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Structure requirements for anaerobe processing of azo compounds: Implications for prodrug design

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ABSTRACT

This Letter generalizes the metabolism of the azo class of compounds by *Clostridium perfringens*, an anaerobe found in the human colon. A recently reported 5-aminosalicylic acid-based prednisolone prodrug was shown to release the drug when incubated with the bacteria, while the *para*-aminobenzoic acid (PABA) based analogue did not. Instead, it showed a new HPLC peak with a relatively close retention time to the parent which was identified by LCMS as the partially reduced hydrazine product. This Letter investigates azoreduction across a panel of substrates with varying degrees of electronic and steric similarity to the PABA-based compound. Azo compounds with an electron donating group on the azo-containing aromatic ring showed immediate disproportionation to their parent amines without any detection of hydrazine intermediates by HPLC. Compounds containing only electron withdrawing groups are partially and reversibly reduced to produce a stable detectable hydrazine. They do not disproportionate to their parent amines, but regenerate the parent azo compound. This incomplete reduction is relevant to the design of azo-based prodrugs and the toxicology of azo-based dyes.

Azo compounds are characterized by one or more R_1–N=N–R_2 bonds. Over 2000 azo dyes are used in the textile, paper, food, cosmetic and pharmaceutical industries. Ingested azo compounds are metabolized by azoreductases in the gastrointestinal tract, skin and liver to their component aromatic amines.[1,2] This has important implications for the safety of some azo-based dyes.[1] The activation of anti-inflammatory agents such as olsalazine, sulfasalazine and balsalazide by colonic microflora continues to inspire efforts to target various drugs to the colon using prodrug and related azoreductase sensitive polymer approaches.

Azoreductases are flavin mononucleotide (FMN)-dependent enzymes whose biological role is not fully understood. Recent research has suggested that they are involved in resistance to thiol-specific stress.[3] There have been numerous investigations into the mechanism of azoreduction, and a hydrazine intermediate has long been suspected.[4,5] The first evidence for hydrazine intermediacy in the reduction process came from findings that administered azobenzene can be partially recovered in the urine as hydrazobenzene.[6] It was later shown that electron-donating groups *ortho*- or *para*-to the azo linkage cause instability of the hydrazo-intermediates and their immediate disproportionation to the parent dye and reduced amine metabolites.[7,8] Spectroscopic evidence was reported for the formation of a hydrazine during reduction of azo-containing polyurethane films.[9,10] The presence of a hydrazine intermediate of azo dyes has been detected in reaction mixtures with *Rhodobacter sphaeroides*.[11]

It has been suggested that azoreduction occurs via a ping pong bi bi mechanism.[5,12] This involves two cycles of NADPH-dependent reduction of FMN to $FMNH_2$, reducing the azo substrate to a hydrazine intermediate in the first cycle, and reducing the hydrazine to two amines in the second cycle. There has been some progress in recent years on elucidating the mechanism of azoreduction using X-ray crystallography of *Pseudomonas aeruginosa* azoreductase paAzoR1 bound to the azo prodrug balsalazide, which suggested that it is the balsalazide hydrazone rather than the azo tautomer which is reduced.[13,14]

We recently reported a novel 5-aminosalicylic acid (5-ASA) double prodrug approach that exploits azoreductase to deliver prednisolone to the colon (**1a**,**b**, Fig. 1a).[15] The role of the 5-ASA group in the design is to depress enteral absorption and to act as a trigger point for azoreductase activity, rather than for its pharmacological actions, there being a significant potency/efficacy disparity between the two agents. At the outset of this work we were interested in examining possibilities for other pedant groups besides 5-ASA that could be used to modulate prodrug pharmaceutical properties, without influencing pharmacological activity. We synthesized the *para*-aminobenzoic acid (PABA) analogue of the 5-ASA compound (**1c**) and some related PABA-based compounds

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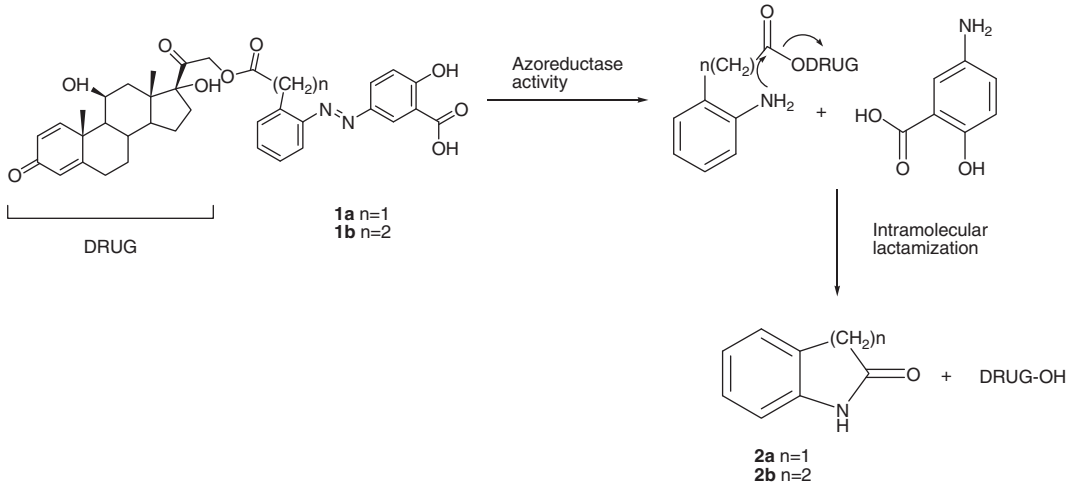


Figure 1a. Prednisolone conjugates **1a**,**b** and their activation mechanism.[15]

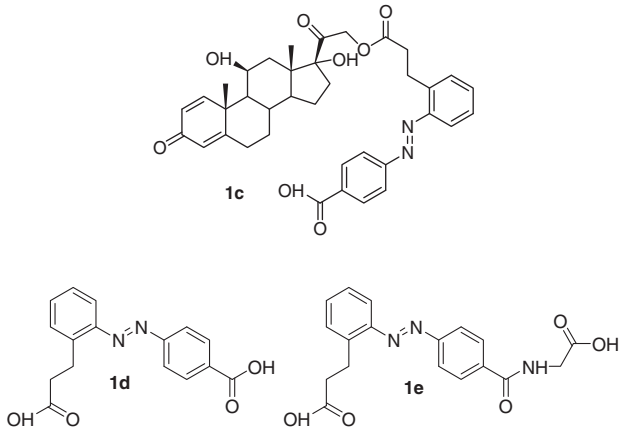
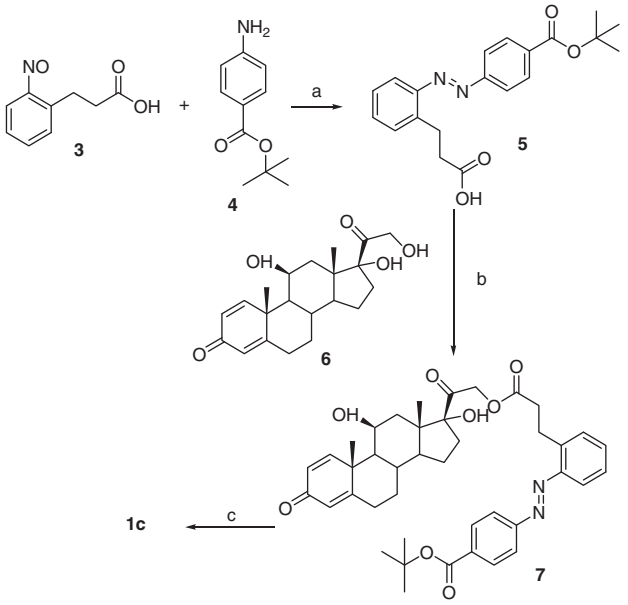


Figure 1b. PABA compounds: PABA-based prednisolone analogue (**1c**). Aminopropionic acid azo conjugates with PABA (**1d**) and aminohippuric acid (**1e**).

(**1d–e**, Fig. 1b). **1c** was tested for its capacity to release the steroid in the presence of cultures of *Clostridium perfringens*, an anaerobe found in the human colon. Unexpectedly, it did not release prednisolone. Instead we observed an unknown peak by HPLC with a relatively close retention time to the parent. The related PABA-based compounds **1d–e** showed a similar behavior. In this Letter, we generalize the behavior of this azo class in the presence of *C. perfringens* using a panel of substrates with varying degrees of steric/electronic similarity to these incompletely-releasing PABA-based compounds. We are able to make observations about the metabolism of these substrates by anaerobic colonic bacteria and the implications for azo-based prodrug development.

The synthesis of **1a**,**b** was carried out previously.[15] The approach to **1c** followed the same synthetic logic but incorporating PABA into the active phenylethyl unit rather than 5-ASA (Scheme 1). This revolved around nitroso-amine condensation between **3** and **4** at room temperature to yield **5**. This chemistry was required as coupling with diazonium salts of aminophenyl propionic acid esters had not been successful in our hands. Protection of the acid component of **4** as the *tert*butyl ester prevented competition during the attachment of the carrier group later in the sequence. Esterification of **5** with DCC and DMAP was used to attach prednisolone (**6**) at the 21-OH. Finally, removal of the *tert*-butyl group of **7** afforded **1c**.[16] Related PABA-based compound **1d** was synthesized by removal of the *tert*butyl group of **5** using TFA



Scheme 1. Synthetic route to **1c**. Reagents: (a) AcOH; (b) DMAP, DCC in THF; (c) TFA, DCM.

in DCM. Compound **1e** was produced by condensation between nitroso compound **3** and 4-aminohippuric acid in glacial acetic acid.

A previously reported *C. perfringens* assay was used for analysis of reduction.[17] This organism was chosen as it is widely distributed in the human colon; has high levels of an azoreductase that exhibits wide cross immunoreactivity with other human flora azoreductase; it can process substrate without the need for additional cofactors; and its azoreductase is secreted, obviating the need for cell fractionation.[17–22] Compounds **1a**,**b** were consumed rapidly under these assay conditions.[17] In contrast to **1a**,**b**, PABA analogue **1c** did not produce prednisolone or the expected DHQ byproduct. Instead, **1c** (15.6 min) disappearance was incomplete (55% at 6 h) and this appeared to be related to the formation of an earlier eluting compound (14.8 min) which later disappeared, regenerating the parent (Fig. 2). The distinctive strongly absorbing PDA UV spectrum between 300 and 400 nm associated with the extended azo chromophore was not evident in the transient product. When the samples were reanalysed immediately by LCMS (Figs. 3a and b) the transient compound could be identified as the hydrazine

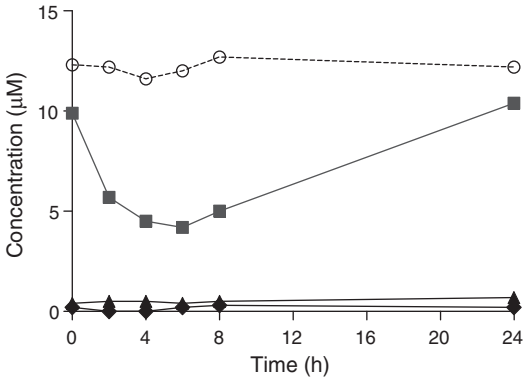


Figure 2. Progress curve for **1c** (■) in the presence of *C. perfringens* culture (37 °C) and formation of prednisolone (▲), DHQ (▼). Also shown is **1c** in BHI (○).

(643.3040 amu) resulting from partial hydrogenation of **1c** (641.2883 amu) (Fig. 3c).

Azo compounds can exhibit cis- and trans isomerism.[23,24] As **1c** eluted as two peaks of similar mass in the LCMS chromatogram (Figs. 3a and b), we concluded that the two peaks corresponded to cis/trans stereoisomers. The cis-isomer, which possesses a greater dipole moment would be expected to appear at lower retention time in a RP-HPLC system. Interestingly, the greater ratio of the cis- to trans-isomer in the 6 h sample suggests that the trans-isomer was reduced to the hydrazine rather than the cis-isomer.

We initially thought that the divergent behavior of the PABA and 5-ASA-based compound as azoreductase substrates was due to an enzymatic preference for the *meta* carboxylate in **1a**,**b** rather than the *para* group in **1c**. Therefore the corresponding methyl and ethyl esters of **1c** were prepared. The PABA-based azo compounds **1d** and related **1e** were also prepared and tested to assess the role of the steroid in the incomplete reduction of the steroidal analogues. Each of these compounds showed a similar behavior to **1c**: incomplete reduction with LCMS evidence of reversible introduction of a single molecule of H_2 (data not shown).

In order to investigate the behavior of the *C. perfringens* azoreductase and SAR of substrates, it was decided to diversify our panel of azo compounds. The poor substrates at this point had in common a PABA group linked through an azo bond to an alkyl benzene; they lacked an electron-donating group on the azo-linked benzene ring. With this in mind, two groups of substituted azobenzene-based substrates were synthesized (Table 1):

Group A: Those containing an electron-donating group on the benzene ring (**8a**–**f**).

Group B: Those without a strong electron donor group on the benzene ring (**9a**–**e**).

Compounds **8a**–**c** were synthesized using a diazonium coupling method (Scheme 2).[25] Compounds **8d**, **9a**–**e** were synthesized via a nitroso-based intermediate using a previously described procedure (Scheme 3).[26] The component amine was oxidized using Oxone® and the intermediate condensed with an aryl amine in glacial acetic acid to form the substituted azobenzene. The nitroso-based chemistry allows for the formation of azobenzene-based substrates containing only electron-withdrawing substituents on the benzene

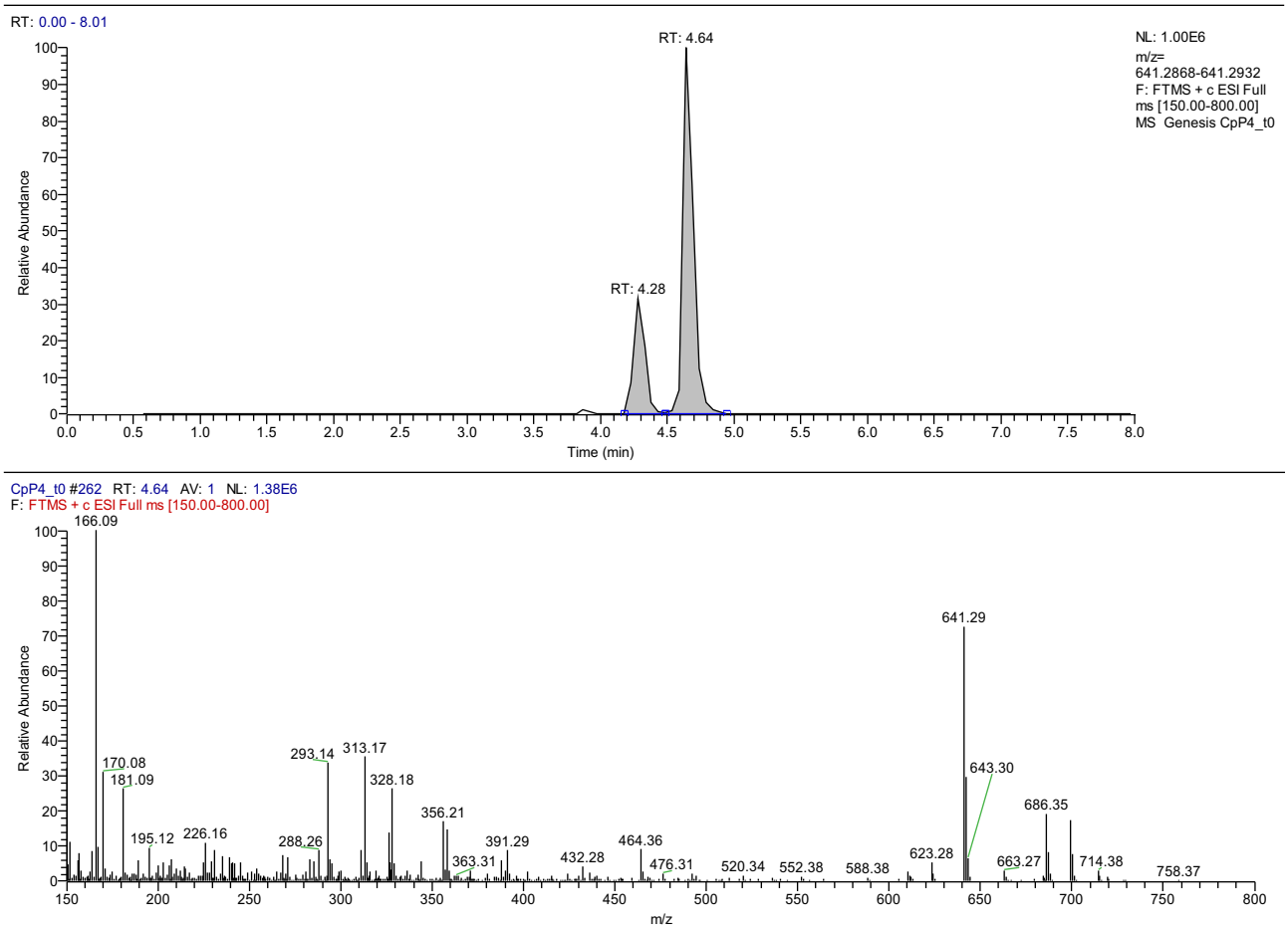


Figure 3a. LCMS chromatogram of **1c** in *C. perfringens* culture at 0 h. Compound **1c** retention time 4.28 and 4.64 min.

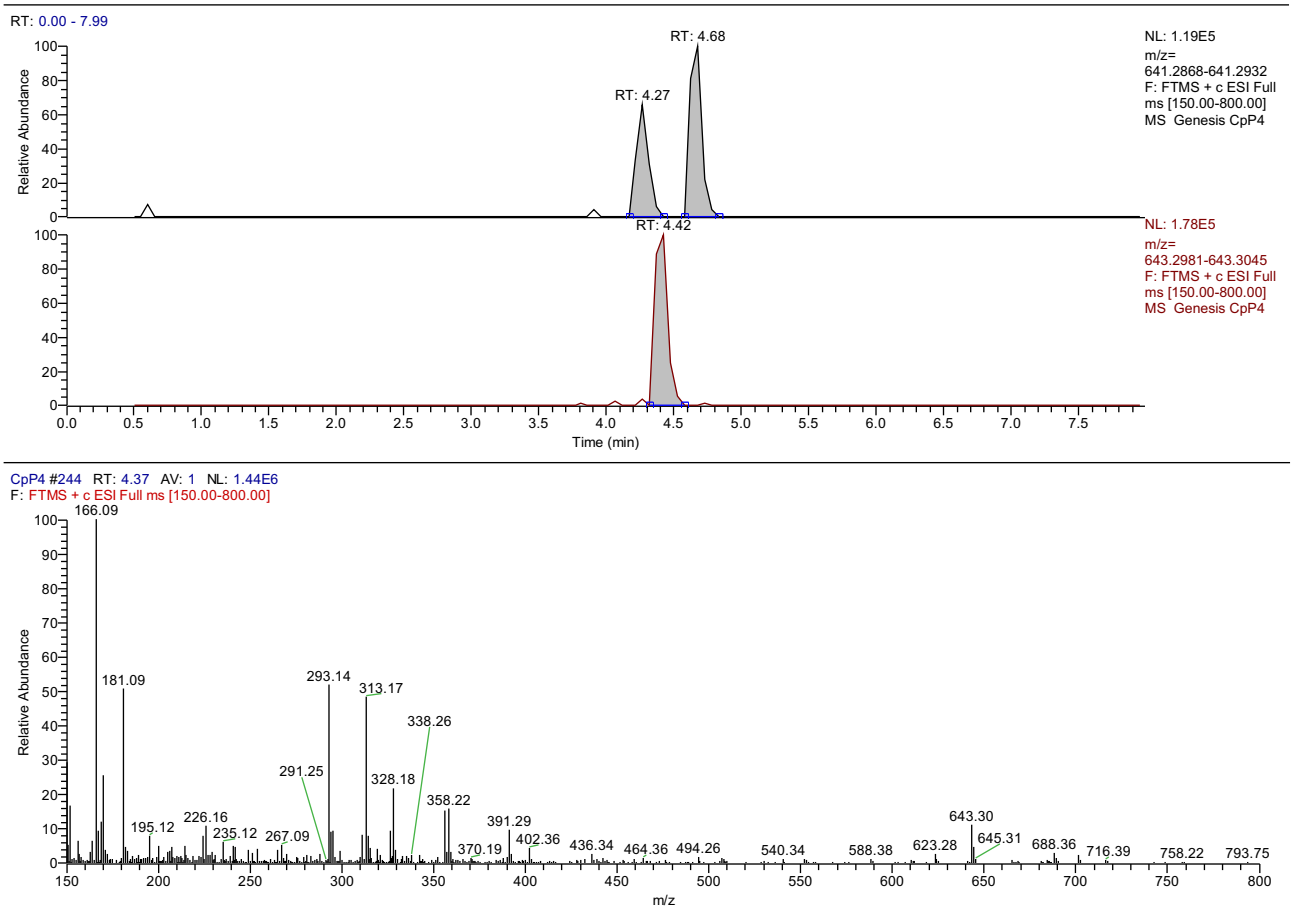


Figure 3b. LCMS chromatogram of **1c** in *C. perfringens* culture at 6 h. Compound **1c** retention time 4.28 and 4.64 min; hydrazine peak 4.42 min.

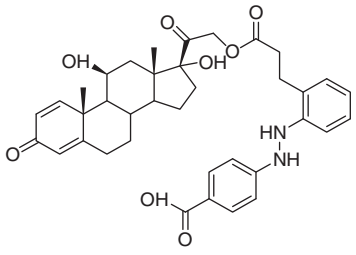
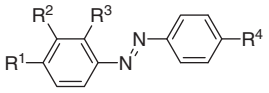


Figure 3c. Hydrazine from partial reduction of **1c** by *C. perfringens*.

ring whereas the more conventional diazonium chemistry leads to products substituted with at least one electron donor group.

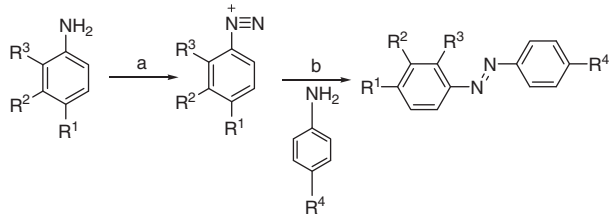
The azobenzene-based compounds were incubated with *C. perfringens* and showed evidence of stereospecific reduction of trans-substrates, similar to that found for **1c** (Figs. 3a and b). Compounds in solution that underwent prolonged exposure to light developed a smaller, earlier eluting peak that exhibited a shorter λ_{max} in the PDA UV spectrum than that of the original substrate (270–290 nm compared to 320–340 nm). It is known that ultraviolet light can cause photoisomerization of azobenzenes and that the cis-isomers display a shorter λ_{max}.[23,27] This suggests that the new peak was a result of partial conversion to the cis-isomer. After incubation for 4 h, it was found that the trans-isomer peaks were affected by the azoreductase activity while the cis-peaks remained unaffected. In order to avoid complication by cis-isomers, test compounds were dissolved immediately before incubation and protected from light during analysis.

Table 1
Azobenzene-based azo compounds

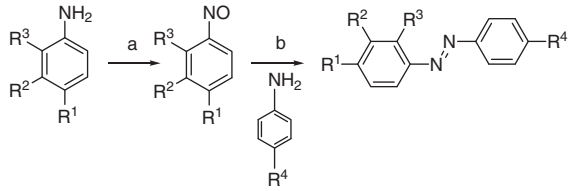


	R^1	R^2	R^3	R^4	Synthetic method
8a	NO_2	H	H	OH	Scheme 2
8b	COOH	H	H	OH	Scheme 2
8c	Br	H	H	OH	Scheme 2
8d	CN	H	H	OH	Scheme 3
8e	H	H	H	$N(CH_3)_2$	—
8f	H	H	COOH	$N(CH_3)_2$	—
9a	H	COOH	H	H	Scheme 3
9b	H	H	H	COOH	Scheme 3
9c	Br	H	H	H	Scheme 3
9d	H	H	H	CN	Scheme 3
9e	H	H	H	CF_3	Scheme 3

Group A compounds were completely consumed in the presence of *C. perfringens* within 4 h (data not shown). Group B compounds (except **9e**) showed a similar pattern of behavior to **1c** when analysed by HPLC that is, incomplete consumption of the azo compounds coupled with the formation of an earlier eluting peak which later disappeared, regenerating the parent (Figs. 4a and b). The published literature on azoreductase activity[28] suggested that the reduction of the azo group was carried out only partially, reversibly producing a hydrazine which was reoxidized



Scheme 2. Diazonium-coupling synthesis of substituted azobenzenes. Reagents and conditions: (a) $NaNO_2$, HCl, 0–5 °C; (b) NaOH, 0–5 °C.



Scheme 3. Nitroso-based synthesis of substituted azobenzenes. Reagents: (a) Oxone®, DCM/H_2O; (b) AcOH.

in situ and/or on standing for analysis. In order to demonstrate this reversibility, samples containing hydrazine peaks were allowed to stand in air for one week and reanalysed. The hydrazine peaks were absent in HPLC chromatograms and the azo peaks were regenerated, indicating auto-oxidation of the hydrazine compounds on exposure to air. To further prove the identity of the HPLC peak, compound **9c** was chemically reduced to form its hydrazo-derivative **10c** (Scheme 4). Compound **10c** eluted with the same retention time as the hydrazine peak formed after incubation of **9c** with *C. perfringens*.

Hydrazine peak formation was associated with Group B substrates **9a**,**d** only, that is, those that do not contain a strong electron donor group on either aromatic ring linked by the azo bond. This suggests that the *C. perfringens* azoreductase completed a two electron transfer to the azo group to form the partially reduced hydrazine intermediate. The second electron transfer stage of reduction did not occur. Instead, the reaction spontaneously reversed with reformation of the parent azo compound. The interpretation of these results must take into account what is already known about hydrazine chemistry and the mechanism of azoreductase.

Compounds resembling Group A have been shown to generate unstable hydrazo-intermediates in the presence of various preparations. Numerous studies have attempted to detect and investigate the stability of the hydrazine intermediates of azo reduction

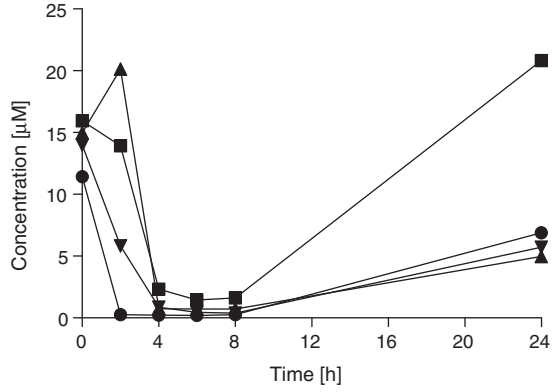


Figure 4a. Partial reduction and re-oxidation of **9a** (■), **9b** (▲), **9c** (▼), **9d** (•) in the presence of *C. perfringens*.

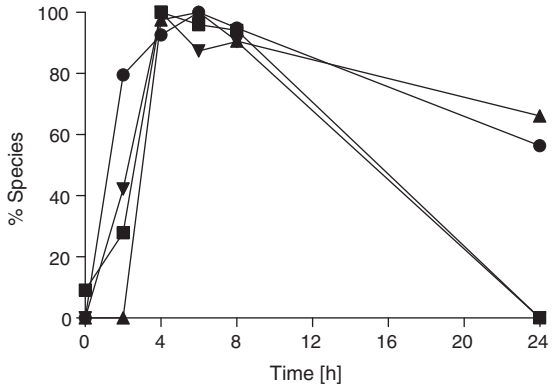
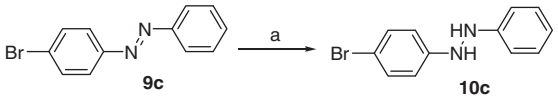


Figure 4b. Formation and reoxidation of hydrazine intermediates **10a** (■), **10b** (▲), **10c** (▼), **10d** (•) from the corresponding azo compounds.



Scheme 4. Chemical reduction of **9c** to form 4-bromohydrazobenzene (**10c**). Reagents: (a) Zn dust, EtOH, AcOH.

via both enzymatic and chemical methods. Bin et al. detected and identified the hydrazine intermediate of methyl red during reduction by the phototrophic bacterium *Rhodobacter sphaeroides* using MS/MS.[11] The low MS abundance suggested that reduction from azo to a hydrazo intermediate in that case might be a rate limiting step with the unstable hydrazine being reduced quickly after formation. Using rat caecal bacteria, with a different set of substrates, Saphier and Karton demonstrated that disappearance of azo compounds by HPLC was faster than appearance of their anilinic products.[29] The intermediates in this work were not identified. Chemically generated hydrazo-derivatives of Group A type compounds were shown to be detectable only at pH >12 with immediate disproportionation upon neutralization towards a pH expected at enzymatic conditions.[30] Group B type compounds on the other hand have demonstrated a much more stable hydrazo-intermediate.[8] This stability has been attributed to the lack of electron donating groups on the aromatic ring.[31]

The findings of the above studies are largely consistent with our observations. Following partial reduction associated with *C. perfringens*, Group A compounds containing an electron donating group showed immediate disproportionation to the parent amines without hydrazine accumulation. Group B compounds **9a**,**d**, which lack an electron donating group produce a stable and readily detectable hydrazine. Instead of disproportionation, these lose a molecule of H_2 to regenerate the more conjugated azo parent. Therefore, the inability of *C. perfringens* associated azoreductase to cause complete reduction of Group B substrates can be attributed to the stability of their corresponding hydrazines towards symmetrical scission rather than any intrinsic substrate preference of the enzyme. Conversely, Group A substrate hydrazo-intermediates spontaneously decay in a non-enzymatic reaction rather than requiring a second two-electron transfer from azoreductase. This is in agreement with a recent discussion by Burger and Stolz.[32]

Compound **9e** which lacks the capacity to tautomerise belongs to group B. HPLC analysis showed degradation of the azo compound with transient appearance of a hydrazine peak, consistent with the other Group B compounds. However in the case of **9e** there was no observable regeneration of the parent azo compound. This behavior may be related to the nature of the *para*-trifluoromethyl substituent, which has been shown to have a weakly

electron donating effect on the benzene ring.[33] This substituent effect appears to promote disproportionation of the hydrazine intermediate to the parent amine compounds akin to that of Group A compounds, while still imparting enough stability to permit its detection via HPLC.

Several other studies have shown insight into the mechanistic features of azoreductases of species which differ from that of the colonic anaerobe *C. perfringens*. Studies on mixed aerobic/anaerobic rabbit liver microsomal P450 azoreductase have reported that polar electron-donating substituents are obligatory for the reduction of azo dyes,[34] and that compounds without such constituents will not bind to cytochrome P450 or undergo any reduction. Ferrous cytochrome P450 enzymes have shown a one-electron reduction mechanism involving a free-radical intermediate.[35] Flavin-dependent enzymes such as *C. perfringens* have shown a two-electron mechanism forming no free-radical intermediates, and have shown different substrate specificity to flavin-independent enzymes.[32]

A separate study on the mechanism of azoreduction of balsalazide by aerobic *Pseudomonas aeruginosa* proposed that tautomerization of azo substrates to the hydrazone form is necessary for azoreduction.[14] This phenomenon has been demonstrated in azo compounds with an electron donor group[36] such as the Group A compounds in this Letter. Group B compounds however do not contain a protic donor group on either phenyl ring and therefore are unable to tautomerize. It can therefore be assumed that the hydrazine is generated by direct transfer of hydride to the azo group in the first step, and this stable intermediate is not subject to further enzymatic electron transfer.

The presence and prevalence of the anaerobic *C. perfringens* in the human colon makes it an important organism as an in vitro model of azo prodrug activation. Despite their widespread application, there is little published literature on the SAR of azoreduction by colonic microflora. The observations in this Letter were made possible because of two differences between our approach and that generally adopted in the literature:

The use of HPLC to separate the intermediate from the reaction product. Colorimetric methods have been reported for monitoring substrate consumption by *C. perfringens.*[37] However this method of monitoring substrate consumption is not ideal to distinguish cases that stop at the hydrazo stage from those that proceed to aniline formation.[10,29]

1. The use of substrates lacking an electron-donating group. Azo compounds usually present an electron-donating substituent, as this is required for diazonium coupling of the precursors. The nitroso-based chemistry permitted the synthesis of substituted azobenzenes without mesomeric donors.

The inability of *C. perfringens* azoreductase activity to completely reduce azo compounds lacking an electron donating group on the phenyl ring has some implications for the design of azo-based prodrugs. It would be interesting to know if these limitations are shared by other bacteria in the colonic microflora. There are additional consequences regarding the toxicology of azo dyes as stabilizing their hydrazine intermediates might be a useful strategy in reducing the toxic liability related to their amino reduction products.

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16. *Compound* **1c**. Mp: 184–186 °C. IR_{vmax} (KBr): 3468.24, 1638.01, 1618.44 and 1384.43 cm^{-1}. ^1H NMR δ (CD_3OD): 8.21 (2H, d, *J* 8.28 Hz), 7.99 (2H, d, *J* 8.28 Hz), 7.74 (1H, d, *J* 8.04 Hz), 7.49 (3H, m), 7.36 (1H, m), 6.29 (1H, dd, *J* 10.04 and 1.76 Hz), 6.02 (1H, s) 4.99 (1H, d, *J* 17.56 Hz, one hydrogen is masked by the water peak), 4.42 (1H, s), 3.53 (2H, t, *J* 7.76 Hz), 2.85 (2H, t, *J* 8.52 Hz) 2.69–0.94 (19H, prednisolone envelope) 1.71 (9H, s). ^{13}C NMR δ (MeOD): 205.73 (C=O, C-20), 187.61 (C=O, C-3), 173.43 (C=O, C-22), 172.71 (CH, C-1), 171.45 (C, C-5), 165.79 (C, C-10′), 158.83 (C=O, C-13′), 154.48 (C, C-2′), 149.95 (C, C-7′), 145.41 (C, C-1′), 140.81 (CH, C-5′), 131.21 (CH, C-6′), 130.46 (CH, C-9′), 130.21 (CH, C-11′ and C-3′), 127.06 (CH, C-2), 126.41 (CH, C-4), 122.18 (CH, C-8′), 121.08 (CH, C-12′), 114.93 (CH, C-4′), 89.08 (C, C-17), 69.37 (CH, C-11), 67.94 (CH_2, C-21), 55.94 (CH, C-9), 51.44 (CH, C-14), CD_3OD residual peak is masking the peak of C, C-13, 44.68 (C, C-10), 38.85 (CH_2, C-12), 35.76 (CH_2, C-23), 34.21 (CH_2, C-6), 33.25 (CH_2, C-7), 31.81 (CH_2, C-16), 31.26 (CH, C-8), 26.79 (CH_2, C-24), 23.45 (CH_2, C-15), 20.22 (CH_3, C-19), 15.91(CH_3, C-18). HRMS: Found: $(M-H)^+$ = 641.2863, Required: $(M-H)^+$ = 641.2850.
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