food quality control) and the environment (*e.g.* monitoring of water contamination).[1-3] The utilisation of synthetic enzyme substrates is an important protocol for bacterial detection and identification. Relevant to this paper is the reductive transformation of weakly fluorescent nitro-aromatic substrates **1** by bacterial nitroreductase enzymes into highly fluorescent aromatic amines **2** (or hydroxylamines) as depicted in Scheme 1. Such a change in fluorescence intensity can frequently offer a practicable method for analysing biological and environmental samples because many bacteria are capable of reducing nitro-aromatic compounds.[4] A bioluminescent nitroreductase probe has been reported and its capacity to detect nitroreductase activity in the Gram-negative *Escherichia coli* and the Gram-positive *Bacillus subtilis* was demonstrated. [5] Chromogenic substrates, including halogenated nitrophenoxazinones[6] and 4-(4-nitrostyryl)quinolines and related compounds,[7] have been evaluated for their ability to detect nitroreductase activity across a range of microorganisms. A substrate derived from 3*H*-phenoxazin-3-one (resorufin) linked to a nitrothiophene moiety has been used to detect *E. coli*.[8] In these chromogenic substrates, weakly coloured nitro-heterocycles are transformed by a nitroreductase enzyme into highly coloured amine derivatives.



Scheme 1. Reduction of nitro-aromatics by nitroreductase enzymes.

The synthesis of a series of several 7-nitrocoumarin derivatives has been described previously by one of us (ALJ) and these fluorogenic nitroreductase substrates were evaluated against 30 bacterial strains which encompassed 24 distinct microbial genera, including a wide range of clinically important species.[9,10] We have also described the synthesis of a series of 2-(2-nitroaryl)benzothiazoles **3a-d**, 2-(2-nitroaryl)benzoxazoles **4a-d** and 2-(2-nitroaryl)benzimidazoles **5a** and **5b** (Figure 1) and their evaluation against a panel of 20 microorganisms in agar media.[11] In general, these heterocycles gave either blue or purple fluorescent colonies with the majority of Gram-negative bacteria and also with two Gram-positive strains, methicillin sensitive *Staphylococcus aureus* (MSSA) and methicillin resistant *Staphylococcus aureus* (MRSA). The benzothiazoles **3a-d** and benzoxazoles **4a-d** usually produced highly fluorescent bacterial colonies, whereas in comparison, the fluorescence intensity of the bacterial colonies in the presence of the benzimidazole derivatives **5a** and **5b** was weak. We also demonstrated the benzoxazole derivative **4c** produced a fluorescent solution when incubated with a *Escherichia coli* BL21 cell-free extract in a Tris buffer solution (pH 7.4) thus confirming that enzyme activity was responsible for the generation of fluorescence. Furthermore, the corresponding amine derivative was identified as the product

by HPLC; the structure of the amine was confirmed by comparison with an authentic sample which was prepared by chemical reduction of compound **4c**.[11]

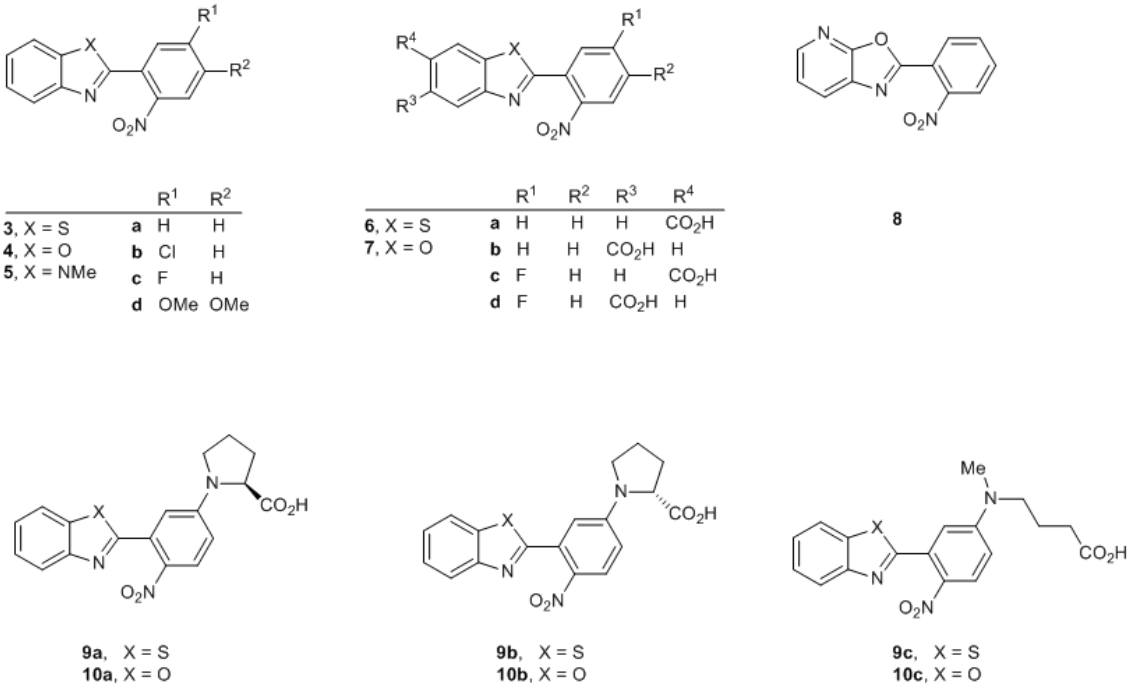


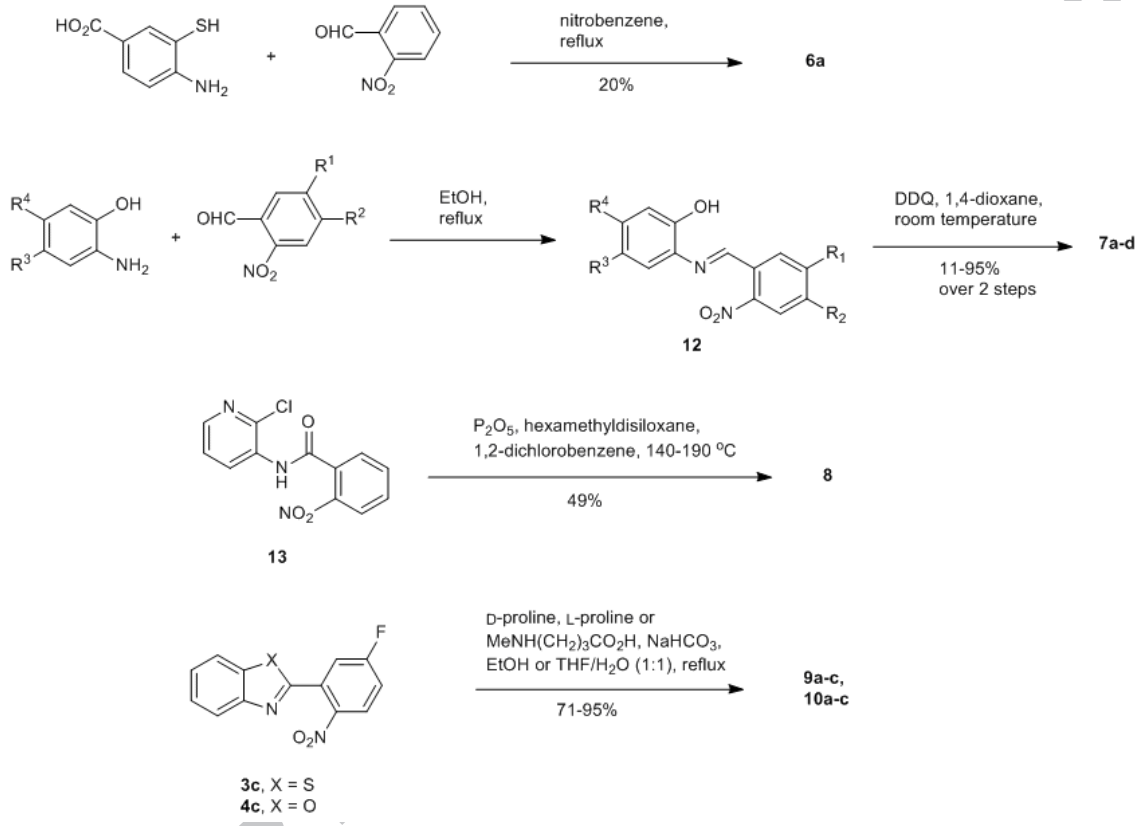
Figure 1. Nitroreductase substrates.

In this paper, we report the synthesis of a series of 2-(2-nitroaryl)benzothiazole **6a**, **9a-c** and 2-(2-nitroaryl)benzoxazole **7a-d** and **10a-c** derivatives that all possess a carboxylic acid group as part of their structure (Figure 1). These substrates have been prepared because the presence of the carboxy-group (either as the free acid or an appropriate salt) would be expected to enhance aqueous solubility; an important consideration for applications in the preparation of microbiological media. Both broth and agar media are frequently prepared by addition of water to the powdered media (containing the substrate) and hence for commercial applications the substrate should be able to dissolve quickly and completely in water. In view of our previous work on fluorogenic nitroreductase substrates,[11] it was also of general interest to investigate and compare the selectivity of these new heterocycles with existing compounds.

The nitroreductase substrates prepared in this paper have been evaluated in agar media because multi-point inoculation of plates allows a wide range of microorganisms to be evaluated concomitantly. Structurally related to the 2-(2-nitroaryl)benzoxazole substrates is 2-(2-nitrophenyl)-[1,3]oxazolo[5,4-*b*]pyridine (**8**).[12] To our knowledge, this compound has not been evaluated as a nitroreductase substrate and hence it has been included in this study.

The synthetic routes to the nitroreductase substrates used in this study are shown in Scheme 2. 3-Mercapto-4-aminobenzoic acid was heated with 2-nitrobenzaldehyde in nitrobenzene solution at reflux following a literature procedure affording substrate **6a**.[13] Substrates **7a-d** were prepared by condensing 2-aminophenol-3/4-carboxylic acids with an appropriately substituted 2-nitrobenzaldehyde derivative in ethanol at reflux to give the Schiff bases **12** which were subjected to an oxidative cyclisation yielding the required

substrates **7a-d**. Substrate **8** was produced following a method described in the patent literature;[8] 3-amino-2-chloropyridine was treated with 2-nitrobenzoyl chloride under basic conditions giving the amide **13** which was subsequently cyclised by heating in a mixture of 1,2-dichlorobenzene, phosphorus pentoxide and hexamethyldisiloxane. The reaction of the fluorinated benzothiazole **3c** and the fluorinated benzoxazole **4c** with an amino acid (D-proline, L-proline or *N*-methyl-γ-aminobutyric) under basic conditions furnished the substrates **9a-c** and **10a-c**.



Scheme 2. Synthesis of the potential nitroreductase substrates.

A preliminary evaluation of each substrate was carried out in Columbia agar media (37 °C in air for 18 hours) against 20 clinically important microorganisms, including 10 Gram-negative bacteria, 8 Gram-positive bacteria and 2 yeasts. Columbia agar media was selected because this enabled a direct comparison of the substrates described in this paper with those previously reported by us and, additionally, each substrate could be evaluated simultaneously against the complete panel of 20 microorganisms on a single plate. The growth of the microorganisms was compared to control plates in which no substrate was present. The Gram-negative microorganisms all grew well on the control plates whereas the Gram-positive microorganisms and the yeasts showed only moderate growth.

Table 1 depicts the results obtained for the evaluation of the substrates **6a**, **7a** and **8** against the panel of 20 microorganisms. Photographs of agar plates for substrates **6a** and **7a** are shown in Figure 2. The majority of the Gram-negative bacteria as well as the two Gram-positive strains, MSSA and MRSA, produced strongly fluorescent colonies with all of these

substrates. These nitroreductase profiles are similar to those previously reported by us for the substrates **3a-d** and **4a-d**, and illustrate that the presence of the carboxy-groups in substrates **6a** and **7a** are not detrimental to the substrates' effectiveness. The results obtained for the evaluation of the substrates **7b-7d** (data not shown) were broadly similar to those of substrate **7a**.

		6a		**7a**		**8**	
	Microorganism / Reference[a]	**Growth**[b]	**Fluorescence**[c]	**Growth**[b]	**Fluorescence**[c]	**Growth**[b]	**Fluorescence**[c]
	Gram-negative microorganisms						
1	*Escherichia coli* NCTC 10418	++	++ green	++	++ blue	++	++ blue
2	*Klebsiella pneumoniae* NCTC 9528	++	++ green	++	++ blue	++	++ blue
3	*Providencia rettgeri* NCTC 7475	++	++ green	++	++ blue	++	++ blue
4	*Enterobacter cloacae* NCTC 11936	++	++ green	++	++ blue	++	++ blue
5	*Serratia marcescens* NCTC 10211	++	++ green	++	++ blue	++	++ blue
6	*Salmonella typhimurium* NCTC 74	++	++ green	++	++ blue	++	++ blue
7	*Pseudomonas aeruginosa* NCTC 10662	++	+/- green	++	-	++	++ blue
8	*Yersinia enterocolitica* NCTC 11176	+	+/- green	++	-	++	+ blue
9	*Burkholderia cepacia* NCTC 10743	++	++ green	++	++ blue	++	+ blue
10	*Acinetobacter baumannii* NCTC 12156	++	++ green	++	++ blue	++	++ blue
	Gram-positive microorganisms						
11	*Streptococcus pyogenes* NCTC 8306	+	-	+	-	+	-
12	*Staphylococcus aureus* (MRSA) NCTC 11939	+	++ green	+	++ blue	+	++ blue
13	*Staphylococcus aureus* (MSSA) NCTC 6571	+	++ green	+	++ blue	+	++ blue
14	*Staphylococcus epidermidis* NCTC 11047	+	Tr. green	+	-	+	-
15	*Listeria monocytogenes* NCTC 11994	+	Tr. green	+	-	+	-
16	*Enterococcus faecium* NCTC 7171	+	Tr. green	+	-	+	-
17	*Enterococcus faecalis* NCTC 775	+	Tr. green	+	-	+	-
18	*Bacillus subtilis* NCTC 9372	+	+ green	+	+ blue	+	+ blue
	Yeasts						
19	*Candida albicans* ATCC 90028	+	Tr. green	+	-	+/-	-
20	*Candida glabrata* NCPF 3943	+	-	+	-	+	-

[a]NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection; NCPF: National Collection of Pathogenic Fungi.

[b] ++ strong growth, + moderate growth, +/- weak growth, Tr. trace of growth.

[c] ++ strong fluorescence, + moderate fluorescence, +/- weak fluorescence, Tr. trace of fluorescence.

Table 1. Evaluation of substrates **6a**, **7a** and **8**. Substrate concentration = 100 mg L^{-1}; inoculum = 100 000 colony-forming units (cfu)/spot.

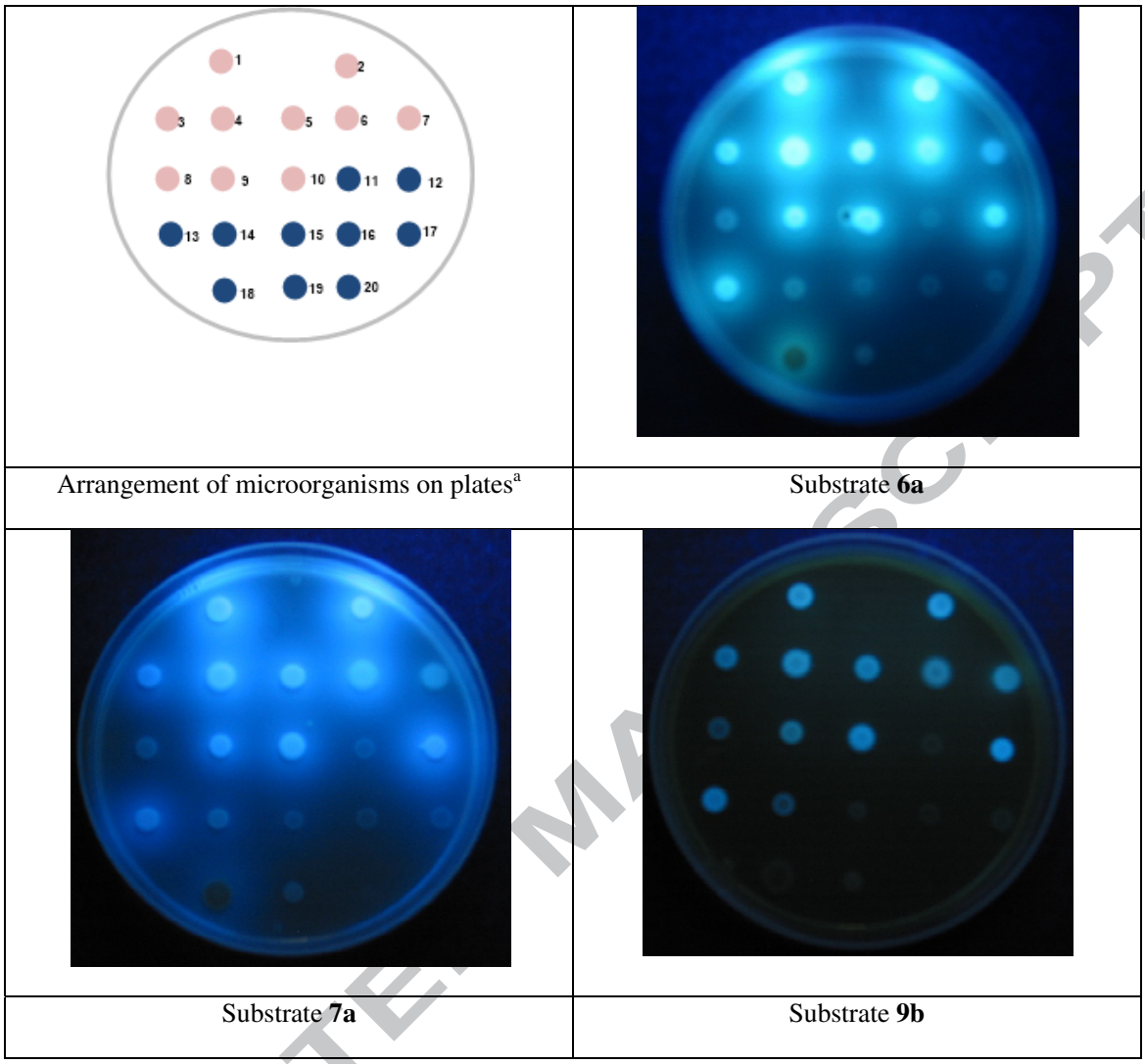


Figure 2. Columbia agar plates depicting fluorescence generated by substrates **6a**, **7a** and **9b** with various microorganisms. [a] Microorganisms are numbered in the sequence shown in the Tables. Pink spots represent Gram-negative bacteria, blue spots represent Gram-positive bacteria and the yeast species

		9a		9b		9c	
	Microorganism / Reference[a]	**Growth[b]**	**Fluorescence[c]**	**Growth[b]**	**Fluorescence[c]**	**Growth[b]**	**Fluorescence[c]**
	Gram-negative microorganisms						
1	*Escherichia coli* NCTC 10418	++	+ yellow	**++**	++ blue	++	+ yellow
2	*Klebsiella pneumoniae* NCTC 9528	++	+ yellow	**++**	++ blue	++	+ yellow
3	*Providencia rettgeri* NCTC 7475	++	+ yellow	**++**	++ blue	++	+ yellow
4	*Enterobacter cloacae* NCTC 11936	++	+ yellow	**++**	++ blue	++	+ yellow

5	*Serratia marcescens* NCTC 10211	++	+ yellow	++	++ blue	++	+ yellow
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12	*Staphylococcus aureus* (MRSA) NCTC 11939	+	+ yellow	+	++ blue	+	+ blue
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17	*Enterococcus faecalis* NCTC 775	+	Tr. yellow	+	-	+	-
18	*Bacillus subtilis* NCTC 9372	+	-	+	-	+	-
	Yeasts						
19	*Candida albicans* ATCC 90028	+	+ yellow	+	-	+	-
20	*Candida glabrata* NCPF 3943	+	-	+	-	+	-

[a]NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection; NCPF: National Collection of Pathogenic Fungi.

[b] ++ strong growth, + moderate growth, +/- weak growth, Tr. trace of growth.

[c] ++ strong fluorescence, + moderate fluorescence, +/- weak fluorescence, Tr. trace of fluorescence.

Table 2. Evaluation of substrates **9a-c**. Substrate concentration = 100 mg L^{-1}; inoculum = 100 000 colony-forming units (cfu)/spot.

Of the three substrates **9a-c**, only the L-prolyl derivative **9b** produced strongly fluorescent colonies (Table 2, Figure 2). This substrate gave the expected pattern of nitroreductase activity with the panel of microorganisms, *i.e.* fluorescent colonies were formed with all of the Gram-negative bacteria and also with the MSSA and MRSA strains. Substrate **9c** gave a similar nitroreductase profile to substrate **9b** but the colonies were yellow fluorescent and the fluorescence intensity was only moderate. There was an interesting and unexpected contrast between the prolyl substrates **9a** and **9b**. The substrate **9a** produced yellow fluorescent colonies with most microorganisms although the fluorescence intensity was very weak in many cases, with the exception of *Acinetobacter baumannii* which gave strongly fluorescent colonies. The observed yellow fluorescence of substrate **9a** is in contrast to the strong blue fluorescence produced by substrate **9b** and the reason for this is as yet unclear.

Unexpected evaluation results were also obtained for the benzoxazole substrates **10a-10c**. These substrates only produced weakly fluorescent colonies (blue) with three of the panel of 20 microorganisms [*Pseudomonas aeruginosa*, *A. baumannii* and MRSA (data not shown)].

Substrate **10a** also gave weakly fluorescent colonies with *Burkholderia cepacia*. This suggests that reduction of these substrates is not occurring with the majority of microorganisms. This observation is surprising in view of our previous work on benzothiazole and benzoxazole nitroreductase substrates in which the nitroreductase activity profiles of both of these heterocyclic systems with the majority of microorganisms has generally been comparable.[11]

The potassium salt of substrate **7a** was prepared by addition of an equimolar quantity of methanolic potassium hydroxide solution to the substrate followed by evaporation of the solvent. The potassium salt (30 mmol) was added to 100 mL of a potassium phosphate buffer (0.1 M, pH 7.4) in order to simulate the pH of typical culture media. The potassium salt was completely soluble in the buffer, whereas in contrast, the corresponding substrate **4a**, lacking the carboxy-group, was visibly insoluble at the same concentration.

We have evaluated substrate **7a** in brain heart infusion broth over a range of substrate concentrations against the Gram-negative *E. cloacae* (NCTC 11936) and the Gram-positive *S. aureus* (NCTC 6571) (Figure 3). The broths were incubated at 37 ^{o}C for 18 hours and bacterial activity could be detected at concentrations as low as 0.02 mmol/L^{-1}.

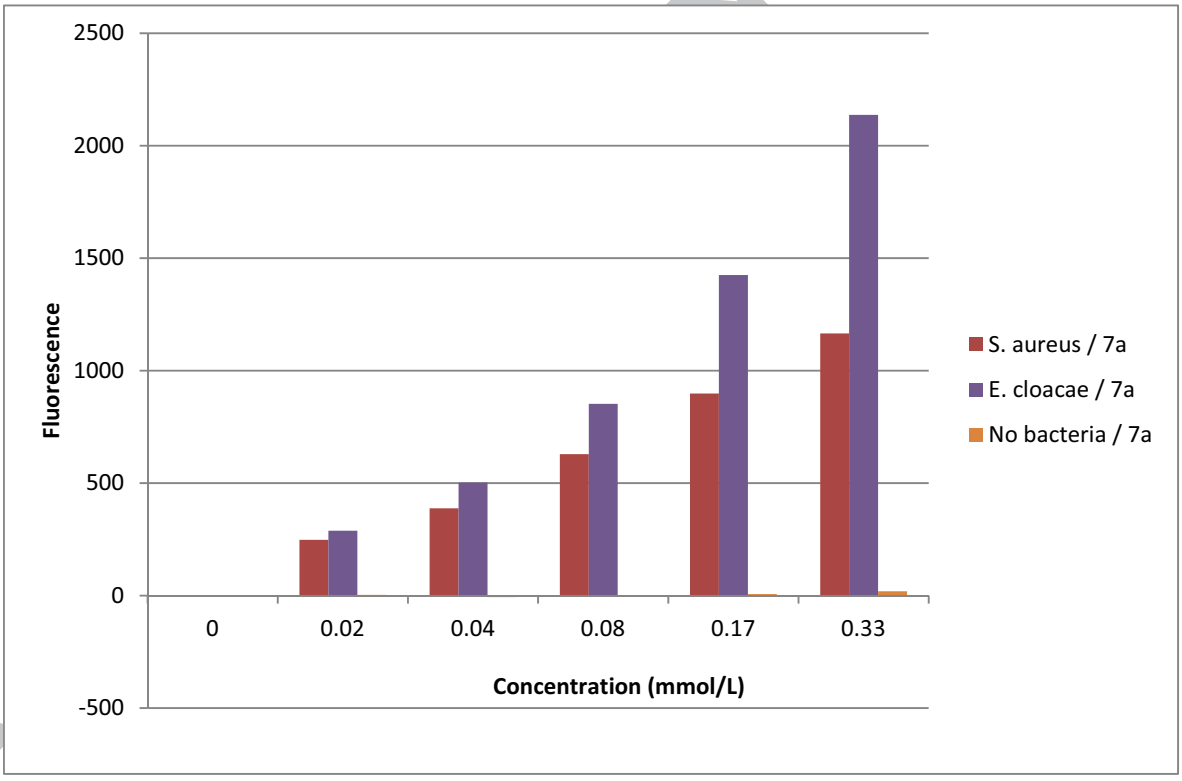

Figure 3. Fluorescence generated from substrate **7a** by two pathogenic bacteria at a range of substrate concentrations.

In conclusion, the nitroreductase substrates **6a** and **7a-d**, which bear a carboxy-group in the benzothiazole/benzoxazole ring, all exhibit a pattern of activity against a selection of

microorganisms in agar media which is broadly similar to that previously observed for the substrates **3a-d** and **4a-d**. All of these new substrates **6a** and **7a-d** produce highly fluorescent colonies with the majority of Gram-negative bacteria in most cases. The pyridine-ring containing substrate **8** also conforms to this pattern of activity. In contrast, the heterocycles **9a-c** and **10a-c** are less effective substrates, generally showing diminished fluorescent intensities and in the cases of substrates **10a-c** a greatly reduced nitroreductase activity profile. The presence of the carboxy-groups in these substrates has been shown to be beneficial to their aqueous solubility, thus allowing potential applications of these substrates in culture media.

Supplementary information

Synthetic and microbiological experimental procedures are given in the supplementary information.

Acknowlegements

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