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Phenothiazinium photosensitisers: choices in synthesis and application

Mark Wainwright[a,*], Richard M. Giddens[b]

[a]Centre for Photobiology and Photodynamic Therapy, Department of Colour Chemistry, Leeds University, Leeds LS2 9JT, UK
[b]Centre for Material Science, University of Central Lancashire, Preston PR1 2HE, UK

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Abstract

The use of phenothiazinium dyes in the photodynamic therapy of cancer and its related antimicrobial protocols, e.g. blood product decontamination, has been mainly limited to a few commercially available dyes, such as Methylene Blue, the Azure stains and Toluidine Blue O. Novel Methylene Blue derivatives are scarce in the literature, and yet there are various synthetic routes available to furnish sufficient candidate compounds for properly organised programmes of photosensitiser design and development to be carried out. In this review, consideration is given to the types of phenothiazinium derivative required for the various photodynamic applications currently under examination, and also to the synthetic strategies involved in their preparation.

Keywords: Antimicrobial; Blood; Cancer; Methylene Blue; Phenothiazinium; Photosensitiser; Synthesis

1. Introduction

The modern renaissance in the use of photosensitising compounds in medicine is based on the porphyrin nucleus—the only FDA-licensed drug for the photodynamic therapy of cancer remains Photofrin®, a mixture of porphyrin oligomers [1]. However, improved, second-generation photosensitisers are derived from a variety of chromophoric types, not just porphyrin analogues.

The phenothiazinium dyes were first synthesised in the late 19th century—e.g. both Methylene Blue (Caro) and Thionin (Lauth) in 1876—during what might be considered to be a "gold rush" period of chemical experimentation after the discovery of the first aniline dyes. These syntheses usually entailed the oxidation of an intended substance which was in fact impure—the occurrence of sulfur in coal tar often made this inevitable. Thus oxidation of anilines in the presence of sulfur gave rise to coloured species, among them the first, crude phenothiazinium dyes.

In terms of the biomedical use of phenothiazinium dyes, this was begun in specimen staining for microscopy by various medical scientists, among whom were famous men such as Romanovsky, Koch and Ehrlich. The idea of structure—activity relationships in stains developed in this era, particularly by Paul Ehrlich, laid the foundations for modern medicinal chemistry, and these principles should be followed by those attempting properly

* Corresponding author.
E-mail address: ccdmw@leeds.ac.uk (M. Wainwright).

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organised photosensitiser synthesis. Cellular uptake is determined by a combination of charge type/distribution and lipophilicity, both of which characteristics may be controlled by informed synthesis.

Due to the expansion of PDT into the antimicrobial milieu, there now exists far greater scope for photosensitiser design. For example, in the field of blood product disinfection, an ideal candidate photosensitiser would be effective in the inactivation of bacteria, viruses, yeasts and protozoa, but would remain non-toxic and non-mutagenic in a human recipient. It is hardly surprising that none of the currently available agents fits all of these criteria.

Where phenothiazine derivatives have been used, the reliance of contemporary researchers both in PDT and in the related antimicrobial approach on commercial phenothiazinium stains (Table 1), rather than novel molecules, underlines the lack of involvement of chemists in photosensitiser development. Many PDT groups, inter alia, have investigated the behaviour of Methylene Blue or occasionally Toluidine Blue O, but where novel analogues have been prepared these are merely auxochromic side chain derivatives. Such an approach to drug discovery could not be countenanced in the pharmaceutical industry—lead structure optimisation requires consideration of the whole molecule, not only selected, straightforward variations.

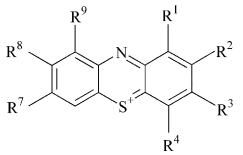
Phenothiazinium salts offer more scope in terms of the therapy of disease states than other dye types—and certainly more so than other photosensitisers. Thus dyes such as Methylene Blue and Toluidine Blue O have been lead compounds in drug research against local bacterial infection [2,3], tuberculosis [4], trypanosomiasis [5], malaria [6,7], rickettsial illness [8], yeast infection [9], viral blood colonisation [10] and cancer [11,12], not to mention their part in the development of the massive phenothiazine neuroleptic family [13]. Among other dye types, only the acridines approach such versatility [14].

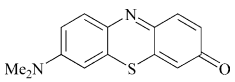
The current review covers the methods of synthesis of the phenothiazinium chromophore, also of related phenothiazine-based photosensitisers, and strategies for drug development in the various photosensitiser-involved protocols for this important class of compounds.

2. Photosensitiser requirements

In general the anatomy of a phenothiazinium photosensitiser may be thought of in two parts:

Table 1
Commercially-available phenothiazine derivatives tested as photosensitisers





MV

	R^1	R^2	R^3	R^4	R^7	R^8	R^9
Methylene Blue (MB)	H	H	NMe_2	H	NMe_2	H	H
Azure A (AA)	H	H	NH_2	H	NMe_2	H	H
Azure B (AB)	H	H	NHMe	H	NMe_2	H	H
Azure C (AC)	H	H	NH_2	H	NHMe	H	H
Thionin (Th)	H	H	NH_2	H	NH_2	H	H
Methylene Green (MG)	H	H	NMe_2[a]	NO_2	NMe_2	H	H
Toluidine Blue O (TBO)	H	Me	NH_2	H	NMe_2	H	H
New Methylene Blue (NMB)	H	Me	NHEt	H	NHEt	Me	H
Dimethyl Methylene Blue (DMMB)	Me	H	NMe_2	H	NMe_2	H	Me
Methylene Violet (MV)							

[a] Probably NHMe, see text.

the chromophore (i.e. the oxidised ring system) and its peripheral modifications (side chains, auxochromes). The former endows the light absorption/emission and photosensitising properties, whereas the latter are important in physical properties such as solubility and lipophilicity, which affect both drug distribution and uptake.

The delocalised phenothiazinium ring system (Fig. 1) offers considerable scope for alteration in terms of both chemical and physical properties. At the outset, however, it should be realised that the alteration of one part of a lead molecule (e.g. Methylene Blue) may have an effect on the electronic distribution in the chromophore, and thus on its photosensitising ability (Table 2). For example, it is known that the iodination of photosensitisers increases singlet oxygen yield, but the inclusion of a single iodine atom in the phenothiazinium chromphore causes a significant increase in lipophilicity [15,16]. Thus although the resulting cation will exhibit improved photosensitising ability, its biological distribution (and toxicity) may also be considerably altered (Table 3).

Some key criteria for the use of phenothiazinium compounds as useful photosensitisers are considered here.

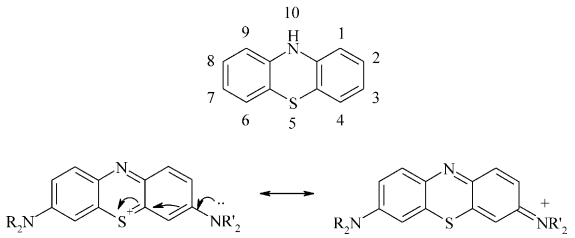


Fig. 1. Numbering of the phenothiazine ring system and delocalisation in the phenothiazinium chromophore.

Table 2
Photoproperties and Log*P* values for commercially-available phenothiazine-based photosensitisers

Photosensitiser	λ_{max} (H_2O, nm)	Relative $^1O_2$ yield[a]	Log*P*
MB	656	1.00	−0.10
AA	623	0.86	+0.70
AB	648	0.41	−0.09
AC	616	0.71	–
Th	595	1.16	–
MG	654	0.50	−0.28
TBO	625	0.86	−0.21
NMB	630	1.35	+1.20
DMMB	648	1.21	+1.01
MV	580	0.73	–

For compound abbreviations, see Table 1.

[a] Spectrophotometric measurement of the oxidation of 1,3-diphenylisobenzofuran, $^1O_2$ yield relative to that of MB.

2.1. Light absorption

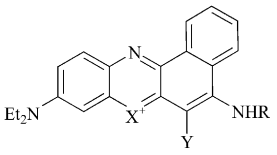
Generally for the PDT of cancer, long wavelength absorbers are required in order to gain maximal penetration of tissue. A typical wavelength range for the useful "therapeutic window" for PDT is 600–750 nm. However, the normal range for conventional phenothiazinium salts (in water) is 590–680 nm (Table 2). Extending the absorption beyond this requires functionalisation outside the auxochromic moieties.

Blood products may be generalised as three basic fractions. Plasma is a colourless suspension of proteins; platelet concentrates are also colourless; red blood cell concentrates approximate a very intense haem-type spectrum. In the first two types, visible light, or long wavelength ultraviolet is sufficient for the activation of photosensitisers. In pigmented red blood cell fractions the photoactivation profile requirement is much more stringent, 620–750 nm, similarly to that for cancer PDT. However, this range is suitable for the majority of the phenothiazinium photosensitisers. Shorter wavelength absorbers such as Thionin (λ_{max} 595 nm) have been proposed for use in plasma decontamination [17].

There are likewise absorption considerations to be made in the application of photosensitisers to antimicrobial disinfection, e.g. wounds, surface sterilisation etc. In wounds endogenous absorption is due mainly to blood, whereas in surface contamination light absorption by microbes is generally in the ultraviolet, except for pigmented species such as *Pseudomonas aeruginosa*, *Aspergillus niger* etc.

Structurally, increasing the maximum wavelength of absorption of phenothiazinium dyes should follow standard rules for an odd-alternant

Table 3
Changes in physicochemical properties of Nile Blue analogues with halogenation



X	Y	R	λ_{max} (nm)	$^1O_2$ quantum yield	Log*P*	pK_a	Ref
O	H	H	623	0.005	2.25	10.0	20
O	Br	H	643	0.007	–	8.0	20
O	I	H	642	0.025	2.55	10.0	20
S	I	H	660	0.23	3.70	6.5	20
O	H	Et	632	0.005	2.69	–	19
S	H	Et	652	0.025	2.76	–	19
Se	H	Et	659	0.650	2.10	–	19

chromogenic system [18], when considering electronic factors although these may be altered by steric crowding. Thus while λ_{max} values may be elevated slightly by judicial substitution of +I/−I groups, significant change is associated with heteroatom (chalcogen) variation and with increased π-character. For example, benzo[*a*]phenothiazinium analogues are generally red-shifted by ca. 10–20 nm (Table 3) [19]. However, the increased aromatic character also leads to decreased singlet oxygen yields (Table 3) [20]. A similar situation pertains with *N*-aryl rather than *N*-alkyl auxochromic character [21].

2.2. Photosensitising efficacy

Since the phenothiazinium compounds discussed in this review are intended for the eradication of non-economic cells, it is logical to suggest that, for each application, the maximum production of toxic species is attained. The quantum yield of singlet oxygen for the widely used lead compound Methylene Blue is 0.44 [15]. Thus, in synthesising derivatives it is important that the resulting candidates do not fall too short of this—for example Methylene Green (*q.v.*), derived from facile nitration of MB was reported to have only 50% of the singlet oxygen yield of the parent compound (Table 2) [22].

Increased singlet oxygen yields have been reported for chromophorically methylated derivatives such as Dimethyl Methylene Blue, New Methylene Blue (DMMB and NMB respectively, Table 2), and 1-methyl Methylene Blue [23,24]. In addition, the tetrahydroquinoline derivative DO15 (effectively with 2,8-dialkyl character, similarly to New Methylene Blue, Fig. 2) is a superior photosensitiser to MB [25].

The "heavy atom effect" is a well established method in the improvement of photosensitising efficiency, depending on the increased stabilisation of the triplet excited state of the chromophore when substituted by atoms of greater atomic mass. This phenomenon is simply demonstrated in comparing the singlet oxygen yield of e.g. MB with the corresponding phenoxazinium dye, sulfur in position 5 of the chromophore giving approximately 10 times the yield of the corresponding oxygen analogue [26]. This effect has also been shown in selenium analogues [27], and in halogenated photosensitisers [15]. It is worthwhile considering the effect of such substitution on the molecule as a whole. Although increased photosensitising efficacy should result, varying the chalcogen leads to a symmetrical change in the electron cloud of the chromophore (being a central atom), whereas halogenation, usually at position 4 of the phenothiazinium ring system, introduces significant asymmetry. The possibility of a change in cellular uptake or in the site of action is therefore more likely using the latter approach.

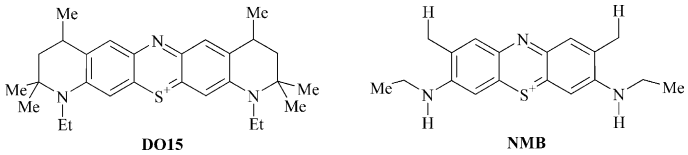


Fig. 2. Structural similarity between DO15 and New Methylene Blue.

2.3. Photobleaching

For any proposed photosensitiser, the effects of light and reactive oxygen species produced on the compound itself must be considered. For therapeutic use, it may be beneficial for photobleaching of the chromophore to occur post-treatment in order to minimize patient photosensitivity, whereas long term disinfection may require minimal photofading for extended photosensitising efficacy. Such considerations must be a part of the approach to rationale photosensitiser design and development programmes.

The illumination of MB can also cause photofading/photobleaching, i.e. the formation of breakdown products via photochemical mechanisms—indeed MB is often employed as a model example for dye photodestruction protocols, e.g. using TiO_2 photocatalysis. Thus, as with chemical methods, demethylation of the molecule at the amino residues leads to the formation of the Azure stains and Thionin [28], and further decomposition to benzenoid species has been proposed [29] (Scheme 1). However, the degree of decomposition is governed by the quantity of illumination—i.e. high intensity light will cause more breakdown than low intensity, and similarly for long periods of illumination compared to short. In terms of any clinical treatment, illumination conditions should thus be optimized to simplify the resulting toxicological spectrum and to ensure a high concentration of the more effective photosensitiser (MB). The decomposition of stored MB (solid or solution) should also be considered in its preparation for a proposed clinical end use.

In addition, as with MB, its demethylated photoproducts may undergo reduction. Thus the therapeutic/toxicological profile of MB in any clinical photodynamic role is considerably more complex than it initially appears. Such considerations can be minimised by local application, but, for example, protocols for the reinfusion of blood plasma which has undergone MB-phototreatment must account for any breakdown species formed during the procedure [30]. The degradation of higher MB homologues in this fashion has not been thoroughly investigated.

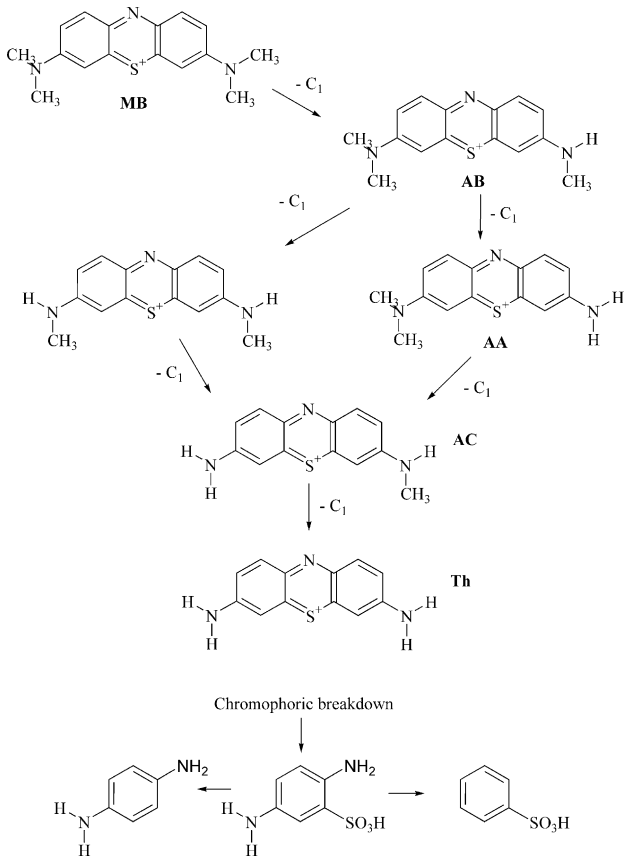
2.4. Redox potential

Photosensitisation requires the promotion of electrons to excited states, and in the case of the type I mechanism involves electron transfer reactions. The phenothiazinium chromophore is well-suited to this behaviour, although most associated photosensitiser processes appear to involve the production of singlet oxygen as the major cell killing agent.

The ease of reduction of some phenothiazinium salts, while useful e.g. in decolorisation tests for bacteria, may be deleterious to photodynamic action, i.e. the reduced (leuco) form is colourless and a poor photosensitiser. However, the steric crowding of *N*-10 in the structure, e.g. by methyl groups, appears to inhibit reduction [23].

2.5. Hydrophilic/lipophilic balance

Commonly used phenothiazinium photosensitisers, such as MB and Toluidine Blue O are highly water soluble (hydrophilic), and this is reflected in short pharmacological half lives. Unfortunately such hydrophilic nature is a barrier to lipid partitioning and explains the poor cellular uptake of these compounds. In quantitative terms, hydrophilic character is associated with poor distribution from water into octanol, the logarithm of the distribution coefficient (Log*P*, Table 2) for such compounds as MB and TBO being <0. Lipophilic character is associated with compounds having Log$P > +1.5$, with species in the intermediate range being considered amphiphilic. From a practical viewpoint, highly lipophilic



Scheme 1. Photodegradative pathways of Methylene Blue. MB = Methylene Blue; AB = Azure B; AA = Azure A; AC = Azure C; Th = Thionin.

photosensitisers can be problematic in that they do not easily dissolve in aqueous media (i.e. for patient administration), although this problem may be solved via the use of benign co-solvents or liposomal formulation.

Simple changes to the MB structure can endow more lipophilicity, for example increasing the alkyl content, either by chromophoric or auxochromic alteration. However, derivatives having long alkyl chains are often insoluble in water. Thus in a homologous series of MB derivatives reported by Mellish et al., where the dimethylamino auxochromes were replaced by diethylamino, dipropylamino etc., up to dihexylamino, aqueous solubility was insufficient above the diethylamino analogue, requiring the use of a co-solvent in testing protocols [31].

As might be expected, the inclusion of increased aromatic character, whether fused or side chain, generally leads to an increase in lipophilicity. For example, the benzo[*a*]phenothiazinium analogues were reported to have Log*P* values in the region of +3 (Table 3, c.f. MB = −0.1), while bis(arylamino)phenothiazinium salts were too insoluble in water to attempt meaningful Log*P* measurement [21].

For useful biological activity, a balance of hydrophilic/lipophilic character is usually required. For example Benetto et al. showed that the chromophore reduction of commercially available phenothiazinium dyes by *Escherichia coli* was closely related to the hydrocarbon character of the auxochromic moieties [32]. Although none of the commercial phenothiazinium dyes is lipo-

philic, this study underlines the increased uptake of the series with increasing Log*P*, since bacterial reduction relies on entry of the dye into the cell.

2.6. Planarity

Regardless of the substitution pattern of the derivative, the phenothiazinium chromophore is planar, whereas the reduced 10*H*-phenothiazine system has a dihedral angle of 115°. In targeting nucleic acid, e.g. in blood disinfection programmes, planarity is obviously an advantage [33]. However, the opposite construction may be applied where potential mutagenicity in transfusion recipients is concerned.

Although this may seem to be an impasse, a middle way may be made possible by carefully directed synthesis, since it has been reported that increased alkyl character in the auxochromic moieties of MB furnishes photosensitisers which do not localise in the cell nucleus [31].

The inclusion of electronically inert (alkyl) groups in to the ring system may affect the intercalative properties of the phenothiazinium chromophore. However, this would require the use of relatively large groups, probably $\geqslant C_4$, although changes in the intercalating behaviour of the related aminoacridine chromophore were brought about by bromination alone [34].

2.7. Charge

Traditionally, the phenothiazinium dyes are known as cations (basic dyes). However, the overall charge on the molecule may be cationic, anionic or neutral, depending on the substitution type. For example, anionic groups may be included in the auxochromes to give an overall negative charge [35]. While this would be useful in preventing nucleic acid intercalation (and thus potential mutagenicity), it would also alter cellular uptake. Such an approach could also be employed to furnish an overall neutral (zwitterionic) molecule. This approach also has the advantage of conferring negative charge without significant—and probably deleterious—effects on the chromophore, as would be the case with e.g. direct ring sulfonation.

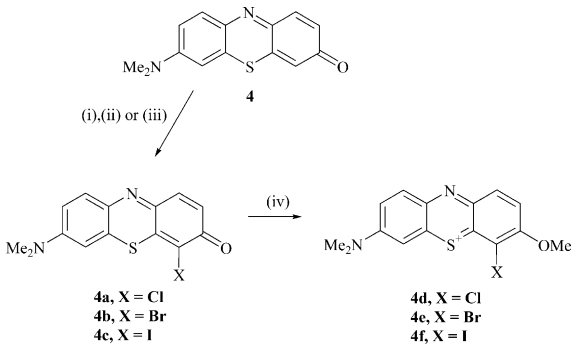
The presence of a delocalised cation as the chromophore can also promote the formation of neutral species where the auxochromic nitrogen has an N–H bond. Although there are more examples of commercial phenothiazinium dyes with this feature (e.g. the Azures, TBO, Thionin etc.) than without, there is scant reference to the formation of the neutral quinoneimine in the literature [36]. Cellular uptake of neutral species is simpler than for charged species.

A related molecular type here is Methylene Violet (MV). This is a neutral species having a double-bonded oxygen in place of one the auxochromic moieties in Methylene Blue. Indeed, alkaline hydrolysis of MB yields MV [37]. A good demonstration of the improved uptake of neutral species is provided by MV, this being far more effective against intracellular viruses in red blood cells than is MB, the latter being mainly excluded from the interior [38]. Such advantage is balanced by the inhibition of action of this photosensitiser (and its halogenated analogues) in the presence of plasma proteins [39]. Greater aqueous solubility was attained on the conversion of the neutral oxo function to alkoxy, regenerating a phenothiazinium salt (Scheme 2), and halogenated versions exhibited both similar singlet oxygen yields to MB in combination with lowered protein binding compared with the parent MV [39].

3. Synthesis

3.1. Ring synthesis: phenothiazinium salts

Since the phenothiazinium derivatives are oxidised species, there must be, by definition, an oxidising agent employed at one stage during the synthetic procedure, and several different reagents have been used for this purpose, e.g. dichromate, silver salts etc. While the choice of reagent is perhaps less important in conventional ring synthesis of the standard Methylene Blue type molecule, where elaboration of the chromophore itself is intended more consideration is required. For example, chromophoric alkyl moieties are likely to be oxidised to carboxylic acid residues, perhaps with subsequent decarboxylation, under the



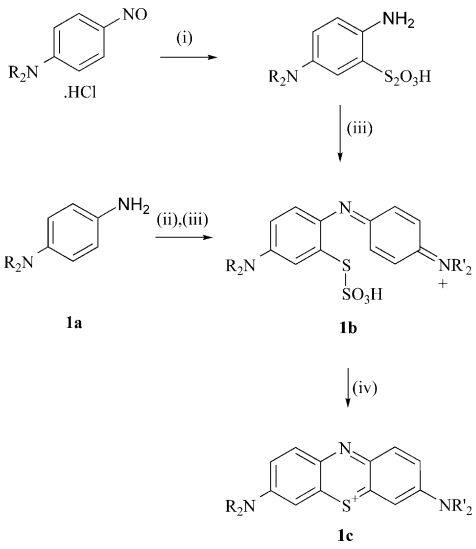
Scheme 2. Synthesis of neutral and cationic Methylene Violet analogues. (i) 1-chloroethyl orthoformate; (ii) *N*-bromosuccinimide/acetic acid; (iii) KI/KIO_3 in absolute ethanol; (iv) methyl triflate.

stringent dichromate/elevated temperature route. Similarly, water-insoluble anilinium salts, e.g. containing halogens or having a high hydrocarbon content, would be better oxidised in a polar organic solvent such as ethanol, which again means that a weaker oxidant than dichromate must be employed, e.g. ferric chloride [40] or silver carbonate, [41].

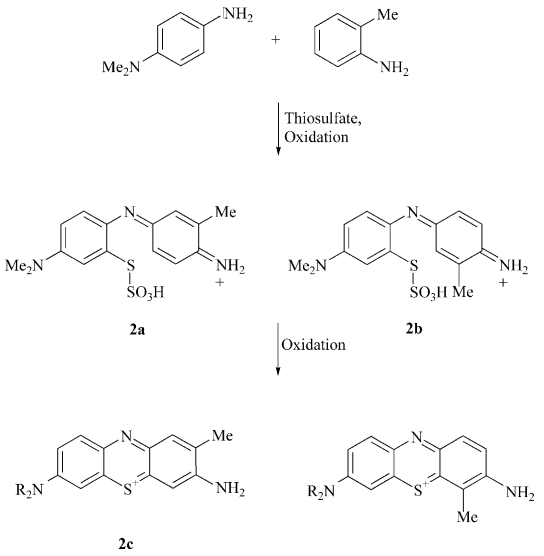
The traditional approach to phenothiazinium synthesis employs a *p*-phenylenediamine derivative (**1a**, Scheme 3) as the starting material. This is dissolved in acidic media and oxidised with thiosulfate and an aniline derivative to give the corresponding thiosulfonic acid derivative of Binschedler's Green (the indaminethiosulfonic acid, **1b**), although the thiosulfate derivative of the *p*-phenylenediamine may also be obtained directly via treatment of the corresponding 4-nitrosoaniline hydrochloride in acetic acid with thiosulfate [42]. Oxidative ring closure then furnishes the phenothiazinium chromophore (**1c**, Scheme 3) [43]. While this method is quite suitable for simple derivatives such as Methylene Blue, care should be taken with chromophoric elaboration, due to the possibility of isomer formation. For example, Toluidine Blue O (**2c**) produced via this route is isomerically impure, since the ring closure step involves an asymmetric intermediate (**2a**/**2b**, Scheme 4). The separation of the resulting 2- and 4-methyl Azure A derivatives—the accepted structure for TBO is 2-methyl Azure A—reportedly requires considerable care [44]. Isomer formation of disulfonated Thionin derivatives by direct ring synthesis has also been reported [45].

On a related matter, the synthesis of functionalised chromophores often requires the presence of atoms or groups which might be lost during ring closure—e.g. halogens, alkoxyls etc. Logically it is sensible to include these groups in the part of the intermediate molecule having the attacking (ring-forming) group, although this is not always synthetically possible and constitutes a limiting factor on the number and range of derivatives available. For example, it is unlikely that the 1-methoxy analogue of Azure A can be produced via this route, since the methoxy group should be lost in the ring closure step, being replaced by sulfur (i.e. S-5 in the ring system), similarly to the situation encountered in the synthesis of Basic Blue 4 [46].

Since the use of phenothiazinium derivatives as photosensitisers in the clinical milieu requires a very high degree of purity, it is sensible to remove by-products during synthesis, rather than relying on chromatographic methods afterwards. This is made more difficult by the one-pot method approach advocated by Fierz-David and Blangey



Scheme 3. Synthesis of Methylene Blue derivatives. Reagents: (i) $Na_2S_2O_3$, AcOH; (ii) $Na_2S_2O_3$, $Na_2Cr_2O_7$, H^+; (iii) $C_6H_5NR'_2$, $Na_2Cr_2O_77$, H^+; (iv) $CuSO_4$. N.B. benzo[*a*]phenothiazinium derivitives (Nile Blue analogues) may also be synthesised via this route, replacing the aniline derivative in (iii) with a 1-naphthylamine derivative.



Scheme 4. Toluidine Blue O isomer formation.

[43], though the material produced here was not intended for contemporary use in humans. However, more recent workers in this field have employed the isolation of the initial *p*-phenylenediaminethiosulfonic acid and its recrystallisation [41,47].

A related method of ring synthesis utilises separate attack on both auxochrome-containing rings. Thus an aniline derivative having a nucleophilic group *meta*- to the amino function will form the indamine with a 4-nitrosoaniline derivative in the usual way, followed by nucleophilic attack of the nucleophile and ring closure by oxidation. Such a synthesis is shown in Scheme 5+ for seleno-toluidine blue (**5**) [48].

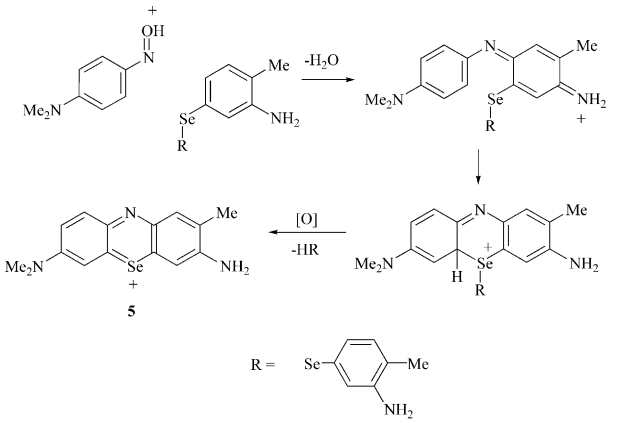
Ring synthesis may also be preferable to functionalisation of phenothiazinium salts, giving a purer product. For example, Azure B is better prepared by the ring synthesis route from *N,N*-dimethyl-*p*-phenylenediamine/*N*-methylaniline than by the oxidative demethylation of MB itself, since this also yields mixtures of the other Azure stains and Thionin [49].

One of the governing factors in choosing any synthetic route is the availability of precursors. This is often problematic for the *N*-alkylated anilines required for the synthesis of this class of heterocycle, mainly due to the steric effects of the dialkylamino function itself. For example, it is difficult to synthesise phenothiazinium salts via this route having *N,N*-dialkyl auxochromes and substituents in positions 2- or 4- (see Section 3.3). Commercially available photosensitisers with a 2-(8-) substituent are either mono *N*-alkylated (New Methylene Blue, Table 1) or non-alkylated (Toluidine Blue O, Table 1).

Logically, it is unlikely that the nitrogen of a crowded *N,N*-dialkyl auxochrome would be able to achieve coplanarity of its lone pair with the remainder of the phenothiazinium chromophore, thus the photosensitising potential of such a compound would be low. However, this is further complicated by the fact that often the same auxochrome is required to contribute sufficiently to the electron density of the precursor aniline to facilitate e.g. nitrosation. For crowded species such as *N,N*-dimethyl-*o*-toluidine cannot be nitrosated using the normal approach, and indeed are only nitrated (sulphuric/nitric acids) in the *meta*-position to the auxochrome [50].

3.2. Functionalisation-oxidation of 10H-phenothiazine

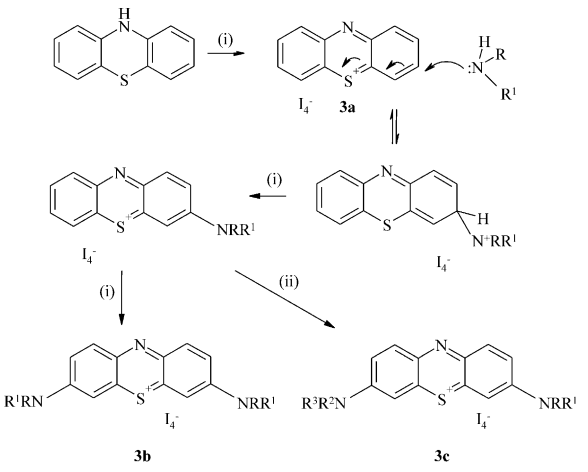
The propensity for redox reaction in phenothiazine derivatives is, as mentioned above, an important factor in their use. The oxidation of 10*H*-phenothiazine itself is also highly convenient in the synthesis of simple MB-type derivatives. Thus, oxidation of the parent compound with, for example, iodine furnishes the phenothiazinium tetraiodide (**3a**) [51], a useful electrophile. This salt is attacked at position 3- and 7- by amines, and thus can be used to furnish homologous series of derivatives and offers considerable scope in the alteration of physicochemical properties (Scheme 6). For example, series of symmetrical compounds (**3b**) having arylamino [21] and dialkylamino functionality [31] have been reported recently. Asymmetric derivatives (**3c**) based on similar methodology are also obtainable, this route relying on the isolation of the mono (3-) adduct [52] (Scheme 6). In both cases, the products formed are easily purified by chromatographic methods, and may also be recrystallised at various stages throughout the synthesis [51,52-phenothiazines [53].



Scheme 5. Synthesis of seleno-toluidine blue.

In a similar fashion, halogenation of the 10*H*-phenothiazine furnishes the 3,7-dihalo derivative. This undergoes substitution by amines, again yielding symmetrical MB-type analogues on oxidation [54]. Obviously, the use of either of the above routes allows important parameters such as aqueous solubility to be improved (e.g. bismorpholino analogues etc.), although some attention may be required with the counter ion resulting, iodide salts being less soluble than e.g. chlorides in aqueous media.

While the above methodologies allow the synthesis of a wide range of analogues, simple amino derivatives are not produced in this way. Although it is perhaps surprising that these compounds are still required in photosensitiser research, examples such as Thionin are under current investigation for the disinfection of blood plasma [17]. While phenothiaziniums having one amino group may be synthesised via the thiosulfate/oxidation route employing aniline or a substituted derivative, Thionin may be produced via the direct nitration of 10*H*-phenothiazine. The 3,7-dinitro derivative is then reduced to the diamino compound conventionally and this is easily oxidised to the product [55].



Scheme 6. Symmetrical and asymmetrical phenothiazinium synthesis using iodine oxidation. (i) Iodine (oxidation); isolate, recrystallise; $HNRR^1$; (ii) isolate recrystallise; iodine, HNR^2R^3.

3.3. Direct functionalisation of the phenothiazinium chromophore—Methylene Green and Methylene Violet

The use of the phenothiazinium salts as precursors for new photosensitisers is not a widely investigated area. As mentioned above, facile demethylation of MB furnishes mixtures of the Azure stains and Thionin, but chemical substitution is rare. Methylene Green (MG, CI 52020, Table 1) is formally the 4-nitro derivative of MB. However, inspection of the accepted structure suggests an anomaly due to the steric crowding entailed by the adjacent positioning of the nitro- and dimethylamino-groups (Table 1). In addition, greenish blue aqueous solutions of MG contain significant quantities of ether-extractable damson coloured species on basification. This behaviour is

strongly indicative of quinoneimine formation, which does not occur with MB, i.e. requiring an NH moiety in the parent molecule.

As has been stated above, MG is not an efficient photosensitiser, so in terms of the current study its chemical identity may seem less than pertinent. However, MG may be used to furnish active compounds by virtue of the reduction of the nitro group to amino, and subsequent reactions which include halogenation via the Sandmeyer route. For example, Brown et al. have synthesised Iodomethylene Blue for use in radiolabelling investigations of metastatic melanoma employing MG as the starting material and following this procedure [56]. The identical product was produced by iodate/iodide treatment of MB [57]. However, NMR analysis of the product—amounting to 85%—showed the presence of an NH peak, suggesting that the substance was in fact the 4-iodo-3-methylamino - 7 - dimethylaminophenothiazinium salt (i.e. Iodoazure B) [58]. In fact, the demethylation of Methylene Blue under nitrating (oxidising) conditions was reported by Gnehm almost a century ago [59,60].

The recent investigations by Morrison et al. involving Methylene Violet (MV) and its analogues for the disinfection of blood products utilised MV (**4**) as the starting material, rather than de novo ring synthesis. Halogenation of the parent chromophore is achieved with 1-chloroethyl orthoformate [61], *N*-bromosuccinimide in acetic acid or iodidate/iodide in absolute ethanol [62] for the chloro-, bromo or iodoMV derivative respectively (**4a**–**c**, Scheme 4). Each MV analogue may be converted into the cationic methoxy derivative (4d-f) by reaction with methyl triflate (Scheme 4) [39].

3.4. *Ring synthesis: 10*H*-phenothiazines*

Conventional syntheses of the 10*H*-phenothiazine system, normally for use in neuroactive compounds (e.g. antipsychotic drugs) have been covered in depth elsewhere [63]. Additionally, since most analogue synthesis in phenothiazinium photosensitiser research is based on varying the auxochromic moieties at positions 3- and 7-, there has been—thus far—little need for de novo ring synthesis. However, given the potential for complication due to isomer formation via the ring closure route, on occasion the reduced (10*H*) phenothiazine precursor may be required. In their recent investigations into alkylated MB derivatives for blood disinfection, Foley et al. synthesised the requisite 1,9-dimethyl-10*H*-phenothiazine via methylation of the benzyne adduct formed by elimination from the 8-chloro-1-methyl precursor [64]. However, further reaction of the dialkyl species via the iodine/oxidative route covered in Section 3.2 furnished only low yields of either the symmetrical or unsymmetrical MB derivatives [53]. New Methylene Blue, having chromophoric methyl groups at positions 2- and 8- (Table 1), is conventionally synthesised via the indaminethiosulfonic acid route.

3.5. *Attachment to biomolecules*

While the major research effort in this field has been that using commercial phenothiazinium photosensitisers and some auxochromic analogues, the preparation of macromolecule-linked examples has also been carried out. Such research can be divided into biomolecule- and synthetic polymer-linked phenothiazinium photosensitisers, since the chemistries involved are dissimilar.

The synthesis of phenothiazinium bioconjugates, as with other photosensitiser types, depends on the presence in the side chain of a labile group (usually carboxylic acid or amino) which will react with the biomolecule under mild conditions (ambient temperature, neutral pH, aqueous media). Usually the governing factor here is the synthesis of phenothiazinium salts having suitable functionality in the auxochromic side chain, although attachment through a 4-amino group (derived from Methylene Green) is also possible [65]. For example, protein attachment via a carboxylic ester function remote from the chromophore has been reported, but the phenothiazinium bearing the carboxylic acid group was synthesised from a suitably substituted aniline and a thiosulfonic acid derivative as in Section 3.1 rather than the carboxyl bearing analogues produced from the 10*H*-phenothiazine/iodine method [66,67].

Where the attachment of the phenothiazinium chromophore to synthetic polymers has been carried out, the chemical link has usually been from

the 3-amino function (e.g. in Thionin) in an amide bond with acrylic type monomers [68]. This allows copolymerisation with other olefinic monomers. However, the formation of an amide moiety directly attached to the phenothiazinium chromophore must influence the π-cloud and thus alter the photosensitising potential of the resulting polymer compared to the parent dye molecule [69].

In both of the above cases there are novel phenothiazinium derivatives with functionality in the side chain which should allow attachment to either biomolecular or synthetic polymer, e.g. with silyl or sulfonyl moieties [70,71].

4. Conclusions

Since Methylene Blue has been known as a useful biomedical dye for well over a century it remains surprising that there is such a scarcity of novel derivatives of this compound—i.e. having chromophoric rather than auxochromic elaboration—in the literature. In consequence, little is yet known for certain concerning more complex photosensitisers based on the phenothiazinium chromophore. It is doubly mystifying that such a situation should pertain given the relative ease of synthesis of the phenothiazinium system and the range of possible end uses for new photosensitisers. It is to be fervently hoped that new avenues of research emanating from cancer PDT, e.g. pathogen inactivation in blood products, will continue to encourage the small amount of pioneering work begun in this area in the recent past.

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