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Antimalarial peroxides: the first intramolecular 1,2,4,5-tetraoxane

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An intramolecular steroidal 1,2,4,5-tetraoxane has been synthesised in six steps starting from methyl 3-oxo-7α,12α-diacetoxy-5β-cholan-24-oate. The synthesised 1,2,4,5-tetraoxane has moderate *in vitro* antimalarial activity against *P. falciparum* strains (IC_{50} (D_6) = 0.35 μg/mL; IC_{50} (W2) = 0.29 μg/mL).

Keywords: tetraoxane, malaria, peroxide, steroid, intramolecular.

INTRODUCTION

Tropical malaria, a major health problem in many southern countries, is caused by multiplication of the protozoan parasite *Plasmodium falciparum* in erythrocytes. More than 400 million disease cases with over 1.5 million deaths are the annual toll of *P. falciparum* infections. *Roll back malaria* programs[1] are hampered *inter alia* by the spreading resistance of the parasite to standard antimalarial drugs, in particular to chloroquine (CQ), which had been the affordable and effective antimalarial mainstay for 50 years. The antimalarial properties of artemisinin[2] and of other peroxides such as 1,2,4,5-tetraoxacycloalkanes[3] opened new avenues in the fight against malaria.

Our research in this area exploited the steroid carrier of cholestane[4] and a cholic acid-derived[5] series: bis-steroidal tetraoxanes were explored. Now, the synthesis and preliminary antimalarial activity of the first intramolecular steroidal tetraoxane derived from cholic acid is presented.

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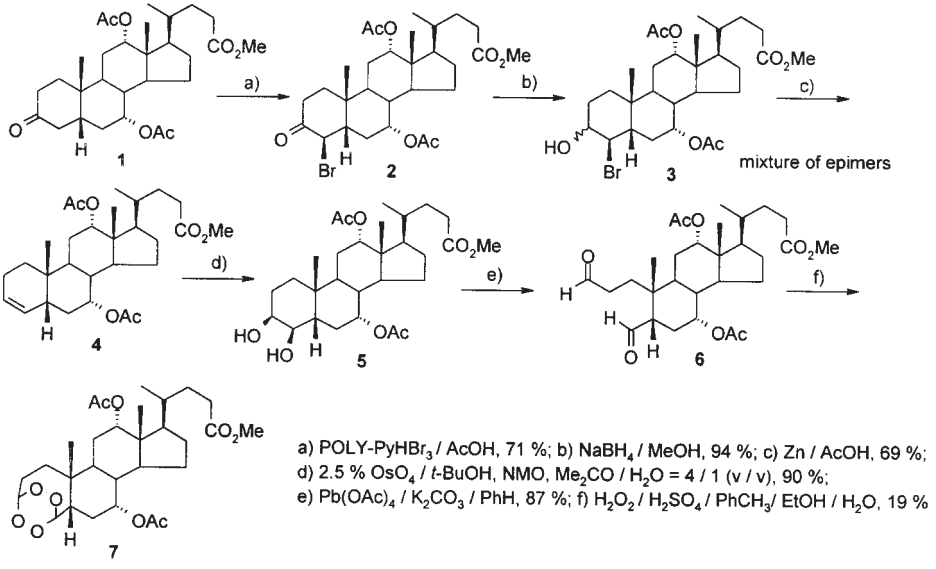
RESULTS AND DISCUSSION

Chemistry

The starting ketone **1** was brominated using polymer-bound $PyHBr_3$ (POLY-$PyHBr_3$) in acetic acid at 80 ºC (Scheme 1). As expected, the 4β-bromo derivative **2** was obtained (71 %) which was further treated with hydrazine/AcOK[6] in order to obtain directly the Δ^3-alkene **4** needed for ring-opening to the dialdehyde **6**. Unfortunately, the reaction was sluggish and a significant amount of unreacted **2** remained. Thus, alkene **4**[7] was obtained by an alternative route involving the reduction of bromoketone **2** into the epimeric bromohydrine mixture **3** (94 %; (1:1)-ratio) which was further treated with Zn/AcOH (69 %). Oxidation of alkene **4** with OsO_4/NMO afforded the *cis*-diol **5**[7] in 90 % yield. Oxidation with LTA/K_2CO_3 afforded the desired dialdehyde **6** in 87 % yield, which was immediately used in the next step to avoid its relatively fast polycondensation even at low temperatures.[8]



Scheme 1

The peroxyacetalization reaction was carried out in a toluene / ethanol / water mixture using 32 % H_2O_2 as described earlier.[4] Based on spectral data, it was concluded that the obtained tetraoxane **7** is a monomeric compound, *i.e.*, the intramolecular tetraoxane was obtained. Thus, the ^{13}C-NMR spectrum excluded the existence of aldehyde functionality (confirmed in ^{1}H-NMR spectrum) and the two distinct C signals at 108.9 and 106.6 ppm are indicative for peroxyacetal carbons (with differing neighbours). This, together with MS (TOF) and m.a. data is sufficient for assigning the structure of **7** (Fig. 1).

Antimalarial activity

Tetraoxane was screened against *Plasmodium falciparum* D6 and W2 clones. D6 is a clone from the Sierra I/UNC isolates and is susceptible to chloroquine and pyrimethamine,

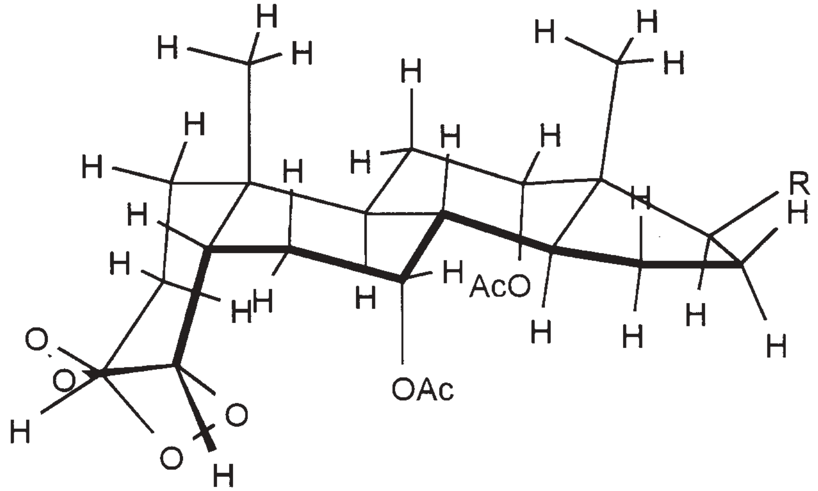


Fig. 1. The MM 2-optimised structure of tetraoxane **7**. For clarity, the cholic acid side chain R is omitted.

but has reduced susceptibilities to mefloquine and halofantrine. W2 is a clone of the Indochina I isolate and is resistant to chloroquine and pyrimethamine, but susceptible to mefloquine.

The activity of **7** against both *P. falciparum* clones (IC_{50} (D6) = 0.35 μg/mL; IC_{50} (W2) = 0.29 μg/mL) is rather low as compared to other steroidal tetraoxanes of cholic acid[5] and artemisinin (IC_{50} (D6) = 2.37 ng/mL; IC_{50} (W2) = 2.06 ng/mL) but, interestingly, it is more active against CQ resistant W2 clone, exhibiting similar IC_{50} (W2)/IC_{50} (D6) ratio (RI = 0.83) as artemisinin (RI = 0.82).

In conclusion, for the first time, an intramolecular steroidal tetraoxane with medium antimalarial activity has been developed which gives a whole range of possibilities for further structure (and acitivity) development, particularly of incorporation of other functionalities into the steroid core and by altering the position of the tetraoxane moiety.

EXPERIMENTAL

General

Melting points were determined on a Boetius PMHK apparatus and were not corrected. Specific rotations were determined on a Perkin-Elmer 141-MC instrument at the given temperatures. IR spectra were recorded on a Perkin-Elmer spectrophotometer FT-IR 1725X. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Gemini-200 spectrometer (at 200 and 50 MHz, respectively) in the indicated solvent using TMS as the internal standard. Chemical shifts are expressed in ppm (δ) values and coupling constants (*J*) in Hz.

EI and CI mass spectra were recorded on a MS Finnigan-MAT 8230 spectrometer with double focusing reverse geometry, using isobutane (CI). Electrospray ionisation mass spectra were acquired on a QStar Pulsar (Applied Biosystems) quadrupole-orthogonal time of flight (QqQ-TOF) hybrid instrument. The samples were dissolved in pure acetonitrile (HPLC grade) to obtain a solution at 1.5 pmol/μL concentration, which was directly introduced into the ion source using a built-in syringe pump. Spectrum acquisition was made in the positive ion mode in the mass range of *m*/*z* 300–1500 using the following ion source parameters: 5500 V accelerating voltage, 100 V orifice voltage and 40 V skimmer voltage. The resolution of the instrument in the TOF-MS mode was measured to be 8500 at 50 % valley definition.

Thin-layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates, using *N,N*-dimethyl-*p*-phenylenediammonium dichloride reagent for peroxide moiety detection.[9] Lobar LichroPrep Si 60 (40–63 μm) columns coupled to a Waters RI 401 detector were used for column chromatography.

*Methyl 4*β*-bromo-3-oxo-7*α*,12*α*-diacetoxy-5*β*-cholan-24-oate (**2**)*

A mixture of **1** (10.10 g, 20 mmol) and polymer bound $PyHBr\cdot Br_2$ (10.0 g, 20 mmol; Fluka) in 500 mL glacial acetic acid was stirred for 1 hour at 80 ºC. Then the reaction mixture was cooled, diluted with water, left overnight, and filtered. The remaining solid was dissolved in $CHCl_3$, and after removal of the polymer the crude product was purified by dry-flash chromatography (eluent: toluene / EtOAc = 85 / 15). Yield 8.30 g (71 %), m.p. = 221–223 ºC (colourless prisms, benzene - hexane). $[\alpha]_D^{20}$ = +92.20 (*c* = 1.00, $CHCl_3$). IR (KBr): 3439m, 2961m, 2878w, 1737s, 1640w, 1436m, 1378m, 1314w, 1244s, 1222m, 1191m, 1176m, 1027m cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$): 5.32 (*d*, *J* = 12.0 Hz, H-C(4)), 5.13 (*s*, H-C(12)), 5.07 (*d*, *J* = 2.8 Hz, H-C(7)), 3.67 (*s*, CH_3O_2C(24)), 2.13 (*s*, CH_3COO–), 2.12 (*s*, CH_3COO–), 1.08 (*s*, H_3C-C(10)), 0.82 (*d*, *J* = 6.2 Hz, H_3C-C(20)), 0.78 (*s*, H_3C-C(13)). ^{13}C-NMR (50 MHz, $CDCl_3$): 201.4, 174.4, 170.4, 170.1, 75.0, 70.1, 62.7, 52.5, 51.5, 45.0, 43.3, 37.8, 37.6, 36.0, 34.5, 30.7, 30.6, 30.2, 29.2, 27.0, 25.8, 22.6, 22.2, 21.5, 21.2, 17.4, 12.2 MS (DCI, *m/z*, %): 585 [$(M^{81}Br)^+$, 1], 583 [$(M^{79}Br)^+$, 1], 525 [$(M^{81}Br)^+$ - AcOH, 12], 523 [$(M^{79}Br)^+$ - AcOH, 12], 505 [$(M^{79}Br)H^+$ - ^{79}Br, 2], 466 [$(M^{81}Br)H^+ - 2 \times$ AcOH, 40], 465 [$(M^{81}Br)^+ - 2 \times$ AcOH, 75], 464 [$(M^{79}Br)H^+ - 2 \times$ AcOH, 60], 463 [$(M^{79}Br)^+ - 2 \times$ AcOH, 100], 443 [$(M^{79}Br)^+$ - AcOH - H^{79}Br, 4], 385 [$(M^{79}Br)H^+ - 2 \times$ AcOH - ^{79}Br, 10], 384 [$(M^{79}Br)^+ - 2 \times$ AcOH - ^{79}Br, 10], 269 (0.5). Anal. calc. for $C_{29}H_{43}BrO_7$ (583.57): C 59.69, H 7.43. Found: C 59.82, H 7.34 %.

*Methyl 4*β*-bromo-3-hydroxy-7*α*,12*α*-diacetoxy-5*β*-cholan-24-oate* (**3**)

Solid $NaBH_4$ (0.60 g, 16 mmol) was added over 30 min to an ice-cooled mixture of **2** (6.40 g, 11 mmol) in MeOH (120 mL) under stirring. The reaction was quenched by 50 % acetic acid and poured onto an ice / water mixture. After filtration and drying, the crude product was obtained (6.10 g, 94 %) and used in the next step without further purification. 3-Hydroxy epimers (**3a** and **3b**) were separated only for analytical purposes by column chromatography (Lobar B, LichroPrep Si 60, eluent: heptane / EtOAc = 8 / 2).

*Methyl 4*β*-bromo-3*β*-hydroxy-7*α*,12*α*-diacetoxy-5*β*-cholan-24-oate (**3a**)*. Mp = 169–172 ºC (colourless prisms, ether-hexane). IR (KBr): 3507s, 3483s, 3459s, 3235m, 2954m, 2876w, 1734s, 1636m, 1445m, 1379m, 1248s, 1027s cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$): 5.09 (*s*, H-C(12)), 5.00–4.90 (*m*, H-C(4), H-C(7)), 4.15 (*bs*, H-C(3)), 3.66 (*s*, CH_3O_2C(24)), 2.17 (*s*, CH_3COO–), 2.12 (*s*, CH_3COO–), 1.00 (*s*, CH_3-C(10)), 0.81 (*d*, *J* = 6 Hz, H_3C-C(20)), 0.74 (*s*, H_3C-C(13)). ^{13}C-NMR (50 MHz, $CDCl_3$): 174.5, 170.4, 75.3, 70.5, 66.8, 51.5, 47.3, 45.0, 43.8, 43.4, 38.6, 37.9, 34.5, 30.8, 30.7, 29.4, 28.7, 28.2, 27.1, 26.6, 25.7, 23.2, 22.6, 21.7, 21.3, 17.4, 12.2.

*Methyl 4*β*-bromo-3*α*-hydroxy-7*α*,12*α*-diacetoxy-5*β*-cholan-24-oate (**3b**)*. M.p. = 163–166 ºC (colourless prisms, ether-hexane). IR (KBr): 3512s, 3499s, 3445s, 3410s, 2988m, 2954s, 2926s, 2861m, 1740m, 1707m, 1644w, 1452m, 1380m, 1274s, 1240s, 1080w, 1025m cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$): 5.07 (*s*, H-C(12)), 4.97 (*d*, *J* = 3 Hz, H-C(7)), 4.67 (*dd*, *J* = 11.8 Hz, *J* = 9.8 Hz H-C(4)), 3.66 (*s*, CH_3O_2C(24)), 3.67–3.50 (*m*, H-C(3)), 2.13 (*s*, CH_3COO–), 2.12 (*s*, CH_3COO–), 1.00 (*s*, H_3C-C(10)), 0.80 (*d*, *J* = 6.2 Hz, H_3C-C(20)), 0.73 (*s*, H_3C-C(13)). ^{13}C-NMR (50 MHz, $CDCl_3$): 174.5, 170.4, 75.3, 70.5, 66.8, 51.5, 47.3, 45.0, 43.8, 43.4, 38.6, 37.9, 34.5, 30.8, 30.7, 29.4, 28.7, 28.2, 27.1, 26.6, 25.7, 23.2, 22.6, 21.7, 21.3, 17.4, 12.2.

*Methyl 7*α*,12*α*-diacetoxy-5*β*-chol-3-en-24-oate* (**4**)

To a solution of **3** (6.50 g, 11 mmol) in glacial AcOH (190 mL) and H_2O (9 mL), Zn powder (37.5 g) was added within 30 min at 45–50 ºC. The reaction mixture was vigorously stirred until completion of the reaction. After cooling and filtration, the solvent was removed under reduced pressure. The remaining viscous oil was dissolved in $CHCl_3$, worked-up in the usual manner, dried over anh. Na_2SO_4 and evaporated to dryness. The crude product was purified by dry-flash chromatography (eluent toluene / EtOAc = 9 / 1) and crystallised from hexane to afford alkene **4**. Yield 3.70 g (69 %), m.p. = 116–118 ºC (colourless prisms, hexane), lit. m.p. 115–116 ºC[7]. $[\alpha]_D^{20}$ = +18.78 (*c* = 1.00, $CHCl_3$). IR (KBr): 3556m, 3507m, 3465s, 3410s, 2964m, 2923m, 1734s, 1619w, 1378m, 1253s, 1028w cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$): 5.53 (*dd*, *J* = 10.1 Hz, *J* = 3 Hz, H-C(3)), 5.31 (*dd*, *J* = 10 Hz, *J* = 1.6 Hz, H-C(4)), 5.08 (*s*, H-C(12)), 4.83 (*d*, *J* = 2.6 Hz, H-C(7)), 3.66 (*s*, CH_3O_2C(24)), 2.12 (*s*, CH_3COO–), 2.02 (*s*, CH_3COO–), 0.95 (*s*, H_3C-C(10)), 0.82 (*d*, *J* = 6 Hz, H_3C-C(20)), 0.74 (*s*, H_3C-C(13)).

^{13}C-NMR (50 MHz, $CDCl_3$): 174.6, 170.8, 170.5, 133.0, 123.4, 75.6, 70.7, 51.5, 47.3, 44.9, 43.1, 41.5, 37.7, 34.6, 33.0, 32.9, 31.1, 30.8, 30.7, 28.3, 27.1, 25.7, 22.8, 22.1, 21.7, 21.2, 21.1, 17.5, 12.1. EI-MS , (*m*/*z*, %): 428 (M^+-AcOH, 1), 368 (M^+-2×AcOH, 30), 315 (28), 282 (2), 253 (100), 227 (12), 199 (12), 145 (12), 84 (12), 43 (25). Anal. calcd. for $C_{29}H_{44}O_6$ (488.67): C 71.28, H 9.08. Found: C 70.64, H 8.60 %.

*Methyl 3*β*,4*β*-dihydroxy-7*α*,12*α*-diacetoxy-5*β*-cholan-24-oate* (**5**)

To a solution of alkene **4** (100 mg, 0.2 mmol) in an acetone / water mixture (4 mL; 4 / 1, v / v) were added NMO·H_2O (110 mg, 0.8 mmol) and a 2.5 % solution of OsO_4 in *t*-BuOH (0.1 mL, 0.01 mmol). The mixture was stirred at room temperature until completion of the reaction, followed by addition of $NaHSO_3$ (10 mg) and further stirring for a further 30 min. The acetone was removed under reduced pressure, brine was added and the water layer was extracted with EtOAc (3×20 mL). The combined organic layers were washed with brine, dried over anh. Na_2SO_4, and evaporated to dryness. The crude product was purified by column chromatography (Lobar, LichroPrep Si 60, eluent: heptane / EtOAc = 3 / 7) and crystallized to afford the dihydroxy compound **5**. Yield 96 mg (90 %), m.p. = 83–85 ºC (colourless prisms, hexane-EtOAc), lit m.p. 74–76 ºC[7]. $[\alpha]_D^{20}$ = +61.10 (*c* = 1.00, $CHCl_3$). IR (KBr): 3548m, 3490s, 2956m, 2877w, 1735s, 1705m, 1638w, 1439w, 1379m, 1254s, 1028m cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$): 5.08 (*s*, H-C(12)), 4.91 (*d*, *J* = 2.6 Hz, H-C(7)), 4.05–3.95 (*m*, H-C(3), H-C(4)), 3.66 (*s*, CH_3O_2C(24)), 2.11 (*s*, CH_3COO–), 2.01 (*s*, CH_3COO–), 0.96 (*s*, H_3C-C(10)), 0.81 (*d*, *J* = 6.0 Hz, H_3C-C(20)), 0.74 (*s*, H_3C-C(13)). ^{13}C-NMR (50 MHz, $CDCl_3$): 174.6, 170.6, 170.5, 75.4, 71.1, 70.9, 69.3, 51.5, 47.3, 45.0, 43.4, 42.7, 37.5, 36.1, 34.6, 30.8, 30.7, 29.7, 28.7, 27.1, 25.8, 24.8, 22.7, 21.6, 21.3, 21.0, 17.4, 14.1, 12.2. Anal. calcd. for $C_{29}H_{46}O_8$·H_2O (540.70): C 64.42, H 8.95. Found: C 64.34, H 9.20 %.

*Methyl 7*α*,12*α*-diacetoxy-3,4-dioxo-3,4-seco-5*β*-cholan-24-oate* (**6**)

To a stirred suspension of **5** (500 mg, 0.96 mmol) and anh. K_2CO_3 (280 mg, 2.0 mmol) in dry benzene (20 mL) under argon, $Pb(AcO)_4$ (440 mg, 0.99 mmol) was added in small portions within 1 h. The mixture was stirred at room temperature for an additional 60 min, filtered through a sinter funnel and the remaining solid was washed with benzene (80 mL). The filtrate was washed with sat. $NaHCO_3$, water, brine and dried over anh. Na_2SO_4. The crude product was used without further purification in the next reaction step. The analytical sample of the dialdehyde **6** was obtained upon column chromatography (Lobar A, LichroPrep Si 60, eluent: toluene / EtOAc = 7/3). Yield 453 mg (87 %). IR (film): 3020m, 2956s, 2877m, 1733s, 1441m, 1378m, 1245*s*, 1025m cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$): 9.87 (*d*, *J* = 2.2 Hz, HCO-C(5)), 9.73 (*s*, HCO-C(2)), 5.11 (*s*, H-C(12)), 4.97 (*d*, *J* = 2.4 Hz, H-C(7)), 3.66 (*s*, CH_3O_2C(24)), 2.12 (*s*, CH_3COO–), 2.02 (*s*, CH_3COO–), 0.96 (*s*, H_3C-C(10)), 0.81 (*d*, *J* = 6.2 Hz, H_3C-C(20)), 0.76 (*s*, H_3C-C(13)). ^{13}C-NMR (50 MHz, $CDCl_3$): 204.3, 201.4, 174.5, 170.4, 170.0, 74.8, 69.2, 52.3, 51.5, 47.3, 44.8, 43.0, 37.7, 36.5, 35.5, 34.5, 30.8, 30.6, 29.7, 29.6, 27.0, 26.0, 22.8, 21.3, 21.1, 18.8, 17.4, 12.1.

*Methyl 7*α*,12*α*-diacetoxy-3,4-diperoxy-3,4-seco-5*β*-cholan-24-oate* (**7**)

To an ice-cooled mixture of 30 % H_2O_2 (0.23 mL, 2.1 mmol), EtOH (1.40 mL), H_2O (1.32 mL) and conc. H_2SO_4 (2.54 mL), a pre-cooled solution (ice-bath) of dialdehyde **6** (470 mg, 0.9 mmol) in 40 mL toluene was added dropwise. After 30 min of stirring the reaction was quenched with water, the layers were separated and the water layer was extracted with toluene (3×20 mL). The combined organic layers were washed with sat. $NaHCO_3$, brine, and dried over anh. Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by SiO_2 chromatography (Lobar, LichroPrep Si 60, eluent heptane / EtOAc = 6/4) to afford tetraoxane **7**. **7**: Yield 97 mg (19 %), m.p. = 108–111 ºC (colourless prisms, ether). $[\alpha]_D^{20}$ = +101.07 (*c* = 1.03, $CHCl_3$). IR (KBr): 3452s, 2955m, 1736s, 1636w, 1442w, 1379m, 1244s, 1027*s* cm^{-1}. ^{1}H-NMR (200 MHz, $CDCl_3$): 10.04, 9.88 (both *s*, residual acidic H, exchangeable with D_2O), 5.79 (*dd*, *J* = 9.8 Hz, *J* = 3.2 Hz, H-C(4)), 5.29 (*s*, H-C(3)), 5.05 (*s*, H-C(12)), 4.92 (*d*, *J* = 2 Hz, H-C(7)), 3.66 (*s*, CH_3O_2C(24)), 2.18 (*s*, CH_3COO–), 2.14 (*s*, CH_3COO–), 0.99 (*s*, H_3C-C(10)), 0.79 (*d*, *J* = 5.8 Hz, H_3C-C(20)), 0.75 (*s*, H_3C-C(13)). ^{13}C-NMR (50 MHz, $CDCl_3$): 174.7, 171.0, 170.6, 108.9, 106.6, 75.1, 69.8, 51.5, 44.8, 43.3, 43.1, 37.9, 37.1, 35.4, 34.5, 32.1, 31.8, 30.8, 30.6, 27.0, 26.3, 25.6, 22.6, 21.4, 21.2, 20.0, 17.4, 14.0, 12.1. ESI-MS (*m*/*z*, %): 625.24 ($M + K^+ + H_2O_2$, 10), 609.28 ($M + Na^+ + H_2O_2$, 43), 603 ($M + NH_4^+ + H_2O_2$, 8), 575.27 ($M + Na^+$, 8), 467.24 (6), 413.25 (3), 284.32 (100).

Animalarial activity

The *in vitro* antimalarial drug susceptibility screen is a modification of the procedures first published by Desjardins *et al.*,[10] with modifications developed by Milhous *et al.*.[11] In brief, the assay relies on the incorporation of radiolabeled hypoxanthine into the parasites. The inhibition of isotope incorporation is attributed to the activity of known or candidate antimalarial drugs. For each assay, proven antimalarials are used as controls. The incubation period is 66 hours and the starting parasitemia is 0.2 % with 1 % hematocrit. The media used is RPMI-1640 culture media with no folate or *p*-aminobenzoic acid (PABA) and 10 % normal heat inactivated human plasma. For quantitative *in vitro* drug susceptibility testing, two well-characterized *P. falciparum* malaria clones are normally used, W2 and D6.[12] W2 is a clone of the Indochina I isolate and is resistant to chloroquine and pyrimethamine, but susceptible to mefloquine. D6 is a clone from Sierra I/UNC isolates and is susceptible to chloroquine and pyrimethamine, but has reduced susceptibilities to mefloquine and halofantrine.

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ИЗВОД

АНТИМАЛАРИЈСКИ ПЕРОКСИДИ: ПРВИ ИНТРАМОЛЕКУЛСКИ 1,2,4,5-ТЕТРАОКСАН

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Полазећи од метил-3-оксо-7α,12α-диацетокси-5β-холан-24-оата синтетисан је у шест фаза интрамолекулски стероидни 1,2,4,5-тетраоксан. Производ има умерену *in vitro* антималаријску активност према *P. falciparum* сојевима (IC_{50} (D6) = 0.35 μg/mL; IC_{50} (W2) = 0.29 μg/mL).

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