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Phenothiazinium photosensitisers VII: Novel substituted asymmetric *N*-benzylphenothiaziniums as photoantimicrobial agents

Mark Wainwright *, Simon D. Brandt, Andrew Smith, Andrew Styles, Katie Meegan, Ciara Loughran

School of Pharmacy & Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, United Kingdom

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ABSTRACT

The synthesis of asymmetrical analogues of methylene blue, in which one of the dimethylamino groups is replaced by a diethylamino or di-*n*-propylamino group, and the other by benzylamino or 4-substituted benzylamino, is reported, the substituents being alkyl, alkoxyl or halogen. As expected, because of their longer alkyl chains these diethylamino- and di-*n*-propylamino derivatives proved to be considerably more lipophilic than the parent compound methylene blue, while maintaining suitable maximum absorption wavelengths and singlet oxygen efficiencies for photoantimicrobial use.

Also as expected, in screening tests against Gram-positive and Gram-negative bacteria, the substituted benzylamino derivatives were highly active on illumination, presumably via singlet oxygen damage, and exhibited considerably increased activity against both classes relative to that of the standard, methylene blue. In addition, the more lipophilic derivatives exhibited greater activity against *Escherichia coli*. This may be due to increased interaction with the lipid-rich outer membrane of this Gram-negative bacterium. DNA binding of the derivatives was also increased, relative to methylene blue, showing large bathochromic shifts (>10 nm) on binding typical of strong intercalators.

1. Introduction

Modern research into the biological application of photosensitisers, was initially based on porphyrin derivatives from natural sources, along with several synthetic dyes established as vital stains [1,2]. The realisation that first generation porphyrin derivatives had shortcomings in terms of non-optimal photoproperties and side effects encouraged efforts in the synthesis of new, improved derivatives from porphyrin and dyestuff leads.

The selective tumour staining activity of several cationic dyes, such as Nile blue and methylene blue provided a sound rationale for photosensitiser development, although the provision of large series of compounds required for rapid drug discovery was not straightforward due to synthetic chemistries based on obsolete and unsuitable textile dye production. For example, the oxidative nature of phenothiazinium dye production traditionally required the use of dichromate (chromium(VII)), but the strength of this oxidant is such that anilines employed as starting materials are invariably converted to a variety of products, including those having substituent alteration in the resulting chromophore. Routes to phenothiaziniums via the oxidative coupling of anilinethiosulphonic acids and anilines using a weaker oxidant such as silver(I) carbonate and the halogen-mediated oxidation of 10*H*-phenothiazine itself followed by amination have led to a greatly improved range of structures [3].

Recent investigations of the activity of methylene blue derivatives – in the fields of both photodynamic therapy (PDT) and photodynamic antimicrobial chemotherapy (PACT) – have mainly been based on symmetrical auxochromic variation – i.e. the dimethylamino groups at positions C-3 and C-7 of the phenothiazinium chromophore being replaced by higher dialkylamino functionality groups [4,5], although some asymmetric derivatives have also been reported [6].

In a previous publication, it was demonstrated that 3-dialkylaminophenothiazinium derivatives having simple 7-anilino- and 7-benzylamino moieties exhibited greatly improved photobactericidal activity, coupled with low dark toxicity, against *Escherichia coli* and *Staphylococcus aureus*, compared to that of the lead compound methylene blue [7]. Such activity in the arylamino derivatives was unexpected, given the lack of associated singlet oxygen production in the standard *in vitro* chemical screen. However, as part of an ongoing programme of photosensitiser drug discovery, the increased activity of these compounds has encouraged the synthesis of peripherally-substituted analogues, the presence of the pendant aryl groups allowing considerable potential for chemical/physicochemical variation without affecting the chromophore and singlet oxygen production. The current paper covers the initial chemical testing and antibacterial screening of a group of derivatives as described, having standard aromatic substitution in the 7-benzyl pendant.

* Corresponding author.
E-mail address: mark_wainwright@hotmail.com (M. Wainwright).

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2. Materials and methods

2.1. Chemicals

10*H*-Phenothiazine, iodine, dialkylamines, benzylamines and solvents were purchased from Sigma–Aldrich, UK, and used without further purification. Both methylene blue and dimethyl methylene blue were also purchased from Sigma–Aldrich, but were purified by column chromatography on silica gel (Fisher Scientific, UK) using gradient elution in dichloromethane/methanol. Photophysical characterisation of the products was carried out using a Hewlett Packard 8452A diode array spectrophotometer. This was also used for the determination of lipophilicity. Accurate molecular ion masses for the derivatives were obtained using a Micromass LCT TOF mass spectrometer.

2.2. Synthesis

Both the precursors, phenothiazin-5-ium tetraiodide and the 3-dialkylaminophenothiazinium triiodides were synthesised as described previously [7].

2.2.1. 3-Dialkylamino-7-benzylaminophenothiazinium iodides (**1a–e**, **2a–e**)

3-Diethylamino- or 3-di-*n*-propylaminophenothiazinium triiodide (0.75 mmol) was suspended in methanol (10 ml) and the requisite benzylamine (1.8 mmol) in 10 ml methanol was added dropwise, the reaction being monitored by TLC (SiO_2, 3% aqueous NH_4OAc/CH_3OH 1:17). Reaction times to the exhaustion of the triiodide salt were in the region of 1.5–2 h. Products were isolated by evaporation of the methanol *in vacuo*, redissolution in dichloromethane, extraction with 5% v/v hydroiodic acid then water, drying of the organic layer over anhydrous sodium sulphate, evaporation to a small volume and repeat precipitations in dry diethyl ether. Compounds impure by thin-layer chromatography at this stage were chromatographed on silica gel (Fisher Scientific, UK) using gradient elution in dichloromethane/methanol.

Synthetic yields and analytical data for the derivatives are given in Table 1.

2.3. Singlet oxygen testing

Singlet oxygen production by the photosensitisers was assayed as in previous work [7], except that the decolourisation of 2,3,4,5-tetraphenylcyclopentadienone (TPCPD) in dichloromethane was employed rather than that of 1,3-diphenylbenzisofuran in methanol. Thus the decrease in absorption at 500 nm was monitored spectrophotometrically with time, using methylene blue as a standard photosensitiser. By assuming that the decrease in absorption of TPCPD at 500 nm is directly proportional to its reaction with singlet oxygen, the time for a 50% decrease in absorption caused by each of the derivatives under identical conditions ($t_{1/2}$MBD) thus gives a measure of its photosensitising efficiency. Thus, if the time for the DPIBF absorption to decrease by 50% due to MB photosensitisation is $t_{1/2}$MB, relative singlet oxygen yields for the derivatives are given by:

$$\text{Relative } {}^1O_2 \text{ yield} = \frac{t_{1/2}\text{MB}}{t_{1/2}\text{MBD}}$$

i.e. the lower the $t_{1/2}$ value for the derivative, the greater its $^1O_2$ yield.

2.4. 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 [8] based on the relationship:

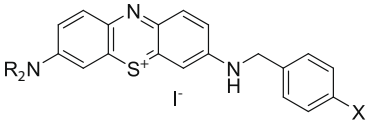
$$\text{Log } P = \text{Log}\left\{\frac{A - A^1}{A^1} \cdot \frac{V_w}{V_o}\right\}$$

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.

2.5. Antibacterial screening

The photobactericidal efficacies of the derivatives in addition to that of the known photosensitiser methylene blue were measured

Table 1
Analytical data for the derivatives.



	R	X	*m/z*[a]		% Yield	λ_{max} (nm)[b]	Log ε_{max}[b]	Relative $^1O_2$[c]	Log *P*
			Calc.	Found					
MB	–	–	–	–	–	656	4.88	1.00	−0.1
1a	Et	H	374.17	374.18	29	644	4.62	0.69	+0.8
1b	Et	Cl	408.13	408.12	35	642	4.66	0.71	>2.0
1c	Et	F	392.16	392.10	26	644	4.71	0.53	>2.0
1d	Et	MeO	404.18	404.18	34	646	4.78	0.54	>2.0
1e	Et	Me	388.18	388.18	36	648	4.63	0.57	+1.9
2a	*n*-Pr	H	402.20	402.22	32	646	4.65	0.47	>2.0
2b	*n*-Pr	Cl	436.16	436.16	37	648	4.63	1.23	>2.0
2c	*n*-Pr	F	420.19	420.11	28	650	4.77	0.46	>2.0
2d	*n*-Pr	MeO	432.21	432.21	30	650	4.69	0.90	>2.0
2e	*n*-Pr	Me	416.22	416.22	33	654	4.67	0.38	>2.0

[a] By ICP-MS.
[b] Measured in MeOH.
[c] Yield of singlet oxygen relative to that of MB.

against a Gram-positive and a Gram-negative bacterium, *S. aureus* (National Culture Tissue Collection, NCTC 6571) and *E. coli* (NCTC 10418) respectively. Both strains were grown overnight in Mueller–Hinton Broth and then diluted to a concentration of 10^6 colony-forming units/ml. Aliquots of the strains in the growth phase were then incubated for 1 h at 37 °C in microtitre trays with various concentrations of photosensitiser ranging from 100 to 3 μM, with zero photosensitiser concentrations in each case for control purposes. The trays were then either illuminated for 20 min using an array of 126 light-emitting diodes (660 nm) giving a light dose of 6 J cm^{-2} or alternatively foil-covered to provide dark controls. From each well apparently showing a complete absence of growth of the micro-organism (i.e. total growth inhibition), 1 μl was subcultured on nutrient agar, using the Miles-Misra method [9], and incubated for 18 h at 37 °C. The minimum bactericidal concentrations (MBCs) were thus determined as the lowest concentration for each photosensitiser giving no bacterial growth. Each test was repeated to ensure an absolute value for the cited MBC with $n = 6$. Due to the absolute nature of the assay, i.e. complete absence of growth, rather than fractional kill, no statistical treatment of the resulting data was applied.

2.6. DNA interaction

A solution of DNA sodium salt Type XIV (Sigma–Aldrich) was made up in pH 7.4 (PBS) buffer. This solution was diluted to 100 μM, according to the method of Huang et al. [10]. Similarly, 100 μM solutions of methylene blue and compounds **1a**–**e** and **2a**–**e** were made up in pH 7.4 (PBS) buffer. A 100 μM solution of 1,9-dimethyl methylene blue (Sigma–Aldrich) was also made up for comparison purposes. The photosensitiser solutions were either diluted 1:8 v/v with buffer, or mixed in this ratio with DNA solution. The spectra of the resulting solutions were then measured for λ_{max} comparison.

3. Results

As with previous work [7], the synthesis of the asymmetrical derivatives (Fig. 1) was straightforward, with product yields typically around 30% (Table 1). The 4-phenyl substituents in the benzyl moieties employed did not cause significant shifts in the absorption spectra of the series, λ_{max} values remaining within 10 nm of that of the original benzyl derivative in each case. Several of the 3-dipropylaminophenothiazinium series exhibited λ_{max} values close to that of the parent photosensitiser, methylene blue (656 nm). Similarly, singlet oxygen yields *in vitro* were in the same range as in the previous study [7], although the dipropylamino/4-chlorobenzylamino derivative (compound **2b**) produced more singlet oxygen than did methylene blue.

All of the benzylamino derivatives, whether ring-substituted or not, were more effective photobactericidal agents than the lead compound, methylene blue, being up to eight times as active (Figs. 2 and 3). Interestingly, the activities of the derivatives were generally higher against the Gram-negative bacterium, *E. coli*, which was unexpected.

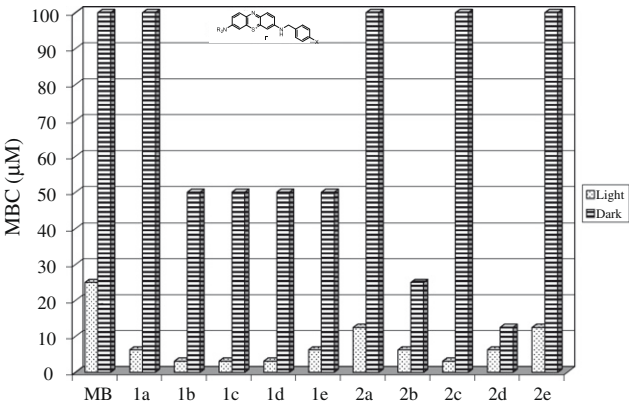


Fig. 2. Antibacterial activities of the compounds against *Staphylococcus aureus*. MBC – minimum bactericidal concentration.

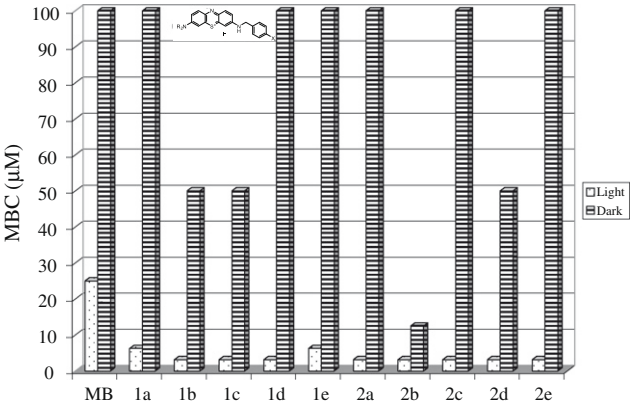


Fig. 3. Antibacterial activities of the compounds against *Escherichia coli*. MBC – minimum bactericidal concentration.

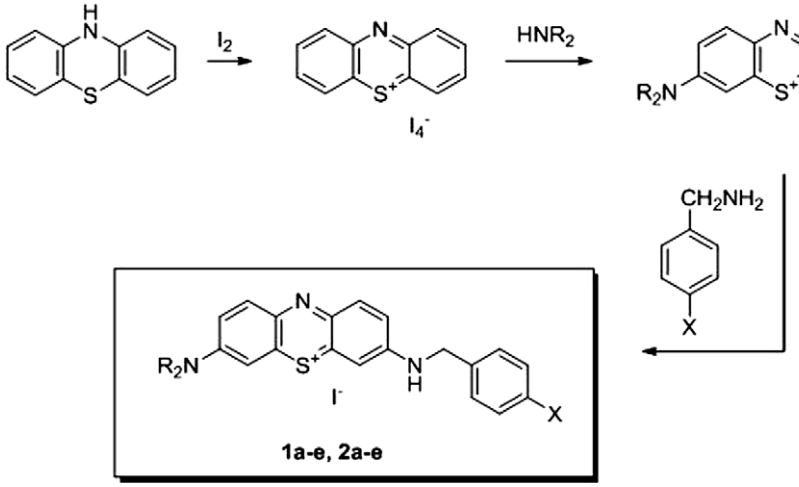


Fig. 1. Synthesis of asymmetrical phenothiazinium derivatives.

Table 2
Bathochromic shifts for the derivatives caused by DNA intercalation.

	λ_{max} (nm) alone	λ_{max} (nm) + DNA	Change
Methylene blue	665	669	+4
1a	652	666	+14
1b	650	662	+12
1c	653	665	+14
1d	654	665	+13
1e	645	659	+14
2a	658	670	+12
2b	656	669	+13
2c	656	666	+10
2d	658	669	+11
2e	658	668	+10
1,9-Dimethyl methylene blue	648	660	+12

As part of the current study, the binding of the photosensitisers to DNA was measured to investigate possible binding changes caused by molecular alteration in the phenothiazinium derivative. All of the derivatives **1a**–**e** and **2a**–**e** demonstrated considerable bathochromic shifts compared to the known intercalator, methylene blue. The shifts observed for the derivatives were in the range 10–14 nm, compared to the 4 nm seen for the lead compound (Table 2).

4. Discussion

The increases in absorption wavelength observed for the benzyl derivatives were, in fact, due to the increased alkyl chain length included in the dialkylamine auxochrome (i.e. diethylamino or dipropylamino, compared to dimethylamino), since – as a comparison – the mono-demethylated analogue of methylene blue, azure B, has a λ_{max} value of 645 nm in methanol – i.e. the aromatic residue in the benzyl group is too far removed to have an electronic effect on the phenothiazinium chromophore. Such increases in λ_{max} have been reported previously for the homologous series of methylene blue derivatives having bis(diethylamino-) up to bis(dihexylamino-) auxochromes [4].

Although each of the benzyl derivatives **1a**–**e** and **2a**–**e** produced singlet oxygen *in vitro*, this data did not correlate with photobactericidal efficacy. Such data should, of course, be used carefully, since cellular environments are quite different to those encountered in a test tube.

That the mode of action of cationic photosensitisers against bacteria is multifactorial is attested to by the various reports of post-treatment damage to nucleic acid, ribosomes and cell wall in the same organism [11].

In work reported by Wagner et al., the intercalation of DNA by 1,9-dimethyl methylene blue caused a 12 nm bathochromic shift, reportedly due to a considerably stronger binding interaction with the nucleic acid [12]. Increased interaction with bacterial DNA would support the increased photobactericidal activities exhibited by the derivatives in the current work, compared with that of methylene blue. Increased lipophilicity in the derivatives might also explain the higher relative activity against *E. coli*, given the lipid-rich outer membrane associated with Gram-negative bacteria, a multiple site of action hypothesis being currently favoured for photoantimicrobials, as mentioned above.

While the number of compounds in the present study is not great enough to produce robust structure–activity data, it may be seen from the results of the antibacterial screen that greater photobactericidal activity was associated with derivatives where the pendant aryl group contained a methoxyl group or halogen atom. In addition, the noticeably increased activity of the 3-di-*n*-propyl derivatives against *E. coli* relative to that against *S. aureus* supports the increased outer membrane interaction of more hydrophobic compounds (Figs. 2 and 3). It is intended that future work will explore greater functional and positional variation in benzyl analogues, for example including those of a hydrophilic nature.

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