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Phenothiazinium photosensitisers XI. Improved toluidine blue photoantimicrobials

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ABSTRACT

The phenothiazinium derivative toluidine blue O (TBO) is widely employed as a photoantimicrobial agent in clinical trialling, particularly in dentistry. However, its activity against a range of pathogenic microbial species is not significantly different to that of the standard photoantimicrobial methylene blue. In the current study, derivatives of TBO with varying hydrocarbon substitution in chromophore position 2 were synthesised via the established anilinethiosulphonic route, using the mild oxidant silver(II) carbonate to allow substituent preservation.
The resulting series of analogues demonstrated the expected increases in visible absorption wavelength and lipophilicity with increasing hydrocarbon content, as well as decreased aggregation for derivatives with bulkier substituents, and all produced singlet oxygen on illumination in vitro. Screening against a range of bacterial and fungal pathogens relevant to infection control showed remarkable increases in activity relative to the parent compound, particularly against the clinically important Gram-negative bacterium *Pseudomonas aeruginosa*. In addition, in order to demonstrate clinical relevance, the photoactivities of the new derivatives against microbial targets were compared to conventional antibacterial and antifungal drugs, as well as biocides commonly used for local disinfection. Activity here was also generally greater than that of the conventional agents used for comparison, considerably so relative to the local disinfectant agents.

1. Introduction

Toluidine blue O (tolonium chloride, TBO, Fig. 1) is a phenothiazinium derivative related to methylene blue (MB, Fig. 1). It has been used widely in the biological arena as a stain in the pathology of both cancer and microbial disease [1,2]. More recently TBO has been widely examined as a photoantimicrobial agent aimed at oral disinfection [3,4], and is a lead compound in azine photosensitiser research and development [5].

Where comparative testing of the photoantimicrobial efficacies of MB and TBO have been carried out, little overall difference has been reported [6]. However, as the principal lead compound for photoantimicrobial research, MB has a significantly fuller pedigree in terms of the range of bacterial, fungal, viral and protozoal challenges reported [7]. Logically, both are established to interact with anionic groups at the external surfaces of the microbial target, e.g. with lipoteichoic acids [8], and to displace stabilising metal cations in membrane structures [9].

The use of TBO in biological staining often utilises the property of metachromasia. This is exhibited as a change in colour of the stained tissue due to the significant aggregation of the highly planar, substituted phenothiazinium cation [10]. As the chromophore concerned is established as a planar moiety, this infers that neither the auxochromic dimethylamino- or amino- groups, nor the methyl at position 2 of the ring system cause any deviation from this. Aggregates can be observed in the visible spectrum of aqueous TBO —– and similarly for other phenothiazinium cations — as a broad shoulder to lower wavelengths extending from the λ_{max} [6].

As part of the ongoing programme of photosensitiser discovery, improved derivatives of toluidine blue were sought — particularly for photoantimicrobial analoguing — by variation of the group at C-2 of the chromophore. Previous work has demonstrated that the use of increased chromophore methylation leads to greater photoantimicrobial activity in derivatives of both methylene blue and azure A [11,12].

2. Materials & Methods

2.1. Synthesis

Substituted anilines, *p*-phenylenediamine derivatives, silver carbonate (5% *w/w* on celite) and solvents were purchased from Sigma-Aldrich UK, and used without further purification. Photophysical characterisation of the products was carried out using a Hewlett Packard 8452A diode array spectrophotometer. This was also used for the determination of singlet oxygen yield and of lipophilicity. Accurate molecular ion masses (m/z) for the derivatives were obtained using a Micromass LCT TOF mass spectrometer.

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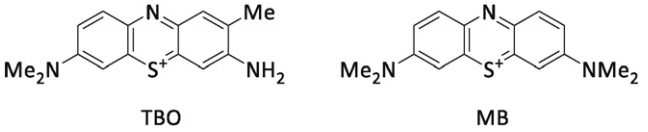


Fig. 1. Accepted structures for toluidine blue O and methylene blue cations.

2.1.1. 2-Amino-5-dimethylaminophenylthiosulphonic Acid

N,N-Dimethyl-*p*-phenylenediamine sulphate (130 mmol) was added to a mechanically stirred solution of aluminium sulphate octadecahydrate/water (43.6 g, 65 mmol/100 ml). To this was added a solution of sodium thiosulfate in water (22.0 g, 139 mmol/80 ml) followed by zinc chloride in water (8.8 g, 63 mmol/12 ml). The reaction solution was cooled to 0 °C and aqueous potassium dichromate (5.0 g, 17 mmol in 20 ml water) was added dropwise over 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. Yield = 15.87 g (49%), m.p. 190 °C (dec.)

2.1.2. 2-Alkyl-3-amino-7-dimethylaminophenothiazinium Derivatives

2-Amino-5-dimethylaminophenylthiosulphonic acid (4 mmol) and 2-alkylaniline (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 was extracted with dichloromethane and purified by column chromatography on silica.

2.1.3. 3-Amino-7-dimethylamino-2-methylphenothiazinium Hydrogensulphate (toluidine blue O, 1a)

Prepared as above from 2-amino-5-dimethylaminophenylthiosulphonic acid and 2-methylaniline. Blue-black powder, yield = 460 mg, 31%; m/s $C_{15}H_{16}N_3S$ requires 270.11, found 270.11; λ_{max} (MeOH) 629 nm.

2.1.4. 3-Amino-7-(dimethylamino)-2-ethylphenothiazinium Hydrogensulphate (1b)

From 2-amino-5-dimethylaminobenzenethiosulphonic acid and 2-ethylaniline as violet-blue crystals, yield = 273 mg, 18%; m/z, $C_{16}H_{18}N_3S$ requires 284.40, found 284.42; λ_{max} (MeOH) 634 nm.

2.1.5. 3-Amino-7-(dimethylamino)-2-n-propylphenothiazinium Hydrogensulphate (1c)

From 2-amino-5-dimethylaminobenzenethiosulphonic acid and 2-*n*-propylaniline as blue-black powder, yield = 302 mg, 19%; m/z, $C_{17}H_{20}N_3S$ requires 298.43, found 298.40; λ_{max} (MeOH) 636 nm.

2.1.6. 3-Amino-2-tert-butyl-7-(dimethylamino)phenothiazinium Hydrogensulphate (1d)

From 2-amino-5-dimethylaminobenzenethiosulphonic acid and 2-*tert*-butylaniline as black powder, yield = 487 mg, 30%; m/z, $C_{18}H_{22}N_3S$ requires 312.45, found 312.41; λ_{max} (MeOH) 637 nm.

2.1.7. 3-Amino-7-(dimethylamino)-2-phenylphenothiazinium Hydrogensulphate (1e)

From 2-amino-5-dimethylaminobenzenethiosulphonic acid and 2-aminobiphenyl as dark blue powder, yield = 455 mg, 26%; m/z, $C_{20}H_{18}N_3S$ requires 332.44, found 332.41; λ_{max} (MeOH) 639 nm.

2.2. Singlet Oxygen Testing

Singlet oxygen production by the photosensitisers was assayed as in previous work [13], using the decolourisation of 2,3,4,5-tetraphenylcyclopentadienone (TPCPD) in dichloromethane. Thus the decrease in absorption of TPCPD 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 toluidine blue derivatives under identical conditions ($t_{½}$TBD) thus gives a measure of its photosensitising efficiency. Thus, if the time for the DPIBF absorption to decrease by 50% due to TBO photosensitisation is $t_{½}$TBO, relative singlet oxygen yields for the derivatives are given by:

$$\text{Relative } {}^1O_2 \text{ yield} = \frac{t_{1/2}\text{TBO}}{t_{1/2}\text{TBD}}$$

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

2.3. 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 [14] based on the relationship:

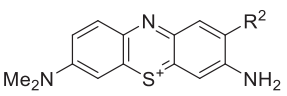
$$\text{Log}P = \text{Log}\left\{\frac{\left(A - A^1\right)}{A^1} \cdot \frac{V_W}{V_O}\right\}$$

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.

2.4. Antimicrobial Screening/Comparison

The photobactericidal efficacies of the derivatives in addition to that of the known photosensitiser methylene blue were measured against both Gram positive *Staphylococcus aureus* (NCTC 6571) and *Enterococcus faecalis* (NCIMB 13280) and Gram negative *Escherichia coli* (NCTC 10418), *Proteus mirabilis* (NCIMB 5887) and a clinical strain of *Pseudomonas aeruginosa* bacteria (courtesy of the Clatterbridge Hospital, Bebington, UK). 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 1 h at 37 °C in microtitre trays with various concentrations of photosensitiser in doubling dilutions from 100 μM, with zero photosensitiser concentrations in each case for control purposes. The trays were then either illuminated for 20 minutes using an array of light-emitting diodes (660 nm) 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, 1 μl was sub-cultured on nutrient agar, using the Miles–Misra method,

Table 1
Relevant properties for the toluidine blue derivatives (* relative to toluidine blue; †Lit. −0.21 [15]).



R^2	λ_{max} (nm, MeOH)	$^1O_2$ yield*	LogP
Me	629	1.00	−0.20†
Et	634	1.49	−0.46
n-Pr	636	1.24	+0.29
t-Bu	637	1.79	+0.84
Ph	639	1.87	+0.63