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Synthesis and evaluation of halogenated nitrophenoxazinones as nitroreductase substrates for the detection of pathogenic bacteria

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ABSTRACT

The synthesis and microbiological evaluation of 7-, 8- and 9-nitro-1,2,4-trihalogenophenoxazin-3-one substrates with potential in the detection of nitroreductase-expressing pathogenic microorganisms are described. The 7- and 9-nitrotrihalogenophenoxazinone substrates were reduced by most Gram-negative microorganisms and were inhibitory to the growth of certain Gram-positive bacteria; however, the majority of Gram-positive strains that were not inhibited by these agents, along with the two yeast strains evaluated, did not reduce the substrates. These observations suggest there are differences in the active site structures and substrate requirements of the nitroreductase enzymes from different strains; such differences may be exploited in the future for differentiation between pathogenic microorganisms. The absence of reduction of the 8-nitrotrihalogenophenoxazinone substrates is rationalized according to their electronic properties and correlates well with previous findings.

1. Introduction

The ability of nitroreductase and other enzymes to reduce an aromatic nitro group to the corresponding hydroxylamine and/or amine is of significant interest, with great potential for biomedical, biocatalyst and bioremediation applications.[1–4] For example, the reduction of a nitroaromatic prodrug by a bacterial nitroreductase is central to the GDEPT anticancer therapeutic approach,[5,6] for which the mechanism of the *Escherichia coli* nitroreductase activation has been elucidated.[7]

Nitroreductase enzymes have been subdivided into two different classes,[8,9] namely type I and type II nitroreductases, based on their relative sensitivity to oxygen. The type I nitroreductases are oxygen insensitive and can reduce nitro compounds to their corresponding amines via the nitroso and hydroxylamine intermediates, using a two-electron transfer mechanism.[9–11] With the type II nitroreductases, which are oxygen sensitive, the reduction of nitro groups is initiated via a single electron transfer process, forming a nitro radical anion, which either accepts a second electron to form the nitroso intermediate and then follows the two-electron reduction process, or is rapidly reoxidised to a nitro group in the presence of oxygen.[8,9]

The widespread distribution of nitroreductase enzymes in pathogenic bacteria and yeasts offers the potential to enhance the detection of such microorganisms in both clinical and food applications.[11,12] The successful detection of pathogenic bacteria as a result of their nitroreductase action has been demonstrated in over 30 Gram-negative and Gram-positive microorganisms,[11] using the fluorogenic 7-nitrocoumarin-3-carboxylic acid **1a** or 7-nitro-4-methylcoumarin **1b** (Fig. 1). These compounds emit a strong

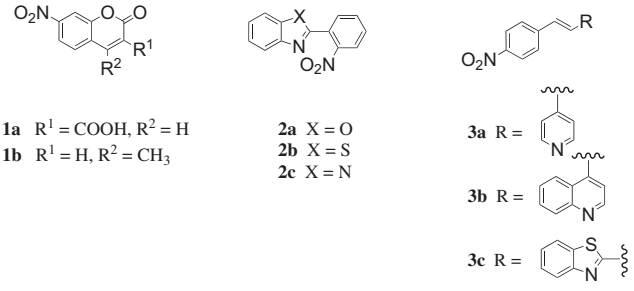

Fig. 1. Examples of established fluorogenic and chromogenic nitroreductase substrates.

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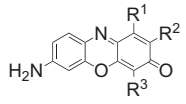
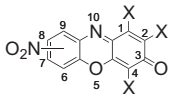
fluorescent signal upon exposure to UV light (λ=365 nm) after incubation with the bacteria. Moreover, other fluorogenic compounds, such as the 4-nitrobenzylcarbamate derivatives of 7-aminocoumarins,[13] and derivatives of nitrobenzoxazole **2a**, nitrobenzothiazole **2b** and nitrobenzimidazole **2c** (Fig. 1), were also shown to respond to nitroreductase activity, although with greater selectivity for Gram-negative bacteria.[14,15] The extension of this approach to chromogenic nitroaromatic substrates, including derivatives of 4-(4′-nitrostyryl)-pyridine **3a**,[16] 4-(4′-nitrostyryl)-quinoline **3b**[17] and 2-(4′-nitrostyryl)-benzothiazole **3c**[18] (Fig. 1), suggests a general application of nitroreductase enzyme substrates as detection agents, particularly for Gram-negative pathogenic bacteria. It is interesting to note that more spatially compact nitroreductase substrates, such as nitrobenzenes and nitrocoumarins **1a** and **1b**,[11,13] are reduced by a wider range of both Gram-negative and Gram-positive bacteria, while the more bulky nitroaromatic substrates, for example, the nitrobenzoxazole **2a**, nitrobenzothiazole **2b** and nitrobenzimidazole **2c** derivatives, are less well reduced by the Gram-positive bacteria,[14–18] which may suggest differences in the active sites of Gram-negative nitroreductases compared to those of Gram-positive bacteria.

We previously reported the synthesis and the microbiological evaluation of the chromogenic 7-aminophenoxazinones **4** and their corresponding aminopeptidase substrates, which indicate the presence of certain bacteria by the intense colour released upon bacterial enzymatic action.[19] By analogy, the corresponding 7-nitrophenoxazinone was a desirable target substrate as a potential marker of nitroreductase activity. Herein we report the preparation and evaluation of 7-, 8- and 9-nitro-1,2,4-trihalogenophenoxazin-3-ones **5**–**7**, (Fig. 2), for their ability to detect pathogenic microorganisms through nitroreductase activity.



4a R^1 = pentyl, R^2 = R^3 = H
4b R^1 = R^2 = R^3 = CH_3
4c R^1 = R^2 = CH_3, R^3 = H



5a-c 7-NO_2
6a-c 8-NO_2
7a,b 9-NO_2
a X = F; **b** X = Cl; **c** X = Br

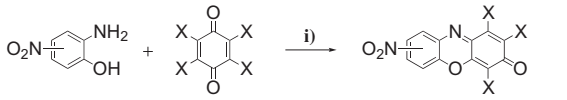
Fig. 2. 7-Aminophenoxazin-3-one chromogens **4** and nitrotrihalogenophenoxazinones **5**–**7**.

2. Results and discussion

2.1. Synthesis

The nitrotrihalogenophenoxazinones were synthesized using the route described previously for the synthesis of 7-nitrohalogeno phenoxazinones **5b**, **5c** and 8-nitro-1,2,4-trichlorophenoxazinone **6b**[20] without modification. With the availability of several 2-aminonitrophenol isomers, the opportunity was taken to include the synthesis of 8-nitro and 9-nitrophenoxazinones in this study. Following the general procedure, 2-aminonitrophenols **8**–**10** were condensed with tetrahalogeno-1,4-benzoquinones **11a**–**c** in an ethanolic solution containing sodium acetate (Scheme 1). 7-Nitro **5a**–**c**, 8-nitro **6a**–**c** and 9-nitro-1,2,4-trihalogenophenoxazin-3-ones **7a**,**b** were isolated in low to high yield (Table 1). The formation of the desired products was confirmed mostly by ^{13}C NMR spectroscopy, with the characteristic C–F coupling observed in the ^{13}C NMR spectra of fluorinated nitrophenoxazin-3-ones **5a**, **6a** and **7a** assisting the characterization of these novel products.

The synthesis of fluorinated derivatives **5a**, **6a** and **7a** proved to be problematic and all were isolated in poor yield, along with



8 5-NO_2
9 4-NO_2
10 3-NO_2

11a X = F
11b X = Cl
11c X = Br

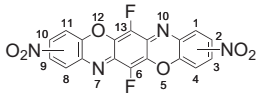
5a-c 7-NO_2
6a-c 8-NO_2
7a,b 9-NO_2
a X = F; **b** X = Cl; **c** X = Br

Scheme 1. Synthesis of nitro-1,2,4-trihalogenophenoxazin-3-ones **5**–**7**. Reagents and conditions: (i) EtOH, NaOAc, rt.

Table 1
Yields of synthesized nitrohalogenophenoxazin-3-ones substrates **5a**–**c**, **6a**–**c** and **7a**,**b**

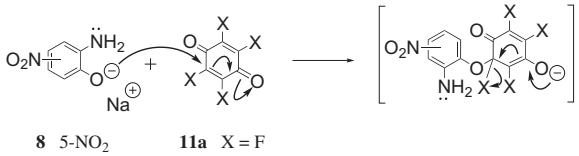
Compound	Nitro group position	X	Yield (%)
5a	7-	F	12
5b	7-	Cl	90
5c	7-	Br	95
6a	8-	F	39
6b	8-	Cl	99
6c	8-	Br	72
7a	9-	F	15
7b	9-	Cl	45

a highly insoluble solid, suspected to be triphenodioxazines **12a**–**c**, respectively. IR spectroscopy and mass spectrometry evidence for structure **12a** was obtained alongside nitrophenoxazinone **5a**. Mital and Jain have described the formation of triphenodioxazines under conditions similar to those described here (Scheme 1).[21]



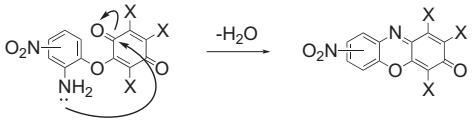
12a 3,10-NO_2
12b 2,9-NO_2
12c 1,8-NO_2

A reaction mechanism for an analogous condensation of various aminophenols with 2,3-dichloro-1,4-naphthoquinone in an ethanolic solution containing sodium or potassium acetate has been reported by Agarwal and Schäfer.[22] Deprotonation of the nitroaminophenol **8**–**10** is followed by a 1,4-Michael-type nucleophilic attack of the resulting phenoxide on tetrahalogenobenzoquinone **11a**–**c**, resulting in the formation of the corresponding 2-phenoxy-3,5,6-trihalogenoquinones **13a**–**c**, **14a**–**c** and **15a**,**b**. These intermediates then undergo ring closure to form the nitro-1,2,4-trihalogenophenoxazin-3-ones **5a**–**c**, **6a**–**c** and **7a**,**b** (Scheme 2).



8 5-NO_2
9 4-NO_2
10 3-NO_2

11a X = F
11b X = Cl
11c X = Br



13a-c 5'-NO_2
14a-c 4'-NO_2
15a,b 3'-NO_2

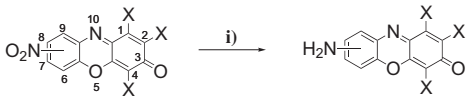
a X = F
b X = Cl
c X = Br

5a-c 7-NO_2
6a-c 8-NO_2
7a,b 9-NO_2

Scheme 2. Suggested mechanism for the formation of nitrohalogenophenoxazin-3-ones[19] **5a**–**c**, **6a**–**c** and **7a**,**b**.

2.2. Microbiological evaluation

The chromogenic substrates **5**–**7** were evaluated for their suitability as nitroreductase substrates for the detection of microbial nitroreductase activity. The reduction of the weakly coloured compounds **5**–**7** by an unspecified nitroreductase was expected to produce the corresponding, intensely coloured, aminophenoxazin-3-ones **16a**–**c**, **17a**–**c** and **18a**,**b**. This would result in a dramatic and readily visualised change in colour at the site of reduction (Scheme 3).



5a-c 7-NO_2 **a** X = F **16a-c** 7-NH_2
6a-c 8-NO_2 **b** X = Cl **17a-c** 8-NH_2
7a,b 9-NO_2 **c** X = Br **18a,b** 9-NH_2

Scheme 3. Expected nitroreductase enzyme activity and reduction of substrates **5a**–**c**, **6a**–**c** and **7a**,**b**. Conditions: (i) bacterial nitroreductase enzymatic activity.

The halogenated nitrophenoxazin-3-ones **5**–**7** were evaluated for their suitability as nitroreductase substrates with a range of pathogenic microorganisms from the National Collection of Type Cultures (London, UK) and from the Freeman Hospital Microbiology Department (Newcastle upon Tyne, UK), including 10 Gram-negative and 8 Gram-positive strains and 2 yeasts (Table 2). All of these microorganisms had previously been shown to express nitroreductases and to have the ability to reduce a nitro-substituted aromatic fluorogenic compound.[11]

these organisms. The most toxic substrate was 8-nitro-1,2,4-trifluorophenoxazin-3-one **6a**, which was universally toxic to all Gram-positive bacteria and the yeasts, while 9-nitro-1,2,4-trifluorophenoxazin-3-one **7a** was toxic to most of these strains and 7-nitro-1,2,4-trifluorophenoxazin-3-one **5a** also exhibited toxicity across many of the Gram-positive bacteria. The growth of the two Enterococcal strains tested and *Listeria monocytogenes* (No. 18) was not inhibited by any of the substrates except **6a**. The trifluorinated derivatives **5a**, **6a** and **7a** were the most toxic compounds in the series.

Most Gram-negative bacteria successfully reduced substrates **5a**–**c** and substrates **7a**,**b**. The enzymatic activity was witnessed by the generation of a red to orange colouration in the bacterial colonies for substrates **5a**–**c**, brown for substrate **7a** and lilac for substrate **7b**. The most encouraging results were obtained with 7-nitro-1,2,4-trifluorophenoxazinone **5a**, as this substrate produced the brightest and most strongly contrasting colour in the bacterial colonies, with minimal diffusion into the medium (Fig. 3). Absence (or low diffusion) of the reduced substrate in the medium is essential for distinction and accurate localization of individual bacterial colonies expressing nitroreductase enzymes when several microorganisms are present in the culture medium.

None of these substrates were reduced at all by *Burkholderia cepacia* (No. 4), which can also metabolise certain nitroaromatic compounds, such as 2,4-dinitrotoluene, by an alternative defined oxidative pathway, leading to oxidative cleavage of the aromatic system.[2] Burkholderia species exhibit high-level intrinsic resistance to many antibacterial agents, even to colistin and gentamicin, due to their particularly impermeable membrane,[23–26] so these substrates may not even reach the cytoplasm, where nitro-

Table 2
Reduction of substrates **5**–**7** by selected clinically relevant Gram-positive and Gram-negative bacteria, and yeasts

Bacterial strain	Substrate[a]	**5a**	**5b**	**5c**	**6a**	**6b**	**6c**	**7a**	**7b**
	Reference	Visualization of colouration after 24 h[b]							
Gram-negative bacteria									
1 *Escherichia coli*	NCTC 10418	+[c]	+	+	–	–	–	+	+
2 *Serratia marcescens*	NCTC 10211	+	+	+	–	–	–	+	+
3 *Pseudomonas aeruginosa*	NCTC 10662	+	+	–	–	–	–	+	–
4 *Burkholderia cepacia*	LMG 1222	–	–	–	–	–	–	–	–
5 *Yersinia enterocolitica*	NCTC 11176	+	–	–	–	–	–	–	+
6 *Salmonella typhimurium*	NCTC 74	+	+	+	–	–	–	+	+
7 *Citrobacter freundii*	Wild type 46262	+	+	–	–	–	–	+	+
8 *Morganella morganii*	Wild type 462403	+	+	+	–	–	–	+	+
9 *Enterobacter cloacae*	NCTC 11936	+	+	+	–	–	–	+	+
10 *Providencia rettgeri*	NCTC 7475	+	+	+	–	–	–	+	+
Gram-positive bacteria									
11 *Bacillus subtilis*	NCTC 9372	NG[d]	–	–	NG	–	–	NG	–
12 *Enterococcus faecalis*	NCTC 775	–	–	–	NG	–	–	–	+
13 *Enterococcus faecium*	NCTC 7171	–	–	–	NG	–	–	–	–
14 *Staphylococcus epidermidis*	NCTC 11047	NG	NG	NG	NG	–	NG	NG	NG
15 *Staphylococcus aureus*	NCTC 6571	NG	NG	NG	NG	–	–	NG	NG
16 *Staphylococcus aureus* (MRSA)	NCTC 11939	NG	–	–	NG	–	–	NG	+
17 *Streptococcus pyogenes*	NCTC 8306	NG	NG	NG	NG	NG	–	NG	NG
18 *Listeria monocytogenes*	NCTC 11994	+	–	–	NG	–	–	–	–
Yeasts									
19 *Candida albicans*	ATCC 90028	–	–	–	NG	–	–	NG	–
20 *Candida glabrata*	NCPF 3943	–	–	–	NG	–	–	–	–

[a] Each substrate was tested at a concentration of 50 mg/L.
[b] Results after 24 h of incubation at 37 °C.
[c] Positive indicates colour observed; negative indicates no colour observed.
[d] NG, no growth.

None of the substrates were toxic to the Gram-negative bacteria and growth was observed in the presence of all substrates; however, toxicity was observed towards some of the Gram-positive bacteria and yeasts, resulting in the inhibition of the growth of

reductase activity would be expected to occur. *B. cepacia* (No. 4) was the only Gram-negative strain that did not reduce 7-nitro-1,2,4-trifluorophenoxazin-3-one **5a** and thus distinguishes this bacterium from the other pathogenic strains tested. This substrate **5a**

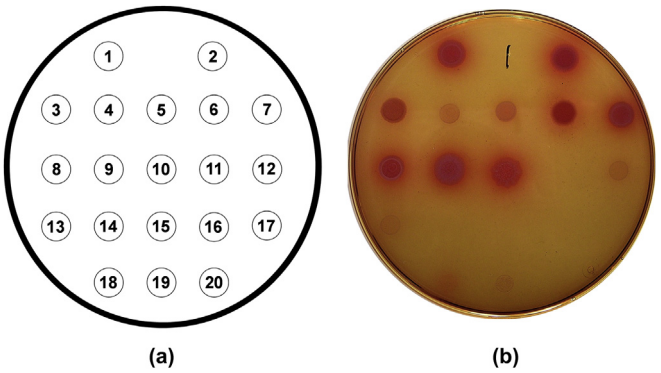


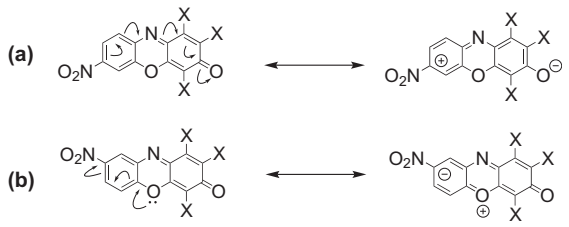
Fig. 3. (a) Inoculation pattern, referenced to the numbers of strains in Table 2; (b) screening of 7-nitro-1,2,4-trifluorophenoxazinone **5a** with 10 Gram-negative and 8 Gram-positive bacterial strains and 2 yeasts.

also differentiates *L. monocytogenes* (No. 18) from the other Gram-positive strains, being the only substrate reduced by this bacterium.

The chlorinated and brominated derivatives of 7-nitropheno xazinone **5b** and **5c** and the chlorinated derivative of 9-nitro phenoxazinone **7b** are able to differentiate MRSA (No. 16) from β-lactam susceptible *Staphylococcus aureus* (No. 15) and *Staphylococcus epidermidis* (No. 14); of these three strains, only MRSA was not inhibited by substrates **5b**, **5c** and **7b**. Although few of the Gram-positive bacteria and the yeasts were unable to reduce the nitro substrates, it was not due to a lack of expression of nitroreductase, as it has been previously shown that they reduce other nitroreductase substrates.[11]

The three 8-nitrophenoxazinone substrates **6a**–**c** were not reduced by nitroreductases from Gram-negative bacteria, unlike their analogues **5a**–**c** and **7a**,**b**, despite good bacterial growth (Table 2). Catalytic reduction of the 8-nitrophenoxazinones **6a**–**c** using hydrogen and either Pd/C or PtO_2, followed by re-oxidation of the initial aminophenol product (**19a**–**c**) with manganese (IV) oxide, produced deeply coloured 8-aminophenoxazinones (**17a**–**c**), of which the most soluble example, 1,2,4-trifluoro-8-aminopheno xazin-3-one **17a**, was isolated and characterized (Scheme 4). The lack of any colour after incubation of the 8-nitrophenoxazinones **6a**–**c** with bacteria thus provides evidence that the three compounds were not substrates for nitroreductase activity.

imine system in the *para*-position. In compounds **7a**,**b**, the 9-nitro group is conjugated at the *ortho*-position to the quinone-imine system and C9 may be considered to be similarly electron deficient to C7 in **5a**–**c**. Conversely, in compounds **6a**–**c**, the 8-nitro group is *para* to the potentially electron releasing ether oxygen. Overall then, the electron density at C8 in the case of substrates **6a**–**c** can be expected to be considerably higher than C7 and C9 in the case of substrates **5a**–**c** and **7a**,**b**, respectively. The electron withdrawing effect of the quinone-imine portion of the phenoxazinone ring affects the 7- and 9-positions primarily and would thus be expected to facilitate the reduction of substrates **5** and **7** (Scheme 5), the oxygen bridging atom can contribute to some extent to the higher electron density at the 8-position, thereby reducing the propensity for reduction of substrates **6a**–**c** (Scheme 5).



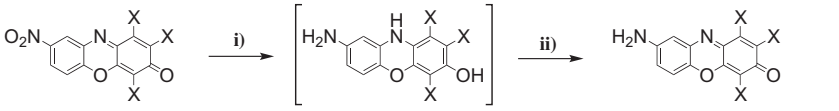
Scheme 5. Key resonance forms that contribute to differentiation of nitroreductase activity in (a) 7-nitrohalogenophenoxazin-3-ones **5a**–**c** and (b) 8-nitrohalogenophenoxazin-3-ones **6a**–**c**.

The ^{13}C NMR data provide support for these electronic differences (Table 3): in compounds **5a**–**c** and **7a**,**b**, the chemical shifts of C7 and C9, respectively, show greater deshielding than C8 of the

Table 3
^{13}C NMR data for C–NO_2 of nitrotrihalogenophenoxazin-3-ones **5a**–**c**, **6a**–**c**, and **7a**,**b**

Compound	Chemical shift of C bearing nitro group (DMSO-d_6; δ/ppm)			
	C–NO_2	**a**X=F	**b**X=Cl	**c**X=Br
5	C7	149.1 (147.9[a])	149.8	149.7
6	C8	145.2 (143.4[a])	145.3	145.3
7	C9	147.8 (146.4[a])	147.8	N.D.[b]

[a] NMR solvent was THF-d_8.
[b] N.D.: not done.



Scheme 4. Catalytic reduction of 8-nitrophenoxazinones **6a**–**c** and oxidation of the resulting aminophenols **19a**–**c** to 8-aminophenoxazinones **17a**–**c**. Reagents and conditions: (i) H_2, Pd/C 10% or PtO_2 (used for the reduction of **6c**), EtOAc/MeOH (1:1); (ii) MnO_2, MeOH.

Early work by McCormick[27] and later studies by Kitamura,[28] testing a wide range of variously substituted nitro compounds for their suitability as nitroreductase substrates, showed that the position of the nitro group with respect to other substituents, alongside their electronic properties, is a determining factor in the bacterial reduction of nitroaromatic compounds. For example, nitroreductase activity was generally higher for nitrobenzenes bearing a *para*-electron withdrawing group. Consideration of the substituents at the *para*-position to the nitro group in each of the present substrates reveals that compounds **5a**–**c**, with the nitro group in the 7-position, have the electron withdrawing quinone-

corresponding halogenated compounds **6a**–**c**.

These observations correlate well with Kitamura's experiments[28] and help to explain the absence of visible reduction for substrates **6a**–**c**, while substrates **5a**–**c** and **7a**,**b** were reduced to some extent.

3. Conclusions

Although some substrate selectivity has been previously observed with the nitroreductase from the food and gastrointestinal bacterium, *Lactobacillus plantarum*,[29] the differences in the

reduction of the substrates, particularly by the bacteria used in the current work, suggest that, rather than detecting a wide range of Gram-negative and Gram-positive microorganisms, nitroreductase activity on substituted 7- or 9-nitrophenoxazinones could be used to distinguish certain pathogenic strains. Furthermore, differences in the nitroreductase enzymes, or in the nitro group metabolic pathways, between strains can be inferred by the variations in reduction of a particular nitroaromatic substrate.

8-Nitro-1,2,4-trihalogenophenoxazin-3-ones **6a**–**c** proved to be unsuitable for the detection of bacterial nitroreductase; the electronic factors associated with the position of the 8-nitro group on the phenoxazin-3-one ring appear to be very unfavourable for enzymatic reduction. These observations correlate with results previously published.[27,28]

7-Nitro-1,2,4-trihalogenophenoxazin-3-ones **5a**–**c** and 9-nitro-1,2,4-trihalogenophenoxazin-3-ones **7a**,**b** showed positive results for the general detection of Gram-negative bacterial nitroreductase, excepting *B. cepacia*, which was distinguished by its lack of reduction of 7-nitro-1,2,4-trifluorophenoxazin-3-one **5a**. Exploration and exploitation of the differences in substrate structure and properties, such as replacement of the halogen atoms by other substituents, could reduce the toxicity of the substrates and improve enzymatic reduction. Such modifications could also assist in distinguishing pathogenic strains by conferring even greater selectivity for the nitroreductase enzymes of particular microorganisms.

4. Experimental section

4.1. General methods

All commercially available reagents and solvents were obtained from Sigma–Aldrich, Fluka or Riedel-de-Haan and were used without further purification. Melting points were recorded on a Reichart–Kofler hot-stage microscope apparatus and are uncorrected. Infrared spectra were recorded in the range 4000–600 cm^{-1} using a Perkin–Elmer Spectrum BX FTIR instrument with internal calibration and a Pike sampling system. NMR spectra were obtained using a Bruker Ultrashield 300 spectrometer at 300 MHz for ^{1}H spectra, 75 MHz for ^{13}C spectra or at 282 MHz for ^{19}F spectra or a Bruker Ultrashield 500 spectrometer at 500 MHz for ^{1}H spectra or at 125 MHz for ^{13}C spectra. High-resolution mass spectra were obtained from the EPSRC National Mass Spectrometry Facility Swansea, using a Thermofisher LTQ Orbitrap XL. Elemental analyses were performed using an Exeter Analytical CE-440 Elemental Analyzer.

4.2. Synthesis of nitrohalogenophenoxazin-3-ones 5–7: general procedure[20,30]

2-Aminonitrophenol **8**–**10** (1 mol equiv), *p*-tetrahalogenobenzoquinone **11a**–**c** (1.1 mol equiv) and sodium acetate (1.2 mol equiv) were suspended in ethanol. The solution was stirred for 24 h or until no more 2-aminonitrophenol was detected by TLC. For the brominated and chlorinated derivatives, a red precipitate progressively appeared as the reaction neared completion. Water was then added to precipitate completely the red solid out of solution. The solid was recovered by filtration and washed with water and a little ethanol, then either recrystallised from acetic acid or purified by column chromatography.

*4.2.1. 7-Nitro-1,2,4-trifluoro-3H-phenoxazin-3-one (**5a**) and 3,10-dinitro-6,13-difluorotriphenodioxazine (**12a**).* The title compounds were prepared from *p*-fluoranil **11a** (1.90 g, 10.57 mmol) and 2-amino-5-nitrophenol **8** (1.42 g, 9.61 mmol) according to the general procedure. Water (100 mL) was added to the reaction mixture and the resulting mixture was extracted with EtOAc (3×60 mL). The emulsion was filtered through a sintered funnel and the insoluble residue was washed several times with EtOAc, giving **12a** as a dark violet solid (1.91 g, 4.63 mmol, 48%). A dark violet microcrystalline solid was obtained from nitrobenzene, mp: 315 °C (dec); (found: $(MH)^+$, 413.0324. Calcd for $C_{18}H_7F_2N_4O_6$: $(MH)^+$, 413.0328); ν_{max}/cm^{-1} 3104 (C–H), 1622 (C=N), 1595 and 1579 (C=C), 1515 and 1332 (NO_2). The aqueous layer was discarded and the combined EtOAc filtrates and layers were washed with water (150 mL) and brine (150 mL), and dried ($MgSO_4$). The solvent was removed in vacuo and the residue subjected to column chromatography on silica, eluting with petroleum ether 60–80 °C/EtOAc (80:20). Nitrophenoxazinone **5a** was isolated as a dark purple solid (0.35 g, 1.19 mmol, 12%). Dark red-purple needle-like crystals were obtained from glacial acetic acid, mp: 218–220 °C; (found: C, 48.5; H, 1.05; N, 9.15%. $C_{12}H_3F_3N_2O_4$ requires C, 48.7; H, 1.0; N, 9.5%); (found: $(MH)^+$, 297.0118. Calcd for $C_{12}H_4F_3N_2O_4$: $(MH)^+$, 297.0118); ν_{max}/cm^{-1} 3104 (C–H), 1660 (C=O), 1596 (C=C), 1531 and 1312 (NO_2), 1003 (C–F); δ_H (300 MHz, THF-d_8) 6.41 (1H, d, J=8.7 Hz, 9-H), 6.58 (1H, dd, J=8.7, 2.4 Hz, 8-H), 6.74 (1H, d, J=2.4 Hz, 6-H); δ_C (75 MHz, THF-d_8) 110.3 (CH, 6-C), 118.6 (CH, 8-C), 129.4 (CH, 9-C), 130.1 (quat, ddd, J=8.5, 5.2, 1.4 Hz, 10a-C), 134.1 (quat, 9a-C), 136.0 (quat, dd, J=262.7, 6.1 Hz, 4-C), 141.3 (quat, dt, J=273.4, 6.9 Hz, 2-C), 142.2 (quat, dd, J=273.4, 12.2 Hz, 1-C), 141.4 (quat, d, J=1.5 Hz, 5a-C), 141.7 (quat, ddd, J=18.1, 4.8, 1.2 Hz, 4a-C), 147.9 (quat, 7-C), 166.7 (quat, td, J=21.4, 5.4 Hz, 3-C); δ_F (282 MHz, THF-d_8) −282.17 (1F, t, J=2.3 Hz, 4-F), −271.89 (1F, dd, J=9.3, 2.0 Hz, 2-F), −265.41 (1F, dd, J=9.3, 2.0 Hz, 1-F); δ_H (300 MHz, DMSO-d_6) 8.23 (1H, d, J=8.7 Hz, 9-H), 8.31 (1H, dd, J=8.7, 2.4 Hz, 8-H), 8.54 (1H, d, J=2.4 Hz, 6-H); δ_C (75 MHz, DMSO-d_6) 112.3 (CH, 6-C), 120.8 (CH, 8-C), 131.4 (CH, 9-C), 132.1 (quat, dd, J=9.4, 3.0 Hz, 10a-C), 135.4 (quat, 9a-C), 136.0 (quat, dd, J=262.7, 6.1 Hz, 4-C), 142.6 (quat, dt, J=268.2, 6.1 Hz, 2-C), 143.6 (quat, dd, J=270.4, 11.7 Hz, 1-C), 142.5 (quat, 5a-C), 141.7 (quat, ddd, J=18.1, 4.8, 1.2 Hz, 4a-C), 149.1 (quat, 7-C), 168.9 (quat, td, J=20.3, 5.6 Hz, 3-C); δ_F (282 MHz, DMSO-d_6) −280.43 (1F, s, 4-F), −268.27 (1F, d, J=11.9 Hz, 2-F), −263.04 (1F, d, J=11.3 Hz, 1-F).

*4.2.2. 7-Nitro-1,2,4-trichloro-3H-phenoxazin-3-one (**5b**).*[31] The title compound was prepared from *p*-chloranil **11b** (3.52 g, 14.32 mmol) and 2-amino-5-nitrophenol **8** (1.97 g, 12.78 mmol); **5b** was isolated as a purple solid (4.06 g, 11.75 mmol, 90%). Purple lustrous plates of **5b** were obtained from glacial acetic acid; mp: 239–241 °C [lit.: 238–240 °C[31] (from AcOH)]; (found: C, 41.7; H, 0.9; N, 7.9%. $C_{12}H_3Cl_3N_2O_4$ requires: C, 41.7; H, 0.9; N 8.1%); (found: $(MH)^+$, 344.9226. Calcd for $C_{12}H_3^{35}Cl_3N_2O_4$: $(MH)^+$, 344.9231; found: $(MH)^+$, 346.9195. Calcd for $C_{12}H_3^{35}Cl_2^{37}ClN_2O_4$: $(MH)^+$, 346.9202; found: $(MH)^+$, 348.9162. Calcd for $C_{12}H_3^{35}Cl^{37}Cl_2N_2O_4$: $(MH)^+$, 348.9172); ν_{max}/cm^{-1} 3094 (C–H), 1646 (C=O), 1584 (C=C), 1519 and 1347 (NO_2); δ_H (300 MHz, DMSO-d_6) 8.24 (1H, d, J=8.7 Hz, 9-H), 8.32 (1H, dd, J=8.7, 2.4 Hz, 8-H), 8.51 (1H, d, J=2.4 Hz, 6-H); δ_C (75 MHz, DMSO-d_6) 111.6 (quat, 4-C), 112.7 (CH, 6-C), 121.5 (CH, 8-C), 132.1 (CH, 9-C), 136.6 (quat, 5a-C), 137.2 (quat, 1-C or 2-C), 138.5 (quat, 1-C or 2-C), 143.5 (quat, 9a-C), 146.4 (quat, 2× C, 4a-C and 10a-C), 149.8 (quat, 7-C), 171.0 (quat, 3-C).

*4.2.3. 7-Nitro-1,2,4-tribromo-3H-phenoxazin-3-one (**5c**).* The title compound was prepared from *p*-bromanil **11c** (1.03 g, 2.42 mmol) and 2-amino-5-nitrophenol **8** (0.34 g, 2.20 mmol); **5c** was isolated as a bright red solid (1.10 g, 2.30 mmol, 95.0%). A microcrystalline dark red solid of **5c** was obtained from glacial acetic acid, mp: 263–264 °C; (found: C, 30.1; H, 0.7; N, 5.6%. $C_{12}H_3Br_3N_2O_4$ requires: C, 30.1; H, 0.6; N 5.85%); (found: $(MH)^+$, 476.7714. Calcd for $C_{12}H_3^{79}Br_3N_2O_4$: $(MH)^+$, 476.7716; found: $(MH)^+$, 478.7692. Calcd for $C_{12}H_3^{79}Br_2^{81}BrN_2O_4$: $(MH)^+$, 478.7696; found: $(MH)^+$, 480.7669. Calcd for $C_{12}H_3^{79}Br^{81}Br_2N_2O_4$: $(MH)^+$, 480.7676; found: $(MH)^+$, 482.7646. Calcd for $C_{12}H_3^{81}Br_3N_2O_4$: $(MH)^+$, 482.7658); ν_{max}/cm^{-1} 3101 (C–H), 1641 (C=O), 1529 and 1346 (NO_2); δ_H (300 MHz,

DMSO-d_6) 8.21 (1H, d, J=9.0 Hz, 9-H), 8.29 (1H, dd, J=8.7, 2.4 Hz, 8-H), 8.44 (1H, d, J=2.4 Hz, 6-H); δ_C (75 MHz, DMSO-d_6) 103.2 (quat, 4-C), 112.5 (CH, 6-C), 121.4 (CH, 8-C), 131.9 (CH, 9-C), 134.9 (quat, 1-C or 2-C), 137.0 (quat, 1-C or 2-C), 137.1 (quat, 5a-C), 143.6 (quat, 9a-C), 147.2 (quat, 4a-C or 10a-C), 148.3 (quat, 4a-C or 10a-C), 149.7 (7-C), 171.5 (quat, 3-C).

*4.2.4. 8-Nitro-1,2,4-trifluoro-3H-phenoxazin-3-one (**6a**).* The title compound was prepared from *p*-fluoranil **11a** (1.51 g, 8.39 mmol) and 2-amino-4-nitrophenol **9** (1.27 g, 8.22 mmol) according to the general procedure. The precipitate was taken into EtOAc (100 mL) and the resulting mixture filtered through a sintered funnel. The insoluble residue was washed several times with EtOAc and discarded. The combined organic filtrates were washed with 10% aq NaOH solution (4×100 mL) and water (1×100 mL), and dried ($MgSO_4$). The solvent was removed under reduced pressure and the residue subjected to column chromatography, eluting with a gradient mixture of petroleum ether 60–80 °C/Et_2O (50:50–0:100). Compound **6a** was isolated as a red solid (0.94 g, 3.17 mmol, 38.5%). An orange crystalline solid of **6a** was obtained from glacial acetic acid, mp: 212–214 °C; (found: C, 48.6; H, 1.05; N, 9.1%. $C_{12}H_3F_3N_2O_4$ requires C, 48.7; H, 1.0; N, 9.5%); ν_{max}/cm^{-1} 3093 (C–H), 1648 (C=O), 1608 (C=C), 1518 and 1308 (NO_2), 1004 (C–F); δ_H (300 MHz, THF-d_8) 6.03 (1H, d, J=9.3 Hz, 6-H), 6.80 (1H, dd, J=9.0, 2.7 Hz, 7-H), 7.06 (1H, d, J=2.7 Hz, 9-H); δ_C (75 MHz, THF-d_8) 115.4 (CH, 6-C), 123.8 (CH, 9-C), 126.2 (CH, 7-C), 129.9 (quat, ddd, J=9.6, 5.1, 1.3 Hz, 10a-C), 130.2 (quat, 9a-C), 136.0 (quat, dd, J=262.4, 6.5 Hz, 4-C), 141.3 (quat, dt, J=272.7, 6.9 Hz, 2-C), 142.2 (quat, dd, J=273.9, 12.0 Hz, 1-C), 140.8 (quat, ddd, J=18.3, 5.5, 1.1 Hz, 4a-C), 143.4 (quat, 8-C), 145.3 (quat, d, J=1.6 Hz, 5a-C), 166.8 (quat, td, J=21.0, 5.6 Hz, 3-C); δ_F (282 MHz, THF-d_8) −282.05 (1F, t, J=2.8 Hz, 4-F), −271.73 (1F, dd, J=9.6, 1.7 Hz, 2-F), −266.07 (1F, dd, J=9.3, 2.5 Hz, 1-F); δ_H (300 MHz, DMSO-d_6) 7.93 (1H, d, J=9.0 Hz, 6-H), 8.56 (1H, dd, J=9.0, 2.4 Hz, 7-H), 8.76 (1H, d, J=2.4 Hz, 9-H); δ_C (75 MHz, DMSO-d_6) 118.4 (CH, 6-C), 125.9 (CH, 9-C), 129.0 (CH, 7-C), 132.1 (quat, 9a-C), 132.5 (quat, dd, J=8.8, 5.2 Hz, 10a-C), 137.8 (quat, dd, J=256.3, 6.45 Hz, 4-C), 143.2 (quat, dt, J=267.75, 6.8 Hz, 2-C), 144.2 (quat, dd, J=270.4, 11.9 Hz, 1-C), 142.8 (quat, d, J=14.1 Hz, 4a-C), 145.2 (quat, 8-C), 147.3 (quat, 5a-C), 169.5 (quat, td, J=20.0, 4.9 Hz, 3-C); δ_F (282 MHz, DMSO-d_6) −279.65 (1F, s, 4-F), −268.13 (1F, d, J=11.3 Hz, 2-F), −264.22 (1F, d, J=11.8 Hz, 1-F).

*4.2.5. 8-Nitro-1,2,4-trichloro-3H-phenoxazin-3-one (**6b**).*[21] The title compound was prepared from *p*-chloranil **11b** (1.74 g, 7.07 mmol) and 2-amino-4-nitrophenol **9** (0.991 g, 6.43 mmol); **6b** was isolated as a red solid (2.19 g, 6.34 mmol, 99%). Red plates of **6b** were obtained from glacial acetic acid; mp: 261–263 °C [lit.: 248–250 °C[21] (from acetone)]; (found: C, 41.7; H, 0.9; N, 7.9%. $C_{12}H_3Cl_3N_2O_4$ requires: C, 41.7; H, 0.9; N 8.1%); found: $(MH)^+$, 344.9229. Calcd for $C_{12}H_3^{35}Cl_3N_2O_4$: $(MH)^+$, 344.9231; found: $(MH)^+$, 346.9197. Calcd for $C_{12}H_3^{35}Cl_2^{37}ClN_2O_4$: $(MH)^+$, 346.9202; found: $(MH)^+$, 348.9164. Calcd for $C_{12}H_3^{35}Cl^{37}Cl_2N_2O_4$: $(MH)^+$, 348.9172); ν_{max}/cm^{-1} 3088 (C–H), 1649 (C=O), 1597 (C=C), 1510 and 1331 (NO_2); δ_H (300 MHz, DMSO-d_6) 7.95 (d, 1H, J=9.0 Hz, 6-H), 8.57 (dd, 1H, J=9.0, 2.7 Hz, 7-H), 8.73 (d, 1H, J=2.7 Hz, 9-H); δ_C (75 MHz, DMSO-d_6) 111.8 (quat, 4-C), 118.3 (CH, 6-C), 125.9 (CH, 9-C), 129.1 (CH, 7-C), 132.6 (quat, 9a-C), 137.2 (quat, 1-C or 2-C), 138.2 (quat, 1-C or 2-C), 145.3 (quat, 8-C), 145.6 (quat, 4a-C or 10a-C), 146.2 (quat, 4a-C or 10a-C), 147.7 (quat, 5a-C), 171.1 quat, 3-C).

*4.2.6. 8-Nitro-1,2,4-tribromo-3H-phenoxazin-3-one (**6c**).*[21] The title compound was prepared from *p*-bromanil **11c** (4.05 g, 9.57 mmol) and 2-amino-4-nitrophenol **9** (1.34 g, 8.70 mmol). Elution with petroleum ether 60–80 °C/EtOAc (60:40) gave **6c** as a red solid (3.01 g, 6.29 mmol, 72%). Lustrous cardinal red crystals of **6c** were obtained from glacial acetic acid, mp: 285–287 °C [lit.: 287 °C[21] (from acetone)]; found: C, 30.1; H, 0.6; N, 5.6%. $C_{12}H_3Br_3N_2O_4$ requires: C, 30.1; H, 0.6; N 5.85%; found: $(MH)^+$, 476.7716. Calcd for $C_{12}H_3^{79}Br_3N_2O_4$: $(MH)^+$, 476.7716; found: $(MH)^+$, 478.7694. Calcd for $C_{12}H_3^{79}Br_2^{81}BrN_2O_4$: $(MH)^+$, 478.7696; found: $(MH)^+$, 480.7670. Calcd for $C_{12}H_3^{79}Br^{81}Br_2N_2O_4$: $(MH)^+$, 480.7676; found: $(MH)^+$, 482.7646. Calcd for $C_{12}H_3^{81}Br_3N_2O_4$: $(MH)^+$, 482.7658); ν_{max}/cm^{-1} 3078 (C–H), 1638 (C=O), 1581 (C=C), 1508 and 1328 (NO_2); δ_H (300 MHz, DMSO-d_6) 7.82 (1H, d, J=9.0 Hz, 6-H), 8.47 (1H, dd, J=9.3, 2.7 Hz, 7-H), 8.60 (1H, d, J=2.7 Hz, 9-H); δ_C (75 MHz, DMSO-d_6) 103.4 (quat, 4-C), 118.1 (CH, 6-C), 125.6 (CH, 9-C), 129.0 (CH, 7-C), 133.0 (quat, 9a-C), 134.8 (quat, 1-C or 2-C), 136.5 (quat, 1-C or 2-C), 145.3 (quat, 8-C), 146.5 (quat, 4a-C or 10a-C), 147.9 (quat, 4a-C or 10a-C), 148.2 (quat, 5a-C), 171.6 (quat, 3-C).

*4.2.7. 9-Nitro-1,2,4-trifluoro-3H-phenoxazin-3-one (**7a**).* The title compound was prepared from *p*-fluoranil **11c** (0.90 g, 5.00 mmol) and 2-amino-3-nitrophenol **10** (0.74 g, 4.82 mmol) according to the general procedure. The precipitate was taken into EtOAc (100 mL) and the resulting mixture filtered through a sintered funnel. The insoluble residue was washed several time with EtOAc and discarded. The combined organic filtrates were washed with water (1×100 mL), brine (1×100 mL) and dried ($MgSO_4$). The solvent was removed under reduced pressure and the residue subjected to column chromatography, eluting with petroleum ether 60–80 °C/EtOAc (80:20). Compound **7a** was isolated as a dark red solid (0.22 g, 0.73 mmol, 15%). Ruby red prisms of **7a** with a metallic lustre were obtained from glacial acetic acid, mp: 216–218 °C; (found: C, 48.5; H, 1.0; N, 9.2%. $C_{12}H_3F_3N_2O_4$ requires C, 48.7; H, 1.0; N, 9.5%); (found: $(MH)^+$, 297.0118. Calcd for $C_{12}H_4F_3N_2O_4$: $(MH)^+$, 297.0118); ν_{max}/cm^{-1} 3084 (C–H), 1649 (C=O), 1603 (C=C), 1530 and 1313 (NO_2), 1004 (C–F); δ_H (300 MHz, THF-d_8) 6.04–6.13 (m, 3H, 6-H, 7-H and 8-H); δ_C (75 MHz, THF-d_8) 117.6 (CH, 8-C), 117.9 (CH, 6-C), 123.0 (quat, 9a-C), 130.0 (quat, ddd, J=10.0, 5.0, 1.2 Hz, 10a-C), 131.5 (CH, 7-C), 135.8 (quat, dd, J=261.9, 6.3 Hz, 4-C), 140.7 (quat, ddd, J=18.2, 4.8, 1.2 Hz, 4a-C), 141.4 (quat, dt, J=273.2, 6.8 Hz, 2-C), 141.5 (quat, d, J=1.7 Hz, 5a-C), 142.1 (quat, dd, J=274.7, 12.2 Hz, 1-C), 146.4 (quat, 9-C), 166.7 (quat, td, J=21.1, 5.5 Hz, 3-C); δ_F (282 MHz, THF-d_8) −282.53 (1F, t, J=2.3 Hz, 4-F), −271.43 (1F, dd, J=9.0, 2.3 Hz, 1-F), −265.36 (1F, dd, J=9.3, 2.3 Hz, 2-F); δ_H (300 MHz, DMSO-d_6) 7.95 (1H, t, J=8.4 Hz, 7-H), 8.01 (1H, dd, J=8.4, 1.8 Hz, 8-H), 8.08 (1H, dd, J=7.5, 1.8 Hz, 6-H); δ_C (75 MHz, DMSO-d_6) 120.5 (CH, 8-C), 120.7 (CH, 6-C), 124.8 (quat, 9a-C), 132.8 (quat, dd, J=9.0, 4.5 Hz, 10a-C), 134.6 (CH, 7-C), 137.5 (quat, dd, J=255.0, 6.0 Hz, 4-C), 142.7 (quat, ddd, J=17.3, 4.5, 1.5 Hz, 4a-C), 143.2 (quat, d, J=0.8 Hz, 5a-C), 143.3 (quat, dt, J=267.8, 7.5 Hz, 2-C), 144.0 (quat, dd, J=270.0, 10.5 Hz, 1-C), 147.8 (quat, 9-C), 169.4 (quat, td, 21.0, 6.0 Hz, 3-C); δ_F (282 MHz, DMSO-d_6) −280.98 (1F, t, J=2.3 Hz, 4-F), −268.59 (1F, dd, J=11.0, 2.3 Hz, 1-F), −262.77 (1F, dd, J=11.0, 2.3 Hz, 2-F).

*4.2.8. 9-Nitro-1,2,4-trichloro-3H-phenoxazin-3-one (**7b**).* The title compound was prepared from *p*-chloranil **11c** (1.17 g, 7.61 mmol) and 2-amino-3-nitrophenol **10** (1.07 g, 6.92 mmol); **7b** was isolated as a dark purple solid (1.08 g, 3.13 mmol, 45%). Lustrous deep purple needles of **7b** were obtained from glacial acetic acid, mp: 254–258 °C; (found: C, 41.6; H, 0.9; N, 7.8%. $C_{12}H_3Cl_3N_2O_4$ requires C, 41.7; H, 0.9; N, 8.1%); (found: $(MH)^+$, 344.9227. Calcd for $C_{12}H_3^{35}Cl_3N_2O_4$: $(MH)^+$, 344.9231; found: $(MH)^+$, 346.9197. Calcd for $C_{12}H_3^{35}Cl_2^{37}ClN_2O_4$: $(MH)^+$, 346.9202; found: $(MH)^+$, 348.9165. Calcd for $C_{12}H_3^{35}Cl^{37}Cl_2N_2O_4$: $(MH)^+$, 348.9172); ν_{max}/cm^{-1} 3100 (C–H), 1647 (C=O), 1590 (C=C), 1532 and 1331 (NO_2); δ_H (300 MHz, DMSO-d_6) 7.97 (1H, t, J=8.4 Hz, 7-H), 8.02 (1H, dd, J=8.4, 2.1 Hz, 8-H), 8.09 (1H, dd, J=7.5, 2.1 Hz, 6-H); δ_C (75 MHz, DMSO-d_6) 111.2 (quat, 4-C), 120.6 (CH, 8-C), 120.8 (CH, 6-C), 125.3 (quat, 9a-C), 134.6 (CH, 7-C), 136.9 (quat, 1-C or 2-C), 138.6 (quat, 1-C or 2-C),

143.7 (quat, 5a-C), 145.5 (quat, 4a-C or 10a-C), 146.3 (quat, 4a-C or 10a-C), 147.8 (quat, 9-C), 171.0 (quat, 3-C).

4.3. Catalytic hydrogenation of 8-nitrohalogenophenoxazin-3-ones 6a–c: general procedure

Nitro-1,2,3-trihalogeno-3*H*-phenoxazin-3-ones **6a–c** were dissolved in a solvent mixture of EtOAc/MeOH (1:1) and either Pd/C 10% or PtO_2 (10% of the reactant mass) was added to the solution. The reaction mixture was then hydrogenated for 2–20 h, the initial hydrogen pressure being set at 2.4 bars. Hydrogenation was continued until the hydrogen pressure was steady, after which the catalyst was removed from the reaction mixture by filtration through Celite 451. The Celite cake was washed several times with methanol. MnO_2 (3 mol equiv) was added to the clear solution and the resulting mixture stirred until no more amino-3-hydroxyphenoxazine was observed by TLC. The reaction mixture changed to a deep colour, ranging from blue to blue-violet, and was again filtered through Celite 451 to remove inorganic insoluble materials and the cake was washed several times with THF, until the filtrate was clear of colour. The solvent mixture was evaporated in vacuo and the residue was subjected to column chromatography on silica.

*4.3.1. 8-Amino-1,2,4-trifluoro-3H-phenoxazin-3-one (**17a**).* The title compound was prepared from 8-nitro-1,2,3-trifluoro-3H-phenoxazin-3-one **6a** (0.38 g, 1.29 mmol) using Pd/C 10%. Elution used a gradient mixture of petroleum ether (60–80 °C)/EtOAc (30:70–10:90). 8-Amino-1,2,4-trifluoro-3*H*-phenoxazin-3-one **17a** was isolated as a dark violet solid (0.34 g, 1.27 mmol, 98.0%); mp: 277 °C (dec); (found: C, 54.2; H, 2.1; N, 10.1%. $C_{12}H_5F_3N_2O_2$ requires C, 54.15; H, 1.9; N, 10.5%); (found: $(MH)^+$, 267.0373. Calcd for $C_{12}H_6F_3N_2O_2$: $(MH)^+$, 267.0376); ν_{max}/cm^{-1} 3503, 3358 and 3230 (NH_2), 1638 (C=O), 1610, 1575 and 1513 (C=C), 1002 (C–F); δ_H (500 MHz, TFA-d_1) 8.17 (1H, d, J=9.0 Hz, 6-H), 8.35 (1H, dd, J=9.0, 2.0 Hz, 7-H), 8.74 (1H, d, J=1.5 Hz, 9-H); δ_C (125 MHz, TFA-d_1) 119.1 (CH, 6-C), 125.7 (CH, 9-C), 127.1 (quat, 9a-C), 129.4 (CH, 7-C), 132.5 (quat, dd, J=16.5, 3.9 Hz, 10a-C), 132.8 (quat, 8-C), 138.2 (quat, dd, J=263.5, 5.4 Hz, 4-C), 141.6 (quat, dd, J=18.0, 7.8 Hz, 4a-C), 143.9 (quat, dt, J=275.0, 5.4 Hz, 2-C), 144.2 (quat, 5a-C), 144.4 (quat, dd, J=279.2, 13.2 Hz, 1-C), 172.3 (quat, td, J=19.0, 6.6 Hz, 3-C).

4.4. Microbiological methods

Test compounds **5–7** were dissolved in a small volume of *N*-methylpyrrolidone (100 μL) and Tween 20 (100 μL) and incorporated into molten Columbia agar (Oxoid, Basingstoke) to give a final concentration of 50 mg/L; the use of a surfactant (Tween 20) was necessary to avoid precipitation of the substrates during incorporation into the agar medium. The resulting chromogenic media were poured into Petri dishes and each solution dried to a gel. The resultant agar plates were each inoculated with 10 Gram-negative, 8 Gram-positive bacteria and 2 yeasts (from the National Collection of Type Cultures, London, UK): colonies of each strain were suspended in sterile deionised water to generate a suspension with a turbidity equivalent to 0.5 McFarland units (approximately 1.5×10^8 colony forming units per mL), as confirmed with a densitometer. One microlitre of this suspension was inoculated onto the agar plates containing the various substrates using an automated multipoint inoculator. All inoculated plates were incubated at 37 °C for 24 h and then examined visually for the presence of growth and colouration of bacterial colonies.

Conflict of interest

No conflicts of interest are noted. The synthetic chemistry and characterisation were conducted at the University of Sunderland; the microbiological evaluation was carried out (by Alexandre Bedernjak) at Freeman Hospital (Newcastle upon Tyne, UK); the analysis and rationalisation of data were the responsibility of the team at University of Sunderland; the final decision to submit these data and the rationalisation for publication were taken by bioMérieux.

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