Tricyclic Cationic Chromophores as Models for New Photoantimicrobials

Mark Wainwright

School of Pharmacy & Biomolecular Sciences

Liverpool John Moores University

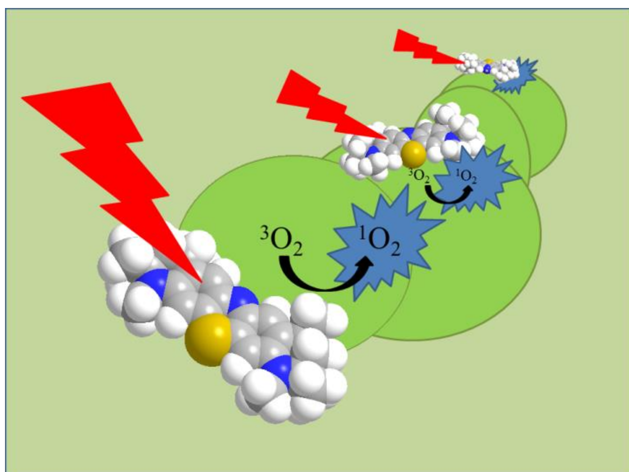
Byrom Street

Liverpool L3 3AF

United Kingdom

Author for correspondence: Email mark_wainwright@hotmail.com

Graphical Abstract



Cationic tricyclic photosensitisers such as the phenothiazinium S137 (shown) are highly effective bacterial killers when activated by the correct wavelength light. Microbial killing by reactive oxygen species such as singlet oxygen ($^1O_2$) is unaffected by conventional antimicrobial resistance.

Abstract

Despite the preponderance of literature pertaining to photosensitisers based on the porphyrin system, many other chemical classes are available with similar or improved characteristics and potential for use in photodynamic medicine. Several of these classes are based on small, tricyclic, heteroaromatic chromophores, often originally developed from textile dyes or biological stains. The latter classification is useful in providing a basis for biological uptake and antimicrobial activity.

The current review covers the chemistry and photoantimicrobial applications of established and novel cationic bisamino derivatives of the acridine, phenazine, phenoxazine, phenothiazine and xanthene systems and related compounds. The range covered is considerable and demonstrates photodynamic performance to rival the porphyrin class. In addition, the chemical synthesis of new analogues of lead compounds such as methylene blue or acridine orange is relatively straightforward and inexpensive, and compound series with varying physicochemical profiles have been produced for structure-activity studies in order to furnish improved photosensitisers for clinical trialling.

Keywords: acridine, benzologue, chalcogens, phenazine, phenoxazine, phenoselenazine, phenothiazine, rosamine, photosensitiser

Running title: Tricyclic cationic photosensitisers

1. History

Dyes and pigments are very important in the modern world – from the colouration of the human environment with paints, plastics and textiles, to dyed clothing and food colours. The significance of the chemicals used in the various processes leading to colouration is mostly taken for granted, if appreciated at all. Similarly, the non-traditional, high-technology use of dye molecules has applications in electronics, light-emitting diodes, photovoltaics, lasers and biosensors, again normally unappreciated by the end user. The application of dyes as biological stains remains highly important in the establishment of infectious disease aetiology via the ubiquitous use of the Gram stain, and there are many examples of established stains in histochemistry and histopathology.[1]

Another aspect of synthetic dye usage is their part in the development of modern drugs. The growth of the pharmaceutical industry can be traced back to the initial essays in aniline dye synthesis in the middle part of the nineteenth century, following Perkin's accidental synthesis of the phenazine derivative, mauveine in 1856. The oxidative reagents used with the various available (but impure) anilines led to the production of a range of colours due to the oxidised heteroaromatic chromophores produced, often involving sulphur as well as nitrogen. Among the earliest chromophores thus obtained were oxidised phenothiazines (phenothiaziniums), methylene blue and thionine being first reported in 1876.[2,3] Early research into the use of such compounds to differentiate cells by Koch, Ehrlich and others eventually led to selective toxicity studies and, in turn, to chemotherapy. Here, dyes which were employed directly as drugs – e.g. the aminoacridines, the azo dye Prontosil and methylene blue itself, became lead compounds for future drug development.[4]

2. Photosensitisation

Photosensitisers – as opposed to conventional dyes – are light-absorbing molecules which are able to populate the excited electronic triplet state ($*T_1$) significantly, thus allowing electron transfer reactions with, or energy transfer to, molecules – typically oxygen - in the environment. The basis of cytotoxic photosensitisation is the generation of reactive oxygen species *in situ*. The superoxide anion, hydroxyl radical and peroxides are produced via electron transfer and singlet oxygen via energy transfer. (Figure 1).

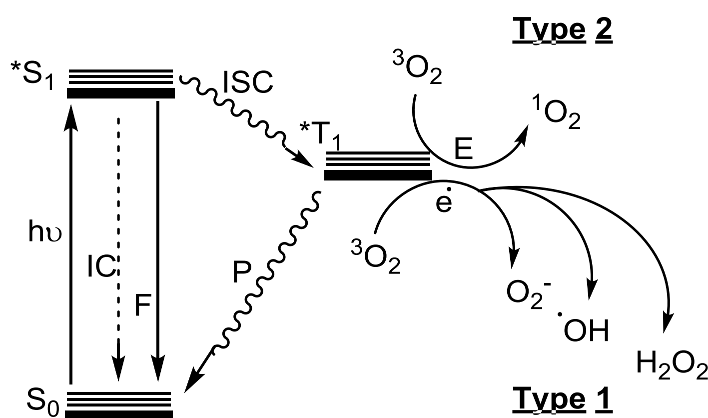


Figure 1. Excitation/relaxation pathways involved in photosensitisation. Key: S_0 – singlet electronic ground state of photosensitiser; $*S_1$ - singlet excited state; $*T_1$ - triplet excited state; hυ - energy of absorbed photon; IC – internal conversion; F - fluorescence; P - phosphorescence; ISC – inter-system crossing.

Tricyclic lead compounds, such as the phenothiazinium derivative methylene blue, produce singlet oxygen efficiently, but it is also possible to design analogue molecules

based on the enhancement of factors supporting this – e.g. heavy atom inclusion (*q.v.*).

For the triplet excited state to be sufficiently populated, a relatively long-lived excited singlet state allowing efficient conversion from $*S_1 \rightarrow *T_1$ is essential. Deactivation of the $*S_1$ state may occur radiatively (fluorescence) and non-radiatively (internal conversion), as with conventional dye molecules.

Stabilisation of the $*T_1$ state itself by the inclusion of atoms of a high atomic number is due to large associated spin-orbital coupling constants and is known as the "heavy atom" effect. The peripheral inclusion of bromine or iodine in the chromophore, or (usually) of sulphur or selenium as ring heteroatoms is common modern practice in the maximisation of singlet oxygen production.

2.1 Dyes as Photoantimicrobials

Raab's initial reporting of the photosensitising action of dyes in 1900 was based on the simple nitrogen tricyclic molecule acridine and the xanthene derivative eosin,[5] the latter also featuring in mammalian cell (anticancer) studies within a few years.[6] Although various discoveries were made with synthetic dyes throughout the next sixty years, the proper development of photodynamic therapy (PDT) as a cancer treatment, beginning in the 1960s,[7] was firmly based on naturally-occurring porphyrin derivatives, and this remains the case to date, with synthetics constituting something of a lower class. To a certain extent, this is also true in the related field of photodynamic antimicrobial chemotherapy (PACT),[8] despite the fact that there is far

less rationale for the use here of the anionic porphyrins used in cancer PDT. If PDT did not exist, PACT would be firmly based on cationic dyes such as methylene blue and the benzo[*a*]phenoxazine Nile blue. However, there remains what amounts almost to a "porphyrin panacea" approach to all aspects of potential photodynamic medicine among a large section of researchers.

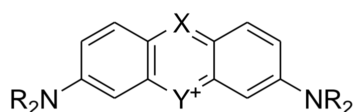
The lead compounds for synthetic photosensitisers proposed for use in biomedicine, particularly in the area of photoantimicrobials, constitute, in the main, a sub-class of established biological stains. Undoubtedly, methylene blue is premier among these, but it is important to recognise that it is not the *sine qua non*, despite its many medical applications in the past.[9] Substituting a methylene blue panacea for the porphyrin version is equally unsuitable in taking forward photodynamic science or its medical applications.

There are many candidate chromophores for novel photosensitisers from the field of biological staining, and these underpin the "stain and kill" approach to the development of fit-for-purpose photoantimicrobial agents. Several lead compounds may thus be found in the tricyclic cationic class which, due to the large number of starting points for photosensitising drug discovery, is the subject of the present work. A section of the xanthene class has also been included among the tricyclic cationic compounds: the diamino functionality and cationic nature of the pyronin and rosamine derivatives offer considerable structural commonality with methylene blue and its congeners.

In the 21st Century, it is incumbent on scientific researchers to ensure that their work is relevant to modern requirements. Photosensitiser discovery, particularly relating to infection control applications, satisfies this criterion since it addresses a serious, often fatal, gap in conventional antimicrobial chemotherapy, *viz*. drug-resistant infection.[10]

2.2 Structures

The fundamental chemical structure involved in tricyclic cationic chromophores consists of a fused, linear, tricyclic arrangement of six-membered aromatic rings, the central one containing one – or, more usually, two - heteroatoms. Traditionally, the combination of heteroatoms is N/NR, N/O, and N/S, but this has been extended to combinations of nitrogen with the lower period, Group VI atom selenium, and also to the CH/N, RCH/O, RCH/S etc. couples, in order to encompass the acridine, pyronine and rosamine photosensitisers (Table 1).



Class	**X**	**Y**
Acridinium	CR	NR
Xanthylium (Pyronin, Rosamine)	CR	O
Thioxanthylium	CR	S
Selenoxanthylium	CR	Se
Telluroxanthylium	CR	Te
Phenazinium	N	NR
Phenoxazinium	N	O
Phenothiazinium	N	S
Phenoselenazinium	N	Se

Table 1. Structures of tricyclic photosensitiser chromophores (R = H, alkyl, aryl, heteroaryl)

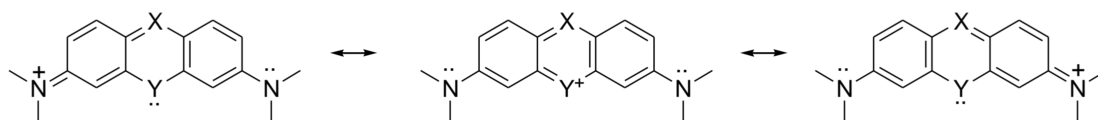


Figure 2. General azinium-type structures, showing the delocalisation of cationic charge. X/Y = CH/NR; RCH/O; RCH/S; RCH/Se; RCH/Te; N/NR; N/O; N/S; N/Se.

The representative structures given in Table 1 provide an idea of general molecular similarity, although it should be remembered that the different X/Y atomic combinations govern the electron density and distribution of the resulting delocalised chromophore (Figure 2), thus explaining some of the varying activities among representative examples, this being of particular relevance to photosensitising capability. On a more obvious note, this variation in electron density/distribution may be visibly observed in solution colour. For example, acridine *orange* and methylene *blue* are identical structures save for the central chromophoric heteroatoms, CH/N and N/S respectively. This can also be observed spectrophotometrically for direct phenoxazinium and phenothiazinium analogues (i.e. N/O → N/S), usually demonstrating a bathochromic shift of λ_{max} of ca. 20 nm.

2.3. Photosensitising activities

Among research groups involved in new photosensitiser development there has been considerable effort committed to measuring the reactive oxygen species (ROS) generation of synthesised candidates as one of the necessary performance indicators. Usually this consists of mixing the candidate with a quencher, illuminating and measuring the decay of the latter spectrophotometrically. Conversely, singlet oxygen can be measured directly via its phosphorescence (delayed fluorescence) at 1270 nm.[11] However, the generation, or otherwise, of ROS - since this is governed by the lifetime of the electronic excited state - depends to a large extent on the molecular environment of the photosensitiser. Thus measurements made in solution may not reflect behaviour in the clinical situation. For example, the production of singlet oxygen by methylene blue can be observed in alcoholic solution via the decrease in

visible absorption of a quencher such as 1,3-diphenylisobenzofuran or 4-nitroso-*N,N*-dimethylaniline.[12] Such photosensitising activity depends on the absorption of light by the methylene blue molecule. Similarly, the binding of methylene blue to deoxyribonucleic acid (DNA), an important and often cited cellular target, can be demonstrated spectrophotometrically, since the λ_{max} of methylene blue shifts by approximately 4 nm.[13] This is a clear indication that there has been a change to the π-electron cloud of the molecule, compared to that in the free state in solution. Since the generation of singlet oxygen also depends on electronic transitions in the photosensitiser, there is clearly potential for biomolecular binding to influence photosensitising activity. Related molecules, such as the acridines proflavine and acridine orange produce no measurable singlet oxygen in the standard spectrophotometric test but are excellent cellular photosensitisers. This may also support the preceding argument or indicate an electron transfer/redox mode of action (Type I photosensitisation).

Such behaviour merely underlines the fact that the testing of potential photosensitisers should be realistic. A ranking order of photosensitising ability constructed using a conventional spectrophotometric assay will, in all likelihood, be completely dissimilar to the order of efficiency of photokilling, for example, in a bacterial strain for the same series of compounds. As the intended target is bacterial infection, it thus makes more sense to use the relevant biological challenge, rather than the *in vitro* assay, as a principal performance indicator. By similar reasoning, a photosensitiser which is an excellent candidate for clinical anticancer PDT may have severe shortcomings in anti-infective terms.

For the tricyclic, heteroaromatic range of photosensitisers, as with other groups, efficiency in cell killing depends on structure since, although there are many well-known examples of biological photosensitisers contained here, there are basic structures which are inactive, whether in the *in vitro* assay or in cell culture. However, there is sufficient knowledge of chemistry and structure-function relationships to be able to remedy such situations via suitable chromophoric substitution patterns, as detailed in the sections below.

3. Nucleic acid intercalation and deleterious effects in humans

The planar structure, surface area and cationic charge of the photosensitisers covered here provide many examples of potential nucleic acid intercalators – i.e. molecules which, because of their geometry and charge, are able to fit between consecutive base-pairs in the double helical structure of DNA. As mentioned, this interaction can be observed easily using a spectrophotometric approach, since the interaction causes a fluctuation in the adjacent molecular electron clouds, leading to absorption wavelength shifting. The importance of a sufficient molecular planar area and positive charge in this interaction was ably demonstrated by Albert in the 1940s.[14]

In terms of photodynamic efficacy, such exquisite targeting of a biomolecule is obviously highly appealing. However, in reality it does not constitute a magic bullet. It is true that adding a photosensitiser such as methylene blue to a solution of DNA leads to observable intercalation. Subsequent red-light irradiation of the interacting

pair causes oxidative breakdown of the nucleic acid, which is again easily observable via HPLC of the resulting mixture, 8-hydroxyguanosine being a major product.[15] Conversely, interaction between cellular DNA and such photosensitisers is a much more difficult proposition, the degree of difficulty being in proportion to cellular complexity. Thus intercalation may occur with viruses in media, or in bacterial cells in culture, and DNA damage following photodynamic action has been reported.[16] However, nuclear DNA in human cell lines is compartmentalised in the cell nucleus and, while there are vital nuclear stains, it is more likely that most of the tricyclic cations currently under consideration, if localised in the nucleus, would only do so after cell death. DNA damage in the photosensitised cell requires careful analysis, as this may occur indirectly, after critical damage to the cell elsewhere, e.g. in the lysosome, followed by relocalisation, as is the case for methylene blue.[17]

It is equally important to consider this proposition objectively. Biological stains such as methylene blue and acriflavine have been used in medicine and clinical laboratories for well over 100 years. There have been no documented associated cases of cancer. A similar argument is possible for the photosensitisers discussed below. While it is imperative to carry out efficacy/safety testing on novel drugs, overall objectivity is also essential. The above-mentioned methylene blue, for example, is clearly safe for human use and yet produces a positive Ames (bacterial mutagenicity) test result.[18]

4. Photosensitiser requirements – 'fit for purpose' photoantimicrobials

Regardless of molecular type, there are certain criteria which must be satisfied for photosensitisation. Simply, this is the combination of the target, a photosensitiser

molecule, oxygen and light. Any of the various applications is merely an elaboration of this and may be deconstructed to provide problem-solving where required in practice. For example, killing bacteria in planktonic culture (i.e. non-biofilm) using a standard photosensitiser such as methylene blue should be a straightforward process. This is only useful if relevant information is provided towards a clinical end-point – e.g. what is the lowest photosensitiser concentration at which complete bacterial kill is achieved? Equally, it is important that the target is relevant – for example, broad-spectrum photobactericidal activity cannot be extrapolated merely from demonstrable efficacy against the standard Gram-positive organism, *Staphylococcus aureus*. The resounding failure of the otherwise excellent anionic porphyrin photosensitisers used in anticancer PDT has been their absence of activity against Gram-negative bacteria.[19]

Light absorption by the photoantimicrobial, at its target cell, is an essential part of the killing process. Consequently, anything which diminishes the amount of incident light makes the process less efficient. Thus, light absorption by biomolecules such as melanin, haem or cytochromes, known as *endogenous* absorbers, should be avoided. This means that where there is the risk of interaction with such species (e.g. in wounds), photoantimicrobials must absorb outside their spectral range, typically at longer wavelengths. This is normally taken as the 630-900 nm region and should inform the molecular design of potential photoantimicrobials.

Similarly, the period of illumination required to satisfy microbial kill targets should be reasonable with respect to patient treatment. A period of tens of seconds or a few minutes is reasonable. One of several hours is not.

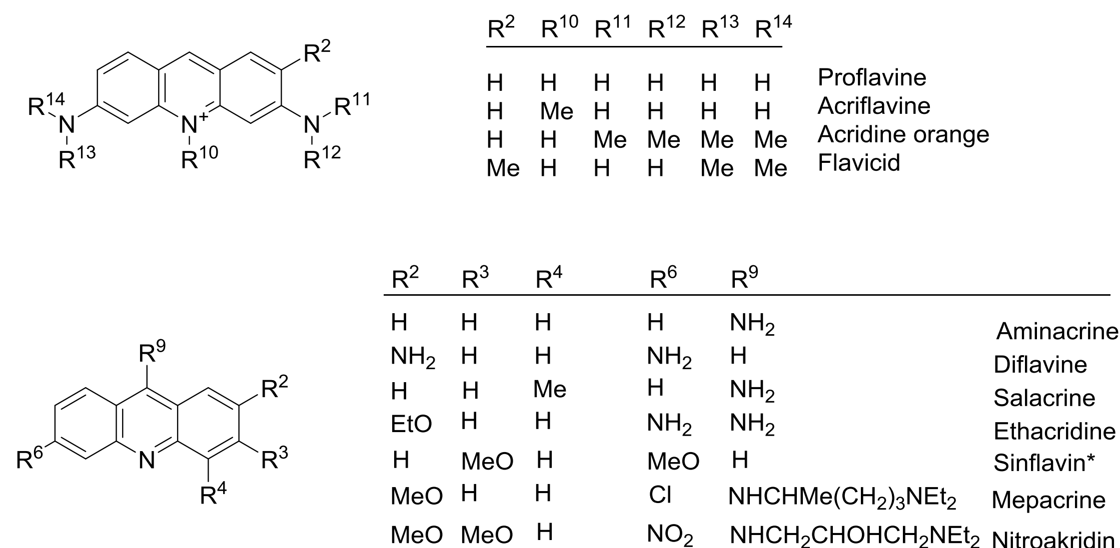
One of the greatest hurdles encountered in moving a drug molecule from bench to bedside remains that of human toxicity. Clearly this has been the main problem in developing new clinical photosensitisers, once improved structures have been decided on the basis of laboratory testing. For this reason it may be more prudent, initially, to return to the lead compounds themselves as putative agents, given that many – methylene blue, crystal violet, acriflavine etc. – possess a considerable history in terms of direct human application as anti-infectives.[20] Consequently, many such examples are covered in this review.

5.1. Acridines

Although as mentioned above, the initial laboratory demonstration of the photodynamic effect involved acridine itself [5], in the main, the photosensitising activity of this family of compounds has been limited to the early bisamino derivatives proflavine and acriflavine. Much of the associated literature covers mutagenic effects in yeast strains as evidence of the deleterious potential of acridine-based drugs against nucleic acid.[21]

The acridines represent an important class of agents with respect to the current argument. Several members of this class have been used clinically in a conventional antimicrobial capacity, without significant side effects. Examples include the antibacterials aminacrine, proflavine, acriflavine (mixture of proflavine hydrochloride and its 10-methyl quaternary salt) and Nitroakridin and the antimalarial agent mepacrine (Figure 3). As mentioned, given the considerable use of these examples as drugs, there should be fewer problems with their regulatory approval as clinical

photosensitisers than would be the case for completely new agents. Also as noted above, the correlation of DNA binding capability *in vitro* with mutagenicity/carcinogenicity in humans is often an assumed relationship.



R^2	R^{10}	R^{11}	R^{12}	R^{13}	R^{14}	
H	H	H	H	H	H	Proflavine
H	Me	H	H	H	H	Acriflavine
H	H	Me	Me	Me	Me	Acridine orange
Me	H	H	H	Me	Me	Flavicid

R^2	R^3	R^4	R^6	R^9	
H	H	H	H	NH_2	Aminacrine
NH_2	H	H	NH_2	H	Diflavine
H	H	Me	H	NH_2	Salacrine
EtO	H	H	NH_2	NH_2	Ethacridine
H	MeO	H	MeO	H	Sinflavin*
MeO	H	H	Cl	$NHCHMe(CH_2)_3NEt_2$	Mepacrine
MeO	MeO	H	NO_2	$NHCH_2CHOHCH_2NEt_2$	Nitroakridin

Figure 3. Clinically-used antimicrobial acridines. (*10-methyl quaternary salt)

Using the amount of literature concerning nucleic acid interactions of acridines in yeasts also as strong evidence for photodynamic activity, photobactericidal investigations have been carried out on the effects of a range of structurally-related acridines against pathogenic bacteria commonly encountered in the hospital milieu. Many of the compounds employed were examples which were, or had been, in use in humans as topical antibacterial agents.[22]

	MBC (μM)			
	Staphylococcus aureus		*Pseudomonas aeruginosa*	
Acridine	Dark	Light*	Dark	Light*
Ethacridine	10	5	100	25
Aminacrine	50	2.5	500	500
Salacrine	25	2.5	500	500
Acridine orange	25	1	500	100
Proflavine	25	2.5	250	10

Table 2 Toxicity and phototoxicity of aminoacridines against bacteria. MBC = minimum bactericidal concentration; * fluence rate = 1.7 mW cm^{-2}, fluence = 6.3 J cm^{-2} (white light).

The resulting photobactericidal effects reported were in stark contrast – particularly against Gram-positive bacteria (e.g. *S. aureus*, Table 2) to the related singlet oxygen efficiencies of the compounds used in the study, since none of the acridine derivatives produced any measurable $^1O_2$ *in vitro*.[22] Dark toxicities were also measured, at much increased concentrations, in agreement with previous work reported to be due to bacterial DNA interference via an intercalative mechanism.[23] Given this mode of action, the increased activity of the acridines used in the study may be considered in terms of the disastrous consequences of critically-localised photosensitisers. In other words, bacterial DNA intercalation leads to interference with cellular reproduction, but is reversible on removal of the agent; photoactivation of the agent *in situ* leads to oxidation at various points, catastrophic breakdown, and is thus irreversible.

Albert and co-workers synthesised many simple amino acridines, which enabled their work on the structure-activity relationships of antibacterial examples.[24,25] Very few have been examined from the photodynamic aspect, so this is another potentially rich area for drug discovery.

In terms of drug design, the physicochemical of aminoacridines can be adjusted with relative simplicity. Just as it has been shown that chain length alteration in 10-alkyl derivatives of acridine orange leads to differences in mammalian subcellular organelle localisation,[26] logically the relationship to lipophilicity can also be used to synthesise derivatives which do not enter mammalian cells. For example, the use of quaternising chains containing basic character, i.e. as would be protonated in the physiological range. Similar, auxochromic amino substitution having basic functionality has, of course, been employed in a great many of the aminoacridines tested for their antimalarial potential.[27]

From a practical point of view, given that such proposed compounds are aimed at photodynamic infection control, the aminoacridines generally exhibit their longest absorption wavelengths in the 400-500 nm range, so as noted previously, this might lead to problems with endogenous absorption. However, riboflavin (vitamin B2) has been reported to retain its photoantimicrobial activity in the presence of red blood cells,[28] and yet has a strong absorption in this region (475 nm), so it may be that there is potential for the aminoacridines here also. In addition, there is certainly potential for their use in infected presentations not involving blood, for example skin or eye bacterial or viral disinfection or fungal skin disinfection. Proflavine was one of the photosensitisers employed in the clinical treatment of genital herpes in the 1970s.[29]

It is also possible, should this be required, to extend the absorption of acridine-based candidates into the red or near infrared regions. This is established chemistry,[30] and utilises the acidic carbon functionality of 9-methyl quaternary acridines to furnish styryl derivatives[31] (Figure 4). This has been extended to cyanine-type analogues having longer wavelength absorption.[32] Little has been reported on the photosensitising potential of such derivatives.

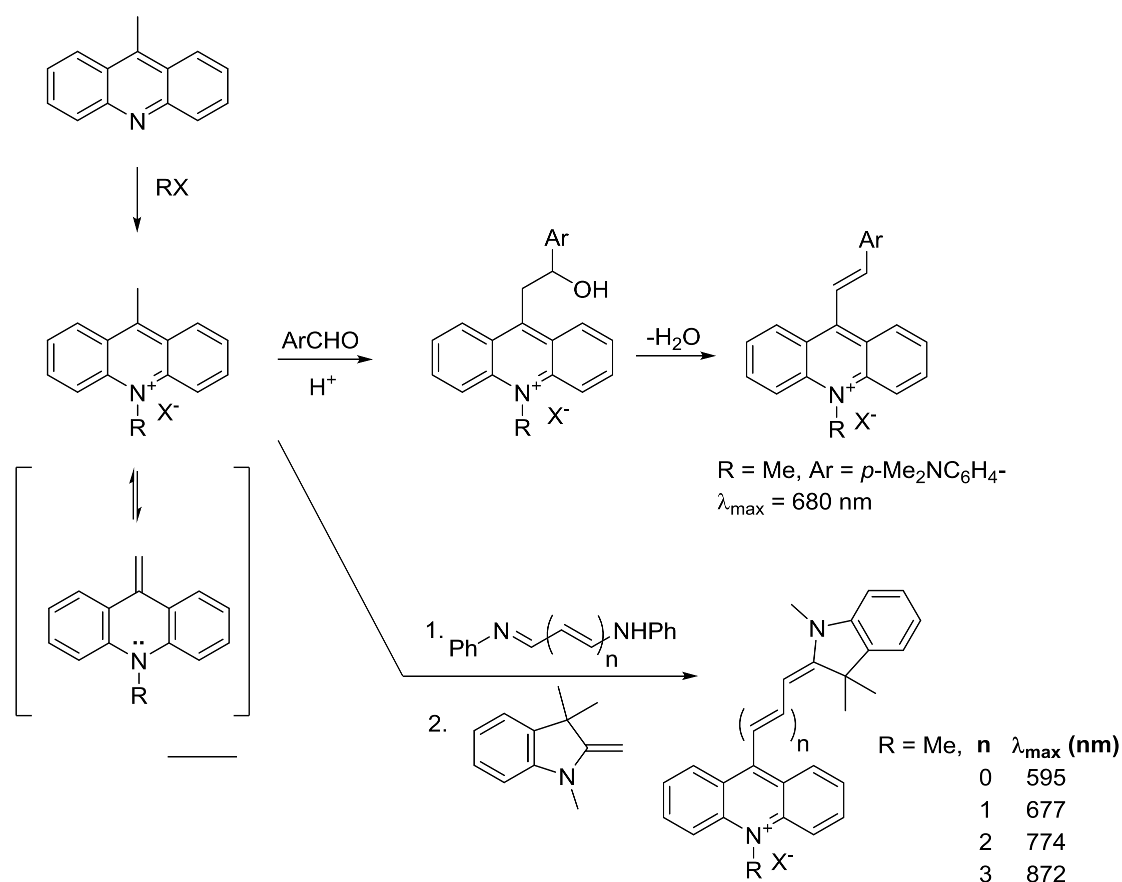


Figure 4. Acridines with extended spectral absorption. λ_{max} values measured in methanol. Typically X = I or Br.

5.2. Phenazines

Given the primogenicity of mauveine among synthetic dyes, as a class of compounds it is perhaps surprising that the phenazines are not more widely investigated. Closely related to the acridines, several examples of the phenazine class also have the capability for DNA intercalation.[33,34] The structural commonality is typified by neutral red (Figure 5) which, as can be seen from comparison with Figure 4, is isosteric with the early clinically-used acridine Flavicid, and also the phenothiazinium photosensitiser, toluidine blue (Figure 9).

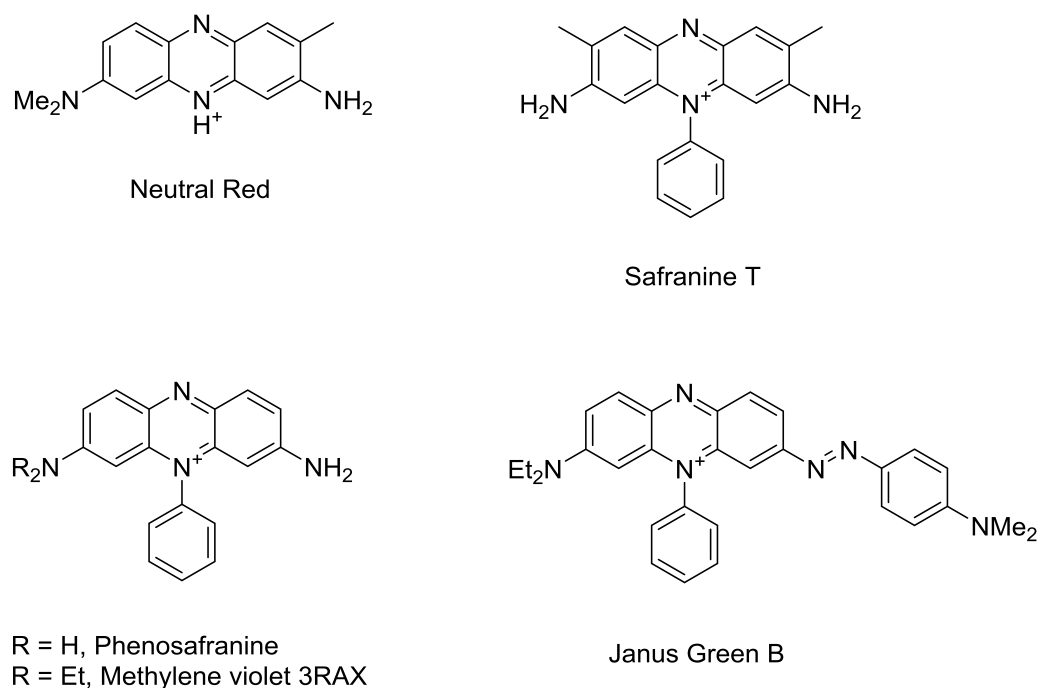


Figure 5. Phenazinium dyes

DNA-intercalating criteria, i.e. sufficient molecular planar area and cationic charge was noted above for the azine class as a whole and unsurprisingly, this interaction has been reported for most well-known derivatives (e.g. neutral red[35] and phenosafranine.[36] Most recently, Methylene violet 3RAX (the 7-*N,N*-diethyl analogue of phenosafranine) has also been shown to intercalate strongly with DNA.[37]

Neutral red is undoubtedly the most commonly tested phenazine in terms of photodynamic activity, mainly owing to its involvement in the clinical phototreatment of genital herpes in the United States during the late 1960s and early 70s.[38] As with the simple acridines, neutral red forms aggregated species due to its high degree of molecular planarity. Thus, in terms of singlet oxygen generation, it is important to ensure that the correct (monomeric) absorption band is targeted on illumination ($\lambda_{max\ monomer}$ = 450 nm; $\lambda_{max\ aggregate}$ = 536 nm).[39] However, it should be remembered that neutral red is also a live stain for mammalian cells, and this must preclude its use as a photoantimicrobial on the grounds of lack of selectivity.

As with other, related azinium-type photosensitisers, the synthesis of neutral red and its derivatives is oxidative. However, derivatives may be prepared easily from a nitroso-containing starting material (Figure 6), allowing considerable scope for derivatisation, for example altering the hydrophilic/lipophilic balance of the molecule via hydroxylation of the auxochromic alkyl groups.[40]

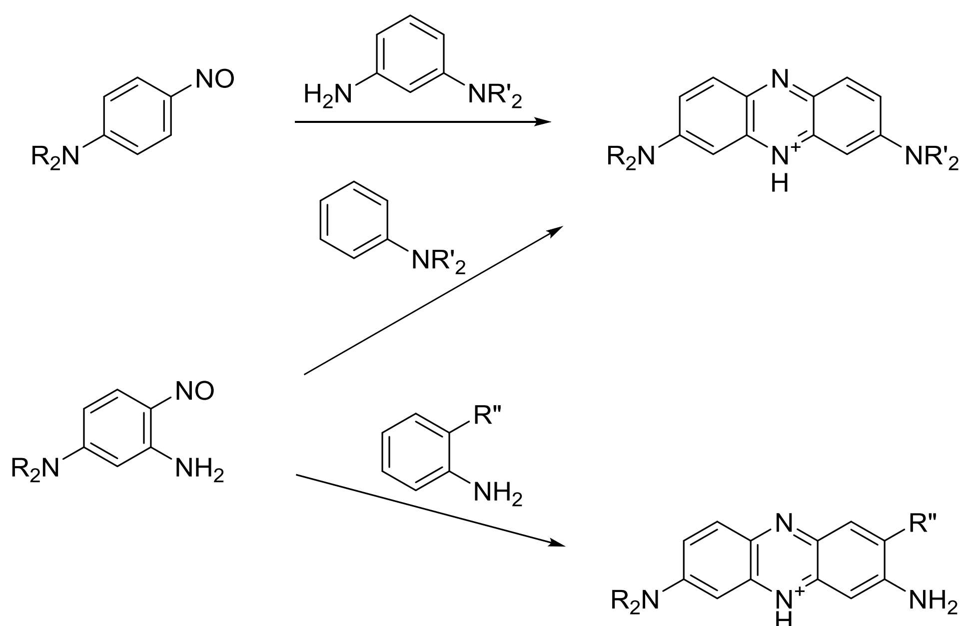


Figure 6. Classical synthesis of phenazinium dyes showing facile variation. Typically R is an alkyl group.

Safranine O was one of the early biological stains to be demonstrated as a Gram-negative photobactericide, a logical use given its part in the Gram-staining procedure.[41] Interestingly, more recently it has also been patented for photoantimicrobial use.[42]

Novel photosensitiser geometries might also be achieved by facile alteration of the azoaryl moiety in Janus green (Figure 5), given that this molecule arises merely from the coupling of diazotised methylene violet 3RAX and *N,N*-dimethylaniline. Again this would allow for alteration of physicochemical properties. As with neutral red, Janus green is taken up by mammalian cells, thus a significant increase in the

hydrophilicity of the molecule would be required in order for microbial selectivity to be achieved.

Janus green is unusual among the phenazinium dyes for its long wavelength absorption. For the most part established phenazinium compounds have associated λ_{max} values short of the therapeutic window normally expected for photoantimicrobial applications. This has been remedied via both extension of the chromophore and rigidification of the amine auxochromic groups, through the use of *N,N*-dialkyltetrahydroquinoxaline as a starting material. The resulting pentacyclic phenaziniums exhibited both increased λ_{max} values and singlet oxygen yields (Figure 7).[43]

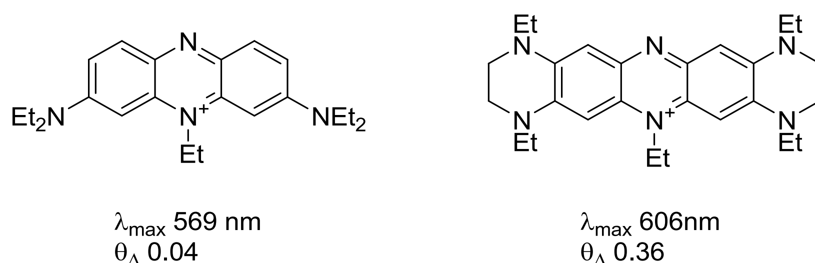


Figure 7. Increased wavelength and singlet oxygen quantum yield in rigidified phenazinium derivatives

5.3. Phenoxazines

Clinical interest in this class comes from long experience with the biological stain Nile blue A. This is, in fact a benzo[*a*]phenoxazine (Figure 8), and it is thus dealt with under *Benzannelated Compounds*, below. Basic blue 3 (Figure 8) is the best

known example of the phenoxazinium class itself but is of no use in terms of the photodynamic effect.

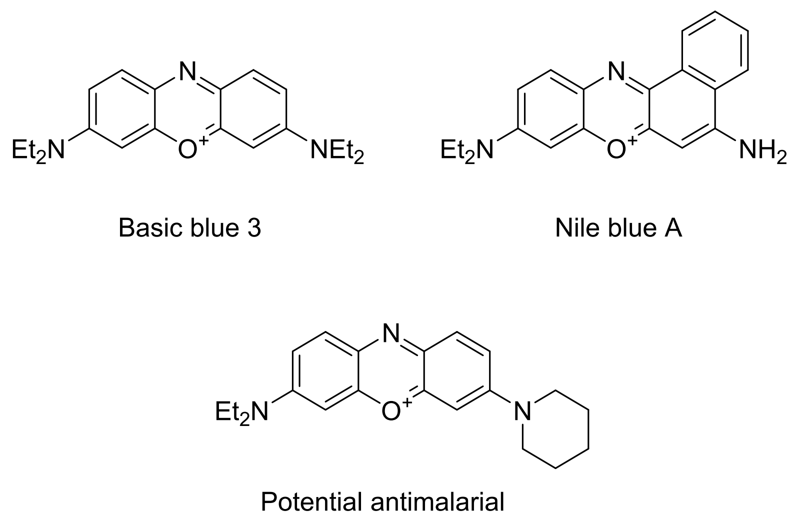


Figure 8. Phenoxazinum derivatives

The phenoxazine dyes, i.e. oxygen analogues of the methylene blue-type phenothiaziniums, do not produce reactive oxygen species on illumination, whether by a Type I or Type II mechanism. The excited state is very short-lived in the parent, undeveloped phenoxazinium chromophore. However, extended lifetimes can be realised via the use of the heavy atom effect, i.e. the inclusion of low-period atoms in the structure which stabilise the excited state molecule, as has been carried out for the realisation of effective Nile blue derivatives (below).

Due to the instability of the excited state chromophore, phenoxazinium dyes find considerable use as fluorescent sensors[44].and, in line with previous work on Nile blue, have also been suggested as conventional antimalarial leads (e.g. Figure 8).[45]

5.4. Phenothiazines

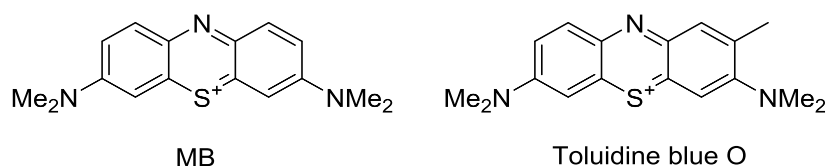


Figure 9. Phenothiazinium photoantimicrobial leads

Given the longevity of the medical use of methylene blue (MB, Figure 9), it is surprising that there has not been more drug development based on this structure. As noted earlier, MB has found use in a variety of roles, in addition to its position as lead compound in antimalarial, antipsychotic and antidepressant research.[9] However, its primogenicity in matters photoantimicrobial has probably inhibited the further clinical introduction of newer phenothiazinium photosensitisers, particularly from a toxicology point of view: MB is safe for human use, whereas any new chemical entity based on its structure would have to undergo extensive testing prior even to pre-clinical trialling. There are many examples of new compounds based on MB which have far greater photoantimicrobial potential – MB itself is only a moderate example – but these face huge regulatory barriers, whereas MB is already licensed for use in blood product photodecontamination,[46] and oral and nasal photodisinfection.[47]

As far as drug development is concerned, the synthesis of novel phenothiaziniums has seen higher productivity than even the benzo[*a*]phenoxazinium (Nile blue) derivatives. In addition, there exists a greater mix of peripheral and auxochromic congeners among MB derivative series.

Synthesis

The traditional, dye industry, synthesis of MB utilises the oxidative coupling of *N,N*-dimethylaniline and *N,N*-dimethyl-*p*-phenylenediaminethiosulphonic acid which is produced *in situ*. The coupled intermediate, Binschedler's green is oxidatively cyclised to provide the phenothiazinium chromophore (Figure 10).

In terms of analogue production for drug design, this is an unsuitable process, principally because of the strong oxidants, e.g. chromium(VI), utilised during the synthesis which cause denaturation of auxochromic groups and peripheral substituents. Similarly, the traditional synthesis is carried out in aqueous media, which limits the lipophilicity of starting materials and products alike.

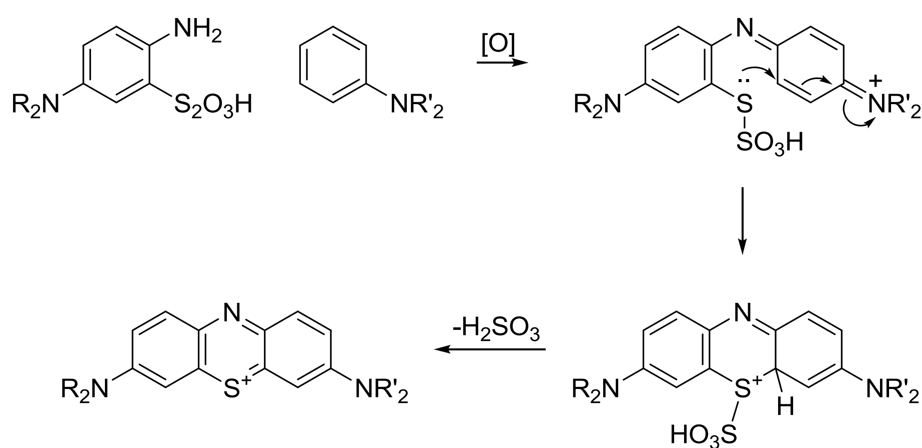


Figure 10. 'Classical' thiosulphonic acid synthesis of methylene blue derivatives. R is typically alkyl or aryl.

Weaker oxidants, such as silver(I) salts in alcoholic media, allow greater product range via a similar route to that described above, while the use of iodine or bromine provides a straightforward method of preparation of direct methylene blue analogues (Figure 11).[48]

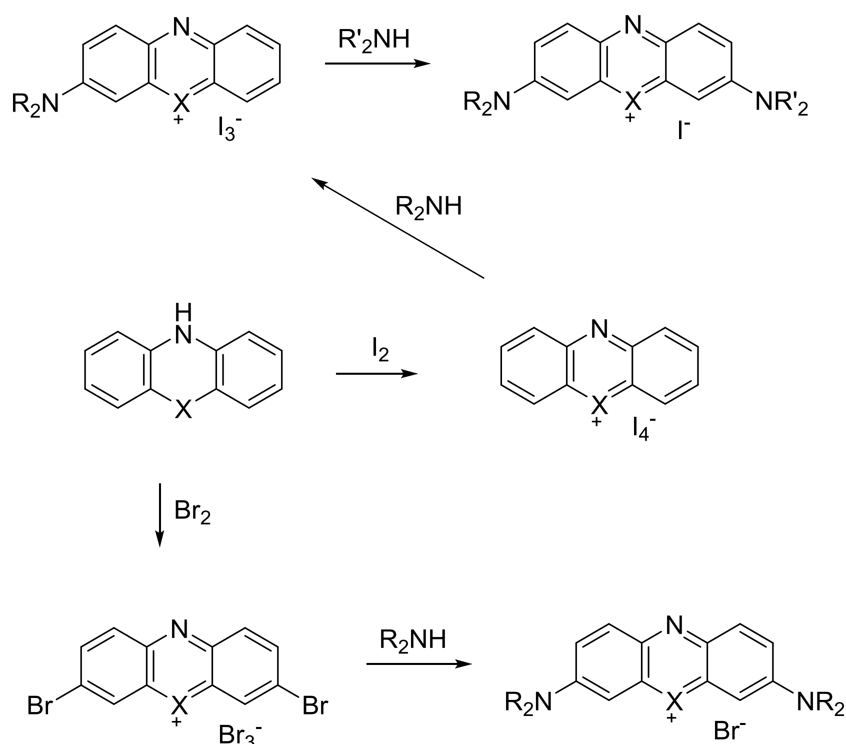


Figure 11. Symmetrical and asymmetrical phenothiazinium/chalcogen analogue formation from the reduced parent (R = alkyl or aryl; X = S, Se)

Despite such advances in synthetic methodology, the preponderance of novel derivatives is of the direct methylene blue type – i.e. auxochromic variants only (Figure 12). Few workers have reported chromophoric substitution of lead compounds, thus limiting proper drug discovery. However, a range of chromophore methylated derivatives[49] and a homologous series based on a second lead, toluidine blue (Figure 9), have been reported.[50] Similarly, the use of indoline and tetrahydroquinoline precursors has allowed the synthesis of tetra- and pentacyclic derivatives (Figure 12).[51,52]

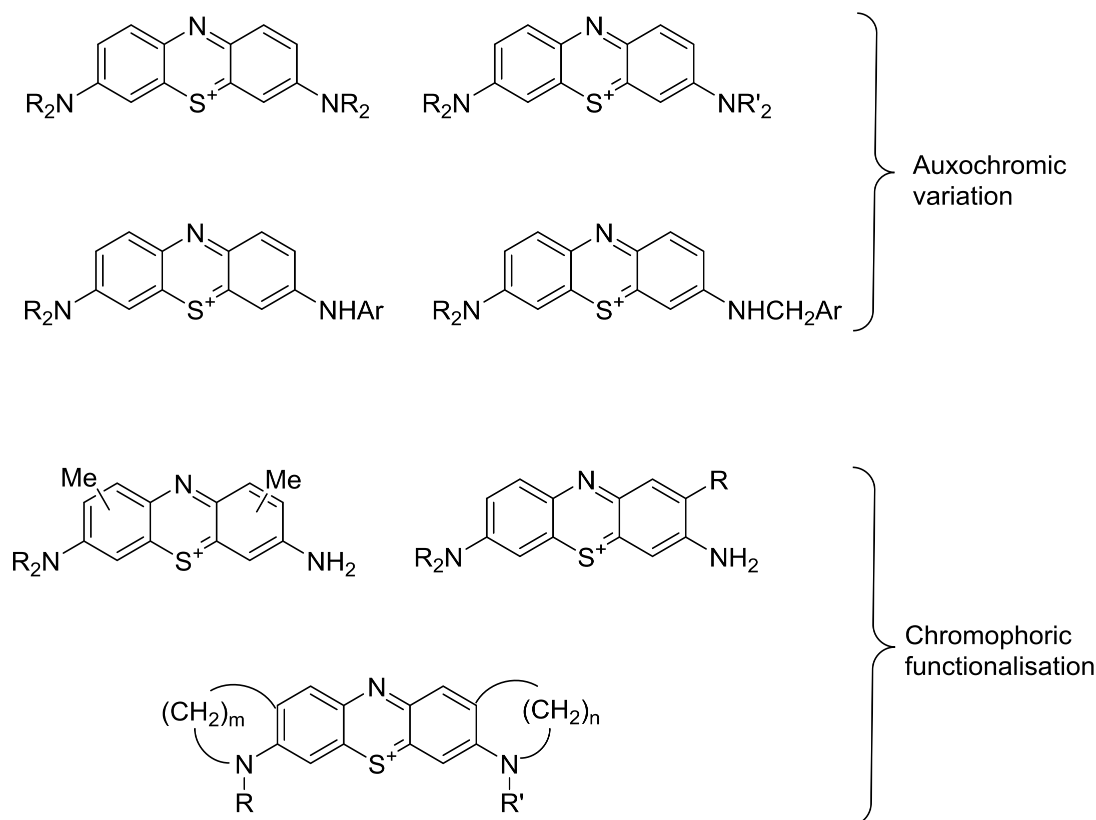


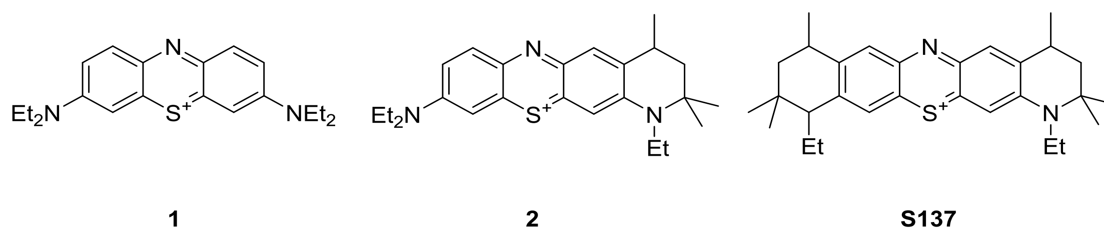
Figure 12. Phenothiazinium photoantimicrobial variants. R, R' = alkyl, aryl.

Activities

Generally, most series based on MB have contained analogues with significantly greater photoantimicrobial activities than the parent. Such increases have not been correlated to singlet oxygen yields, which is not surprising given that – as noted above – singlet oxygen measurement *in vitro* and cell killing in culture are two very different processes, the latter including cellular uptake and potential metabolism of the photosensitiser, neither of which is related to singlet oxygen yield.

A good example of this phenomena is provided by the pentacyclic derivative DO15 (S137). This produces lower yields of singlet oxygen than MB, but is considerably more active as a photoantimicrobial under the same conditions as the parent (Table

3).[52] In addition, its activity against Gram-negative bacteria is greater than usually seen with phenothiazinium photosensitisers and this is due to its lower propensity to aggregate formation (due to bulkier molecular shape) and ability to alter membrane structure.[53]



				MBC (μM)			
				S. aureus		***E. coli***	
Ps	**λ_{max} (nm)**	**Log P**	**Rel. $^1O_2$**	**Light***	**Dark**	**Light***	**Dark**
MB	660	-0.10	1.00	25	100	25	50
1	664	+0.80	0.55	6.25	100	6.25	100
2	678	+1.44	0.87	3.13	100	3.13	100
137	683	+1.54	0.92	0.78	100	3.13	100

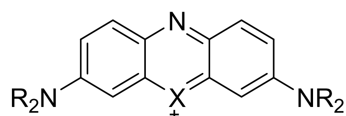
Table 3. Properties and photoactivities of a series of methylene blue derivatives. * fluence rate = 100 mW cm^{-2}, fluence = 6.2 J cm^{-2} (red light)

5.5. Phenoselenazines

As noted under *Phenoxazines*, the heavy atom effect has been utilised in several photosensitiser types in order to increase the occurrence of inter-system crossing and

significant production of the activated photosensitiser triplet state. Given that the change from ring oxygen to ring sulphur (phenoxazinium to phenothiazinium chromophore) generally furnished efficient photosensitisers via this route, the subsequent change to ring selenium should be supposed to provide further increases in singlet oxygen yields. Very little work has been carried out following this line of inquiry, compared to the benzologues or rosamine derivatives (*q.v.*).
However, a series of selenium homologues of MB was synthesised and tested by Griffiths and Gorman, with surprising results from both *in vitro* and photoantimicrobial testing Table 4.[54]

Synthesis of the derivatives followed the earlier halogen oxidation work on phenothiazine (Figure 11), but using 10*H*-phenoselenazine instead as the starting material.



X = Se[54]

R	λ_{max} (nm, H_2O)	LogP	Relative Φ_Δ	10 μM Log Photokill† *S. aureus*	*E. coli*
Me (MB)	660	-0.10	1.00	-	-
Et	664	+1.50	1.50	1.74	3.33
n-Pr	670	>+2	1.11	1.70	3.16
n-Bu	670	>+2	0.92	2.72	3.78

X = S[13]

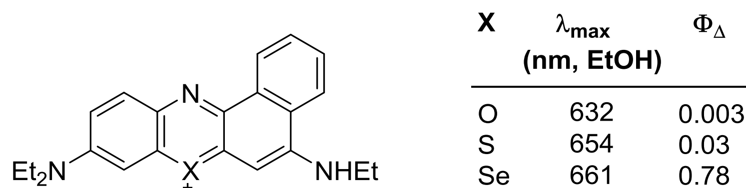
R	λ_{max} (nm, H_2O)	LogP	Relative Φ_Δ	6-Log Photokill (μM)* *S. aureus*	*E. coli*
Me (MB)	660	-0.10	1.00	25	25
Et	664	+0.81	0.55	6.3	6.3
n-Pr	669	+1.10	0.59	3.1	6.3
n-Bu	671	+1.34	0.61	3.1	3.1

Table 4. Properties and photobactericidal activities of MBDs and selenologues. † Fluence rate = 10 mW cm^{-2}, fluence = 3.0 J cm^{-2} (red light)*Fluence rate = 10 mW cm^{-2}, fluence = 6.2 J cm^{-2} (red light)

5.6. Benzannelated Compounds

The selectivity of the benzo[*a*]phenoxazine Nile blue for tumour tissue *in vivo* during the mid-20th Century led to the use of this as a lead compound for anticancer PDT agents by Foley in the 1980s.[55] Interestingly, a significant series of papers by Crossley et al. concerning Nile blue derivatives (NBDs) as potential anti-tubercular agents was published in the 1950s. Although photodynamic testing was not carried out in either the tumour staining or anti-TB work, the biological activity demonstrated in both cases is a strong argument for drug development and photodynamic analogues have resulted.[56]

As with the phenoxazinium parent chromophore, the disadvantage of Nile blue lies in its lack of photodynamic activity, whether by a Type I or Type II mechanism. Similarly bromo- and iodo-analogues have been synthesised with increased yields of singlet oxygen, but perhaps more significantly ring analogues with sulphur or selenium replacing oxygen have provided excellent scaffolds for drug development in the area of photoantimicrobials. As can be seen from Figure 13, the influence of heavy atoms is considerable.



X	λ_{max} (nm, EtOH)	Φ_Δ
O	632	0.003
S	654	0.03
Se	661	0.78

Figure 13. Chalcogen substituted photosensitisers

Due to its relatively high yield of singlet oxygen, the benzo[*a*]phenoselenazinium derivative EtNBSe (X = Se, Figure 13) has been used by several groups to investigate applications in photoantimicrobial activity as well as further synthesis.[57]

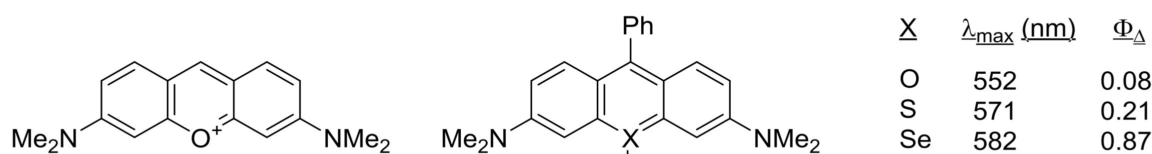
In terms of functionalisation, and as with most of the derivatives discussed in this review, very little has been reported concerning peripheral substitution. Clearly it is much simpler to produce analogues having variation in the auxochromic group, given the wide variety of amines available, but also due to the alteration of the redox characteristics of the parent aromatic constituents on functionalisation, which often inhibits either aromatic coupling in (A+B)-type syntheses, or chromophoric oxidation to yield the cationic heteroaromatic system.

Within this area, the effect of charge on the photobactericidal acitivity (as noted above for the anionic porphyrins) has been reported for the sulphur analogue (Figure 13, X = S). As expected, the presence of an anionic carboxylate residue removed activity against the Gram-negative bacterium *Escherichia coli*.[58] Unsurprisingly, also, the inclusion of a (cationic) guanidinyl moiety on the *N*-ethyl side chain has been found to increase photobactericidal activity against Gram-negative species, including the dangerous pathogen *Acinetobacter baumanii*.[59]

5.7. Xanthyliums (Rosamine-type)

While rhodamines have exhibited reported activity in the field of PDT, as noted above, this is not necessarily an indication of potential for photoantimicrobial action, and certainly the propensity for rhodamine uptake by mammalian mitochondria[60] would argue against the selectivity of such examples for microbial cells. However, the structurally related pyronines – i.e. rhodamines without the 9-aryl moiety are singlet oxygen producers and highly active photobactericides.[61] Given the anionic contribution of the 2'-carboxyl function in rhodamines, broad-spectrum activity against microbial species is unlikely, thus the removal of this group to furnish the cationic rosamines is logical. This class of photosensitiser might therefore be thought of as a 9-arylpyronine or, conversely, a 2,2'-oxygen-bridged triarylmethane. Either scenario provides antimicrobial pedigree.

In relatively recent work by Detty *et al.*, rosamine derivatives containing bridging heteroatoms other than oxygen were reported to have associated singlet oxygen yields similar to those of the phenothiaziniums, once again demonstrating the importance in photosensitiser design of the heavy atom effect (Figure 14).



X	λ_{max} (nm)	Φ_{Δ}
O	552	0.08
S	571	0.21
Se	582	0.87

Figure 14. Pyronin and simple rosamine derivatives

Rosamine analogue synthesis is relatively straightforward[62] and a variety of 9-aryl/heteroaryl substituents may be obtained via organometallic attack on the corresponding xanthone derivative (Figure 15).

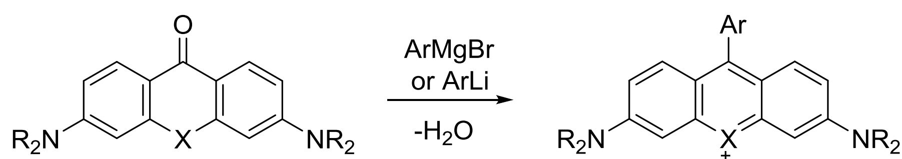


Figure 15. Synthesis of functionalised sulphur and selenium rosamine analogues (X = S, Se, Ar = aryl or heteroaryl nucleus)

For example, the use of 2-lithiothiophene produces the 9-thienoanalogue,[63].which extends the longest absorption wavelength to 590 nm (methanol) for the thiorosamine analogue, compared to 571 nm for the equivalent 9-phenyl derivative.

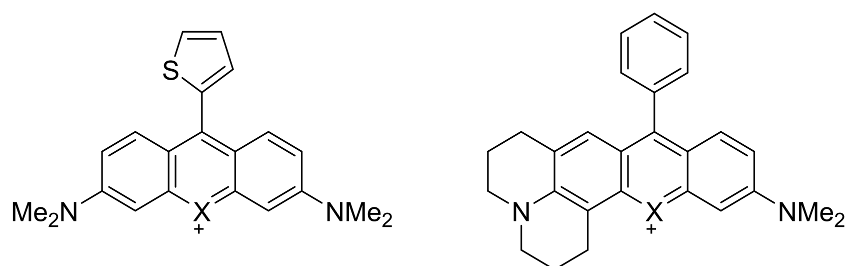
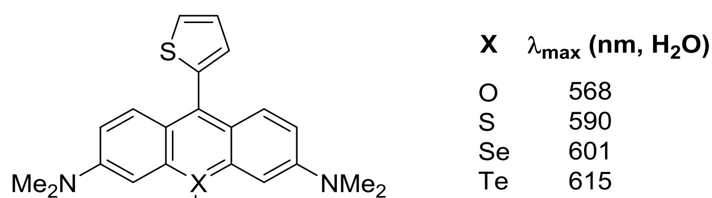


Figure 16. 9-(2'-Thienyl)- and auxochrome-rigidified rosamine derivatives (X = S, Se).

Rigidification of one of the amino auxochromes (e.g. the julolidino derivative, Figure 16, λ_{max} = 582 nm) has a lesser effect on wavelength.[64] As expected, the heavy atom effect also produces longer wavelengths, but the use of structural alterations other than that of heavy atom substitution does not appear to alter the singlet oxygen yield significantly.

Tellurium substitution is, of course the rational extension of this work - although there remain some concerns about the associated potential toxicity of this element - and this has been achieved, in contrast to work carried out in the study of phenothiazinium and benzo[*a*]phenothiazinium photosensitiser analogues. Tellurium-containing analogues have been synthesised which show the expected extension of the absorption spectrum beyond 600 nm.[65] Effects of the heavier atoms on λ_{max} for the 9-(2-thienyl) compound can be seen in Figure 17.



X	λ_{max} (nm, H_2O)
O	568
S	590
Se	601
Te	615

Figure 17. Effect of lower period atom inclusion in rosamine derivatives.

Apart from derivatives intended for use in blood photodecontamination (e.g. the 9-thienyl seleno-derivative mentioned above), the majority have been aimed at PDT application. Consequently, this is an area worth exploring for novel photoantimicrobial derivatives. However, it is noticeable that the wavelength range of

derivatives reported thus far has been significantly shorter than that normally desired for anti-infective work, as noted above (*Photosensitiser Requirements*).

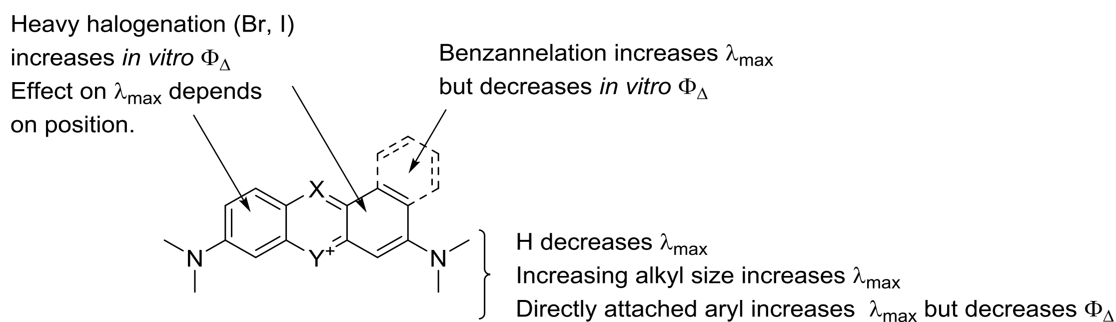
6. Future directions

In the early 21st Century, the photoantimicrobial approach has not yet reached proper clinical acceptance, being largely ignored by the pharmaceutical industry and viewed mainly sceptically by the medical profession in most countries. Consequently, to discuss novel derivatives at such a stage of development might seem somewhat fanciful.

This is not the case, due to the ever-increasing problem of clinical drug resistance among many serious pathogens and the very slow production of new antimicrobials. The ‘treatment gap’ in hospitals worldwide is becoming greater year on year and new approaches, hopefully including photoantimicrobials, must be employed in order at least to slow resistance development. The local application of photoantimicrobials offers considerable potential for the conservation of traditional antimicrobial drugs.

The few instances of current clinical photoantimicrobial use, whether in oral or nasal disinfection or in blood product photodecontamination employ methylene blue for the most part. It is hoped that this use will extend in these areas and that others in infection control will be developed. Novel photoantimicrobials will be required at this stage on the grounds of efficacy in the different presentations, but also for the commercialisation of the product, in the same way as conventional antimicrobials.

Given the very close similarities between the various derivatives discussed here, it is possible to derive a general structure-activity picture, as shown in Figure 18.



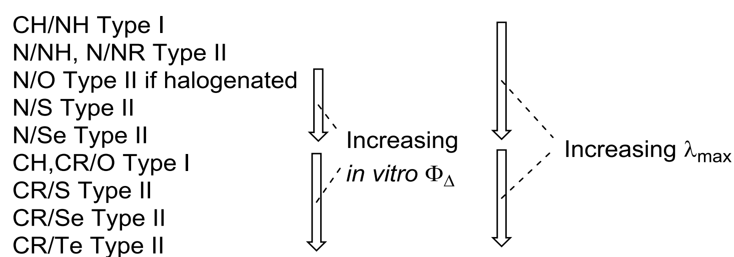


Figure 18. Structure-photophysical relationships in cationic, azine-type photosensitisers

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