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Phenothiazinium photosensitisers: V. Photobactericidal activities of chromophore-methylated phenothiazinium salts

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Abstract

The synthesis of chromophoric di- and trimethyl derivatives of azure A has been carried out along with the physicochemical profiling of the resulting derivatives. Structure–function relationships of the derivatives are proposed with a view to the development of novel photosensitisers with antimicrobial applications. All derivatives exhibited similar magnitudes of photosensitising efficacy in vitro to the standard dyes, azure A and toluidine blue O, but were generally more lipophilic in nature. Chromophoric methylation furnished more active photoantibacterials compared to the lead compounds, both against Gram-positive and Gram-negative organisms.

Keywords: Azure A; Gram-negative; Gram-positive; PACT; Phenothiaziniums; Photobactericide; Photosensitiser; Toluidine blue O

1. Introduction

Photoantimicrobial compounds – i.e. those which exhibit increased inactivation of microorganisms when exposed to light – have been known for over a century [1]. While much research has been reported on the use of photosensitisers against bacterial and viral targets, the clinical use of photosensitisers in antimicrobial therapy is being realised very slowly through small scale trials. This is particularly surprising in view of the efficacy exhibited, especially by cationic photosensitisers, against pathogenic drug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* [2,3].

Among photobactericidal compounds, the phenothiazinium photosensitisers methylene blue and toluidine blue O (MB and TBO, respectively, Fig. 1) have often been used as lead structures [4–6]. In addition to being effective photosensitisers with singlet oxygen quantum yields of approximately 0.40, they exhibit low toxicity levels in mammalian cells [7]. Toluidine blue has been shown to destroy several species of bacteria responsible for dental caries, of the causative organisms in oral candidosis and *Helicobacter pylori* implicated in stomach ulcers [8]. Conversely, effective photobactericidal and photovirucidal activities have led to the current use of methylene blue by several European agencies in the disinfection of blood plasma [9].

The light absorption properties of the phenothiazinium dyes are excellent for the local therapy of microbial disease. Most examples have intense long wavelength absorption in the region 620–660 nm which lies outside the light absorption by endogenous pigments such as haem that might otherwise interfere with the photosensitisation process.

There are several closely related commercial analogues of MB: the demethylated azure stains (e.g. azure A, Fig. 1) and thionin, and nuclear-substituted derivatives such as TBO and Taylor's blue (1,9-dimethyl methylene blue, Fig. 1). The majority of photobiological and clinical work has involved MB and TBO, once again reflecting their widespread use in vital staining [10].

In terms of cellular activity, both MB and TBO are reduced by ubiquitous cellular coenzymes. The reduced or leuco-form, LMB, is colourless, and is thus inactivable by the long

Abbreviations: AA, Azure A; DPIBF, 1,3-Diphenylisobenzofuran; MB, Methylene blue; MBC, Minimum bactericidal concentration; PDT, Photodynamic therapy; TBO, Toluidine blue O.

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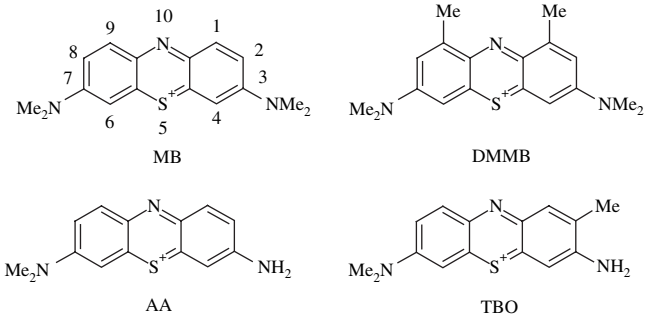


Fig. 1. Standard phenothiazinium photosensitisers showing the numbering of the chromophore.

wavelength light used in PDT. In addition, the pK_a value of LMB is low (5.8) compared with MB, leading to a low level of ionisation of the reduced species (3% ionised at pH 7.3). High ionisation is essential for efficient DNA intercalation, and photodamage to DNA is thought to be important in the photocytotoxicity of MB and its close analogues [11] although other sites of action are possible.

Such activity suggests the rational design and synthesis of improved analogues based on MB or TBO as lead compounds. Thus far, there have been few reports of chromophoric alteration [12], such analogue work which has appeared concentrating on changing the amino functionality at positions 3- and 7- of the phenothiazinium chromophore [13–16]. While any change in molecular structure has the potential to lead to an improved photosensitiser, alteration of the amino groups, e.g. via the use of larger homologous alkyl chains, might yield compounds which exhibit enhanced cellular uptake on the grounds of increased lipophilicity, but which suffer from twisting of the amino function out of coplanarity with the chromophore to minimise steric repulsions, thus reducing inherent photosensitising ability. Low aqueous solubility is also a potential problem for those derivatives containing multiple long chain alkyl groups. It therefore seems logical to attempt to improve the therapeutic potential of this class of compound through the functionalisation of the phenothiazine chromophore itself.

The author has in the past reported the improvement of the photosensitising properties of methylene blue by chromophoric methylation [17]. Methylation led to increased singlet oxygen production, greater lipophilicity and to the inhibition of intracellular chromophore reduction in vitro. Steric crowding due to the dimethylamino auxochromes meant that only methylene blue itself and derivatives bearing methyl groups at positions 1- and 9- were available. Conversely, with azure A, the primary amino function at position 3-, having far less steric bulk, allows more extensive methylation (Fig. 1). The present work concerns several closely related phenothiazinium derivatives, based on azure A and toluidine blue O (Fig. 1).

2. Materials and methods

2.1. Photosensitisers

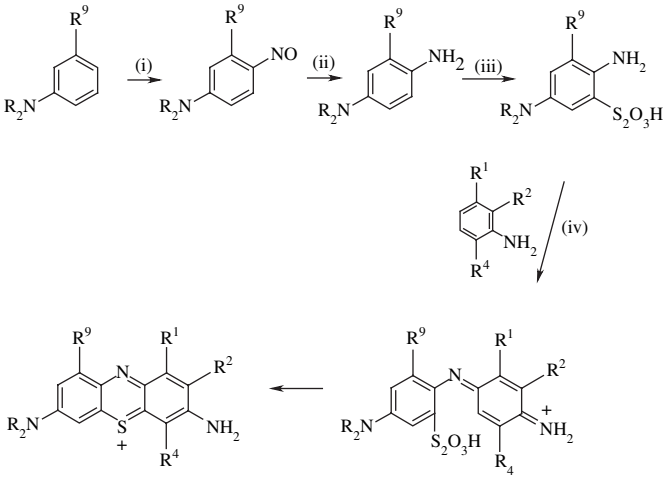
The following anilines were purchased from Aldrich (Gillingham, UK) and were used without further purification: 2- and 3-methylaniline; 2,3-, 2,5- and 2,6-dimethylaniline; *N*,*N*-dimethylaniline and *N*,*N*-dimethyl-*m*-toluidine. Similarly, the reagents silver carbonate on celite, aluminium sulfate, zinc chloride, sodium thiosulfate, sodium dichromate and copper sulfate were purchased from BDH (Poole, UK). 1,3-Diphenylisobenzofuran (DPIBF), methanol (spectrophotometric grade) and 1-octanol were purchased from Aldrich (Gillingham, UK) and used without further purification.

Methylene blue, azure A, and toluidine blue O were purchased from Aldrich (Gillingham, UK) and were purified chromatographically on silica using methanol/dichloromethane (5:95) as eluent. Methylated analogues of AA and TBO were synthesised via oxidation of the requisite diaminoarylthiosulfonic acids and anilines (Scheme 1) as detailed below [18].

All spectrophotometric measurements were carried out on a Hewlett Packard 8452A diode array spectrophotometer. The dyes were found to obey Beer's law in the concentration range 10^{-5}–10^{-7} M.

2.1.1. 2-Amino-5-dimethylaminophenylthiosulfonic acid

N,*N*-Dimethyl-*p*-phenylenediamine sulfate (30.3 g, 130 mmol) was added to a mechanically stirred solution of aluminium sulfate octadecahydrate/water (43.6 g, 65 mmol/100 ml). To this was added sodium thiosulfate/water (22.0 g, 139 mmol/80 ml) followed by zinc chloride/water (8.8 g, 63 mmol/12 ml). The solution was cooled to 0 °C and potassium dichromate/water (5.0 g, 17 mmol/20 ml) was added dropwise for a 30 min period. Following this addition, the mixture was allowed to stir for 2 h. During the last 30 min the temperature was allowed to rise to 10 °C causing the formation of a viscous precipitate. This was isolated by filtration and washed with water followed by acetone. 2-Amino-5-dimethylaminophenylthiosulfonic acid, yield = 15.87 g (49%), m.p. 190 °C (dec.)



Scheme 1. Formation of azure derivatives via the traditional thiosulfonic acid route, R = Me, Et, R^1–R^9 = H or Me. Reagents: (i) conc. HCl, $NaNO_{2(aq)}$, ≤5 °C; (ii) Zn dust, c. HCl, ≤20 °C; (iii) $Na_2S_2O_{3(aq)}$, $K_2CrO_{7(aq)}$, room temp.; (iv) Ag_2CO_3, MeOH, reflux.

2.1.2. 3-Methyl-2-amino-5-dimethylaminophenylthiosulfonic acid

2-Methyl-*N*,*N*-dimethyl-*p*-phenylenediamine sulfate (32.2 g, 130 mmol) was added to a mechanically stirred solution of aluminium sulfate octadecahydrate/water (43.6 g, 65 mmol/100 ml). To this was added sodium thiosulfate/water (22.0 g, 139 mmol/80 ml) followed by zinc chloride/water (8.8 g, 63 mmol/12 ml). The solution was cooled to 0 °C and potassium dichromate/water (5.0 g, 17 mmol/20 ml) was added dropwise over a 30 min period. Following this addition, the mixture was allowed to stir for 2 h. During the last 20 min the temperature was allowed to rise to 10 °C causing the formation of a viscous precipitate. This was isolated by filtration and washed with ice-water followed by ice-cool acetone. 3-Methyl-2-amino-5-dimethylaminophenylthiosulfonic acid, yield = 14.06 g (41%), m.p. 176 °C (dec.)

2.1.3. General procedure for methylated azure photosensitisers

The requisite thiosulfonic acid (4 mmol) and aniline (5 mmol) were refluxed in 120 ml methanol and silver carbonate on celite (5 g, 50% w/w) was added slowly over 0.5 h. The reaction mixture was refluxed for a further hour, filtered through a celite pad and the filtrates evaporated. The residue wax extracted with dichloromethane and purified by medium pressure liquid chromatography.

2.1.4. 3-Amino-7-dimethylamino-1,2-dimethylphenothiazinium sulfate

From 2-amino-5-dimethylaminophenylthiosulfonic acid 1,2-dimethylaniline, as a purple/black powder (38%), m.p. 180 °C. Found C 51.10; H 4.74; N 10.80; S 16.88%. $C_{16}H_{19}N_3S_2O_4$ requires C 50.38; H 5.02; N 11.02; S 16.81%.

2.1.5. 3-Amino-7-dimethylamino-1,4-dimethylphenothiazinium sulfate

From 2-amino-5-dimethylaminophenylthiosulfonic acid and 2,5-dimethylaniline, as a black amorphous powder (11%), m.p. 200–204 °C. Found C 50.85; H 5.15; N 10.70; S 15.38%. $C_{16}H_{19}N_3S_2O_4$ requires C 50.38; H 5.02; N 11.02; S 16.81%.

2.1.6. 3-Amino-7-dimethylamino-1,9-dimethylphenothiazinium sulfate

From 3-methyl-2-amino-5-dimethylaminophenylthiosulfonic acid and *m*-toluidine, as a deep purple microcrystalline powder (18%), m.p. 191 °C. Found C 51.05; H 5.15; N 11.30; S 16.38%. $C_{16}H_{19}N_3S_2O_4$ requires C 50.38; H 5.02; N 11.02; S 16.81%.

2.1.7. 3-Amino-7-dimethylamino-2,9-dimethylphenothiazinium sulfate

From 3-methyl-2-amino-5-dimethylaminophenylthiosulfonic acid and *o*-toluidine, as a purple crystals (23%), m.p. 196–197 °C. Found C 49.15; H 4.76; N 10.67; S 16.95%. $C_{16}H_{19}N_3S_2O_4$ requires C 50.38; H 5.02; N 11.02; S 16.81%.

2.1.8. 3-Amino-7-dimethylamino-2,4,9-trimethylphenothiazinium sulfate

From 3-methyl-2-amino-5-dimethylaminophenylthiosulfonic acid and 2,6-dimethylaniline, as a blue–black amorphous powder (12%), m.p. 201–202 °C. Found C 50.33; H 5.20; N 11.00; S 15.90%. $C_{17}H_{21}N_3S_2O_4$ requires C 51.62; H 5.35; N 10.62; S 16.21%.

2.2. Singlet oxygen production

Singlet oxygen production by the photosensitisers was assayed using the decolourisation of 1,3-diphenylisobenzofuran (DPIBF) in methanol. Thus, the decrease in absorption at 410 nm was monitored spectrophotometrically with time as in the method of Cincotta et al. [19]. The singlet oxygen yield for the standard photosensitiser, methylene blue ($\Phi_{\Delta MB}$) is given as 0.443 [19]. By assuming that the decrease in absorption of DPIBF at 410 nm is directly proportional to its reaction with singlet oxygen, the time for a 50% decrease in absorption caused by each of the azure A derivatives under identical conditions ($t_{1/2AAD}$) thus gives a measure of its photosensitising efficiency. Thus, the time for the DPIBF absorption to decrease by 50% due to MB photosensitisation ($t_{1/2MB}$) was taken as 1.0. To calculate the singlet oxygen yield for the methylated azure A/toluidine blue O derivatives ($\Phi_{\Delta AAD}$), the following formula was used:

$$\Phi_{\Delta AAD} = \Phi_{\Delta MB} \times \frac{t_{1/2MB}}{t_{1/2AAD}}$$

2.3. Lipophilicity (log P)

The lipophilicities of the photosensitisers were calculated in terms of log *P*, the logarithm of their partition coefficients between phosphate-buffered saline and 1-octanol. The data were calculated using the standard spectrophotometric method [20] based on the relationship:

$$\mathrm{Log}\, P = \mathrm{Log}\left\{\frac{(A - A^1)}{A^1} \times \frac{V_w}{V_o}\right\}$$

where A and A^1 are the absorption intensities before and after partitioning, respectively, and V_w and V_o are the respective volumes of the aqueous and 1-octanol phases. Determinations were repeated three times.

2.4. Photobactericidal activity

The photobactericidal efficacies of a range of novel derivatives, azure A and TBO were measured against a Gram-positive and a Gram-negative organism, *Staphylococcus epidermidis* (NCTC 6513) and *Escherichia coli* (NCTC 9001), respectively. Both strains were grown in Mueller–Hinton Broth and then diluted to a concentration of 10^6 colony-forming units/ml. Aliquots of the strains were then incubated for 18 h at 37 °C in microtitre trays with various concentrations of the photosensitisers giving a final range of

Table 1
Physicochemical and photoproperties of azure A, toluidine blue O and other methylated derivatives

	λ^{a}_{max} (nm)	Log ε_{max} [a]	Log *P*	Relative $^1O_2$ yield[b]
MB	656	4.98	−0.10	1.00
AA	633	4.80	+0.70	0.86
TBO	626	4.91	−0.21	0.86
1-MTB	616	4.78	−0.16	0.80
1,4-DMAA	620	4.68	−0.09	0.81
1,9-DMAA	619	4.69	+0.21	0.95
9-MTB	615	4.77	+0.36	0.90
4,9-MTB	610	4.62	+0.80	0.70

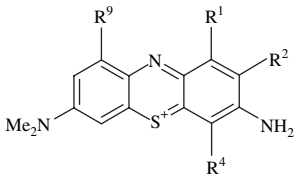
MB, methylene blue.
[a] Measured in ethanol.
[b] Relative to methylene blue as standard comparison.

80–82.5 μM, with zero photosensitiser concentrations in each case for control purposes. The trays were then either illuminated for 0.5 h using an array of white strip lights giving a light dose of 6.3 J/cm^2 or foil-covered (dark controls). This light source has been used in previous work by the author [17]. From each well showing inhibition of growth 1 μl was sub-cultured on horse blood agar and incubated for 18 h at 37 °C. Minimum lethal concentrations were then determined as the lowest concentration for each photosensitiser giving no bacterial growth. Results are given in Table 1.

3. Results

Note on abbreviations: compounds were named after azure A, unless a chromophoric 2-methyl group was present in which case the compound was named after toluidine blue O. Thus, for example, the compound with methyl groups at positions 1- and 9- of the phenothiazinium chromophore was named 1,9-dimethyl azure A (1,9-DMAA), whereas that with methyl groups at positions 2- and 9- was named 9-methyl toluidine blue (9-MTB).

The syntheses of the methylated analogues (Scheme 1) were carried out in reasonable yields using silver carbonate as the oxidising agent, in order to preserve the alkyl groups, furnishing di- and trimethyl azure analogues (Fig. 2). Chromatography was required to separate the blue products from other oxidised materials.



Compound	R^1	R^2	R^4	R^9
AA	H	H	H	H
TBO	H	Me	H	H
1-MTB	Me	Me	H	H
1,4-DMAA	Me	H	Me	H
1,9-DMAA	Me	H	H	Me
9-MTB	H	Me	H	Me
4,9-MTB	H	Me	Me	Me

Fig. 2. Photosensitiser structures based on azure A and toluidine blue O.

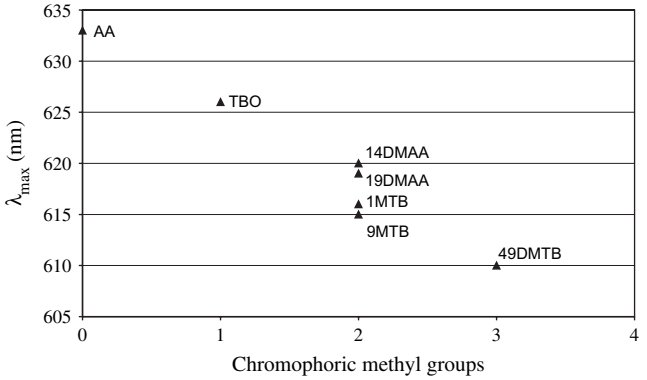


Fig. 3. Variation of λ_{max} in ethanol with degree of methylation.

Given the neutrality of the methyl group, little deviation from the properties of azure A/toluidine blue O was expected. In terms of light absorption, the new derivatives exhibited small hypsochromic shifts (i.e. to shorter wavelengths) in line with the degree of methylation (Table 1 and Fig. 3). Singlet oxygen yields were similar to the lead compounds, but less than methylene blue, while the lipophilicity was found to increase generally with the degree of methylation although methylation in both outside rings of the tricyclic nucleus led to higher lipophilicities than dimethylation in one (Table 1).

The photoantimicrobial effects of the new derivatives were generally greater than the lead compounds (Figs. 4 and 5). However, due to the well-established high activity of phenothiazinium derivatives against Gram-positive bacteria, the most significant finding was the similarly high activity of the derivatives against the Gram-negative organism, *E. coli*. Noticeably, the inherent (dark) antibacterial effects of the derivatives were higher than the lead compounds.

4. Discussion

The use of the standard photosensitiser toluidine blue O in research programmes and clinical trials aimed at clinical local

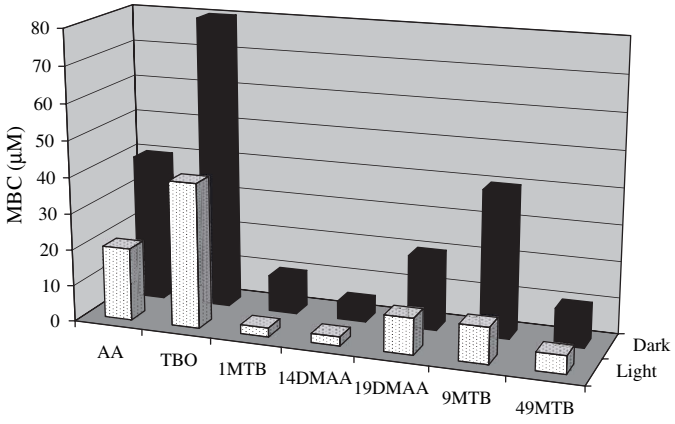


Fig. 4. Bactericidal and photobactericidal activities of the methylated derivatives against *Staphylococcus epidermidis*, compared with those of azure A and toluidine blue. MBC – minimum bactericidal concentration. Compound abbreviations as in Fig. 2.

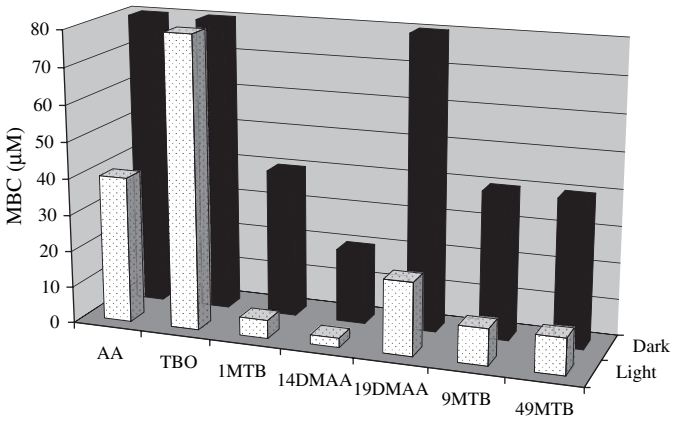


Fig. 5. Bactericidal and photobactericidal activities of the methylated derivatives against *Escherichia coli*, compared with those of azure A and toluidine blue. MBC – minimum bactericidal concentration. Compound abbreviations as in Fig. 2.

disinfection constitutes a significant section of photoantimicrobial endeavour over the past 15 years, particularly in the field of oral disease [21]. However, there have been very few chemically comparable photosensitisers tested so that structure optimisation might be achieved. The current series of methylated azure photosensitisers represents a contribution in this respect.

The production of methylated analogues of azure A furnished a number of derivatives which were similar to both the parent compound and toluidine blue O, since this is also a methylated derivative, i.e. 2-methyl azure A (Fig. 1). In addition, the inclusion of extra methyl groups was thought unlikely to alter greatly important factors for photosensitiser action, such as absorption wavelength and singlet oxygen production. Conversely, previous experience with methylated derivatives of methylene blue suggested the potential for improved antibacterial activity [2,3,18].

None of the new photosensitisers produced in the current work offered any increase in absorption wavelength compared to that of azure A/toluidine blue O (Fig. 2). Rather, there was generally a slight decrease, although not sufficient to discourage the use of the compounds in the presence of biological media. However, in terms of singlet oxygen production, several of the new compounds exhibited higher yields than the parent compounds. This was particularly obvious with 1,9-DMAA and 9-MTB, i.e. those derivatives having a single methyl group in each of the outer rings (Fig. 3). Both the wavelength shift and singlet oxygen trend were observed previously with methylated derivatives of methylene blue [17].

The exception here was the trimethylated compound, 4,9-MTB. In this compound, the amino group at position 3- had two *ortho*-methyl groups which caused steric crowding of the auxochrome and thus interfered with its coplanarity with the chromophore. This led both to the greatest hypsochromic shift (to 610 nm) and the lowest singlet oxygen yield.

Toluidine blue O has been shown to be a highly active photobactericidal agent against both Gram-positive and Gram-negative organisms in vitro [5,22]. However, in the current work, the activities of the lead compounds against model bacteria *S. epidermidis* (Gram-positive) and *E. coli* (Gram-negative) were noticeably weaker than the new derivatives, toluidine blue O exhibiting no increased activity on illumination against *E. coli* (Fig. 5).

Although it is not possible to infer firm structure–activity relationships from the current small sample of compounds, it was noticeable that 1-MTB and 1,4-DMAA were most active against both bacterial challenges (MBC vs *S. epidermidis* = 2.5 μM vs *E. coli* = 5 μM and 2.5 μM, respectively, Figs. 4 and 5). In both of these compounds, both methyl groups are in the amino-containing ring of the molecule. The next most active compound, 4,9-MTB also features this pattern. The lower singlet oxygen yield of this compound has already been mentioned, suggesting that this may be more critically targeted than the other candidates.

Given the far greater photobactericidal activity observed with 1,9-dimethyl methylene blue compared to methylene blue itself in earlier work [2,3], the weaker effects of 1,9-DMAA and 9-MTB here were unexpected, particularly in view of their greater singlet oxygen yields within the series. Again this may suggest a difference in targeting at the bacterial cell.

The current work has shown that it is possible to produce photosensitisers based on the azure A molecule having increased activities against both Gram-positive and Gram-negative bacteria relative to the lead compound, by the simple step of chromophore methylation. This is in line with earlier work on the methylene blue molecule.

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