Journal of Photochemistry and Photobiology B: Biology 99 (2010) 74–77

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

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

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ARTICLE INFO

Article history:
Received 22 September 2009
Received in revised form 9 February 2010
Accepted 11 February 2010
Available online 16 February 2010

Keywords:
Asymmetric phenothiazinium derivatives
Methylene blue
PACT
Photosensitiser
Photoantimicrobial

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.

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doi:10.1016/j.jphotobiol.2010.02.008

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:

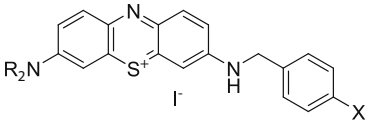
$$\text{Log } P = \text{Log}\left\{\frac{A - A^1}{A^1} \cdot \frac{V_w}{V_o}\right\}$$

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.