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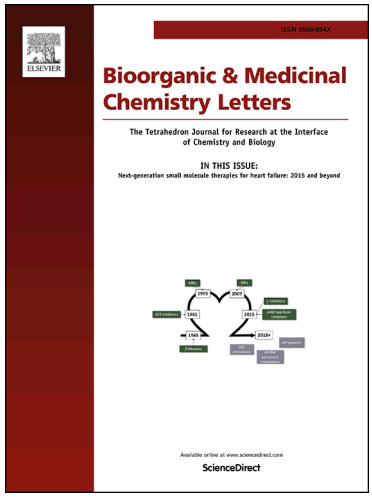

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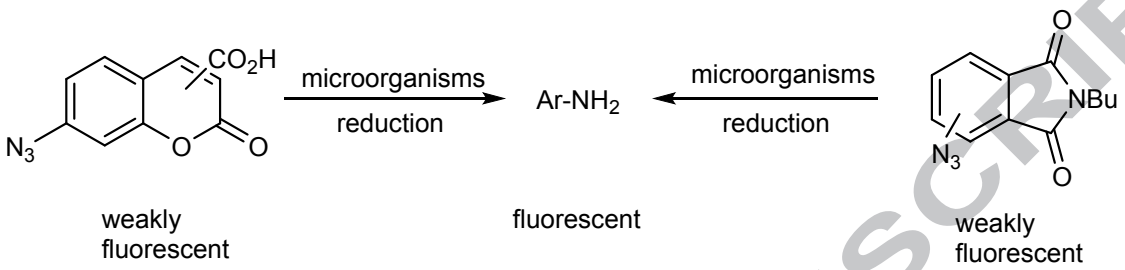

Fluorogenic 7-azidocoumarin and 3/4-azidophthalimide derivatives as indicators of reductase activity in microorganisms.

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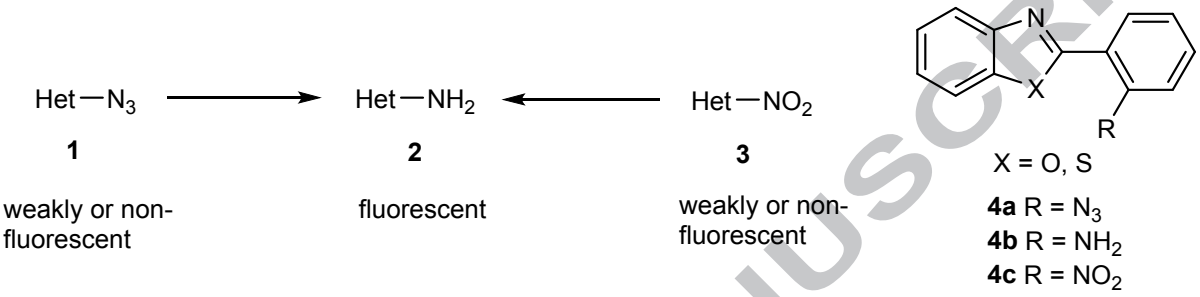
Abstract: A series of fluorogenic heterocyclic azides were prepared and assessed as reductase substrates across a selection of Gram-negative and Gram-positive microorganisms. The majority of these azides showed similar activity profiles to nitroreductase substrates. Microorganisms that do not produce hydrogen sulfide reduced the azides, indicating reductase activity was not linked to hydrogen sulfide production.

Keywords: fluorogenic substrates, fluorogenic azides, nitroreductase, microorganism detection.

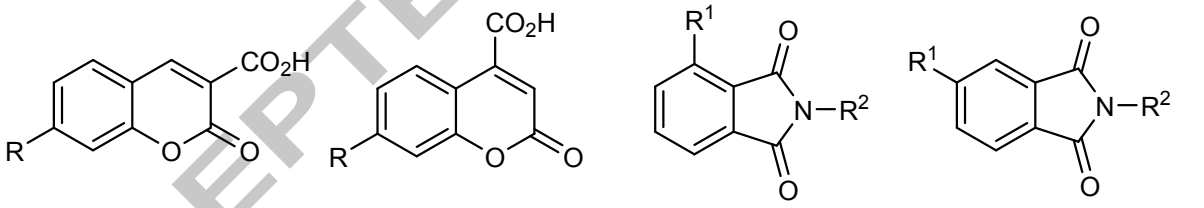
A tremendous number of fluorogenic heterocyclic azides **1** have been described in the literature as reagents for H_2S detection in biological and environmental samples.[1] Reduction of the weakly or non-fluorescent azides **1** by H_2S gives fluorescent amines **2** thus producing a definitive 'off-on' response (Scheme 1). Current methods of H_2S detection in microorganisms tend to rely on a visible colour change when an appropriate metal salt *e.g.* lead acetate[2] or ferric ammonium citrate[3] reacts with the H_2S producing a black precipitate. A more sensitive non-visual approach to H_2S detection in microorganisms using static headspace – multi-capillary column – ion mobility spectrometry (SHS-MCC-GC-IMS) has recently been reported.[4]

We have previously described the application of nitroaromatic compounds **3** for the detection of nitroreductase activity in clinically important microorganisms.[5,6] These substrates, based mainly upon the 2-(2-nitrophenyl)benzoxazole and 2-(2-nitrophenyl)benzothiazole cores **4c** (X = O, S), produced highly fluorescent amines **4b** (X = O, S) upon microbial reduction (Scheme 1). We were therefore interested in comparing the activity profiles of azides **1** with their corresponding nitroreductase substrates **3** in order to establish whether any reduction of these azides is linked to microbial H_2S production.



Scheme 1. Detection of reductase activity (Het = heterocycle)

The azide derivatives chosen for study were the isomeric 7-azidocoumarins **5a** and **6a**, the 3-azidophthalimides **7a** and **8a** and the 4-azidophthalimides **9a** and **10a** (Figure 1). It was anticipated that if these potential non-fluorescent azido probes underwent microbial reduction, then the corresponding fluorescent amines **5b-10b** respectively would be formed resulting in an 'off-on' sensor system.



5a R = N_3
5b R = NH_2
5c R = NO_2

6a R = N_3
6b R = NH_2
6c R = NO_2

	R^1	R^2
7a	N_3	n-Bu
7b	NH_2	n-Bu
7c	NO_2	n-Bu
8a	N_3	CH_2CO_2H
8b	NH_2	CH_2CO_2H
8c	NO_2	CH_2CO_2H

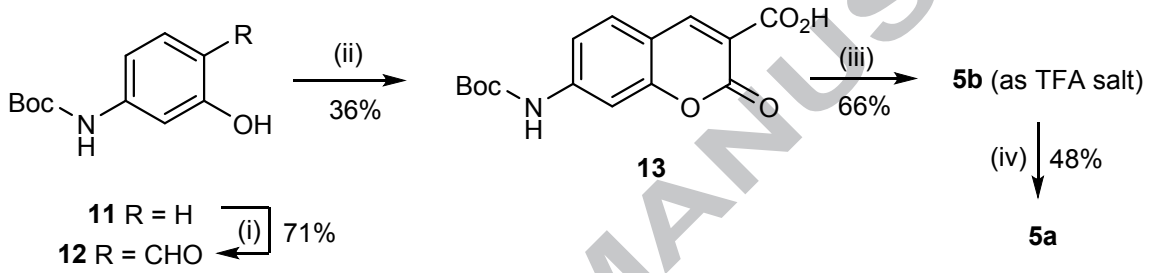
	R^1	R^2
9a	N_3	n-Bu
9b	NH_2	n-Bu
9c	NO_2	n-Bu
10a	N_3	CH_2CO_2H
10b	NH_2	CH_2CO_2H
10c	NO_2	CH_2CO_2H

Figure 1. Structures of the coumarins and phthalimides prepared in this study.

During the course of this work, a six-step synthesis of 7-aminocoumarin-3-carboxylic acid **5b** from 4-amino-2-hydroxybenzoic acid was reported in 2016 from which the azide **5a** was subsequently prepared.[7] More recently (2017), a three-step synthesis of azide **5a** commencing from 4-bromosalicylaldehyde was described.[8] Amide derivatives of azide **5a** have been evaluated as biological probes for H_2S[7,8] and amide derivatives of azide **6a** have been converted into thiol-

detecting reagents.[9] The 3-azidophthalimide derivative **7a** has been prepared from the amine **7b** and subsequently evaluated as a fluorescent probe for imaging H_2S in nasopharyngeal carcinoma cell lines.[10] A series of *N*-substituted 4-azidophthalimide derivatives have been described as photo-affinity labels[11] and cross-linking spin-label reagents.[12] To our knowledge, none of these azides **5a-10a** or their derivatives have been evaluated for applications in diagnostic microbiology.

Our synthesis of azide **5a** followed the novel route outlined in Scheme 2. The Boc-protected 3-aminophenol **11**[13] was formylated giving the salicylaldehyde derivative **12**.[14] Treatment of compound **12** with Meldrum's acid afforded the coumarin derivative **13** from which the Boc-group was removed by treatment with trifluoroacetic acid (TFA) yielding 7-aminocoumarin-3-carboxylic acid **5b** as its TFA salt. Diazotisation of compound **5b** followed by reaction of the resulting diazonium salt with sodium azide produced the required azide **5a**. Amine **6b**, and hence azide **6a**, were prepared using similar methods to those already described.[9]



Scheme 2. Synthesis of azide **5a**. Reagents and conditions: (i) paraformaldehyde, $MgCl_2$, Et_3N, CH_3CN, reflux, 48 h; (ii) Meldrum's acid, EtOH, reflux, 18 h; (iii) CF_3CO_2H, rt, 3 h; (iv) (a) HCl/H_2SO_4, H_2O, $NaNO_2$, 0 °C, 15 min. then (b) NaN_3, NaOAc, H_2O, 0 °C, 5 min.

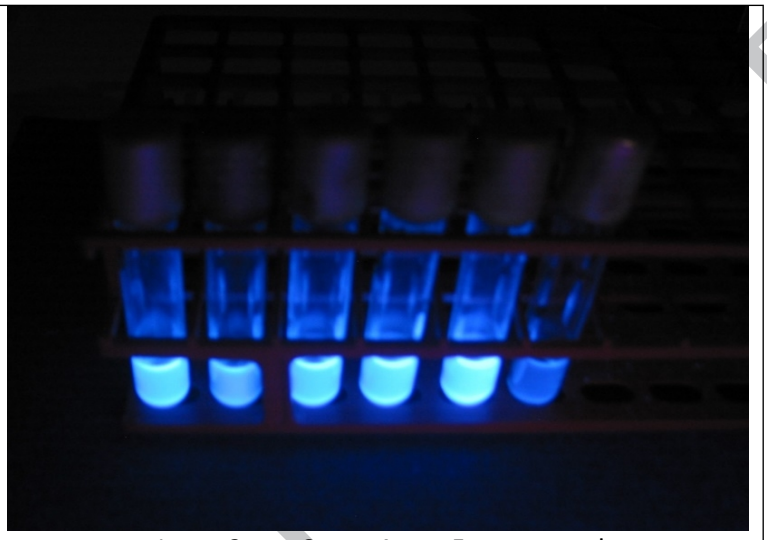
Diazotisation of amine **9b**[15] followed by treatment of the resulting diazonium salt with sodium azide produced the azido derivative **9a** (38% yield). Heating 3- or 4-nitrophthalic anhydride with glycine in the melt afforded the phthalimides **8c** (26%) and **10c** (74%) respectively which were then reduced (H_2/Pd cat.) to the corresponding fluorescent amines **8b** (51%) and **10b** (96%). These two amines were then converted into the required azides **8a** (80%) and **10a** (87%) in the usual manner. Also prepared was the known azide **4a** (X = S)[16] from amine **4b**. The nitrocoumarin **6c** was synthesised to enable a comparison with its azide analogue **6a**. Heterocycle **6c** was prepared by oxidation of the ethyl ester of amine **6b**[9] using a urea-hydrogen peroxide complex in TFA (31%) followed by hydrolysis of the ester group (56%).

As noted in the introduction, fluorogenic azides **1** have been frequently used to detect H_2S. A preliminary study using azide **5a** was therefore conducted in Nutrient Broth (supplemented with sodium thiosulfate as a sulfur source)[2] in order to determine whether microorganisms could be identified as either producers or non-producers of H_2S (Figure 2). This could be particularly useful for the differentiation of *Salmonella* (H_2S positive) and *Escherichia coli* (H_2S negative) because when testing for *Salmonella* in stool samples (*e.g.* as a result of food poisoning), *Escherichia coli* is also typically present as part of the normal gut flora in large numbers.

Five representative microorganisms were chosen of which two were expected to be H_2S negative and three H_2S positive. All five microorganisms produced a strong fluorescence response compared to the microorganism-free control (right-hand tube in Figure 2). Thus, the non-producers of H_2S,

Escherichia coli and *Enterobacter cloacae*, are capable of reducing the azide **5a** suggesting that a different mode of reduction is operative.

	Microorganism/ reference[a]	**Expected H_2S reaction**
1	*Escherichia coli* NCTC 10418	Negative
2	*Enterobacter cloacae* NCTC 11936	Negative
3	*Salmonella typhimurium* NCTC 74	Positive
4	*Salmonella enteritidis* NCTC 6676	Positive
5	*Citrobacter freundii* NCTC 9750	Positive



1 2 3 4 5 control

[a] NCTC: National Collection of Type Cultures

Figure 2. Fluorescence generated by 5 microorganisms in Nutrient Broth supplemented with sodium thiosulfate in the presence of substrate **5a** after 18 hours at 37 °C. Substrate concentration = 50 mg L^{-1}, sodium thiosulfate concentration = 6.8 g L^{-1}, inoculum = 1000 colony-forming units (CFU). Tubes viewed under UV light (365 nm).

Following on from our previous studies on nitroreductase substrates,[5,6] the activity profiles of azides **5a-10a** were evaluated against a panel of clinically important Gram-negative and Gram-positive microorganisms in Columbia agar medium. The azide **4a** (X = S) was not sufficiently soluble in Columbia agar medium. The protocol for carrying out these studies has been described previously[5,6] and the results are presented in Table 1. In both the presence and absence of substrates, the Gram-negative and Gram-positive microorganisms showed good and moderate growth respectively in Columbia agar, thus indicating these substrates were not inhibitory to microorganism growth. Two yeasts were also included in this study (*Candida albicans* and *Candida glabrata*) but their growth was generally poor, both in the presence and absence of substrates, and neither of these yeasts produced any fluorescent response with the selection of substrates (data not shown). Also included in Table 1 for comparison are three nitroreductase substrates; compounds **4c** (X = S),[5] **5c**[17] and **6c**. None of the nitrophthalimide derivatives **7c-10c** produced fluorescent responses with any of the microorganisms listed in Table 1 despite these compounds being non-inhibitory to microorganism growth (data not shown). This may be associated with their lack of uptake by the microorganisms.

		Azide substrates					**Nitroreductases**		
Entry	**Substrate**	**5a**	**6a**	**7a**	**8a**	**9a**	**4c (X = S)**	**5c**	**6c**
	Microorganism / Reference[a]	**Fluorescence[b]**							
	Gram-negative								

	microorganisms								
1	*Escherichia coli* NCTC 10418	++ blue	+ yellow	++ green	+ green	+/- green	++ blue	++ blue	++ yellow
2	*Raoultella planticola* NCTC 9528[c]	+ blue	+ yellow	++ green	+ green	+ green	++ blue	++ blue	++ yellow
3	*Providencia rettgeri* NCTC 7475	+ blue	-	+ green	+ green	+ green	++ blue	++ blue	++ yellow
4	*Enterobacter cloacae* NCTC 11936	++ blue	+ yellow	++ green	+ green	+ green	++ blue	++ blue	++ yellow
5	*Serratia marcescens* NCTC 10211	++ blue	+ yellow	++ green	+ green	+ green	++ blue	++ blue	++ yellow
6	*Salmonella typhimurium* NCTC 74	+ blue	-	+/- green	+/- green	-	++ blue	++ blue	trace
7	*Pseudomonas aeruginosa* NCTC 10662	+ green	-	-	+/- green	+/- green	-	+/- green	-
8	*Yersinia enterocolitica* NCTC 11176	+/- blue	-	-	+/- green	+/- green	-	+ blue	-
9	*Burkholderia cepacia* NCTC 10743	+/- blue	-	++ green	+/- green	+ green	++ blue	+/- blue	trace
10	*Acinetobacter baumannii* NCTC 12156	++ blue	-	++ green	+ green	+ green	++ blue	+ blue	++ yellow
	Gram-positive microorganisms								
11	*Streptococcus pyogenes* NCTC 8306	-	-	-	-	-	-	-	-
12	*Staphylococcus aureus* (MRSA) NCTC 11939	+ blue	-	+ green	-	+ green	++ blue	++ blue	-
13	*Staphylococcus aureus* (MSSA) NCTC 6571	+ blue	-	+ green	-	+ green	++ blue	++ blue	-
14	*Staphylococcus epidermidis* NCTC 11047	-	-	-	-	-	trace	+ blue	-
15	*Listeria monocytogenes* NCTC 11994	-	-	-	-	-	-	++ blue	trace
16	*Enterococcus faecium* NCTC 7171	-	-	-	-	-	-	+ blue	trace
17	*Enterococcus faecalis* NCTC 775	-	-	-	-	-	-	+ blue	trace
18	*Bacillus atrophaeus* ATCC 9372[d]	-	-	-	-	-	-	+ blue	-

[a] NCTC: National Collection of Type Cultures. ATCC: American Type Culture Collection.
[b] ++ strong fluorescence, + moderate fluorescence, +/- weak fluorescence, - no fluorescence.
[c] Formerly named *Klebsiella pneumoniae* NCTC 9528.
[d] Formerly named *Bacillus subtilis* ATCC 9372.

Table 1. Fluorescence generated by microorganisms in the presence of azide and nitroreductase substrates in Columbia agar medium after 18 hours at 37 °C. Substrate concentration = 100 mg L^{-1}, inoculum = 100,000 CFU/spot. Plates viewed under UV light (365 nm).

It is evident from Table 1 that the azide substrates **5a-9a** are giving fluorescent responses mainly with Gram-negative microorganisms and this profile is typical of several nitroreductase substrates, *e.g.* compound **4c** (X = S) and other unpublished nitroaromatics. Of these Gram-negative microorganisms, only *Salmonella typhimurium* is a recognised H_2S producer and, with the exception

of substrate **5a**, the remaining selection of azide substrates showed either no fluorescence response or a weak fluorescence response with this microorganism. This strongly suggests that when a fluorescent response is observed as a consequence of reductase activity, H_2S is not involved in the reduction process.[18] There are obvious similarities between the profiles of the azide substrate **5a** and the corresponding nitroreductase substrate **5c** with the Gram-negative microorganisms. The isomeric azide substrate **6a** was not as effective as compound **5a** across the range of Gram-negative microorganisms with only four of the ten Gram-negative microorganisms producing a fluorescence response. The nitroreductase substrate **6c** gave additional strong fluorescence responses with *Providencia rettgeri* and *Acinetobacter baumannii* compared to its azide analogue **6a**.

The three azidophthalimide substrates **7a-9a** exhibited broadly similar activity profiles with the Gram-negative microorganisms but some minor differences in positive/negative fluorescence responses were observed with *S. typhimurium, Pseudomonas aeruginosa* and *Yersinia enterocolitica* where fluorescence production was either absent or weak.

Within the selection of Gram-positive microorganisms depicted in Table 1, significant fluorescence production is generally (but not always) seen with both methicillin-resistant (MRSA) and methicillin-susceptible (MSSA) strains of *Staphylococcus aureus* in the presence of nitroreductase substrates. This is exemplified by nitroreductase substrates **4c** (X = S), **5c** and other unpublished nitroaromatic compounds. The azides **5a**, **7a** and **9a** are also showing fluorescence responses for *S. aureus*.

In conclusion, reduction of the azide substrates is not attributed to the presence of H_2S. These substrates show broadly similar reductase profiles to nitroreductase substrates across the range of microorganisms studied.

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