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Phenothiazinium photosensitisers IX. Tetra- and pentacyclic derivatives as photoantimicrobial agents

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ABSTRACT

Methylene blue derivatives, synthesised as novel photosensitising agents are usually simple auxochromic variations on the lead compound. The current paper represents the initial report on the synthesis and biological testing of a new class of phenothiazinium derivatives, having either one or two tetrahydropyridine rings fused to the phenothiazinium chromophore. The derivatives exhibited extended absorption wavelengths, increased amphiphilic character and much greater photoantimicrobial efficacies compared to methylene blue, some examples being more effective even than dimethyl methylene blue. The high activities of this class of photosensitiser recommend its use in infection control, both locally and in blood product decontamination.

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1. Introduction

Commercially available photosensitisers based on the phenothiazinium chromophore have found considerable utility in the burgeoning fields of photodynamic therapy (PDT) and photodynamic antimicrobial chemotherapy (PACT) [1]. The major compound involved remains methylene blue, this having been employed both in local cancer therapy [2] and in several photodisinfection protocols, including that of the oral cavity [3] and in blood plasma photodecontamination [4].

Despite such a range of useful activity, analogues based on this lead compound are only slowly emerging and are, in the main, simple derivatives having variation in the auxochromic amino groups at positions 3- and 7- of the phenothiazine ring (Fig. 1).

For example, work published by Mellish and Gorman described the synthesis and properties of both symmetrically and asymmetrically-substituted derivatives [5,6], both types exhibiting altered photoproperties and cytotoxicities relative to the parent compound. Other workers have used hydrophilic ring-based substituents, such as piperidine, morpholine etc. [7] in order to endow increased aqueous solubility.

However, such research does not reflect the full potential of the phenothiazinium ring system for adaptation and novel molecular shape generation since, in most cases, the non-auxochromic positions remain unsubstituted. Foley *et al.* have investigated derivatives having a fused benzene ring across positions C1/C2 (benzo[*a*]phenothiazinium derivatives [8]), but this led to decreased photosensitising efficacy *in vitro* in comparison to methylene blue. Others have reported chromophore methylation as a route to improved photoantimicrobials, but many of the derivatives here exhibited poor light absorption profiles ($\lambda_{max} < 620$ nm), auxochrome alkylation being sterically inhibited by adjacent methyl substitution [9].

The nitrogen-containing heterocycle tetrahydroquinoline may be viewed as a bridged, or rigidified aniline derivative, i.e. *N*-alkylanilines where the alkyl moiety is also attached to the ring carbon adjacent to the amino residue. This type of structural motif inherently features both ring and auxochrome alkylation and, as stated, the auxochromic group is rigidified, thus ensuring more effective overlap of the amino lone pair with the attached aromatic moiety via enforced coplanarity. These features have been utilised in dye chemistry to provide analogues having improved photostability and/or longer wavelength absorption [10,11]. In the present work, the bridged aniline nature of tetrahydroquinolines was attractive in the synthetic development of novel phenothiazinium derivatives as part of an ongoing programme of photoantimicrobial drug discovery [12], particularly as they offer routes to novel four- and five-ring systems, as opposed

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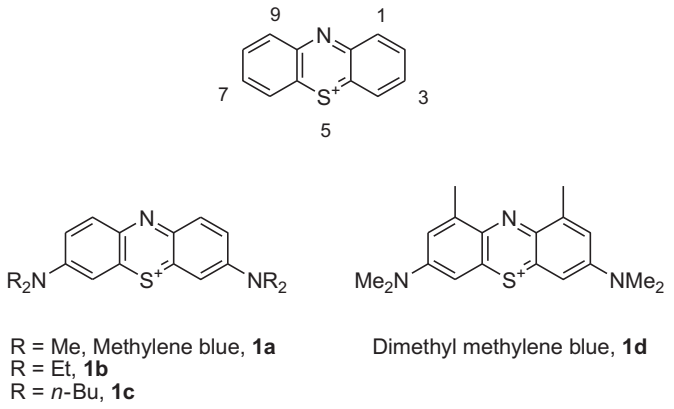

Fig. 1. Standard methylene blue derivatives and the phenothiazinium chromophore, showing numbering.

to the traditional, three ring methylene blue-type. One pentacyclic derivative has been reported in previous work on anti-cancer photodynamic therapy [13]. The current work thus represents the initial report of the synthesis and initial antimicrobial testing of this new class of photosensitiser.

2. Results and discussion

Both four- and five-ring systems were synthesised in similar yields to those for conventional methylene blue derivatives using the oxidative thiosulphonic acid route (Fig. 2), although it was necessary in each case to use column chromatography to achieve purity. Despite the increased hydrocarbon content of the new derivatives, since most were associated with alkylated ring fusion (Fig. 3), none presented any problems in the production of the aqueous solutions required for antimicrobial screening.

In terms of light absorption, the addition of a single six-membered ring did not appear to alter the λ_{max} value significantly. For example, compound **2c** represents the replacement of one methyl group in methylene blue and bridging the auxochrome and its adjacent chromophoric position with a 2-methyl-2,4-pentylene moiety, but both compounds exhibited a maximum absorption at 656 nm in water. A similar situation pertained for the tetraethyl derivative of methylene blue (**1b**) and the analogous tetracyclic derivative **2e**. In both cases, however, the respective pentacyclic derivatives, **3a** and **3b** respectively, exhibited considerable bathochromic shifts to >680 nm (Table 1). The use of the 7-methyltetrahydroquinoline derivative allowed entry into non-linear molecular frameworks (**2g**, **2h**, **3c** and **3d**), since the methyl group blocks one of the ring closure sites, the new C–S bond perforce

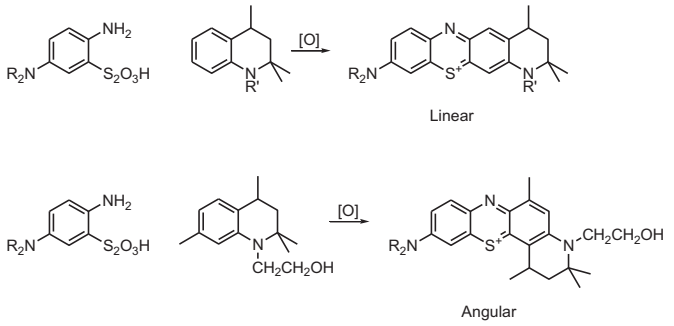

Fig. 2. Thiosulphonic acid synthetic route to linear- and angular-fused phenothiazinium derivatives.

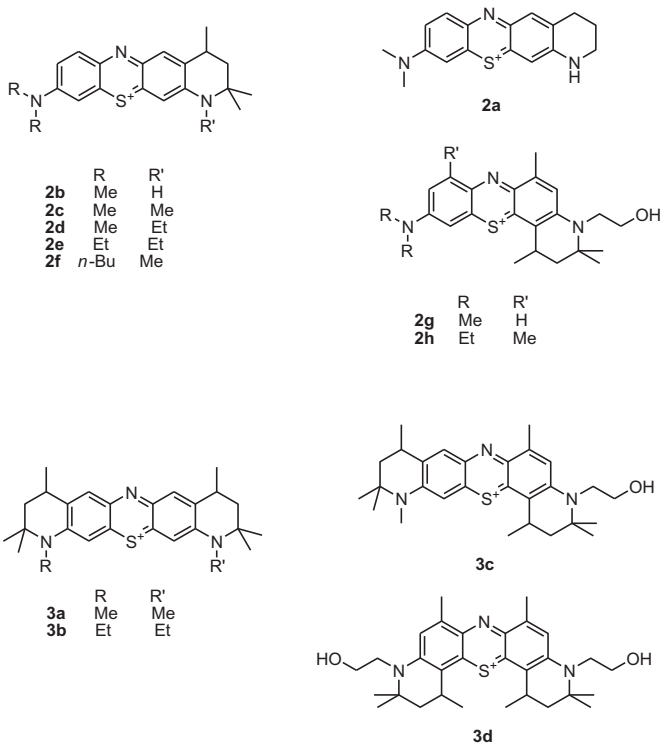

Fig. 3. Chemical structures of the tetra and pentacyclic derivatives ($X = HSO_4^-$).

being formed at C-5. However, it is clear that the pentacyclic derivatives, whether linear or angular, provided the greatest bathochromic shifts (Table 1). As noted, such behaviour has been reported for other tetrahydroquinoline-containing series, including azo dyes [11] and imines [10], but this has not been shown previously for photosensitisers, the only other example being that of tetrahydro-1,4-pyrazine fusion to the phenazine chromophore in work by Foley [14].

All of the new derivatives were efficient singlet oxygen producers in the standard tetraphenylcyclopentadienone spectrophotometric assay. Unusually, two examples, **2g** and **3d**, were more efficient than methylene blue itself although this is merely an indicator of potential photoantimicrobial activity since the molecular environment in free solution is usually quite different to that in the cellular milieu. Indeed the increased *in vitro* singlet oxygen yields for the two derivatives cited were not reflected in their

Table 1
Relevant physicochemical properties for the derivatives. ND = not determined.

Photosensitiser	λ_{max} (nm, H_2O)	Logε_{max} (H_2O)	Rel. $^1O_2$ yield	Log*P*
1a	656	4.69	1.00	−0.10
1b	664	4.87	0.55	+0.80
1c	671	4.46	0.61	+1.30
1d	648	4.51	1.11	+1.01
2a	641	4.26	0.51	+0.94
2b	656	4.60	0.52	ND
2c	656	4.30	0.97	+1.10
2d	667	4.84	0.76	+1.10
2e	664	4.11	0.87	+1.54
2f	684	4.60	0.87	+1.88
2g	665	4.35	1.10	+0.20
2h	658	4.22	0.57	+1.69
3a	684	4.25	0.98	+1.40
3b	687	4.37	0.92	+1.44
3c	680	4.33	0.89	+1.21
3d	670	4.31	1.11	+1.71

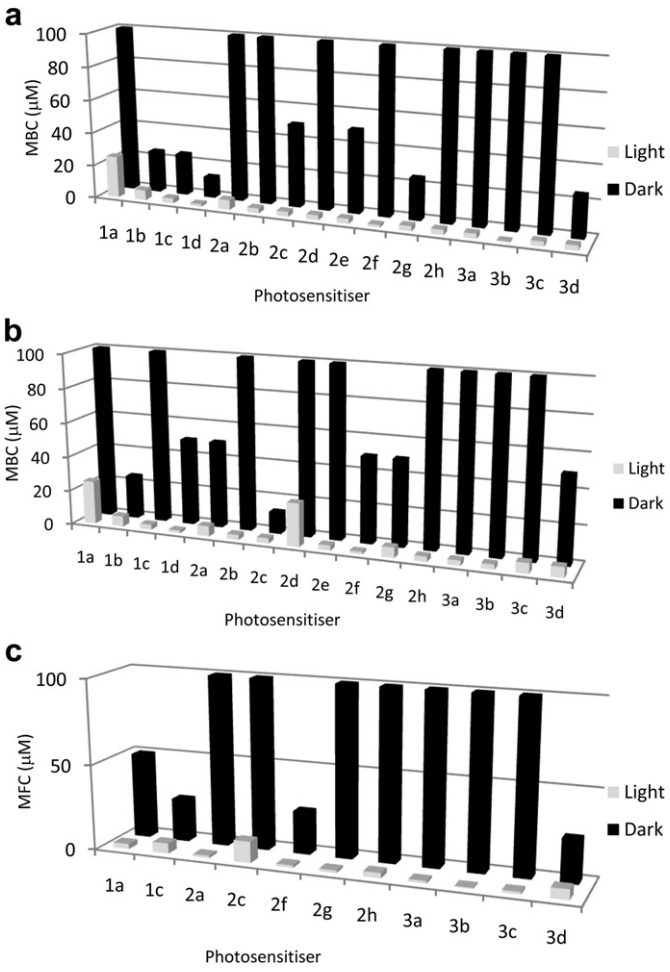

Fig. 4. Photoantimicrobial activities of the derivatives: (a) *S. aureus*; (b) *E. coli*; (c) *C. albicans*. MBC – minimum bactericidal concentration; MFC – minimum fungicidal concentration.

respective photoantimicrobial efficacies (Fig. 4a–c). Similarly, the derivatives exhibited intense absorption bands in the far-red region of the spectrum when in solution, but this may also be altered on biomolecular binding.

The microbiological screen used in the current work was based on *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*, to represent commonly occurring Gram-positive and Gram-negative bacteria and yeasts respectively, since the aim of the work was the production of improved broad-spectrum photoantimicrobials, rather than the class-specific chemotherapeutic agents currently available to healthcare concerns. As mentioned above, the lead compound in this work was methylene blue, but its tetraethyl and tetra(*n*-butyl) analogues (**1b** and **1c** respectively), whose photobactericidal efficacies have been published previously [15], were included to enable more direct comparison with several of the new derivatives. Dimethyl methylene blue (**1d**) is the most powerful commercially available phenothiazinium photosensitiser [16,17]. This was included both for this reason and also for structural comparison, since the 1- methyl- or 1,9-dimethylphenothiazinium fragment also appears in several of the new derivatives (Fig. 3).

It was established in a previous part of the program that the photobactericidal efficacy of methylene blue is not high against either of the standard bacterial challenges used. Thus it was not surprising that all of the new derivatives exhibited considerable improvements on this baseline (Fig. 4 a and b). However, most were also more active in the antibacterial screen than the tetraethyl and tetra(*n*-butyl) analogues, **1b** and **1c**, which had been shown previously to be more effective than the lead compound [15], and several were also of equal or greater potency when compared to **1d**. In the testing against *C. albicans*, however, methylene blue proved considerably more active, and although several of the new derivatives exhibited still higher photofungicidal activities, the differences in activity compared to the lead compound were considerably less than in the bacterial screening. The level of dark (inherent) toxicity for the derivatives was generally low, being in line with that of methylene blue in most cases (Fig. 4 a–c). Several of the derivatives thus proved to be highly photobactericidal and photofungicidal but to have low dark toxicities, conforming to the desired paradigm for photosensitisers. Indeed, compound **3b** was effective at submicromolar levels and was less dark toxic to *C. albicans* than was methylene blue under the conditions used.

The use of auxochrome-chromophore bridging to provide novel four- and five-ring derivatives of methylene blue thus proved advantageous from several standpoints regarding the accepted, necessary properties for a biological photosensitiser, viz. increased λ_{max} and amphiphilic character, while maintaining intense light absorption and high singlet oxygen yields. Given that the only previous example of this class of photosensitiser was synthesised for anticancer PDT application [13], the demonstrated photoantimicrobial utility, across the board, of the new derivatives was not predicted.

In several reported photoantimicrobial screens, 1,9-dimethyl methylene blue (**1d**) was significantly more effective in cell killing than methylene blue against both bacteria and viruses [17,18], much of this work forming the basis for the proposal of this derivative as an agent for pathogen inactivation in blood products. From Table 1 it may be observed that many of the Log*P* values of the current series are of similar magnitude to that of **1d**, i.e. amphiphilic rather than hydrophilic like methylene blue. This alone may explain the increased photoantimicrobial activities of the new derivatives, via improved uptake. In addition, the increases in λ_{max}, particularly in the pentacyclic derivatives, offer advantages in avoiding endogenous light absorption. The influence of haem pigments in the blood and tissues is one of the major obstacles to effective photodynamic action – whether antimicrobial or anticancer in application – thus the thrust to produce effective photosensitising agents which absorb intensely at wavelengths nearer to 700 than to 600 nm. This obstacle is of even greater significance in the use of PACT in the disinfection of red blood cells, for which there is currently no clinically accepted protocol. In terms of red cell disinfection, in addition to being a more powerful photoantimicrobial agent, the advantage of **1d** over the lead compound methylene blue lies in its ability to enter the red cell and thus to combat intracellular pathogens. Given the similarity in physicochemical profile between DMMB and the new derivatives (Table 1), intracellular activity is again indicated.

3. Experimental

N,N-Dimethyl-*p*-phenylenediamine sulphate, *N,N*-diethyl-*p*-phenylenediamine sulphate, 2-methyl-*N,N*-dimethyl-*p*-phenylenediamine sulphate, 1,2,3,4-tetrahydroquinoline, aluminium sulphate, potassium dichromate, silver carbonate (50% w/w on celite), sodium nitrite and zinc chloride were all purchased from Sigma-Aldrich Ltd (Gillingham, UK). Both 1,2,2,4-tetramethyl -1,2,3,4-tetrahydroquinoline and 1-ethyl-2,2,4-tetramethyl -1,2,3,4-tetrahydroquinoline were originally gifts from Eastman-Kodak Ltd. (Speke, UK). Methylene blue was purchased from Sigma-Aldrich but was chromatographically purified on silica. The tetraethyl and tetra (*n*-butyl) methylene blue derivatives were compounds prepared in pure form earlier in the project [15].

All spectrophotometric measurements were made using a Nicolet Evolution 300 spectrophotometer (Thermo Electron Corp.,

Hemel Hempstead, UK). Accurate molecular ion masses were obtained using a Micromass LCT TOF mass spectrometer.

3.1. Precursors

2-Amino-5-dimethylaminophenylthiosulphonic acid, 2-amino-5-diethylaminophenylthiosulphonic acid and 3-methyl-2-amino-5-dimethylaminophenylthiosulphonic acid were synthesised as in previous work [9].

3.2. General procedure for the synthesis of tetracyclic phenothiazinium salts

The requisite components, namely the arylthiosulphonic acid (4 mmol) and tetrahydroquinoline derivative (5 mmol) were brought to reflux 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 then refluxed for a further hour and allowed to cool before being filtered and the filtrates evaporated. The resulting residue was extracted with dichloromethane and purified by medium pressure liquid chromatography on silica using a gradient eluent system of dichloromethane:methanol, beginning with 100% dichloromethane, decreasing to 80% if required.

3.2.1. 9-Dimethylamino-1,2,3,4-tetrahydropyrido[3,2-b] phenothiazinium hydrogensulphate (**2a**)

9-Dimethylamino-1,2,3,4-tetrahydropyrido[3,2-*b*]phenothiazinium hydrogensulphate (**2a**) was prepared following the general procedure as outlined above from 2-amino-5-dimethylaminophenylthiosulphonic acid and 1,2,3,4-tetrahydroquinoline, as a blue-black powder, yield (19%); *m*/*z*, $M^+ = C_{17}H_{18}N_3S$ requires 296.41, found 296.41.

3.2.2. 9-Dimethylamino-2,2,4-trimethyl-1,2,3,4-tetrahydropyrido [3,2-b]phenothiazinium hydrogensulphate (**2b**)

From 2-amino-5-dimethylaminophenylthiosulphonic acid and 2,2,4-trimethyl-1,2,3,4-tetrahydroquinoline, as a blue-black powder, yield (13%); *m*/*z*, $M^+ = C_{20}H_{24}N_3S$ requires 338.17, found 338.50.

3.2.3. 9-Dimethylamino-1,2,2,4-tetramethyl-1,2,3, 4-tetrahydropyrido[3,2-b]phenothiazinium hydrogensulphate (**2c**)

From 2-amino-5-dimethylaminophenylthiosulphonic acid and 1,2,2,4-tetramethyl-1,2,3,4-tetrahydroquinoline, as a blue-black powder, yield (19%); *m*/*z*, $M^+ = C_{21}H_{26}N_3S$ requires 352.18, found 352.80.

3.2.4. 9-Dimethylamino-1-ethyl-2,2,4-trimethyl-1,2,3, 4-tetrahydropyrido[3,2-b] phenothiazinium hydrogensulphate (**2d**)

From 2-amino-5-dimethylaminophenylthiosulphonic acid and 1-ethyl-2,2,4-trimethyl-1,2,3,4-tetrahydroquinoline, as a blue-black powder (19%); *m*/*z*, $M^+ = C_{22}H_{28}N_3S$ requires 366.24, found 366.28.

3.2.5. 9-Diethylamino-1-ethyl-2,2,4-trimethyl-1,2,3, 4-tetrahydropyrido[3,2-b]phenothiazinium hydrogensulphate (**2e**)

From 2-amino-5-diethylaminophenylthiosulphonic acid and 1-ethyl-2,2,4-trimethyl-1,2,3,4-tetrahydroquinoline. A blue-black powder was isolated: yield (22%), *m*/*z*, $M^+ = C_{24}H_{32}N_3S$ requires 394.23, found 394.38.

3.2.6. 10-Dimethylamino-4-(2-hydroxyethyl)-1,3,3,6-tetramethyl-1,2,3,4-tetrahydropyrido [2,3-c]phenothiazinium hydrogensulphate (**2g**)

From 2-amino-5-dimethylaminophenylthiosulphonic acid and 1-(2-hydroxyethyl)-2,2,4,7-tetramethyl-1,2,3,4-tetrahydroquinoline, as a blue-black powder (14%); *m*/*z*, $M^+ = C_{23}H_{30}N_3S$ requires 396.21, found 396.20.

3.2.7. 10-Diethylamino-4-(2-hydroxyethyl)-1,3,3,6,8-pentamethyl-1,2,3,4-tetrahydropyrido[2,3-c]phenothiazinium hydrogensulphate (**2h**)

From 2-amino-3-methyl-5-diethylaminophenylthiosulphonic acid and 2,2,4,7-tetramethyl-1-(2-hydroxyethyl)-1,2,3,4-tetrahydroquinoline, as a blue-black powder, yield, 10%; *m*/*z*, $M^+ = C_{26}H_{36}N_3OS$ requires 438.26, found 438.20.

3.3. Synthesis of compounds from tetrahydroquinolinethiosulphonic acids

Since it was not possible readily to isolate the relevant thiosulphonic acids as required, the material was produced and utilised *in situ*. Thus, 1-ethyl-2,2,4-trimethyl-,2,3,4-tetrahydroquinoline (13.2 g, 65 mmol) was acidified with concentrated hydrochloric acid (200 mL) and ice (300 g) and was nitrosated by the dropwise addition of sodium nitrite solution (48 g in 240 mL water), keeping the reaction temperature below 10 °C with appropriate cooling. The resulting nitroso-compound was reduced with zinc powder (115 g) and concentrated hydrochloric acid (300 mL), to yield a pale pink aqueous solution of aryldiamine.

The diamino solution was added to a stirred solution of aluminium sulphate octadecahydrate/water (43.6 g, 65 mmol/100 mL). To this mixture was further added sodium thiosulphate/water (22.0 g, 139 mmol/80 mL) followed by zinc chloride/water (8.8 g, 63 mmol/12 mL). The solution was then cooled to 0 °C and potassium dichromate/water (5.0 g, 17 mmol/20 mL) was added dropwise over a 30 min period. Following addition of the potassium dichromate/water, the mixture was stirred for 2 h. For the last 30 min, the temperature was allowed to rise to room temperature. The resulting solution was heated to 40 °C and the requisite tetrahydroquinoline or substituted aniline (65 mmol) was added, along with potassium dichromate/water (5.0 g, 17 mmol/20 mL). The resulting dark green solution was then heated to 85 °C and powdered copper(II) sulphate (130 g) added. The final reaction mixture was maintained at 85 °C for 1 h, cooled to 25 °C, followed by the slow addition of concentrated sulphuric acid (5 mL), and the precipitated solid filtered off. Purification of the resultant solid was carried out by column chromatography as described in the general procedure for tetracyclics above.

3.3.1. 1,2,2,4,8,10,10,11-Octamethyl-1,2,3,4,8,9,10, 11-octahydrodipyrido[3,2-b:2′,3′-i]phenothiazinium hydrogensulphate (**3a**)

1,2,2,4,8,10,10,11-Octamethyl-1,2,3,4,8,9,10,11-octahydrodipyrido [3,2-*b*:2′,3′-*i*]phenothiazinium hydrogensulphate (**3a**) was prepared following the general procedure as outlined above from 1,2,2,4-tetramethyl-1,2,3,4-tetrahydroquinoline as metallic red-brown leaflets, yield (21%); *m*/*z*, $M^+ = C_{26}H_{34}N_3S$ requires 420.25, found 420.20.

3.3.2. 1,11-Diethyl-2,2,4,8,10,10-hexamethyl-1,2,3,4,8,9,10, 11-octahydrodipyrido[3,2-b:2′,3′-i]phenothiazinium hydrogensulphate (**3b**)

From 2,2,4-trimethyl-1-ethyl-1,2,3,4-tetrahydroquinoline as metallic red-brown leaflets: yield (22%); *m*/*z*, $M^+ = C_{28}H_{38}N_3S$ requires 448.28, found 448.25.

3.3.3. 4-(2-Hydroxyethyl)-1,3,3,6,9,11,11,12-octamethyl-1,2,3,4,9,10,11,12-octahydrodipyrido[2,3-c:2′,3′-i]phenothiazinium hydrogensulphate (**3c**)

From 1,2,2,4-tetramethyl-1,2,3,4-tetrahydroquinoline and 1-(2-hydroxyethyl)-2,2,4,7-tetramethyl-1,2,3,4-tetrahydroquinoline

(15.2 g, 65 mmol) as a blue-black powder, yield (20%); *m*/*z*, $M^+ = C_{28}H_{38}N_3OS$ requires 464.27, found 464.30.

*3.3.4. 4,10-Di(2-hydroxyethyl)-1,3,3,6,8,11,11,13-octamethyl-1,2,3,4,10,11,12,13-octahydrodipyrido [2,3-c:3′,2′-h]phenothiazinium hydrogensulphate (**3d**)*

From 1-(2-hydroxyethyl)-2,2,4,7-tetramethyl-1,2,3,4-tetrahydroquinoline as a black powder, yield (13%); *m*/*z*, $M^+ = C_{30}H_{42}N_3O_2S$ requires 508.30, found 508.30.

*3.3.5. 9-Di(n-butyl)amino-1,2,2,4-tetramethyl-1,2,3,4-tetrahydrodipyrido[3,2-b]phenothiazinium hydrogensulphate (**2f**)*

From 1,2,2,4-tetramethyl-1,2,3,4-tetrahydroquinoline and *N,N*-dibutylaniline (13.3 g, 65 mmol) as dark blue powder, yield (25%); *m*/*z*, $M^+ = C_{27}H_{38}N_3S$ requires 436.28, found 436.26.

3.4. Photoantimicrobial activity

The activities of the derivatives alongside those of the known photosensitisers methylene blue, dimethyl methylene blue and the two alkylated methylene blue derivatives were measured against a Gram-positive and a Gram-negative organism, *S. aureus* (National Culture Tissue Collection, NCTC 6571) and *E. coli* (NCTC 10418) respectively. Both strains were grown in Mueller-Hinton Broth and then diluted to a concentration of 10^6 colony-forming units mL^{-1} using M^{c}Farland's standard. Aliquots of the strains were then incubated for 1 h at 37 °C in 96-well microtitre trays with various concentrations of photosensitiser in the range 100–0.4 μM, with zero photosensitiser concentrations in each case for control purposes. The trays were then either illuminated for 10 min using an LED array (14 × 9 660 nm, Heelspurs LLC, USA) giving a light dose of 6.2 J cm^{-2} or alternatively foil-covered to provide dark controls. From each well showing an inhibition of growth of the micro-organism, 10 μL was sub-cultured on nutrient agar, using the Miles–Misra method [19], and incubated for 18 h at 37 °C. The minimum bactericidal concentrations were then 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.

The photofungicidal efficacies of a range of derivatives were measured against the yeast organism *C. albicans* (National Collection of Pathogenic Fungi, NCPF 8179) in a similar fashion to that detailed above, except that the strain was initially grown in Sabouraud media and was sub-cultured on Sabouraud-dextrose agar plates.

3.5. Singlet oxygen production

Relative singlet oxygen production by the photosensitisers was assayed using the decolourisation of 2,3,4,5-tetraphenylcyclopentadienone (TPCPD) in dichloromethane. Thus the decrease in absorption by TPCPD at 500 nm due to its interaction with singlet oxygen was monitored spectrophotometrically over time as in the method of Cincotta *et al* [8]. Methylene blue was employed as the standard photosensitiser as in previous work [15].

3.6. Measurement of lipophilicity (LogP)

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 described by Pooler and Valenzo [20].

4. Conclusion

The extension of the phenothiazinium system via ring fusion incorporating both the chromophore and auxochromes furnished both longer linear and angular molecular shapes than is normal with methylene blue derivatives. The improved photoproperties in the resulting series were accompanied by considerably increased photoantimicrobial efficacies and low dark toxicities, thus exhibiting marked improvements over methylene blue.

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