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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

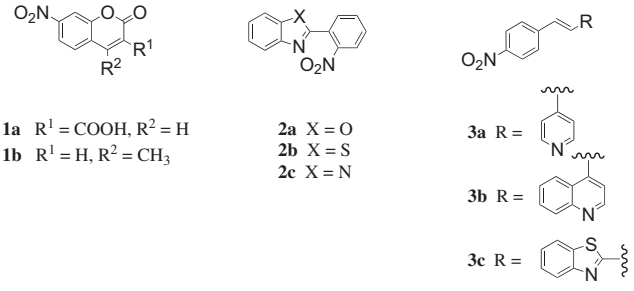

Fig. 1. Examples of established fluorogenic and chromogenic nitroreductase substrates.

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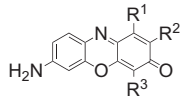
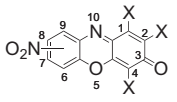
fluorescent signal upon exposure to UV light (λ=365 nm) after incubation with the bacteria. Moreover, other fluorogenic compounds, such as the 4-nitrobenzylcarbamate derivatives of 7-aminocoumarins,[13] and derivatives of nitrobenzoxazole **2a**, nitrobenzothiazole **2b** and nitrobenzimidazole **2c** (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

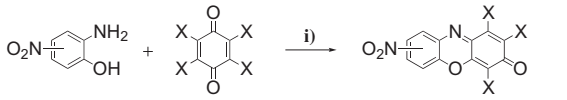
Fig. 2. 7-Aminophenoxazin-3-one chromogens **4** and nitrotrihalogenophenoxazinones **5**–**7**.

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

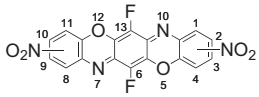
5a-c 7-NO_2
6a-c 8-NO_2
7a,b 9-NO_2
a X = F; **b** X = Cl; **c** X = Br

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**

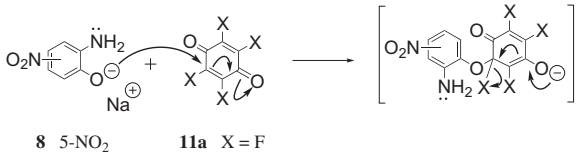
Compound	Nitro group position	X	Yield (%)
5a	7-	F	12
5b	7-	Cl	90
5c	7-	Br	95
6a	8-	F	39
6b	8-	Cl	99
6c	8-	Br	72
7a	9-	F	15
7b	9-	Cl	45

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]



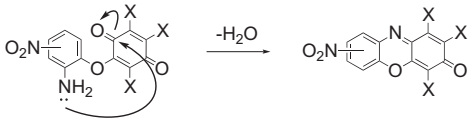
12a 3,10-NO_2
12b 2,9-NO_2
12c 1,8-NO_2

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**.