



HAL
open science

Phenothiazinium photosensitisers, Part VIII: phenothiazinium–fluoroquinolone drug conjugates

Mark Wainwright, Ami Shah, Katie Meegan, Ciara Loughran, Andy Smith,
Nasima Valli, Nicola Dempster

► **To cite this version:**

Mark Wainwright, Ami Shah, Katie Meegan, Ciara Loughran, Andy Smith, et al.. Phenothiazinium photosensitisers, Part VIII: phenothiazinium–fluoroquinolone drug conjugates. *International Journal of Antimicrobial Agents*, 2010, 35 (4), pp.405. 10.1016/j.ijantimicag.2009.11.022 . hal-00567282

HAL Id: hal-00567282

<https://hal.science/hal-00567282>

Submitted on 20 Feb 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Phenothiazinium photosensitisers, Part VIII:
phenothiazinium–fluoroquinolone drug conjugates

Authors: Mark Wainwright, Ami Shah, Katie Meegan, Ciara Loughran, Andy Smith, Nasima Valli, Nicola Dempster



PII: S0924-8579(09)00565-2
DOI: doi:10.1016/j.ijantimicag.2009.11.022
Reference: ANTAGE 3201

To appear in: *International Journal of Antimicrobial Agents*

Received date: 6-10-2009
Revised date: 27-11-2009
Accepted date: 30-11-2009

Please cite this article as: Wainwright M, Shah A, Meegan K, Loughran C, Smith A, Valli N, Dempster N, Phenothiazinium photosensitisers, Part VIII: phenothiazinium–fluoroquinolone drug conjugates, *International Journal of Antimicrobial Agents* (2008), doi:10.1016/j.ijantimicag.2009.11.022

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **Phenothiazinium Photosensitisers VIII. Phenothiazinium-**
2 **Fluoroquinolone Drug Conjugates**

3

4 Mark Wainwright*, Ami Shah, Katie Meegan , Ciara Loughran, Andy Smith and
5 Nasima Valli

6

7

8 School of Pharmacy & Biomolecular Sciences

9 Liverpool John Moores University

10 Byrom Street

11 Liverpool

12 L3 3AF

13 United Kingdom

14

15 ***Author for correspondence**

16 Email mark_wainwright@hotmail.com

17 Tel. (44) 0151 231 2039

18 Fax (44) 0151 231 2170

19

20 **Keywords:** DNA; fluoroquinolone; methylene blue derivatives; phenothiaziniums;
21 photodynamic antimicrobial chemotherapy (PACT).

22

23

24

25

26 **Abstract**

27 The synthesis and antibacterial screening of a homologous series of 3-
28 dialkylaminophenothiazinium-7-norfloxacin conjugates was carried out alongside a
29 corresponding series of symmetrical methylene blue derivatives. The norfloxacin
30 conjugates maintained typical methylene blue derivative photoproperties such as long
31 wavelength absorption, but produced no measurable singlet oxygen in the standard
32 assay and provided no significant increase in the magnitude of photoantibacterial
33 action, this being similar to the methylene blue homologues, although both the
34 conjugates and homologues were considerably more active than methylene blue itself
35 against both *Staphylococcus aureus* and *Escherichia coli*. DNA binding via
36 intercalation was considerably greater for the series of norfloxacin conjugates than for
37 the methylene blue homologues.

38

39

40

41 1. Introduction

42 Methylene blue and a number of structurally-related phenothiazinium derivatives have
43 been reported to be efficacious photoantimicrobials against a wide range of bacteria,
44 fungi and viruses [1]. Red light absorption by such compounds (620-670 nm), is
45 sufficient to circumvent endogenous absorption by natural chromophores e.g. haem
46 and melanin, allowing the use of methylene blue derivatives in body fluids and
47 tissues.

48

49 Other classes of photosensitiser have been similarly investigated. The porphyrin class
50 is chief among these, mainly due to its majority share in the clinical photodynamic
51 therapy of cancer (PDT), although this may not supply a sound rationale for
52 antimicrobial discovery. Indeed, whereas the cationic phenothiazinium class exhibits
53 broad-spectrum antibacterial activity, anionic porphyrins used in PDT, such as
54 protoporphyrin IX or haematoporphyrin derivative, are normally only effective
55 against Gram-positive bacteria [2].

56

57 To remedy this lack of activity, various groups have investigated the attachment of
58 targeting or adjuvant moieties to anionic porphyrin photosensitisers. Thus,
59 polycationic molecules, such as poly(lysine) and poly(ethyleneimine) have been
60 attached to chlorin e_6 (Ce6), the resulting polycationic side chains having the effect of
61 permeabilising the outer membrane of Gram-negative bacteria, allowing
62 photosensitiser ingress, and thus photocytotoxicity [3,4]. Anionic porphyrins have
63 also been examined in conjunction with the cationic peptide antibiotic, polymyxin,
64 with similar intent [5].

65 However, such chromophore attachment to peptides and polymers suffers from the
66 fact that there are multiple sites of attachment, in this case between free carboxylic
67 acid residues in the chlorin ring and numerous amine residues in the polymers, often
68 chemically equivalent. Consequently, product mixtures are formed rather than pure
69 material, and this may be disadvantageous from the point of view both of structure-
70 activity relationships and of future clinical product registration.

71

72 Novel methylene blue derivatives are usually produced by altering the auxochromic
73 amine groups at positions 3- and 7- of the phenothiazinium ring, typically providing
74 homologous series, and both symmetrical and asymmetrical examples have been
75 reported in the literature [6,7]. However, due to the broad-spectrum activity
76 associated with methylene blue derivatives, the use of biomolecule attachment to the
77 phenothiazinium chromophore has received scant attention, although in the anticancer
78 application attachment of methylene blue via an extra amino group at C-4 to
79 oligonucleotides has been reported [8].

80

81 As part of an ongoing photosensitiser discovery program, it was considered that it
82 might be possible to use small bioactive molecules to aid in photoantibacterial
83 targeting. Given the requirement for single, pure active species in drug development,
84 the reactive intermediate cation employed in asymmetrical phenothiazinium synthesis
85 was used as a starting point, since this would guarantee a single active site for
86 coupling with amines.

87

88 The fluoroquinolone class of antibacterial agents is one of the most widely used in
89 modern infection control, the presence of a fluorine atom at position 6 of the 4-

90 quinolone ring, coupled with amino functionality at the neighbouring position (C-7)
91 providing optimal antibacterial activity [9]. Several front-line fluoroquinolones are
92 substituted in this latter position with a 4-piperazinyl moiety, thus offering ideal
93 potential reactive sites for the current proposition, since this would furnish a
94 dialkylamine equivalent auxochrome in the resulting phenothiazinium derivative
95 (Figure 1), optimising light absorption in the 660 nm region. A further reason for the
96 use of fluoroquinolones here is their targeting of enzymes closely associated with
97 bacterial DNA [10].

98

99 The present study covers the investigation of the antibacterial activity of a series of
100 methylene blue analogues, i.e. having increasing size of dialkylamine group at
101 position 3- and attached at position 7- to the fluoroquinolone, norfloxacin, via the 4-
102 piperazinyl moiety.

103

104

105

106

107 2. Materials & Methods

108 2.1. Materials

109 The following amines were purchased from Sigma-Aldrich (Gillingham, UK) and
110 were used without further purification: dimethylamine, diethylamine, di-*n*-
111 propylamine, di-*n*-butylamine, di-*n*-pentylamine and di-*n*-hexylamine, as were 10*H*-
112 phenothiazine, norfloxacin. The standard photosensitiser, methylene blue, was also
113 purchased from Sigma-Aldrich and was purified by column chromatography on silica
114 gel (gradient elution with 0-10% methanol/dichloromethane). Methanol
115 (spectrophotometric grade), 1-octanol, dichloromethane (DCM) and iodine were
116 purchased from Fisher Scientific (Leicestershire, UK) and used without further
117 purification.

118

119 2.2. Symmetrical Photosensitisers

120 2.2.1 3,7-Dibromophenothiazinium tribromide

121 1.0 g of phenothiazine (5 mmol) was dissolved in 70ml of glacial acetic acid, ensuring
122 uniform mixing for about half an hour. To this solution 5ml of bromine in 40ml of
123 glacial acetic acid was added rapidly. 200ml of cold water was then added to this
124 mixture quickly with vigorous stirring, resulting in a dark red solution containing a
125 black precipitate. The solution was then filtered through sintered glass crucible and
126 the resulting solid washed with water and then with diethyl ether, to ensure all
127 bromine was removed. Yield of dark, red-brown powder = 2.72 g (81%).

128

129

130 2.2.2. 3,7-Bis(dialkylamino)phenothiazinium bromides (**1b-1f**)

131 The requisite dialkylamine (15 mmol) in 100 ml of dichloromethane was stirred in a
132 flask followed by the addition of 1.00 g (1.7 mmol) of solid 3,7-
133 dibromophenothiazinium tribromide. The mixture was stirred for about 4 hours,
134 washed with dilute hydrobromic acid followed by water, the organic layer separated
135 and dried (Na_2SO_4) and then concentrated under vacuum and the product precipitated
136 using diethyl ether. Dissolution in dichloromethane and ether precipitation was
137 repeated until spectrophotometric examination of the resulting material gave an
138 absorbance ratio ($A_{\lambda_{\text{max}}}:A_{290}$) of 2.2.

139

140 2.3. Asymmetrical Photosensitisers

141 Asymmetric analogues were prepared by the initial oxidation of 10*H*-phenothiazine
142 with iodine, but with reaction of the resulting cation with a lowered concentration of
143 amine, furnishing a mono-3-substituted derivative (**2a-f**). These preparative steps
144 were carried out as in previous work [11]. 3-Substituted intermediates were further
145 reacted with the second amine, i.e. the 4-piperazinyl unit of norfloxacin to obtain the
146 asymmetrical product.

147

148 2.3.1. Norfloxacin conjugates (**3a-f**)

149 A solution of the 3-dialkylamino- compound (**2a-f**, 0.75 mmol) in methanol (20 ml)
150 was stirred at room temperature and solid norfloxacin (0.57 g, 1.8 mmol) added in
151 several portions over 10 minutes. The progress of the reaction was monitored by TLC
152 as described above. The mixture was stirred for about 5-6 hours at room temperature.
153 The solvent was then removed from the reaction mixture *in vacuo* and the residual
154 black solid was extracted with DCM. The resulting solution was chromatographed on

155 silica with a gradient MeOH/DCM eluent (0-10% v/v) and the blue fraction
156 concentrated *in vacuo* before precipitation using diethyl ether.

157

158 Solutions (100 μM) of phenothiaziniums and conjugates were made up in distilled
159 water for use in the bacterial screen. All spectrophotometric measurements were
160 carried out on a Hewlett Packard 8452A diode array spectrophotometer. The
161 photosensitisers were found to follow the Beer's law in the concentration range of 10^{-5}
162 $- 10^{-7} \text{ mol l}^{-1}$.

163

164 2.4. Antibacterial Screening

165 The photobactericidal activities of the norfloxacin conjugates and methylene blue
166 homologues in addition to that of the known photosensitiser methylene blue and
167 norfloxacin itself were measured against a Gram positive and a Gram negative
168 organism, *Staphylococcus aureus* (NCTC 6571) and *Escherichia coli* (NCTC 10418)
169 respectively. Both strains were grown in Mueller-Hinton Broth and then diluted to a
170 concentration of 10^6 colony-forming units/ml. Aliquots of the strains were then
171 treated in microtitre trays with various concentrations of photosensitiser ranging from
172 100 to 3 μM , with zero photosensitiser concentrations in each case for control
173 purposes. The trays were then either illuminated for twenty minutes using an array of
174 126 light-emitting diodes (660 nm) giving a light dose of 6 J cm^{-2} or alternatively foil-
175 covered to provide dark controls. From each well showing a complete absence of
176 growth of the micro-organism (i.e. total bacterial kill), 1 μl was sub-cultured on
177 nutrient agar, using the Miles-Misra method, and incubated for 18 hours at 37°C .

178

179 The minimum bactericidal concentrations (MBCs) were thus determined as the lowest
180 concentration for each photosensitiser giving no bacterial growth. Each test was
181 repeated to ensure an absolute value for the cited MBC with $n = 6$. Due to the
182 absolute nature of the assay, i.e. the cut-off point was taken as a complete absence of
183 growth, rather than fractional kill, no statistical treatment of the resulting data was
184 applied.

185

186 *2.5. DNA intercalation*

187 Following previous workers [12], a DNA solution was made with 0.75 mg DNA
188 sodium salt type XIV from herring testes (Aldrich, St. Louis, MO, USA), dissolved in
189 10 ml distilled water. Using the extinction coefficient (ϵ) for DNA of $6600 \text{ M}^{-1}\text{cm}^{-1}$,
190 the concentration of the DNA solution was found to be $100 \mu\text{M}$.

191

192 Distilled water solutions of the candidate photosensitisers ($100 \mu\text{M}$) were made up
193 and λ_{max} values measured spectrophotometrically.

194

195 For each of the candidates, 8 ml of $100 \mu\text{M}$ DNA solution was then mixed in a test
196 tube with 1 ml of the $100 \mu\text{M}$ photosensitiser solution. The λ_{max} of the resulting
197 mixture was recorded and compared to that of the original photosensitiser solution.

198 Both methylene blue and norfloxacin were included as comparators.

199

200 *2.6. Singlet Oxygen production and Lipophilicity (LogP)*

201 These properties were measured spectrophotometrically, as recounted in previous
202 work [11].

203

204 **3. Results**

205 The present study represents the first reporting of the synthesis of phenothiazinium-
206 drug conjugates. As expected, reaction between the mono-substituted 3-
207 dialkylaminophenothiazinium intermediate compounds and norfloxacin was
208 straightforward, due to the nucleophilic nature of the 4-piperazinyl moiety, providing
209 deep-blue coloured reaction mixtures (Fig. 1). Products required column
210 chromatography for purification, but yields were in line with previous work involving
211 the synthesis of asymmetrical phenothiazinium derivatives [11]. In the same way, the
212 absorption maxima of the conjugates were similar to those of 3-dialkylamino-7-
213 heterocyclophenothiazinium derivatives reported by Gorman *et al.* [7], since any
214 electronic effects due to the fluoroquinolone moiety are not transmissible to *N*-4 of the
215 piperazinyl sub-unit (Table 1).

216

217 *N*-4-Piperazinyl derivatives of norfloxacin have been synthesised as novel, but
218 conventional, antibacterial agents, using a range of substituents, including
219 heteroaromatics, several of which exhibited increased activity against Gram-negative
220 bacteria including *E. coli* [13]. Simple 4-*N*-alkyl derivatives have also been shown to
221 have increased activity against conventional-resistant bacteria, again including *E. coli*
222 and *S. aureus* [14].

223

224 Series of symmetrical methylene blue derivatives (**1a-f**, Fig. 2), i.e. having identical
225 dialkylamine groups in positions 3- and 7- of the phenothiazinium ring, have been
226 reported previously by different groups [7,11], and exhibited the expected increase in
227 Log*P* and λ_{\max} with auxochrome size along the homologous series.

228

229 **4. Discussion**

230 Although it had been assumed that the presence of the inert piperazinyl linker between
231 the phenothiazinium and fluoroquinolone ring systems would prevent intramolecular
232 interference with singlet oxygen generation by the former under red light conditions,
233 this was not the case. *In vitro* singlet oxygen yields for the conjugates were, in fact,
234 too low to measure using the standard spectrophotometric assay employed, unlike
235 previous asymmetrical examples (Table 2). However, it is again noted that such tests
236 are not necessarily of great utility as performance indicators for photoantimicrobials,
237 due to the potential influence of local electronic effects in the cellular milieu, not
238 replicated in photofading tests. It should also be recalled that the cytotoxic agents
239 produced during PACT are very short-lived - for example, singlet oxygen has a half-
240 life of less than a microsecond before decaying to ground state molecular oxygen [1].

241

242 The loss of *in vitro* singlet oxygen production in the conjugates may be due to
243 molecular aggregation at the concentrations used in the test. The aqueous spectra of
244 the conjugates in the 600-700 nm region were similar in shape to those of the
245 symmetrical derivatives (**1a-f**), i.e. broadened with a small shoulder around 630 nm.
246 However, while this aggregation was not deleterious for the symmetrical species, it is
247 possible that in the conjugates this would allow the close proximity of neighbouring
248 phenothiazinium and quinolone chromophores, thus providing a deactivation route.
249 The photobactericidal effects observed (see below) are not thought to develop from
250 such aggregates.

251

252 In terms of the nucleic acid interaction, it was noticeable that, while the symmetrical
253 series of methylene blue derivatives exhibited similar bathochromic shifts to that of

254 methylene blue itself on *in vitro* mixing with DNA [15], the conjugates exhibited
255 much greater shifts (typically > 10 nm, Table 2). The magnitude of these shifts was
256 more akin to that reported for Taylor's blue (1,9-dimethyl methylene blue),
257 understood to be a much stronger intercalator [15]. The bathochromic shifts observed
258 in the red region of the spectrum underline the hypothesis that intercalation involved
259 the phenothiazinium chromophore, rather than the fluoroquinolone moiety, no shifting
260 of the norfloxacin λ_{\max} (324 nm) was observed.

261

262 It should be noted at the outset that the testing of the photosensitisers against bacteria
263 carried out in this work was aimed at a clinically-realistic approach. Thus the
264 candidate photosensitisers were mixed with the bacterial suspensions and illuminated
265 almost immediately with red light for 20 minutes. The antibacterial screening of
266 conventional agents usually entails a significant incubation time, in order for the drug
267 to be taken up by the bacterial cells, typically of 18 hours. Consequently, under the
268 same conditions as those used for the phenothiazinium derivatives, norfloxacin was
269 much less effective than might normally be expected. In addition, although the
270 fluoroquinolones are also known to be photosensitisers, the use of red light for
271 activation - rather than ultraviolet - elicited the photoactivation only of the
272 phenothiazinium chromophores involved.

273

274 All of the conjugates and symmetrical analogues of methylene blue exhibited greater
275 efficacy in both organisms than either methylene blue itself or norfloxacin (Table 2).
276 The pattern of increasing activity with alkyl group size among the symmetrical
277 derivatives was not repeated in the conjugates, the dibutyl-dihexyl analogues (**3d-f**)
278 among the latter exhibiting much lower efficacies. While these activity profiles may

279 correlate with lipophilicity for the symmetrical derivatives, this was not the case with
280 the conjugates, since little variation in $\text{Log}P$ was observed across the series,
281 presumably due to the highly hydrophilic character of the norfloxacin moiety.

282

283 Dark toxicity among both sets of derivatives was found to be low, most derivatives
284 being toxic at the highest concentrations used (100 μM) or above, in line with
285 methylene blue (Table 2). This suggests a lack of essential targeting, and does not
286 support a specific DNA-localising hypothesis. Bacterial DNA photodamage has been
287 reported for methylene blue itself, but it has been suggested that the initial activity
288 occurs at the cell exterior [1]. In addition, the relatively amphiphilic nature of the
289 norfloxacin derivatives here ($-0.1 < \text{Log}P < +0.5$) suggests some difficulty in reaching
290 the interior of mammalian cell nuclei, since such localisation has not reported for the
291 symmetrical derivatives [7].

292

293 An increasing number of phenothiazinium derivatives is appearing in the literature as
294 potential biological photosensitisers. As far as we are aware, the present study is the
295 first time that conjugation of the phenothiazinium chromophore to a standard drug has
296 been reported.

297

298

299 **Declarations**

300 **Funding:** N/A

301 **Competing Interests:** N/A

302 **Ethical Approval:** N/A

303

304 **References**

305

306 1. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). *J Antimicrob*307 *Chemother* 1998;42:13-28.

308 2. Nitzan Y, Dror R, Ladan H, Malik Z, Kimel S, Gottfried V. Structure-activity

309 relationship of porphines for photoinactivation of bacteria. *Photochem Photobiol*

310 1995;62:342-7.

311 3. Soukos NS, Ximenez-Fyvie LA, Hamblin MR, Socransky SS, Hasan T. Targeted

312 antimicrobial photochemotherapy. *Antimicrob Agents Chemother* 1998;42:2595-

313 2601.

314 4. Tegos GP, Anbe M, Yang C, Demidova TN, Satti M, Mroz P, Janjua S, Gad F,

315 Hamblin MR. Protease-stable polycationic photosensitizer conjugates between

316 polyethyleneimine and chlorin (e6) for broad-spectrum antimicrobial

317 photoinactivation. *Antimicrob Agents Chemother* 2006;50:1402-10.

318 5. Nitzan Y, Gutterman M, Malik Z, Ehrenberg B. Inactivation of Gram-negative

319 bacteria by photosensitized porphyrins. *Photochem Photobiol* 1991;55:89-96.320 6. Mellish KJ, Cox RD, Vernon DI, Griffiths J, Brown SB. *In vitro* photodynamic321 activity of a series of methylene blue analogues. *Photochem Photobiol* 2002;75:392-

322 7.

323 7. Gorman SA, Bell AL, Griffiths J, Roberts D, Brown SB. The synthesis and

324 properties of unsymmetrical 3,7-diaminophenothiazin-5-ium salts: potential

325 photosensitisers for photodynamic therapy. *Dyes Pigments* 2006;71:153-60.

326 8. Moller U, Schubert F, Cech D. Versatile procedure of multiple introduction of 8-

327 aminomethylene blue into oligonucleotides. *Bioconj Chem* 1995;6:174-8.328 9. Anderson MI, MacGowan AP. Development of the quinolones. *J Antimicrob*329 *Chemother* 2003;51:1-11.

- 330 10. Blondeau JM. Fluoroquinolones: mechanism of action, classification, and
331 development of resistance. *Surv Ophthalmol* 2004;49:S73-S78.
- 332 11. Wainwright M, Meegan K, Loughran C, Giddens RM. Phenothiazinium
333 photosensitisers VI. Photobactericidal asymmetric derivatives. *Dyes Pigments*
334 2009;82:387-91.
- 335 12. Huang CZ, Li YF, Zhang DJ, Ao XP. Spectrophotometric study on the
336 supramolecular interactions of Nile blue sulphate with nucleic acids. *Talanta*
337 1998;49:495-503.
- 338 13. El-Din G, Abuo-Rahma AA, Sarhan HA, Gad GFM. Design, synthesis,
339 antibacterial activity and physicochemical parameters of novel *N*-4-piperazinyl
340 derivatives of norfloxacin. *Bioorg Med Chem* 2009;17:3879-86.
- 341 14. Vila J, Sanchez-Céspedes J, Sierra JM, Piqueras M, Nicolas E, Freixas J, et al.
342 Antibacterial evaluation of a collection of norfloxacin and ciprofloxacin derivatives
343 against multi-resistant bacteria. *Int J Antimicrob Agents* 2006;28:19-24.
- 344 15. Wagner SJ, Skripchenko A, Robinette D, Foley JW, Cincotta L. Factors affecting
345 virus photoinactivation by a series of phenothiazine dyes, *Photochem Photobiol*
346 1998;67:343-9.
- 347
348

349 **Legends**

350

351 Fig. 1. Synthetic routes to the phenothiazinium homologues. Reagents: (i) bromine
352 in acetic acid; (ii) dialkylamine in dichloromethane; (iii) iodine in dichloromethane;
353 (iv) dialkylamine in methanol; (v) norfloxacin in methanol.

354

355 Table 1. Analytical data for the derivatives. ^aBy ICP-MS; ^b measured in MeOH.

356

357 Table 2. Antibacterial and related data for the derivatives. ^aMinimum bactericidal
358 concentration; ^b Yield of singlet oxygen relative to that of MB; ^c¹O₂ measurement
359 ≤2% of that of MB.

360

361

	R ³	R ⁷	X ⁻	m/z ^a		% Yield	λ_{\max} (nm) ^b	Log ϵ_{\max} ^b
				Calc.	Found			
1a	NMe ₂	NMe ₂	Cl ⁻	-	-	-	657	4.88
1b	NEt ₂	NEt ₂	Br ⁻	340.18	340.18	23	664	4.87
1c	NPr ⁿ ₂	NPr ⁿ ₂	Br ⁻	396.25	396.24	28	669	4.75
1d	NBu ⁿ ₂	NBu ⁿ ₂	Br ⁻	452.31	452.33	30	671	4.84
1e	NPe ⁿ ₂	NPe ⁿ ₂	Br ⁻	508.37	508.32	21	672	4.69
1f	NHx ⁿ ₂	NHx ⁿ ₂	Br ⁻	564.43	564.44	18	673	4.55
3a	NMe ₂	Norflo.	I ⁻	558.20	558.15	24	658	4.61
3b	NEt ₂	Norflo.	I ⁻	586.23	586.05	25	662	4.66
3c	NPr ⁿ ₂	Norflo.	I ⁻	614.26	614.17	30	665	4.77
3d	NBu ⁿ ₂	Norflo.	I ⁻	642.29	642.09	37	665	4.81
3e	NPe ⁿ ₂	Norflo.	I ⁻	670.32	670.27	34	664	4.65
3f	NHx ⁿ ₂	Norflo.	I ⁻	698.35	698.15	33	665	4.69

Compound	MBC ^a (μM)				Rel. ¹ O ₂ ^b	LogP	DNA shift (nm)
	<i>S. aureus</i>		<i>E. coli</i>				
	Light	Dark	Light	Dark			
Norflo.	100	100	25	25	^c	+0.1	0
1a	25	100	25	50	1.00	-0.1	+4
1b	6.3	100	6.3	100	0.55	+0.8	+3
1c	3.1	50	6.3	100	0.59	+1.1	+1
1d	3.1	50	3.1	100	0.61	+1.3	+2
1e	3.1	100	3.1	100	0.26	+1.6	+5
1f	3.1	100	3.1	100	0.21	+1.8	+6
3a	6.3	100	6.3	100	^c	-0.1	+11
3b	3.1	25	3.1	100	^c	+0.2	+10
3c	3.1	100	3.1	100	^c	+0.2	+12
3d	12.5	100	12.5	100	^c	+0.4	+11
3e	12.5	100	12.5	100	^c	+0.5	+10
3f	12.5	50	12.5	50	^c	+0.4	+4

Table 2. Antibacterial and related data for the derivatives. ^a Minimum bactericidal concentration; ^b Yield of singlet oxygen relative to that of MB; ^c ¹O₂ measurement ≤2% of that of MB.

Phenothiazinium photosensitisers, Part VIII: phenothiazinium–fluoroquinolone drug conjugates

Mark Wainwright *, Ami Shah, Katie Meegan, Ciara Loughran, Andy Smith,
Nasima Valli, Nicola Dempster

*School of Pharmacy and Biomolecular Sciences, Liverpool John Moores
University, Byrom Street, Liverpool L3 3AF, UK*

ARTICLE INFO

Article history:

Received 6 October 2009

Accepted 30 November 2009

Keywords:

DNA

Fluoroquinolone

Methylene blue derivatives

Phenothiaziniums

Photodynamic antimicrobial chemotherapy (PACT)

* Corresponding author. Tel.: +44 151 231 2039; fax: +44 151 231 2170.

E-mail address: mark_wainwright@hotmail.com (M. Wainwright).

ABSTRACT

Synthesis and antibacterial screening of a homologous series of 3-dialkylaminophenothiazinium-7-norfloxacin conjugates was carried out alongside a corresponding series of symmetrical methylene blue derivatives. The norfloxacin conjugates maintained typical methylene blue derivative photoproperties, such as long wavelength absorption, but produced no measurable singlet oxygen in the standard assay and provided no significant increase in the magnitude of photoantibacterial action, this being similar to the methylene blue homologues, although both the conjugates and homologues were considerably more active than methylene blue itself both against *Staphylococcus aureus* and *Escherichia coli*. DNA binding via intercalation was considerably greater for the series of norfloxacin conjugates than for the methylene blue homologues.

1. Introduction

Methylene blue and a number of structurally related phenothiazinium derivatives have been reported to be efficacious photoantimicrobials against a wide range of bacteria, fungi and viruses [1]. Red light absorption by such compounds (620–670 nm) is sufficient to circumvent endogenous absorption by natural chromophores, e.g. haem and melanin, allowing the use of methylene blue derivatives in body fluids and tissues.

Other classes of photosensitiser have been similarly investigated. The porphyrin class is chief among these, mainly due to its majority share in the clinical photodynamic therapy (PDT) of cancer, although this may not supply a sound rationale for antimicrobial discovery. Indeed, whereas the cationic phenothiazinium class exhibits broad-spectrum antibacterial activity, anionic porphyrins used in PDT, such as protoporphyrin IX or haematoporphyrin derivative, are normally only effective against Gram-positive bacteria [2].

To remedy this lack of activity, various groups have investigated the attachment of targeting or adjuvant moieties to anionic porphyrin photosensitisers. Thus, polycationic molecules, such as poly(lysine) and poly(ethyleneimine), have been attached to chlorin e_6 (Ce6), the resulting polycationic side chains having the effect of permeabilising the outer membrane of Gram-negative bacteria allowing photosensitiser ingress and thus photocytotoxicity [3,4]. Anionic porphyrins have also been examined in conjunction with the cationic peptide antibiotic polymyxin, with similar intent [5].

However, such chromophore attachment to peptides and polymers suffers from the fact that there are multiple sites of attachment, in this case between free carboxylic acid residues in the chlorin ring and numerous amine residues in the polymers, often chemically equivalent. Consequently, product mixtures are formed rather than pure material and this may be disadvantageous from the point of view both of structure–activity relationships and of future clinical product registration.

Novel methylene blue derivatives are usually produced by altering the auxochromic amine groups at positions 3 and 7 of the phenothiazinium ring, typically providing homologous series, and both symmetrical and asymmetrical examples have been reported in the literature [6,7]. However, owing to the broad-spectrum activity associated with methylene blue derivatives, the use of biomolecule attachment to the phenothiazinium chromophore has received scant attention, although in the anticancer application attachment of methylene blue via an extra amino group at C-4 to oligonucleotides has been reported [8].

As part of an ongoing photosensitiser discovery programme, it was considered that it might be possible to use small bioactive molecules to aid in photoantibacterial targeting. Given the requirement for single, pure, active species in drug development, the reactive intermediate cation employed in asymmetrical phenothiazinium synthesis was used as a starting point, since this would guarantee a single active site for coupling with amines.

The fluoroquinolone class of antibacterial agents is one of the most widely used in modern infection control, the presence of a fluorine atom at position 6 of the 4-quinolone ring coupled with amino functionality at the neighbouring position (C-7) providing optimal antibacterial activity [9]. Several front-line fluoroquinolones are substituted in this latter position with a 4-piperazinyl moiety, thus offering ideal potential reactive sites for the current proposition, since this would furnish a dialkylamine equivalent auxochrome in the resulting phenothiazinium derivative (Fig. 1), optimising light absorption in the 660 nm region. A further reason for the use of fluoroquinolones here is their targeting of enzymes closely associated with bacterial DNA [10].

The present study covers the investigation of the antibacterial activity of a series of methylene blue analogues, i.e. having increasing size of dialkylamine group at position 3 and attached at position 7 to the fluoroquinolone norfloxacin via the 4-piperazinyl moiety.

2. Materials and methods

2.1. Materials

The following amines were purchased from Sigma-Aldrich (Gillingham, UK) and were used without further purification: dimethylamine; diethylamine; di-*n*-propylamine; di-*n*-butylamine; di-*n*-pentylamine; and di-*n*-hexylamine. 10*H*-phenothiazine and norfloxacin were also purchased from Sigma-Aldrich. The standard photosensitiser methylene blue (Sigma-Aldrich) was purified by

column chromatography on silica gel [gradient elution with 0–10% methanol/dichloromethane (DCM)]. Methanol (spectrophotometric grade), 1-octanol, DCM and iodine were purchased from Fisher Scientific (Leicestershire, UK) and were used without further purification.

2.2. Symmetrical photosensitisers

2.2.1. 3,7-Dibromophenothiazinium tribromide

One gram of phenothiazine (5 mmol) was dissolved in 70 mL of glacial acetic acid, ensuring uniform mixing for approximately 30 min. To this solution, 5 mL of bromine in 40 mL of glacial acetic acid was added rapidly. Then, 200 mL of cold water was quickly added to this mixture with vigorous stirring, resulting in a dark red solution containing a black precipitate. The solution was then filtered through a sintered glass crucible and the resulting solid was washed with water and then with diethyl ether to ensure all bromine was removed. The yield of dark red–brown powder was 2.72 g (81%).

2.2.2. 3,7-Bis(dialkylamino)phenothiazinium bromides (**1b–1f**)

The requisite dialkylamine (15 mmol) in 100 mL of DCM was stirred in a flask followed by the addition of 1.00 g (1.7 mmol) of solid 3,7-dibromophenothiazinium tribromide. The mixture was stirred for approximately 4 h, washed with dilute hydrobromic acid followed by water, the organic layer was separated and dried (Na_2SO_4) and then concentrated under vacuum and the product was precipitated using diethyl ether. Dissolution in DCM and ether

precipitation was repeated until spectrophotometric examination of the resulting material gave an absorbance ratio ($A_{\lambda_{\max}}:A_{290}$) of 2.2.

2.3. Asymmetrical photosensitisers

Asymmetric analogues were prepared by the initial oxidation of 10*H*-phenothiazine with iodine, but with reaction of the resulting cation with a lowered concentration of amine, furnishing a mono-3-substituted derivative (**2a–f**). These preparative steps were carried out as in previous work [11]. 3-Substituted intermediates were further reacted with the second amine, i.e. the 4-piperazinyl unit of norfloxacin, to obtain the asymmetrical product.

2.3.1. Norfloxacin conjugates (**3a–f**)

A solution of the 3-dialkylamino- compound (**2a–f**, 0.75 mmol) in methanol (20 mL) was stirred at room temperature and solid norfloxacin (0.57 g, 1.8 mmol) was added in several portions over 10 min. Progress of the reaction was monitored by thin layer chromatography [11] as described above. The mixture was stirred for approximately 5–6 h at room temperature. The solvent was then removed from the reaction mixture in vacuo and the residual black solid was extracted with DCM. The resulting solution was chromatographed on silica with a gradient MeOH/DCM eluent (0–10% v/v) and the blue fraction was concentrated in vacuo before precipitation using diethyl ether.

Solutions (100 μ M) of phenothiaziniums and conjugates were made up in distilled water for use in the bacterial screen. All spectrophotometric

measurements were carried out on a Hewlett Packard 8452A diode array spectrophotometer (Agilent Technologies, Waldbronn, Germany). The photosensitisers were found to follow Beer's law in the concentration range of 10^{-5} – 10^{-7} mol/L.

2.4. Antibacterial screening

The photobactericidal activities of the norfloxacin conjugates and the methylene blue homologues in addition to those of the known photosensitiser methylene blue and norfloxacin itself were measured against a Gram-positive organism (*Staphylococcus aureus* NCTC 6571) and a Gram-negative organism (*Escherichia coli* NCTC 10418). Both 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 treated in microtitre trays with various concentrations of photosensitiser ranging from 100 μ M to 3 μ M, with zero photosensitiser concentration in each case used for control purposes. The trays were then either illuminated for 20 min using an array of 126 light-emitting diodes (660 nm) giving a light dose of 6 J/cm² or alternatively foil-covered to provide dark controls. From each well showing complete absence of growth of the microorganism (i.e. total bacterial kill), 1 μ L was subcultured on nutrient agar using the Miles–Misra method and incubated for 18 h at 37 °C.

Minimum bactericidal concentrations (MBCs) were thus determined as the lowest concentration of each photosensitiser giving no bacterial growth. Each test was repeated ($n = 6$) to ensure an absolute value for the cited MBC.

Owing to the absolute nature of the assay, i.e. the cut-off point was taken as a complete absence of growth rather than fractional kill, no statistical treatment of the resulting data was applied.

2.5. DNA intercalation

Following previous work [12], a DNA solution was made with 0.75 mg of DNA sodium salt type XIV from herring testes (Aldrich, St Louis, MO) dissolved in 10 mL of distilled water. Using the extinction coefficient (ϵ) for DNA of $6600 \text{ M}^{-1} \text{ cm}^{-1}$, the concentration of the DNA solution was found to be $100 \mu\text{M}$.

Distilled water solutions of the candidate photosensitisers ($100 \mu\text{M}$) were made up and λ_{max} values were measured spectrophotometrically.

For each of the candidates, 8 mL of $100 \mu\text{M}$ DNA solution was mixed in a test tube with 1 mL of the $100 \mu\text{M}$ photosensitiser solution. The λ_{max} of the resulting mixture was recorded and compared with that of the original photosensitiser solution. Both methylene blue and norfloxacin were included as comparators.

2.6. Singlet oxygen production and lipophilicity (logP)

These properties were measured spectrophotometrically, as recounted in previous work [11].

3. Results

The present study represents the first report of the synthesis of phenothiazinium–drug conjugates. As expected, reaction between the monosubstituted 3-dialkylaminophenothiazinium intermediate compounds and norfloxacin was straightforward owing to the nucleophilic nature of the 4-piperazinyl moiety, providing deep-blue coloured reaction mixtures (Fig. 1). Products required column chromatography for purification but yields were in line with previous work involving the synthesis of asymmetrical phenothiazinium derivatives [11]. In the same way, the absorption maxima of the conjugates were similar to those of 3-dialkylamino-7-heterocyclophenothiazinium derivatives reported by Gorman et al. [7], since any electronic effects due to the fluoroquinolone moiety are not transmissible to *N*-4 of the piperazinyl subunit (Table 1).

Previously, *N*-4-piperazinyl derivatives of norfloxacin have been synthesised as novel but conventional antibacterial agents using a range of substituents, including heteroaromatics, several of which exhibited increased activity against Gram-negative bacteria including *E. coli* [13]. Simple 4-*N*-alkyl derivatives have also been shown to have increased activity against conventional resistant bacteria, again including *E. coli* and *S. aureus* [14].

Series of symmetrical methylene blue derivatives (**1a–f**; Fig. 1), i.e. having identical dialkylamine groups in positions 3 and 7 of the phenothiazinium ring, have been reported previously by different groups [7,11] and exhibited the

expected increase in $\log P$ and λ_{\max} with auxochrome size along the homologous series.

4. Discussion

Although it had been assumed that the presence of the inert piperazinyl linker between the phenothiazinium and fluoroquinolone ring systems would prevent intramolecular interference with singlet oxygen generation by the former under red light conditions, this was not the case. In vitro singlet oxygen yields for the conjugates were, in fact, too low to measure using the standard spectrophotometric assay employed, unlike previous asymmetrical examples (Table 2). However, it is again noted that such tests are not necessarily of great utility as performance indicators for photoantimicrobials owing to the potential influence of local electronic effects in the cellular milieu, not replicated in photofading tests. It should also be recalled that the cytotoxic agents produced during photodynamic antimicrobial chemotherapy (PACT) are very short-lived, e.g. singlet oxygen has a half-life of less than a microsecond before decaying to ground state molecular oxygen [1].

The loss of in vitro singlet oxygen production in the conjugates may be due to molecular aggregation at the concentrations used in the test. The aqueous spectra of the conjugates in the 600–700 nm region were similar in shape to those of the symmetrical derivatives (**1a–f**), i.e. broadened with a small shoulder around 630 nm. However, whilst this aggregation was not deleterious for the symmetrical species, it is possible that in the conjugates this would allow the close proximity of neighbouring phenothiazinium and

quinolone chromophores, thus providing a deactivation route. The photobactericidal effects observed (see below) are not thought to develop from such aggregates.

In terms of nucleic acid interaction, it was noticeable that, whilst the symmetrical series of methylene blue derivatives exhibited similar bathochromic shifts to that of methylene blue itself on in vitro mixing with DNA [15], the conjugates exhibited much greater shifts (typically >10 nm) (Table 2). The magnitude of these shifts was more akin to that reported for Taylor's blue (1,9-dimethyl methylene blue), understood to be a much stronger intercalator [15]. The bathochromic shifts observed in the red region of the spectrum underline the hypothesis that intercalation involved the phenothiazinium chromophore, rather than the fluoroquinolone moiety, as no shifting of the norfloxacin λ_{\max} (324 nm) was observed.

It should be noted at the outset that testing of the photosensitisers against bacteria carried out in this work was aimed at a clinically realistic approach. Thus, the candidate photosensitisers were mixed with the bacterial suspensions and illuminated almost immediately with red light for 20 min. Antibacterial screening of conventional agents usually entails a significant incubation time in order for the drug to be taken up by the bacterial cells, typically 18 h. Consequently, under the same conditions as those used for the phenothiazinium derivatives, norfloxacin was much less effective than might normally be expected. In addition, although the fluoroquinolones are also known to be photosensitisers, the use of red light for activation, rather than

ultraviolet, elicited photoactivation only of the phenothiazinium chromophores involved.

All of the conjugates and symmetrical analogues of methylene blue exhibited greater efficacy in both organisms than either methylene blue itself or norfloxacin (Table 2). The pattern of increasing activity with alkyl group size among the symmetrical derivatives was not repeated in the conjugates, the dibutyl-dihexyl analogues (**3d–f**) among the latter exhibiting much lower efficacies. Whilst these activity profiles may correlate with lipophilicity for the symmetrical derivatives, this was not the case with the conjugates, since little variation in $\log P$ was observed across the series, presumably due to the highly hydrophilic character of the norfloxacin moiety.

Dark toxicity among both sets of derivatives was found to be low, most derivatives being toxic at the highest concentrations used (100 μM) or above, in line with methylene blue (Table 2). This suggests a lack of essential targeting and does not support a specific DNA-localising hypothesis. Bacterial DNA photodamage has been reported for methylene blue itself, but it has been suggested that the initial activity occurs at the cell exterior [1]. In addition, the relatively amphiphilic nature of the norfloxacin derivatives here ($-0.1 < \log P < +0.5$) suggests some difficulty in reaching the interior of mammalian cell nuclei, since such localisation has not reported for the symmetrical derivatives [7].

An increasing number of phenothiazinium derivatives is appearing in the literature as potential biological photosensitisers. As far as we are aware, the present study is the first time that conjugation of the phenothiazinium chromophore to a standard drug has been reported.

Funding

None.

Competing interests

None declared.

Ethical approval

Not required.

Accepted Manuscript

References

- [1] Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). *J Antimicrob Chemother* 1998;42:13–28.
- [2] Nitzan Y, Dror R, Ladan H, Malik Z, Kimel S, Gottfried V. Structure–activity relationship of porphines for photoinactivation of bacteria. *Photochem Photobiol* 1995;62:342–7.
- [3] Soukos NS, Ximenez-Fyvie LA, Hamblin MR, Socransky SS, Hasan T. Targeted antimicrobial photochemotherapy. *Antimicrob Agents Chemother* 1998;42:2595–601.
- [4] Tegos GP, Anbe M, Yang C, Demidova TN, Satti M, Mroz P, et al. Protease-stable polycationic photosensitizer conjugates between polyethyleneimine and chlorin(e6) for broad-spectrum antimicrobial photoinactivation. *Antimicrob Agents Chemother* 2006;50:1402–10.
- [5] Nitzan Y, Gutterman M, Malik Z, Ehrenberg B. Inactivation of Gram-negative bacteria by photosensitized porphyrins. *Photochem Photobiol* 1991;55:89–96.
- [6] Mellish KJ, Cox RD, Vernon DI, Griffiths J, Brown SB. In vitro photodynamic activity of a series of methylene blue analogues. *Photochem Photobiol* 2002;75:392–7.
- [7] Gorman SA, Bell AL, Griffiths J, Roberts D, Brown SB. The synthesis and properties of unsymmetrical 3,7-diaminophenothiazin-5-ium iodine salts: potential photosensitisers for photodynamic therapy. *Dyes Pigm* 2006;71:153–60.

- [8] Moller U, Schubert F, Cech D. Versatile procedure of multiple introduction of 8-aminomethylene blue into oligonucleotides. *Bioconjug Chem* 1995;6:174–8.
- [9] Anderson MI, MacGowan AP. Development of the quinolones. *J Antimicrob Chemother* 2003;51:1–11.
- [10] Blondeau JM. Fluoroquinolones: mechanism of action, classification, and development of resistance. *Surv Ophthalmol* 2004;49(Suppl 2):S73–8.
- [11] Wainwright M, Meegan K, Loughran C, Giddens RM. Phenothiazinium photosensitisers, Part VI: photobactericidal asymmetric derivatives. *Dyes Pigm* 2009;82:387–91.
- [12] Huang CZ, Li YF, Zhang DJ, Ao XP. Spectrophotometric study on the supramolecular interactions of Nile blue sulphate with nucleic acids. *Talanta* 1998;49:495–503.
- [13] El-Din G, Abuo-Rahma AA, Sarhan HA, Gad GFM. Design, synthesis, antibacterial activity and physicochemical parameters of novel *N*-4-piperazinyl derivatives of norfloxacin. *Bioorg Med Chem* 2009;17:3879–86.
- [14] Vila J, Sanchez-Céspedes J, Sierra JM, Piqueras M, Nicolas E, Freixas J, et al. Antibacterial evaluation of a collection of norfloxacin and ciprofloxacin derivatives against multiresistant bacteria. *Int J Antimicrob Agents* 2006;28:19–24.
- [15] Wagner SJ, Skripchenko A, Robinette D, Foley JW, Cincotta L. Factors affecting virus photoinactivation by a series of phenothiazine dyes. *Photochem Photobiol* 1998;67:343–9.

Fig. 1. Synthetic routes to the phenothiazinium homologues. Reagents: (i) bromine in acetic acid; (ii) dialkylamine in dichloromethane; (iii) iodine in dichloromethane; (iv) dialkylamine in methanol; and (v) norfloxacin in methanol.

Accepted Manuscript

Table 1

Analytical data for the derivatives

Derivative	R ³	R ⁷	X ⁻	m/z ^a		% Yield	λ_{\max} (nm) ^b	Log ϵ_{\max} ^b
				Calculated	Observed			
1a	NMe ₂	NMe ₂	Cl ⁻	–	–	–	657	4.88
1b	NEt ₂	NEt ₂	Br ⁻	340.18	340.18	23	664	4.87
1c	NPr ⁿ ₂	NPr ⁿ ₂	Br ⁻	396.25	396.24	28	669	4.75
1d	NBu ⁿ ₂	NBu ⁿ ₂	Br ⁻	452.31	452.33	30	671	4.84
1e	NPe ⁿ ₂	NPe ⁿ ₂	Br ⁻	508.37	508.32	21	672	4.69
1f	NHx ⁿ ₂	NHx ⁿ ₂	Br ⁻	564.43	564.44	18	673	4.55
3a	NMe ₂	Norfloxacin	I ⁻	558.20	558.15	24	658	4.61
3b	NEt ₂	Norfloxacin	I ⁻	586.23	586.05	25	662	4.66
3c	NPr ⁿ ₂	Norfloxacin	I ⁻	614.26	614.17	30	665	4.77
3d	NBu ⁿ ₂	Norfloxacin	I ⁻	642.29	642.09	37	665	4.81
3e	NPe ⁿ ₂	Norfloxacin	I ⁻	670.32	670.27	34	664	4.65
3f	NHx ⁿ ₂	Norfloxacin	I ⁻	698.35	698.15	33	665	4.69

^a Molecular ion mass measured by inductively-coupled plasma mass spectrometry.^b Measured in MeOH.

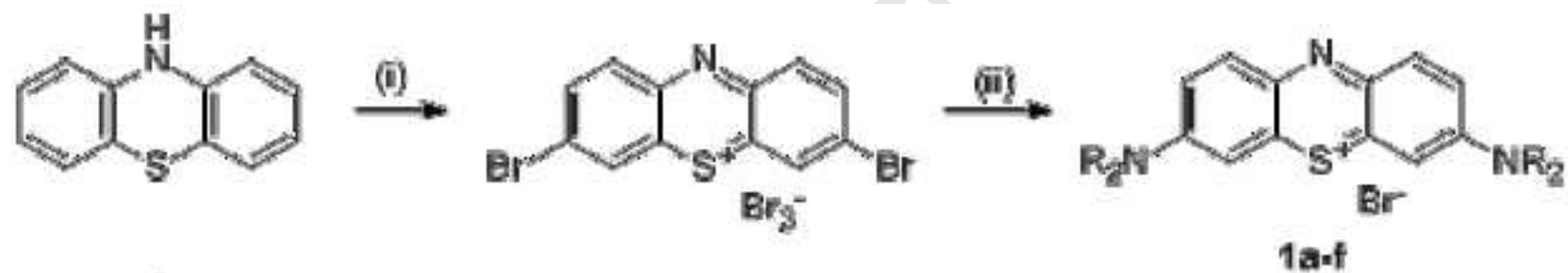
Table 2

Antibacterial and related data for the derivatives

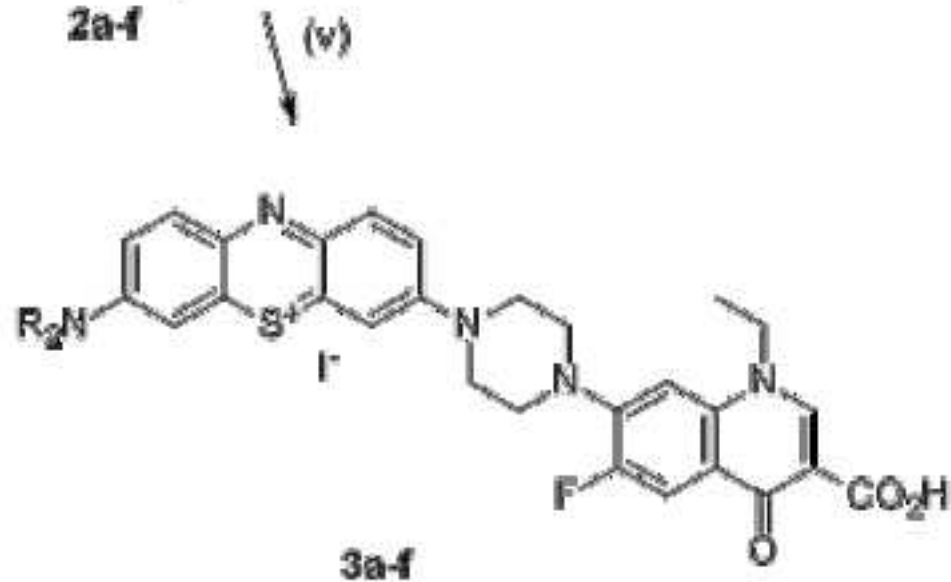
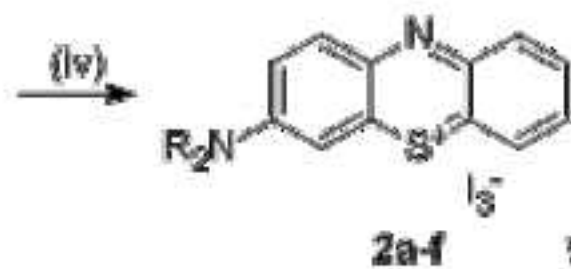
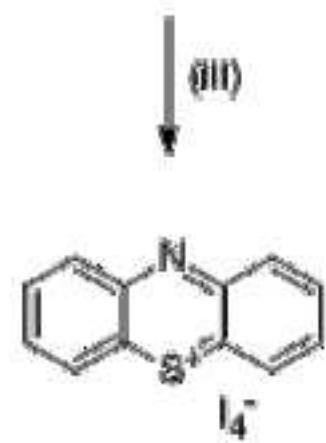
Compound	MBC (μM)				Rel. ¹ O ₂ ^a	Log <i>P</i>	DNA shift (nm)
	<i>Staphylococcus aureus</i>		<i>Escherichia coli</i>				
	Light	Dark	Light	Dark			
Norfloxacin	100	100	25	25	^b	+0.1	0
1a	25	100	25	50	1.00	-0.1	+4
1b	6.3	100	6.3	100	0.55	+0.8	+3
1c	3.1	50	6.3	100	0.59	+1.1	+1
1d	3.1	50	3.1	100	0.61	+1.3	+2
1e	3.1	100	3.1	100	0.26	+1.6	+5
1f	3.1	100	3.1	100	0.21	+1.8	+6
3a	6.3	100	6.3	100	^b	-0.1	+11
3b	3.1	25	3.1	100	^b	+0.2	+10
3c	3.1	100	3.1	100	^b	+0.2	+12
3d	12.5	100	12.5	100	^b	+0.4	+11
3e	12.5	100	12.5	100	^b	+0.5	+10
3f	12.5	50	12.5	50	^b	+0.4	+4

MBC, minimum bactericidal concentration.

^a Yield of singlet oxygen relative to that of methylene blue.^b ¹O₂ measurement $\leq 2\%$ that of methylene blue.



Symmetrical route



Asymmetrical route