# Chemical Stability of the Peroxide Bond Enables Diversified Synthesis of Potent Tetraoxane Antimalarials[1]

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Of 17 prepared 1,2,4,5-tetraoxacyclohexanes stable to reductive and acidic conditions, 3 of them were more active than artemisinin against CQ and MFQ resistant strain TM91C235 and all compounds were more active in vitro against W2 than against D6 strain. In vivo, amines **10** and **11a** cured all mice at higher doses with MCD $\leq$ 37.5 (mg/kg)/day. Triol **13** was exceptionally active against melanoma (LOX IMVI) and ovarian cancer (IGROV1), both with $LC_{50} = 60$ nM.

## Introduction

The development of widespread drug resistance to chloroquine (CQ[a]) has resulted in severe health issues for countries in malaria endemic regions. The antimalarial properties of artemisinin[2] and of other peroxides, such as 1,2,4,5-tetraoxacycloalkanes (tetraoxanes),[3,4] have recently begun to be exploited in the development of new approaches to fighting CQ-resistant strains of malaria. New tetraoxanes employing a steroidal backbone have now been prepared that are highly active, are inexpensive, and demonstrate low toxicity.[5,6]

A part of our research in this field is focused on the development of a new type of tetraoxane with nonidentical substituents[6] that utilize a steroid and small cyclohexylidene carriers possessing secondary amide bonds. Also, during our work in this field we discovered that tetraoxanes are unusually stable, even at pH 1.6,[6c] a characteristic that subsequently allowed the synthesis of many interesting derivatives.

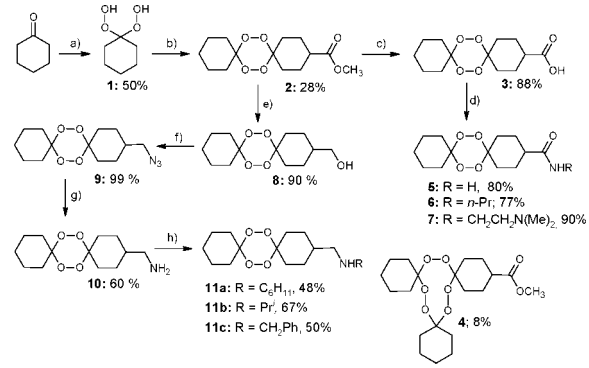
This communication encompasses the synthesis of various amino-functionalized antimalarials based on the appreciable stability of the tetraoxane moiety to reaction conditions such as reductive amination and $LiAlH_4$ reduction. Their respective antimalarial activities and the pronounced antiproliferative activity of certain products are reported along with in vitro metabolism studies.

## Results and Discussion

The discovery of the appreciable stability of tetraoxanes to basic (pH 12, NaOH/*i*-PrOH/$H_2O$, room temp $\rightarrow$ 80 °C)[5a] and acidic (pH 1.6, $CH_3OH$/HCl, 37 °C)[6c] conditions initiated our research into the application of classical reagents for reductive amination conditions ($NaBH_3CN$, $NaBH(OAc)_3$), reduction ($NaBH_4$, $LiAlH_4$), and acetylation (cat. TMSOTf/$Ac_2O$).[7]

As noted previously,[5,6] our approach to functionalized tetraoxanes consists of an ester $\rightarrow$ acid $\rightarrow$ amide sequence. Thus, we prepared 1,1-dihydroperoxycyclohexane (**1**) in 50% yield,[8,9] which was subsequently coupled to methyl 4-oxocyclohexanecarboxylate, affording **2** in 28–35% yield and the side product hexaoxonane **4** (Scheme 1).[10] Upon transformations furnishing tetraoxane amides (**2** $\rightarrow$ **3** $\rightarrow$ **5**−**7**) in 65–79% yield, we explored the stability of the tetraoxane moiety under reducing conditions. We discovered that ester **2** was reduced in very high yield to alcohol **8** (Scheme 1) with no appreciable cleavage of the tetraoxane moiety observed with use of $LiAlH_4$.[11] Stability of this moiety to LAH was confirmed by azide-to-amine reduction (Scheme 1) and the reduction of steroidal tetraoxane **12** to triol **13** (Scheme 2). Established stability[6c] of a tetraoxacyclohexane at pH 1.6 enabled us to use an acidic workup procedure (see Experimental Section). Additionally, we successfully applied a TMSOTf/$Ac_2O$ esterification method en route to mononalcohol **15**, which was further oxidized in 83% yield under aprotic conditions. Finally, $NaBH(OAc)_3$ and $NaBH_4$ were applied for reductive ammination and the reduction of mixed anhydride to alcohol, respectively. Thus, we have shown that the tetraoxane moiety is stable to reducing conditions ($LiAlH_4$, $NaBH(OAc)_3$, and $NaBH_4$) and mild acidic conditions (protic and aprotic).

**Scheme 1**[a]




[a] (a) 30% $H_2O_2$/HCl, $CH_3CN/CH_2Cl_2$; (b) methyl 4-oxocyclohexanecarboxylate, $CH_2Cl_2$, $H_2SO_4/CH_3CN$; (c) NaOH, *i*-PrOH/$H_2O$/Δ; (d) $ClCO_2Et$/$Et_3N$, $R_1NH_2$, (e) $LiAlH_4$, $Et_2O$; (f) (1) MsCl, Py, (2) $NaN_3$, DMF; (g) $LiAlH_4$, $Et_2O$; (h) carbonyl, $NaBH(OAc)_3$, $CH_2Cl_2$.

**Biological Screening. Antimalarial Activity.** All synthesized compounds were screened in vitro against CQ-susceptible, CQ-resistant, and multidrug resistant strains, D6, W2, and TM91C235 (Thailand), respectively.[12] The least active compounds were hexaoxonane **4**, a type of peroxide much less active than tetraoxanes, trioxanes, or trioxolanes,[4c] and the most polar compounds **3** and **13**. The significantly lower in vitro activity

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[a] Abbreviations: CQ, chloroquine; MFQ, mefloquine; ART, artemisinin; CA, cholic acid; DCA, deoxycholic acid; MCD, mimimal curative dose; MAD, minimal active dose; MG_MID, mean graph midpoint.

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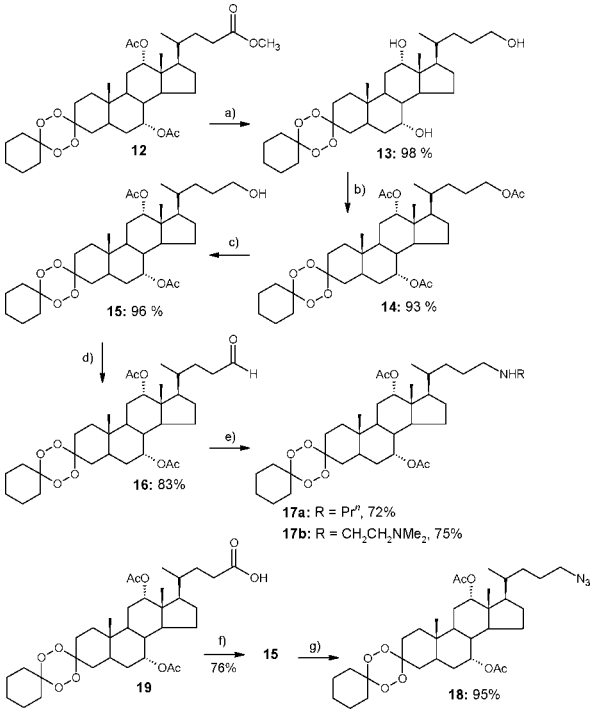
**Scheme 2**[a]




[a] (a) $LiAlH_4$, $Et_2O$; (b) $Ac_2O$, TMSOTf, $CH_2Cl_2$; (c) $K_2CO_3$, MeOH; (d) PCC, $CH_2Cl_2$; (e) $RNH_2$, $NaBH(OAc)_3$, $CH_2Cl_2$; (f) (i) $ClCO_2Et/Et_3N$/THF; (ii) $NaBH_4$; (g) (i) MsCl/Py; (ii) $NaN_3$/DMF.

of acid **3**, in comparison to corresponding methyl ester **2**, was expected on the basis of previous results.[4a,6c] However, one can observe that the differences in activities of the tetraoxane acids and corresponding esters diminish with a decrease of polarity of the molecule. An example is in Table 1, **12** and **19**, with further examples in refs 6a and 6b. Steroidal triol **13** is much less active than its diacetoxy derivative **15**, and this information clearly indicates that the protected hydroxy groups at C(7) and C(12) of cholic acid are needed for good activity. In addition, no effect on activity is excerted by the C(24)-O functionality; triacetate **14**, monoalcohol **15**, and starting ester **12** have very similar activities, and this trend is similar to that seen with trioxolanes.[13] The analysis given above possibly points to the high importance of the substitution pattern at C(7) and C(12) of the steroidal tetraoxanes.

The in vitro antimalarial potency of dicyclohexylidene carboxylic amides **5**–**7**, prepared via mixed anhydrides, is higher than that of the ester **3**; however, they are less active than most amino derivatives. Thus, when directly compared (on the same plate), the activity of primary amide **5** appears to be one-half that of the corresponding amine **10**. Of the amides, the most active was the *N*,*N*-dimethylethan-1,2-diamino derivative **7**, projected to possess a weak base structural subunit. Resitance of the tetraoxane moiety to LAH and applied reductive amination conditions enabled easy approach to amines (**8** → **9** → **10** → **11**). For the first time we tested tetraoxane azides as possible antimalarial candidates. Interestingly, dicyclohexylidene azide **9** and its steroidal analogue **18** (**19** → **15** → **18**) are equipotent antimalarials with activities very similar to that of artemisinin.

In vitro metabolism studies were performed on compounds **7**, **11a**−**c**, **17a**,**b** to assess the bioavailability of possible drug candidates after oral administration. Metabolic stability assays were done using human and mouse liver microsomes.[6c] Stable compounds were defined as having half-lives of >60 min, and the relevant data are given in Table 1. The data showed that **7**, **17a**, and **17b** were metabolically stable. However, **11a**, **11b**, and **11c** were metabolically less stable, with half-lives of 43.9, 9.9, and 3.5 min in mouse, and the lesser in vivo activity of **11b** compared to **11a** might be ascribed to the shorter half-life.

Five achiral dicyclohexylidene tetraoxanes were chosen for further evaluation in vivo against *P. berghei* infected mice using a modified Thompson test.[6c] The amide **7** was tested orally, while tetraoxane azide **9** and amines **10**, **11a**, and **11b** were administered subcutaneously. In both tests the mice were infected on day 0, and the tested compounds were administered accordingly on days 3−5 postinfection. To our surprise, tetraoxane **7**, despite being a metabolically stable compound ($t_{1/2}$ > 60 min, no metabolite produced upon incubation with human, mouse, rat, and rhesus monkey microsomes), was inactive in the in vivo test even at a dose of 320 (mg/kg)/day (MTD > 960 mg/kg, Table 2). However, peroxide azide **9** cured 4 of 5 mice at a dose of 300 (mg/kg)/day, with a mean survival time of 30.6 days versus 7–9 days in the control mice. Cure of 2 of 5 mice and increased survival were also seen in mice dosed with 150 (mg/kg)/day, with a group mean survival time of >26 days. In the present set of compounds, the most active were tetraoxane amines **10**, **11a**, and **11b**, with a minimum curative dose (MCD) of ≤37.5 (mg/kg)/day. Primary amine **10** cured all test animals at doses of 300 and 150 (mg/kg)/day and 2 of 5 at 37.5 (mg/kg)/day, with a minimum active dose (MAD) of 9.3 (mg/kg)/day. Secondary amines **11a** and **11b** were less active than **10**, both with minimal curative dose of 37.5 (mg/kg)/day. Toxicity was not observed at any dose; all animals not cured in the above tests died of malaria.

**Antiproliferative Activity.** The antiproliferative activity of five compounds in Table 3 was tested against a diverse panel of 60 human cancer cell lines at NIH-NCI, starting at $10^{-4}$ M.[14] Compounds **2**, **5**, **7**, and **17b** showed low to moderate activity as exemplified by low MG_MID values (Table 3). However, the most polar, and one of the least active compounds in antimalarial screen, tetraoxane **13**, was found to be a very effective antiproliferative agent against a broad spectrum of cancer cells. The results of the activity against 19 cancer cell lines, shown in Table 4, indicate that triol **13** totally inhibits the cancer growth (TGI) at submicromolar levels, with an average concentration of 0.40 $\mu$M. This pronounced antitumor activity is further accented by very high and selective toxicity of **13** against melanoma (LOX IMVI, $LC_{50}$ = 60 nM) and ovarian cancer (IGROV1, $LC_{50}$ = 60 nM).

## Conclusion

The stability of the tetraoxane moiety to hydride reduction and to acidic conditions (up to pH 1.6) enabled the synthesis of a series of mixed dicyclohexylidene tetraoxanes and a new type of steroidal mixed tetraoxane. In contrast to the steroidal tetraoxanes, the amines of the dicyclohexylidene series were more active in vitro and in vivo than the corresponding carboxylic amides. In vitro, all compounds were more active against the CQ-resistant W2 strain than against the CQ-susceptible D6. Compounds **7**, **11a**, **11b** were more active than ART against the CQ and MFQ-resistant strain TM91C235 (Thailand). In vivo, amines **10** and **11a** cured all mice at higher doses and exhibited MCD ≤ 37.5 (mg/kg)/day. As in our earlier studies, no peroxide bond scission was observed in any in vitro ADME studies. Of the tested compounds, triol **13** is exception-

**Table 1.** In Vitro Antimalarial Activities of Tetraoxanes **2–19** against *P. falciparum* D6,[a] W2,[b] and TM91C235[c] Strains

| | $IC_{50}$ (nM) | | | $IC_{90}$ (nM) | | | met. stab. $t_{1/2}$ (min) | |
|---|---|---|---|---|---|---|---|---|
| compd | D6 | W2 | TM91C235 | D6 | W2 | TM91C235 | human | mouse |
| **2** | 29.20[d] | 40.41[d] | 26.96 | 83.92 | 62.48 | 110.22 | | |
| **3** | 429.27[d] | 413.18[d] | 410.58 | 467.17 | 519.94 | 522.55 | | |
| **4** | >499.41 | >499.41 | >499.41 | | | | | |
| **5** | 23.96 | 20.16 | 27.24 | 42.75 | 40.36 | 55.62 | | |
| **6** | 19.27 | 21.98 | 25.34 | 49.17 | 44.54 | 53.89 | | |
| **7** | 11.24 | 9.72 | 7.24 | 15.10 | 17.87 | 12.79 | >60 | >60 |
| **8** | 15.18 | 6.54 | 12.62 | 22.52 | 11.57 | 23.66 | | |
| **9** | 9.87 | 6.07 | 11.29 | 20.34 | 11.04 | 22.24 | | |
| **10** | 12.84 | 7.79 | 19.75 | 24.14 | 16.00 | 28.98 | | |
| **11a** | 11.18 | 6.17 | 10.78 | 14.02 | 17.68 | 13.93 | 15.9 | 43.9 |
| **11b** | 9.40 | 7.54 | 9.27 | 14.54 | 22.91 | 16.73 | 38.7 | 9.9 |
| **11c** | 13.82 | 8.19 | 13.89 | 34.52 | 20.60 | 39.51 | 42.0 | 3.5 |
| **12** | 21.47[e] | 16.96[e] | | | | | | |
| **13** | 129.72 | 108.88 | 165.77 | 623.30 | 297.96 | 339.88 | | |
| **14** | 15.92 | 11.61 | 19.24 | 28.40 | 26.34 | 29.31 | | |
| **15** | 15.64 | 9.77 | 18.15 | 28.67 | 24.21 | 27.19 | | |
| **17a** | 24.12 | 11.10 | 20.77 | 49.29 | 26.85 | 73.88 | >60 | >60 |
| **17b** | 46.77 | 18.51 | 49.67 | 109.81 | 65.44 | 88.09 | >60 | >60 |
| **18** | 8.60 | 6.30 | 13.93 | 13.31 | 21.60 | 20.56 | | |
| **19** | 30.57[e] | 19.22[e] | | | | | | |
| MFQ[f] | 7.34 | 4.89 | 22.45 | 19.49 | 9.45 | 50.14 | | |
| CQ[f] | 13.17 | 616.94 | 244.76 | 17.58 | 1019.71 | 345.08 | | |
| ART[g] | 9.0 | 6.7 | 13.04 | 12.8 | 11.5 | 17.40 | | |

[a] *P. falciparum* African D6 clone. [b] *P. falciparum* Indochina W2 clone. [c] *P. falciparum* multidrug resistant TM91C23 strain (Thailand). [d] Taken from ref 3b for comparison. [e] Taken from ref 6a for comparison. [f] Control drugs. [g] Average of greater than eight replicates.

ally active in an in vitro antiproliferative screen against a panel of 60 cell lines.

## Experimental Section

For general remarks see ref 6c.

**1,1-Dihydroperoxycyclohexane (1).** Cyclohexanone (1 mL, 10 mmol) was dissolved at room temperature in a $CH_2Cl_2/CH_3CN$ mixture (20 mL, 1:3 v/v) followed by 30% $H_2O_2$ (10.4 mL, 0.1 mol) and 6 drops of concentrated HCl. The reaction mixture was stirred for 2 h at room temperature and quenched with saturated $NaHCO_3$ and $CH_2Cl_2$. The organic layer was separated, and the water layer was additionally extracted with EtOAc (3 × 50 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated to dryness. The obtained crude product (740 mg, 50%) was used in the following step.

**Methyl 7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadecane-3-carboxylate and Methyl 7,8,15,16,23,24-Hexaoxatrispiro[5.2.5.2.5.2]tetracosane-3-carboxylate (2 and 4).** To a cooled solution (ice bath) of dihydroperoxide **1** (0.34 g, 2.3 mmol) in $CH_2Cl_2$ (20 mL) was added ketone **3** (0.36 g, 2.3 mmol). After the mixture was stirred for 30 min at the same temperature, a cooled $H_2SO_4/CH_3CN$ mixture (1.66 mL, 1:10, v/v) was added dropwise. After an additional 50 min of stirring, the mixture was worked up in the usual manner and was purified by $SiO_2$ column chromatography (Lobar B, LichroPrep Si 60, eluent heptane/EtOAc = 95/5) affording 185 mg (28%) **2** and 37 mg (8%) **4**. **2**: colorless foam, softness 75–80 °C. Anal. ($C_{14}H_{22}O_6 \cdot {}^1/_4H_2O$) C, H. **4**: solid oil. Anal. ($C_{20}H_{32}O_8 \cdot H_2O$) C, H.

**7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadecane-3-carboxylic Acid (3).** Methyl ester **2** (142 mg, 0.5mmol) was hydrolyzed at 80 °C with NaOH (29.5 mg, 0.7mmol) in an *i*-PrOH/$H_2O$ mixture (12 mL, 3:1 v/v). After 15 min, the mixture was cooled and diluted with $H_2O$ (20 mL) and $CH_2Cl_2$ (50 mL). The water layer was acidified to pH 2 with diluted HCl, and the layers were separated. The water layer was further extracted with $CH_2Cl_2$ (3 × 20 mL). Then the combined organic layers were washed with water and brine, dried over anhydrous $MgSO_4$, and evaporated to dryness. Trituration with $Et_2O$ afforded 120 mg (88%) of the product. With heating at 144–156 °C, the amorphous powder transforms into rhombic crystals, which melt at 168 °C. Anal. ($C_{13}H_{20}O_6 \cdot {}^1/_3H_2O$) C, H.

**Table 2.** In Vivo Antimalarial Activities of Tested Tetraoxanes against *P. berghei*[a]

| $mg \cdot kg^{-1} \cdot day^{-1}$ | mice dead/day died | mice alive day 31/total | survival time (day)[b] |
|---|---|---|---|
| | Compound **7**[c] | | |
| 320 | 1/14, 1/16, 1/17, 1/19, 1/21 | 0/5 | 17.4 |
| 80 | 3/7, 1/8, 1/9 | 0/5 | 7.6 |
| 20 | 1/6, 3/7, 1/9 | 0/5 | 7.2 |
| | Compound **9**[d] | | |
| 300 | 1/29 | 4/5 | 30.6 |
| 150 | 1/15, 1/27, 1/28 | 2/5 | 26.4 |
| 37.5 | 1/10, 1/17, 1/20, 1/24, 1/25 | 0/5 | 19.2 |
| 9.3 | 1/8, 3/10, 1/12 | 0/5 | 10 |
| | Compound **10**[d] | | |
| 300 | | 5/5 | >31 |
| 150 | | 5/5 | >31 |
| 37.5 | 1/17, 1/20, 1/24 | 2/5 | 24.6 |
| 9.3 | 1/8, 1/10, 1/14, 1/20, 1/31 | 0/5 | 16.6 |
| | Compound **11a**[d] | | |
| 300 | | 5/5 | >31 |
| 150 | 1/21 | 4/5 | 29 |
| 37.5 | 2/17, 2/24 | 1/5 | 22.6 |
| 9.3 | 1/8, 2/11, 1/17, 1/20 | 0/5 | 13.4 |
| | Compound **11b**[d] | | |
| 300 | 1/6, 1/19, 1/29 | 2/5 | 23.2 |
| 150 | 1/17, 1/18, 1/25, 1/27 | 1/5 | 23.6 |
| 37.5 | 1/8, 1/10, 1/16, 1/26 | 1/5 | 18.2 |
| 9.3 | 2/8, 2/9, 1/25 | 0/5 | 11.8 |
| | Infected Controls[e] | | |
| 0 | 7–9 | 0/5 | |

[a] Groups of five *P. berghei* (KBG 173 strain) infected CD-1 mice were treated on days 3−5 postinfection with tetraoxanes suspended in 0.5% hydroxyethylcellulose/0.1% Tween-80 (po) or sesame oil (sc). Mice alive on day 31 with no parasites in a blood film are considered cured. [b] Including cured mice. [c] Compound administered orally. [d] Compounds administered subcutaneously. [e] All noninfected age controls survived (5/5).

**General Procedure for Preparation of Amides 5–7.** A solution of **4** (250 mg, 0.92 mmol) in dry $CH_2Cl_2$ (25 mL) with added $Et_3N$ (130$\mu$L, 0.92mmol) and $ClCO_2Et$ (90 $\mu$L, 0.92mmol) was stirred

**Table 3.** MG_MID (TGI) Values for Compounds **2**, **5**, **7**, **13**, and **17b**

| 2 | 5 | 7 | 13 | 17b |
|---|---|---|---|---|
| −4.34 | −4.13 | −4.00 | −5.92 | −4.64 |

**Table 4.** In Vitro Antiproliferative Activities of Tetraoxane **13** ($\mu$M, after 48 h, Selected Data)

| cell line | | $GI_{50}$[a] | TGI[b] | $LC_{50}$[c] |
|---|---|---|---|---|
| leukemia | CCRF-CEM | 0.10 | 0.24 | 0.56 |
| | HL-60(TB) | 0.15 | 0.31 | 0.62 |
| | MOLT-4 | 0.18 | 0.41 | 0.96 |
| | SR | 0.16 | 0.34 | 0.72 |
| non-small-cell lung cancer | EKVX | 0.14 | 0.31 | 0.65 |
| | HOP-92 | 0.14 | 0.32 | 0.73 |
| | NCI-H460 | 0.24 | 0.62 | 2.68 |
| CNS cancer | SF-295 | 0.17 | 0.33 | 0.66 |
| | SF-539 | 0.17 | 0.32 | 0.59 |
| | U251 | 0.21 | 0.46 | 0.98 |
| melanoma | LOX IMVI | 0.02 | 0.03 | 0.06 |
| | SK-MEL-2 | 0.20 | 0.39 | 0.73 |
| ovarian cancer | IGROV1 | <0.01 | 0.01 | 0.06 |
| renal cancer | 786-0 | 0.23 | 0.54 | 2.01 |
| | UO-31 | 0.04 | 0.17 | 0.61 |
| prostate cancer | PC-3 | 0.29 | 0.99 | 3.41 |
| | DU-145 | 0.11 | 0.23 | 0.48 |
| breast cancer | NCI/ADR-RES | 0.25 | 0.59 | 4.27 |
| | T-47D | 0.33 | 0.93 | 5.30 |

[a] 50% growth inhibitory activity. [b] Total growth inhibition. [c] Concentration of the compound at which 50% of the cells are killed.

for 90 min at 0 °C. Amine was added, and after 30 min of stirring the mixture was warmed to room temperature. After 90 min it was diluted with $H_2O$, the layers were separated, and the organic layer was washed with brine, dried over anhydrous $MgSO_4$, and evaporated to dryness.

**7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadecane-3-carboxamide (5).** By use of the above procedure, **3** was reacted with 10 equiv of $NH_4Cl$ and 10 equiv of $Et_3N$ in dry $CH_2Cl_2$ (20 mL) to afford the primary amide **5** (200 mg, 80%), which was then triturated with $Et_2O$. **5**: mp 168–172 °C. Anal. ($C_{13}H_{21}NO_5 \cdot H_2O$) C, H.

***N*-Propyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane-3-carboxamide (6).** Acid **3** (250 mg, 0.92 mmol) was transformed into amide **6** (220 mg, 77%) using 10 equiv of *n*-$PrNH_2$ in dry $CH_2Cl_2$ (45 mL). Column chromatography: Lobar B, LichroPrep RP-18, eluent MeOH/$H_2O$ = 8/2. Colorless foam, softness at 200–203 °C. Anal. ($C_{16}H_{27}NO_5$) C, H.

***N*-[2-(Dimethylamino)ethyl]-7,8,15,16-tetraoxadispiro[5.2.5.2]-hexadecane-3-carboxamide (7).** Acid **4** (250 mg, 0.92 mmol) was transformed into amide **7** (280 mg, 90%) using 10 equiv of $Me_2NCH_2CH_2NH_2$ in dry $CH_2Cl_2$ (45 mL). Column chromatography: Lobar B, LichroPrep RP-18, eluent MeOH. Colorless foam, softness at 179–182 °C. Anal. ($C_{17}H_{30}N_2O_5 \cdot H_2O$) C, H, N.

**7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadec-3-ylmethanol (8).** A solution of methyl ester **2** (1 g, 3.5 mmol) in dry ether (5 mL) was added in portions to a suspension of $LiAlH_4$ (177 mg, 4.7 mmol) in dry ether (5 mL) at room temperature. After 50 min it was diluted with $H_2O$ and EtOAc. The water layer was acidified to pH 2 with diluted HCl, the layers were separated, and the water layer was further extracted with EtOAc (3 × 50 mL). The combined organic layers were dried over anhydrous $Na_2SO_4$ and evaporated to dryness. The crude product was purified using dry flash chromatography, with an eluent of heptane/EtOAc (8/2). Yield 813 mg (90%). Colorless foam, softness 116–118 °C. Anal. ($C_{13}H_{22}O_5$) C, H.

**3-(Azidomethyl)-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane (9).** To a solution of alcohol **8** (650 mg, 2.5 mmol) in pyridine (5 mL) at room temperature was added methanesulfonyl chloride (250 $\mu$L, 3.0 mmol). The mixture was stirred at room temperature for 2 h, then diluted with $H_2O$ and EtOAc. The water layer was acidified with diluted HCl, and the layers were separated. The water layer was further extracted with EtOAc (3 × 50 mL). The combined organic layers were dried over anhydrous $Na_2SO_4$ and evaporated to dryness. The obtained crude product was used in the following step. To a solution of mesylate (1.4 g, 4.2 mmol) in DMF (15 mL) was added $NaN_3$ (2.7g, 42 mmol). The mixture was stirred at 50 °C for 16 h before being quenched with water and EtOAc, and the layers were separated. The water layer was further extracted with EtOAc (3 × 75 mL). The combined organic layers were washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated to dryness. The crude product was purified using dry flash chromatography with a heptane/EtOAc eluent (8/2). Yield 1.57 g (>99%). Colorless foam, softness 86–87 °C. Anal. ($C_{13}H_{21}N_3O_4$) C, H, N.

**1-(7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadec-3-yl)methanamine (10).** A solution of azide **9** (900 mg, 3.18 mmol) in dry ether (5 mL) was added in one portion to a suspension of $LiAlH_4$ (165 mg, 4.35 mmol) in dry ether (5 mL) at room temperature. After 50 min it was diluted with $H_2O$ and NaOH (10%). The solution was filtered, and the residue was washed with a small portion of ether. The filtrate was extracted with ether (2 × 50 mL), and the combined organic layers were dried over anhydrous $Na_2SO_4$ and evaporated to dryness. The crude product was purified using dry flash chromatography with an EtOAc/MeOH/$NH_3$ eluent (8/1/1). Yield 475 mg (60%). Colorless foam, softness 75–77 °C. Anal. ($C_{13}H_{23}NO_4 \cdot {}^1/_2H_2O$) C, H, N.

**7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadecane-3-methanamine, *N*-cyclohexyl- (11a).** To a mixture of amine **10** (145 mg, 0.56 mmol) and cyclohexanone (59 $\mu$L, 0.56 mmol) in $CH_2Cl_2$ (10 mL) was added sodium triacetoxyborohydride (286 mg, 1.35 mmol). After the mixture was stirred at room temperature for 18 h, it was poured into water and extracted with $CH_2Cl_2$ (2 × 50 mL). The combined organic layers were dried over anhydrous $Na_2SO_4$ and evaporated to dryness. The crude product was purified by dry flash chromatography with an eluent of EtOAc/MeOH/$NH_{3aq}$ = 27/0.5/0.5. Yield 97 mg (48%). Colorless foam, softness 104–106 °C. Anal. ($C_{19}H_{33}NO_4 \cdot {}^1/_3H_2O$) C, H, N.

***N*-(7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadec-3-ylmethyl)propan-2-amine (11b).** Amine **10** (220 mg, 0.85 mmol) was transformed into amine **11b** (172 mg, 67%) using acetone (69 $\mu$L, 0.94 mmol) and $NaBH(OAc)_3$ (473 mg, 2.23 mmol). The crude product was purified using dry flash chromatography with an eluent of EtOAc/MeOH (8/2). Colorless foam, softness 87–89 °C. Anal. ($C_{16}H_{29}NO_4$) C, H, N.

***N*-(Phenylmethyl)-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane-3-methanamine (11c).** Amine **10** (100 mg, 0.39 mmol) was transformed into amine **11c** (45 mg, 50%) using PhCHO (40 $\mu$L, 0.39 mmol) and $NaBH(OAc)_3$ (200 mg, 0.94 mmol). The crude product was purified using dry flash chromatography with an eluent of EtOAc/MeOH (8/2) and repeated dry flash chromatography using EtOAc. Colorless foam, softness 61–62 °C. Anal. ($C_{20}H_{29}NO_4 \cdot {}^1/_2H_2O$) C, H, N.

**5$\beta$-Cholan-7$\alpha$,12$\alpha$,24-triol-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (13).** A solution of methyl ester **12** (100 mg, 0.16 mmol) in dry ether (5 mL) was added in one portion to a suspension of $LiAlH_4$ (21 mg, 0.57 mmol) in dry ether (5 mL) at room temperature. After 50 min the reaction was quenched with $H_2O$ and EtOAc. The water layer was acidified to pH 2 with diluted HCl, and layers were separated. The water layer was further extracted with EtOAc (3 × 50 mL), and the combined organic layers were dried over anhydrous $Na_2SO_4$ and evaporated to dryness. Crude triol **13** was purified using dry flash chromatography using a heptane/EtOAc eluent (2/8). Yield 81 mg (98%). Colorless foam, softness 119–121 °C. $[\alpha]^{20}_D$ +24.0 (*c* 0.2, $CHCl_3$). Anal. ($C_{30}H_{50}O_7 \cdot {}^1/_2H_2O$) C, H.

**7$\alpha$,12$\alpha$,24-Triacetoxy-5$\beta$-cholan-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (14).** Alcohol **13** (1.67 g, 3.19 mmol) was dissolved in a previously prepared solution of $Ac_2O$ (1.7 mL) and TMSOTf (35 $\mu$L, 0.19 mmol) in dry $CH_2Cl_2$ (30 mL) at room temperature. After stirring for 15 min, the reaction was quenched with saturated $NaHCO_3$ and the layers were separated. The water layer was further extracted with $CH_2Cl_2$ (3 × 15 mL), and the combined organic layers were washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated to dryness. Crude triacetate **14** was purified using dry flash chromatography using a heptane/

EtOAc eluent (7/3). Yield 1.92 g (93%). Colorless foam, softness 136–137 °C. $[\alpha]^{20}_D$ +55.5 (*c* 0.2, $CHCl_3$). Anal. ($C_{36}H_{56}O_{10}$) C, H.

**7α,12α-Diacetoxy-5β-cholan-24-ol-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (15). Hydrolysis of Triacetate 14.** Triacetate **14** (1.67 g, 2.57 mmol) was dissolved in dry methanol (50 mL), followed by addition of anhydrous $K_2CO_3$ (640 mg, 4.63 mmol). The suspension was stirred at room temperature for 5 h. The mixture was evaporated to dryness, dissolved in $CH_2Cl_2$ and $H_2O$, and the layers were separated. The organic layer was washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated to dryness. The crude monoalcohol **15** was purified using dry flash chromatography using a heptane/EtOAc eluent (4/6). Yield 1.50 g (96%). Colorless foam, softness 207–210 °C. $[\alpha]^{20}_D$ +57.0 (*c* 0.2, $CHCl_3$). Anal. ($C_{34}H_{54}O_9$) C, H.

**Via Mixed Anhydride.** Acid **19** (50 mg, 0.08 mmol) was dissolved in dry THF (5 mL) and treated with $Et_3N$ (23 μL, 0.16 mmol) and $ClCO_2Et$ (15.34 μL, 0.16 mmol). After 3 h of stirring at 0 °C, $NaBH_4$ (30.5 mg, 0.8 mmol) was added. After an additional 24 h of stirring at room temperature, the mixture was diluted with $H_2O$ and $CH_2Cl_2$, and the layers were separated. The water layer was further extracted with $CH_2Cl_2$(2 × 50 mL) and the combined organic layers were dried over anh. $Na_2SO_4$ and evaporated to dryness. The crude alcohol **15** was purified using dry flash chromatography using a heptane/EtOAc eluent (1/1). Yield 37 mg (76%).

**7α,12α-Diacetoxy-5β-cholan-24-al-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (16).** Alcohol **15** (100 mg, 0.16 mmol) was dissolved in dichloromethane (20 mL) followed by the addition of pyridinium chlorochromate (53 mg, 0.25 mmol). After 2 h the mixture was transferred to a silica gel column and eluted with $CH_2Cl_2$ to afford 83 mg (83%) of **16** as a colorless solid.

***N*-(*n*-Propyl)-7α,12α-diacetoxy-5β-cholan-24-amine-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (17a).** To a mixture of crude aldehyde **16** (83 mg, 0.14 mmol) and *n*-$PrNH_2$ (23 μL, 0.28 mmol) in dichloromethane (20 mL), sodium triacetoxyborohydride (58 mg, 0.28 mmol) was added. The mixture was stirred at room temperature for 18 h. The mixture was then poured onto water and extracted with $CH_2Cl_2$ (2 × 50 mL). The combined organic layers were dried over anhydrous $Na_2SO_4$ and evaporated to dryness. The crude amine **17a** was purified by dry flash chromatography using an eluent of EtOAc/MeOH/$NH_{3aq}$ = 8/1/1. Yield 64 mg (72%). Colorless foam, softness 76–78 °C. $[\alpha]^{20}_D$ +43.0 (*c* 0.2, $CHCl_3$). Anal. ($C_{37}H_{61}NO_8$) C, H, N.

***N*-(2-Dimethylamino)ethyl)-7α,12α-diacetoxy-5β-cholan-24-amine-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (17b).** Aldehyde **16** (200 mg, 0.33 mmol) was transformed into amine **17b** (168 mg, 75%) using $Me_2NCH_2CH_2NH_2$ (72.5 μL, 0.66 mmol) and $NaBH(OAc)_3$ (140 mg, 0.66 mmol). The crude product was purified using dry flash chromatography with an EtOAc/MeOH/$NH_{3aq}$ (8/1/1) eluent. Solid. $[\alpha]^{20}_D$ +45.0 (*c* 0.2, $CHCl_3$). Anal. ($C_{38}H_{64}N_2O_8 \cdot 5H_2O$) C, H, N.

**7α,12α-Diacetoxy-5β-cholan-24-azido-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (18).** To a solution of alcohol **15** (200 mg, 0.33 mmol) in pyridine (4 mL) at room temperature was added methanesulfonyl chloride (31 μL, 0.4 mmol). The mixture was stirred at room temperature for 2 h, then diluted with $H_2O$ and EtOAc. The water layer was acidified with diluted HCl, and layers were separated. The water layer was further extracted with EtOAc (3 × 50 mL), and the combined organic layers were dried over anhydrous $Na_2SO_4$ and evaporated to dryness. The obtained crude product was used in the following step. To a solution of mesylate (226 mg, 0.33 mmol) in DMF (5 mL) was added $NaN_3$ (214 mg, 3.3 mmol). The mixture was stirred at 50 °C for 16 h before being quenched with water and EtOAc, and layers were separated. The water layer was further extracted with EtOAc (3 × 75 mL), and the combined organic layers were washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated to dryness. The crude product was purified using dry flash chromatography using a heptane/EtOAc (7/3) eluent. Yield 198,5 mg (95%). Solid oil. $[\alpha]^{20}_D$ +44.5 (*c* 0.2, $CHCl_3$). Anal. ($C_{34}H_{53}N_3O_8 \cdot 2H_2O$) C, H, N.

**In Vitro Antimalarial Activity.** The in vitro antimalarial drug susceptibility screen is a modification of the procedures first published by Desjardins et al.,[15] with modifications developed by Milhous et al.,[16] and the details are given in ref 5a.

**In Vivo Antimalarial Activity.** The *P. berghei* mouse efficacy tests were conducted using a modified version of the Thompson test. Groups of five mice were inoculated intraperitoneally with erythrocytes infected with a drugsensitive strain of *P. berghei* on day 0. Drugs were suspended in 0.5% hydroxyethylcellulose/0.1% Tween-80 (for po administration) or in sesame oil (for sc administration). Drugs were administered orally once a day beginning on day 3 postinfection. Dosings are given in Table 2. Cure was defined as survival until day 31 posttreatment. Untreated control mice die on day 7–9 postinfection.

**In Vitro Metabolism Studies.** The metabolic stability assay sample preparation was performed in a 96-well plate on a TECAN Genesis robotic sample processor. All incubations were carried out in 0.1 M sodium phosphate buffer (pH 7.4) in the presence of an NADPH-regenerating system ($NADP^+$ sodium salt, $MgCl_2 \cdot 6H_2O$, and glucose 6-phosphate). Test drug (10 μM), microsomes (1 mg/mL total protein), buffer, and NADPH-regenerating system were warmed to 37 °C, and the reaction was initiated by the addition of glucose 6-phosphate dehydrogenase (G6PD). Samples were quenched using an equal volume of cold methanol. Samples were centrifuged to pellet the proteins, and the supernatant was analyzed by LC−MS/MS using fast LC gradient or isocratic methods. Percentages of parent drug remaining at each time point were calculated using the ratio of the peak area at each time point to the area of the time zero point. To calculate the half-life, a first-order rate of decay was assumed. A plot of the natural log (ln) of the drug concentration versus time was generated, where the slope of that line was $-k$. The half-life was calculated as $0.693/k$.

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**Supporting Information Available:** Analytical data of synthesized/isolated compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Preliminary results were presented at the 43rd Meeting of the Serbian Chemical Society, Belgrade, Serbia, January 24–25, 2005, and at the 1st European Chemistry Congress, Budapest, Hungary, August 27–31, 2006.

(2) (a) For recent developments in artemisinin field, see the followin: Posner, G. H.; Paik, I.-K.; Chang, W.; Borstnik, K.; Sinishtaj, S.; Rosenthal, A. S.; Shapiro, T. A. Malaria-Infected Mice Are Cured by a Single Dose of Novel Artemisinin Derivatives. *J. Med. Chem.* **2007**, *50*, 2516–2519. (b) Haynes, R. K.; Chan, W. C.; Lung, C-M.; Uhlemann, A-C.; Eckstein, U.; Taramelli, D.; Parapini, S.; Monti, D.; Krishna, S. The $Fe^{2+}$-Mediated Decomposition, PfATP6 Binding, and Antimalarial Activities of Artemisone and Other Artemisinins: The Unlikelihood of C-Centered Radicals as Bioactive Intermediates. *ChemMedChem* **2007**, *2*, 1480–1497.

(3) Vennerstrom, J. L.; Fu, H.-N.; Ellis, W. Y.; Ager, A. L.; Wood, J. K., Jr.; Andersen, S. L.; Gerena, L.; Milhous, W. K. Dispiro-1,2,4,5-tetraoxanes: A New Class of Antimalarial Peroxides. *J. Med. Chem.* **1992**, *35*, 3023–3027. (b) Todorović, N. M.; Tinant, B.; Declercq, J.-P.; Makler, M. T.; Šolaja, B. A. Steroidal Geminal Dihydroperoxides and 1,2,4,5-Tetraoxanes: Structure Determination and Their Antimalarial Activity. *Steroids* **1996**, *61*, 688–696.

(4) (a) Opsenica, I.; Terzić, N.; Opsenica, D.; Milhous, W. K.; Šolaja, B. 7,8,15,16-Tetraoxa dispiro[5.2.5.2]hexadecane-3-carboxylic Acid Derivatives and Their Antimalarial Activity. *J. Serb. Chem. Soc.* **2004**, *69*, 919–922. (b) Amewu, R.; Stachulski, A. V.; Ward, S. A.; Berry, N. G.; Bray, P. G.; Davies, J.; Labat, G.; Vivas, L.; O'Neill, P. M. Design and synthesis of orally active dispiro 1,2,4,5-tetraoxanes; synthetic antimalarials with superior activity to artemisinin. *Org. Biomol. Chem.* **2006**, *l4*, 4431–4436. (c) Dong, Y.; Creek, D.; Chollet, J.; Matile, M.; Charman, S. A.; Wittlin, S.; Wood, J. K.; Vennerstrom, J. L. Comparative Antimalarial Activities of Six Pairs of 1,2,4,5-Tetraoxanes (Peroxide Dimers) and 1,2,4,5,7,8-Hexaoxonanes (Peroxide Trimers). *Antimicrob. Agents Chemother.* **2007**, *51*, 3033–3035.

(5) (a) Opsenica, D.; Pocsfalvi, G.; Juraniæ, Z.; Tinant, B.; Declercq, J.-P.; Kyle, D. E.; Milhous, W. K.; Šolaja, B. A. Cholic Acid Derivatives as 1,2,4,5-Tetraoxane Carriers: Structure and Antimalarial and Antiproliferative Activity. *J. Med. Chem.* **2000**, *43*, 3274–3282. (b) Opsenica, D.; Angelovski, G.; Pocsfalvi, G.; Juraniæ, Z.; éieak, Z.; Kyle, D.; Milhous, W. K.; Šolaja, B. A. Antimalarial and Antiproliferative Evaluation of Bis-Steroidal Tetraoxanes. *Bioorg. Med. Chem.* **2003**, *11*, 2761–2768.

(6) (a) Šolaja, B. A.; Terziæ, N.; Pocsfalvi, G.; Gerena, L.; Tinant, B.; Opsenica, D.; Milhous, W. K. Mixed Steroidal 1,2,4,5-Tetraoxanes: Antimalarial and Antimycobacterial Activity. *J. Med. Chem.* **2002**, *45*, 3331–3336. (b) Opsenica, D.; Kyle, E.; Milhous, W. K.; Šolaja, B. A. Antimalarial, Antimycobacterial and Antiproliferative Activity of Phenyl Substituted Mixed Tetraoxanes. *J. Serb. Chem. Soc.* **2003**, *68*, 291–302. (c) Opsenica, I.; Terzić, N.; Opsenica, D.; Angelovski, D.; Lehnig, M.; Eilbracht, P.; Tinant, B.; Juraniæ, Z.; Smith, K. S.; Yang, Y. S.; Diaz, D. S.; Smith, P. L.; Milhous, W. K.; Doković, D.; Šolaja, B. A. Tetraoxane Antimalarials and Their Reaction with Fe(II). *J. Med. Chem.* **2006**, *49*, 3790–3799. (d) Terzić, N.; Opsenica, D.; Miliæc, D.; Tinant, B.; Smith, K. S.; Milhous, W. K.; Šolaja, A. B. Deoxycholic Acid-Derived Tetraoxane Antimalarials and Antiproliferatives. *J. Med. Chem.* **2007**, *50*, 5118–5127.

(7) Procopiou, P. A.; Baugh, S. P. D.; Flack, S. S.; Inglis, G. G. A. An Extremely Powerful Reaction of Alcohols with Acid Anhydrides Catalyzed by Trimethylsilyl Trifluoromethanesulfonate. *J. Org. Chem.* **1998**, *63*, 2342–2347.

(8) The yields of these cyclohexane-1,1-diyl dihydroperoxides fall within 50–85%. In this experiment it was 50%. The other cyclohexanones (4-methyl-, 2-methyl-, and 4-*tert*-butylcyclohexanone) were transformed into corresponding dihydroperoxides in 85%, 77%, and 81% yields, respectively.

(9) (a) Žmitek, K.; Zupan, M.; Stavber, S.; Iskra, J. Iodine as a Catalyst for Efficient Conversion of Ketones to gem-Dihydroperoxides by Aqueous Hydrogen Peroxide. *Org. Lett.* **2006**, *8*, 2491–2494. (b) Žmitek, K.; Zupan, M.; Stavber, S.; Iskra, J. The Effect of Iodine on the Peroxidation of Carbonyl Compounds. *J. Org. Chem.* **2007**, *72*, 6534–6540.

(10) Terent'ev, A. O.; Platonov, M. M.; Sonneveld, E. J.; Peschar, R.; Chernyshev, V. V.; Starikova, Z. A.; Nikishin, G. I. New Preparation of 1,2,4,5,7,8-Hexaoxonanes. *J. Org. Chem.* **2007**, *72*, 7237–7243.

(11) This and ensuing $LiAlH_4$ reductions presented here were found to be in accordance with the results of the following: Jin, H.-X.; Liu, H.-H.; Zhang, Q.; Wu, Y. On the Susceptibility of Organic Peroxy Bonds to Hydride Reduction. *J. Org. Chem.* **2005**, *70*, 4240–4247.

(12) CQ and MFQ susceptible strain D6 (clone of Sierra I/UNC isolate), CQ resistant but MFQ susceptible strain W2 (clone of Indochina I isolate), and CQ and MFQ resistant strain TM91C235 (clone of South-East Asian isolate).

(13) Vennerstrom, J. L.; Dong, Y.; Chollet, J.; Matile, H. Spiro and Dispiro 1,2,4-Trioxolane Antimalarials. U.S. Patent 6,486,199, 2002.

(14) Drug Discovery and Development Program, National Cancer Institute, Bethesda, MD (NCI). http://dtp.nci.nih.gov.

(15) Desjardins, R. E.; Canfield, C. J.; Haynes, D. E.; Chulay, J. D. Quantitative Assessment of Antimalarial Activity in Vitro by a Semiautomated Microdilution Technique. *Antimicrob. Agents Chemother.* **1979**, *16*, 710–718.

(16) Milhous, W. K.; Weatherly, N. F.; Bowdre, J. H.; Desjardins, R. E. In Vitro Activities of and Mechanisms of Resistance to Antifol Antimalarial Drugs. *Antimicrob. Agents Chemother.* **1985**, *27*, 525–530.

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