On peroxide antimalarials

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Abstract: Several dicyclohexylidene tetraoxanes were prepared in order to gain a further insight into structure–activity relationship of this kind of antimalarials. The tetraoxanes 2–5, obtained as a cis/trans mixture, showed pronounced antimalarial activity against Plasmodium falciparum chloroquine susceptible D6, chloroquine resistant W2 and multidrug-resistant TM91C235 (Thailand) strains. They have better than or similar activity to the corresponding desmethyl dicyclohexylidene derivatives. Two chimeric endoperoxides with superior antimalarial activity to the natural product ascaridole were also synthesized.

Keywords: mixed tetraoxanes, endoperoxides, malaria, P. falciparum.

INTRODUCTION

Malaria affects more than 500 million people per annum, causing more than one million deaths, mostly in Africa.1 Infants, young children and pregnant women are particularly at risk; in fact, it has been estimated that a child dies of malaria every 30 seconds. Furthermore, the disease has as an immeasurable negative impact, both personally and socioeconomically, on families and communities in endemic areas. Although malaria is treatable, increased resistance of the protozoan parasite Plasmodium falciparum to standard and affordable anti-malarial drugs, such as chloroquine (CQ), complicates the treatment of infected individuals. Peroxide antimalarials of the 1,2,4-trioxacyclohexane class (artemisinin and its derivatives)2 and drugs of the trioxolane class3 offer some new possibilities for treating malaria.

Compounds of another peroxide class, the 1,2,4,5-tetraoxacyclohexanes (tetraoxanes), although less investigated, also have been shown to have potent anti-
malarial activity. Subsequently, the syntheses of mixed tetraoxanes enabled the controlled preparation of a new generation of this promising class of antimalarials. In addition to the steroidal tetraoxanes, a significant number of dicyclohexylidene tetraoxanes have been synthesized and their anti-malarial activity evaluated both \textit{in vitro} and \textit{in vivo}.\textsuperscript{8,9}

Monoterpane ascaridole (Scheme 1), a natural bicyclic [2.2.2] endoperoxide with moderate antimalarial properties, was used as a model for the synthesis of a series of diaryl substituted ascaridole-type endoperoxides, which showed higher activity as compared to ascaridole or dihydroascaridole.\textsuperscript{10} More recently, the syntheses of less volatile ascaridole and dihydroascaridole derivatives have been accomplished starting from perillyl and nopol derivatives.\textsuperscript{11}

![Scheme 1](image)

Here are reported the synthesis and results of anti-malarial screening of two types of anti-malarial peroxides: mixed tetraoxanes, the derivatives of 12-methyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane-3-carboxylic acid, and endoperoxides, monoterpane ascaridole derivatives, bound to another anti-malarial pharmacophore, 4-amino-7-chloroquinoline. In addition, the results of \textit{in vivo} screening of some mixed steroidal tetraoxanes are also discussed.

RESULTS AND DISCUSSION

Chemistry

The class of dicyclohexylidene tetraoxanes (2–5) was designed with varying substituents at the C(4") position in order to obtain further insight into the structure–activity relationship related to spiro-substituents in 1,2,4,5-tetraoxacyclohexane at C(3) and C(6). Gem-dihydroperoxide 1 was prepared from 4-methylocyclohexanone using 30 % hydrogen peroxide and HCl as a catalyst.\textsuperscript{5a} Compound 1 was isolated in 47–85 % yield and was pure enough (Scheme 2) to be directly used in the next step.\textsuperscript{**} This gem-dihydroperoxide was coupled to 4-oxocyclohe-
xanecarboxylate according to a previously developed procedure to yield the parent mixed tetraoxane, achiral methyl 12-methyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane-3-carboxylate (2; 10–35 %). Further transformations using the ester → acid → amide sequence (Scheme 2) afforded the desired amides 4 and 5 in 59 % and 49 % yield, respectively.

Scheme 2.

Ascaridole derivatives 6 and 7 synthesized earlier were hydrolysed with LiOH into the corresponding acids and further coupled to N-(7-chloroquinolin-4-yl)ethane-1,2-diamine to afford the final aminoquinoline products 8 and 9, respectively (Scheme 1).

Antimalarial activity

The synthesized peroxides were screened in vitro against three Plasmodium falciparum strains: D6 (chloroquine-susceptible), W2 (chloroquine-resistant, sus-
ceptible to mefloquine), and TM91C235 (Thailand), a multidrug-resistant strain, following the protocol given in the literature. All the synthesized tetraoxanes exhibited interesting antimalarial activity. In accordance with previous findings, the acid 3 was less active (in this particular case—practically inactive) than the methyl ester 2 and the corresponding amides 4 and 5 against all parasite strains. One of the current hypotheses regarding the mechanism of antimalarial action of peroxides is that they act in the food vacuole (FV) of *P. falciparum*, which has a pH around 5.5. As such, amide 5 was designed to have an N,N-dimethylamino group, expecting that protonation of the basic nitrogen would enhance the flux of the compound through the FV membrane, thereby increasing its concentration at the desired site of action. However, the in vitro antimalarial activity data for tetraoxane 5 (Table I) does not support this hypothesis, since amide 4 (which possesses only an acidic hydrogen at the nitrogen) and ester 2 exhibit very similar activity (both IC₅₀ and IC₉₀) against all the tested parasites. In addition, the activities of the dicyclohexylidene tetraoxanes presented in this paper are better or equivalent to similar desmethyl compounds.

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<td>30.4</td>
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<td>13.0</td>
<td>12.8</td>
<td>11.5</td>
<td>17.4</td>
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</table>

The antimalarial activity of compounds 6–9 appears to be strongly dependent on the degree of saturation: the saturated bicyclic endoperoxides (7, 9) are more potent antimalarials relative to their unsaturated counterparts (Table I), and both unsaturated (6, 8) and saturated (7, 9) endoperoxides are 5–12 times more active than ascaridole and dihydroascaridole themselves. These results indicate that peroxide–aminoquinoline chimeras, in addition to the trioxaquines, might represent a promising addition to the existing antimalarial arsenal.

Finally, the results of an in vivo study on the epimeric mixed steroidal tetraoxanes 10 and 11 (Table II) are presented. The in vitro activity of the com-
pounds differs 20- to 30-fold, with the (4"\textsuperscript{R})-epimer \textbf{10} being more active; this structure-activity relationship qualitatively holds for other epimeric pairs at the same carbon.\textsuperscript{5} Docking calculations of \textbf{10} and \textbf{11} with heme\textsuperscript{14} are consistent with the observation that the proximity of the heme iron to the oxygen atom of the tetraoxane moiety favours potent \textit{in vitro} activity of both compounds. The more potent analogues have much lower energy minimized docked structures. In addition, preliminary metabolic stability assays and metabolite identification were performed using human and mouse liver microsomes to aid the estimation of the first-pass metabolism of the drug candidates in relevant species (Table II).\textsuperscript{5c} It is important to note that no scission of peroxide bond was observed (the tetraoxane moiety is stable in this assay); only monohydroxylations occurred. In this assay,\textsuperscript{5c} stable compounds were defined as having half-lives > 60 min. Both compounds have similar metabolic half-lives ($t_{1/2}$ (mouse) $\cong$ 30 min). Hence, it would be logical that the observed difference in \textit{in vitro} activity would also be seen in the \textit{in vivo} efficacy test in mice. However, the collective \textit{in vivo} results presented in Table II are not consistent with results from the \textit{in vitro} screening and with the docking calculations: epimers \textbf{10} and \textbf{11} exert similar \textit{in vivo} activity in mice, regardless of whether they were administered orally or subcutaneously (Table II). Furthermore, at 600 mg/kg total dose p.o. and 480 mg/kg total dose s.c. of tetraoxane \textbf{10}, 4/5 and 5/5 mice cured were observed and the respective survival times were 28 and 31 days. At a total dose 150 mg/kg p.o. and 120 mg/kg s.c., very similar survival times were observed.

Analogous results were also seen for epimer \textbf{11}. In addition, at comparable total doses of 150 mg/kg p.o. and 120 mg/kg s.c., compounds \textbf{10} and \textbf{11} both cured more animals orally than subcutaneously. This apparent higher bioavailability by the oral route may have resulted from a lack of absorption due to s.c. depot formation: a subcutaneous sterile pocket of oil and unabsorbed test compound was commonly found in each animal. Alternatively, one of the hydroxylated metabolites formed after oral administration may be more active than the administered parent drug. For both compounds, \textbf{10} and \textbf{11}, no toxic effects were observed at any tested concentration or applied protocol.

To conclude, several dicyclohexylidene tetraoxanes were prepared and tested in order to gain additional insight into the structure-activity relationship of this structural class of antimalarials. The tetraoxanes \textbf{2-5} obtained as \textit{cis/trans} mixtures exerted better, or similar activity, than the corresponding desmethyl dicyclohexylidene derivatives.\textsuperscript{6d,9} The initial work on ascaridole-related compounds\textsuperscript{11} enabled the synthesis of two chimeric endoperoxides with superior antimalarial activity to the natural product, ascaridole. The present results indicate that further efforts should be put into research of peroxide–aminoquinoline chimeras as potential antimalarial drug leads. Finally, the results of \textit{in vivo} tests of two epimeric tetraoxanes with a steroidal carrier indicate that subcutaneous testing using an oil vehicle for these compounds may be unsuitable.
TABLE II. *In vivo* activity of tetraoxanes 10 and 11 against *Plasmodium berghei*

<table>
<thead>
<tr>
<th>Compd.</th>
<th>mg/kg per day</th>
<th>mg/kg total</th>
<th>Admin.</th>
<th>Mice dead/day died</th>
<th>Mice alive day 31/total</th>
<th>Survival time days</th>
<th>Metabolic stability t_{1/2}/min</th>
<th>Metabolite identification</th>
<th>In vitro (IC_{50} / nM)</th>
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<td>1800</td>
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<td>4/5</td>
<td>30</td>
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<td></td>
<td>200</td>
<td>600</td>
<td>p.o. ^d</td>
<td>1/16</td>
<td>4/5</td>
<td>28</td>
<td>mouse, 29</td>
<td>hydroxylation (3)</td>
<td></td>
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<tr>
<td></td>
<td>50</td>
<td>150</td>
<td>p.o. ^d</td>
<td>1/12 1/16 1/19</td>
<td>2/5</td>
<td>21.8</td>
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<td></td>
<td>160</td>
<td>480</td>
<td>s.c.</td>
<td>1/15, 1/16, 1/18, 1/23</td>
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<td>20.6</td>
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<tr>
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<td></td>
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<td>150</td>
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<td>2/13 1/20</td>
<td>2/5</td>
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<td>1/8, 1/11, 1/16, 2/17</td>
<td>0/5</td>
<td>13.8</td>
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*Groups of five *P. berghei* (KBG 173 strain) infected CD-1 mice were treated on days 3, 4, and 5 post infection with tetraoxanes suspended in sesame oil. Mice alive on day 31 with no parasites in a blood film are considered cured; ^aIncluding cured mice; ^bTaken from ref. 5a; ^cTaken from ref. 5c.
EXPERIMENTAL

General remarks

For details, please see references 5a, 5b, 5c.

ESI MS spectra of the synthesized compounds were recorded on an Agilent Technologies 6210 Time-of-Flight LC/MS instrument in the positive ion mode using CH₃CN/H₂O = 1/1 with 0.2 % HCOOH as the carrying solvent solution. The samples were dissolved in pure acetonitrile (HPLC grade). The selected values were as follows: capillary voltage 4 kV; gas temperature 350 °C; drying gas 12 l min⁻¹; nebuliser pressure 45 psig; fragmentator voltage 70 V.

1,1-Dihydroperoxy-4-methylcyclohexane (1)

4-Methylcyclohexanone (1.1 ml, 8.9 mmol) was dissolved at r.t. in a CH₂Cl₂/CH₃CN mixture (20 ml, 1:3 v/v), followed by addition of 30 % H₂O₂ (10.4 ml, 0.1 mol) and a few drops of conc. HCl. The reaction mixture was stirred for 2 h at r.t. and then quenched with saturated NaHCO₃ and HCl, and layers were separated. The water layer was further extracted with CH₂Cl₂ (3×20 ml); the combined organic layers were dried over anhydrous MgSO₄ and evaporated to dryness. Triturating with Et₂O afforded 305 mg (80 %) of product. m.p. 134 °C;

Methyl 12-methyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane-3-carboxylate (2)

To a cooled solution (ice bath) of dihydroperoxide 1 (1.2 g, 7.4 mmol) in CH₂Cl₂ (20 ml), methyl 4-oxocyclohexanecarboxylate was added and after stirring for 30 min at the same temperature, 1.66 ml of an ice-bath cooled H₂SO₄/CH₃CN mixture (1:10, v/v) was added dropwise. After an additional 50 min stirring, the reaction mixture was work-up in the usual manner and purified by SiO₂ column chromatography (Lobar B, LichroPrep Si 60, eluent: heptane/EtOAc = 95/5) affording 222 mg (10 %) 2.* 2: Colourless foam, softens at 57–59 °C. IR (KBr, cm⁻¹): 3449w, 2958s, 2930s, 2865s, 1712w, 1637w, 1554m, 1578m, 1357m, 1265m, 1200w, 1158m, 1104m, 1050m, 1017m, 980m, 910m, 861m cm⁻¹. IR (CCl₄, cm⁻¹): 3431m, 2957s, 2930s, 2865m, 1712w, 1551s, 1454m, 1384m, 1357m, 1255s, 1222s, 1162w, 1103m, 1071m, 1012s, 980s.¹H-NMR (200 MHz, CDCl₃, δ, ppm): 9.40–9.00 (m, 2×HOO–C(1)), 2.30–2.00 (m), 1.70–1.30 (m), 1.30–1.10 (m), 0.93 (d, H₃C–C(4), J = 6.2 Hz). ¹³C-NMR (50 MHz, CDCl₃, δ, ppm): 110.73, 40.83, 31.59, 30.58, 29.03, 21.38.

Methyl 12-methyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane-3-carboxylic acid (3)

Methyl ester 2 (400 mg, 1.3 mmol) was hydrolyzed at 80 °C with NaOH (72.8 mg, 1.82 mmol) in an i-PrOH/H₂O mixture (12 ml, 3:1 v/v). After 15 min, the reaction mixture was cooled and diluted with 20 ml H₂O and 50 ml CH₂Cl₂. The water layer was acidified to pH 2 with dilute HCl, and layers were separated. The water layer was further extracted with CH₂Cl₂ (3×20 ml); the combined organic layers were washed with water and brine, dried over anhydrous MgSO₄, and evaporated to dryness. Trifluoracetic acid (TFA) (1 ml) was added to the crude reaction mixture, followed by stirring for 1 h at the same temperature. After removal of the solvent on a rotary evaporator, the residue was triturated with Et₂O and redissolved in CDCl₃, affording 110 mg (30 %) of product. δ, ppm (CDCl₃): 180.73, 108.44, 107.19, 41.30, 30.79, 29.13, 21.38. -ESI–MS (m/z (%)): 285.0 ([M–H]⁻, 89), 104.9 (100). Anal. calcd. for C₁₂H₂₂O₁₁: H 5.90, H 8.10; Found: C 59.52, H 8.39.

*The yields varied within 10–35 %.
General procedure for the preparation of amides

A solution of acid 3 (103 mg, 0.36 mmol) in dry CH2Cl2 (20 ml), with added Et3N (51 µl, 0.36 mmol) and CICO2Et (35 µl, 0.36 mmol) was stirred for 90 min at 0 °C. A given amount of amine was added and after 30 min stirring, the reaction mixture was warmed to r.t. After 90 min it was diluted with H2O, the layers were separated and the organic layer was washed with brine, dried over anh. MgSO4 and evaporated to dryness.

12-Methyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane-3-carboxamide (4)

Acid 3 was transformed into amide 4 (75 mg, 74 %), which upon triturating with Et2O afforded a sample having m.p. 161–165 °C. IR (KBr, cm⁻¹): 3402, 1755, 1519, 1451, 1381, 1357, 1305, 1282, 1249, 1231, 1175, 975, 808. 1H-NMR (200 MHz, CDCl3, δ, ppm): 6.20 (bs, 2H), 6.49 (d, 1H, J = 15.6 Hz), 6.95 (bs, H-NAr), 6.62 (bs, H-NCO), 6.58 (d, 1H, J = 8.7 Hz), 6.49 (d, 1H, J = 8.7 Hz), 6.26 (m, H-C(3)′), 6.14 (d, 1H, J = 15.6 Hz), 3.80–3.70 (m, CONHCH2CH2NHAr), 3.48–3.36 (m, CONHCH2CH2NHAr), 2.20–1.95 (m, 4H), 1.50–1.70 (m, 1H), 1.02 (m, 6H). 13C-NMR (50 MHz, CDCl3, δ, ppm): 167.53, 150.90, 150.54, 147.94, 143.44, 141.80, 135.30, 133.77, 127.36, 125.59, 123.83, 122.30, 117.00, 98.08, 80.79, 75.60, 45.43, 38.93, 32.06, 28.91, 24.54, 17.53, 17.08. (+)ESI–HRMS (m/z (%)): 357.3 ([M+H]+, 100); calculated 357.3 ([M+H]+). 11C-NMR (50 MHz, CDCl3, δ, ppm): 175.56, 151.80, 150.15, 148.93, 134.91, 128.24, 125.43, 122.07, 117.26, 98.17, 79.70, 76.08, 45.61, 38.79, 34.15, 33.23, 30.25, 28.87, 25.68, 16.85. (+)ESI–HRMS (m/z (%)): 432.2050 ([M+H]+). Calculated 432.2048.
In vitro antimalarial activity

The in vitro antimalarial drug susceptibility screen was a modification of the procedures first published by Desjardins et al.,15 with modifications developed by Milhous et al.,16 with the details given in ref. 4c.

In vivo antimalarial activity

The P. berghei mouse efficacy tests were conducted using a modified version of the Thomp- son test. On day 0, each mouse was inoculated intraperitoneally with 0.1 ml, 1.0×10⁶ P. berghei P-line infected red blood cells from donor mice. The test drugs at 10, 20, and 80 mg/kg were sus- pended or dissolved in sesame oil and administered s.c. beginning on day 3 post-infection. Drug administrations were performed twice per day at 12 hours intervals for 3 days. The dose levels are given in Table II. Cure was defined as survival until day 31 post-treatment. Untreated control mice typically died on day 7−9 post-infection.

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ИЗВОД
О ПЕРОКСИДНИМ АНТИМАЛАРИЦИМА
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