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Deoxycholic Acid-Derived Tetraoxane Antimalarials and Antiproliferatives[1]

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The synthesis of deoxycholic acid (DCA)- and cholic acid (CA)-derived mixed tetraoxanes revealed that *N*-(2-dimethylamino)ethyl derivatives are potent antimalarials in vitro and in vivo. The tetraoxanes presented in this paper are dual inhibitors: besides curing mice in vivo without observed toxic effects, they kill cancer cell lines at very low concentrations. For example, DCA and CA derivatives **16** and **25** cured 3/5 (160 mg/kg/day) and 2/5 (40 mg/kg/day, MTD >960 mg/kg), respectively, and they were extremely active against melanoma LOX IMVI cancer, LC_{50} = 22 nM and 69 nM, respectively.

Introduction

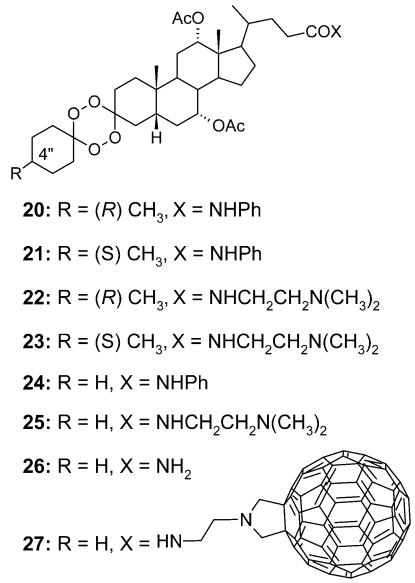
Malaria was successfully reduced after World War II as a consequence of easy access to the cheap insecticide, dichlorodiphenyltrichloroethane (DDT), as well as inexpensive and readily available drugs such as chloroquine (CQ)[a], mefloquine (MFQ), and quinine. CQ and MFQ were both preceded by quinine, which was the first purified natural product used as a drug (in 1821). Malaria is caused by multiplication of the protozoan parasite *Plasmodium* in erythrocytes and is a major health problem in many tropical and subtropical countries. Of the four species of malaria that cause human disease, *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, *P. falciparum* is the most lethal strain. The present resurgence of malaria and the lack of proper treatment affect 300−500 million people annually causing over 1.5 million deaths.[2] The development of resistance to chloroquine has severe health implications for countries in malaria-endemic regions. In a recent genetic study[3] of *P. falciparum*, it was found that this species is unexpectedly diverse; another study[4] points to the multiple independent origins of mutations in one parasite gene that confer resistance to the widely used drug CQ. The results show that, in principle, *P. falciparum* could rapidly develop resistance to multiple drugs (CQ: estimated ∼6−30 years), additionally justifying a further search for new drugs.

For some time, our research has focused on the generation of analogs containing a 1,2,4,5-tetraoxacyclohexane antimalarial pharmacophore with a cholic acid (CA)-derived carrier.[5] On the basis of our accumulated evidence regarding the influence of substitution at the spirocycloalkane moiety, it appears that a methyl group at C(4″), Chart 1 affords the best in vitro and in vivo antimalarial activity.[5b,d]

With concern of the synthesis of mixed tetraoxanes, four distinct procedures based on coupling of a ketone/protected ketone species to a protected/nonprotected *gem*-dihydroperoxide have been used: (a) coupling of TMS-protected *gem*-dihydroperoxide to an aldehyde (catalyst TMSOTf);[6] (b) coupling of nonprotected *gem*-dihydroperoxide to a ketone (catalyst H_2SO_4/CH_3CN);[6b] (c) one-pot *gem*-dihydroperoxide MTO/TFE mediated preparation and coupling to a ketone (catalyst HBF_4);[7] and (d) coupling of nonprotected *gem*-dihydroperoxides to acetals derived from a suitable ketone (catalyst BF_3/Et_2O).[8,9] The best reported yields of desired products were obtained using procedures described in refs 7 and 8 (up to 90%); however, the tetraoxanes with meaningful antimalarial activity were synthesized according to the procedure presented in refs 5b−d and 9, with yields of 18−50%. Our approach to the synthesis of cholic acid-derived tetraoxanes utilizes the coupling of a ketone to steroidal *gem*-dihydroperoxide obtained from a steroidal ketone in high yield and with high purity.

We found that the toxicity of steroidal tetraoxanes against healthy cells (PBMC,[5a,d] VERO[5d]) is low as compared with their antimalarial activity (SI = 826−33 000) and to their activity against certain types of cancers.[5d] In addition, preliminary results on nonhemolytic behavior[5b] as well as their high maximum tolerated doses (MTD) in an antiproliferative screen (400 mg/kg) and an antimalarial screen (>1960 mg/kg)[5d] provide a strong rationale for further research in this area.

Chart 1



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[a] Abbreviations: CQ, chloroquine; MFQ, mefloquine; ART, artemisinin; CA, cholic acid; DCA, deoxycholic acid; MG_MID, mean graph midpoint.

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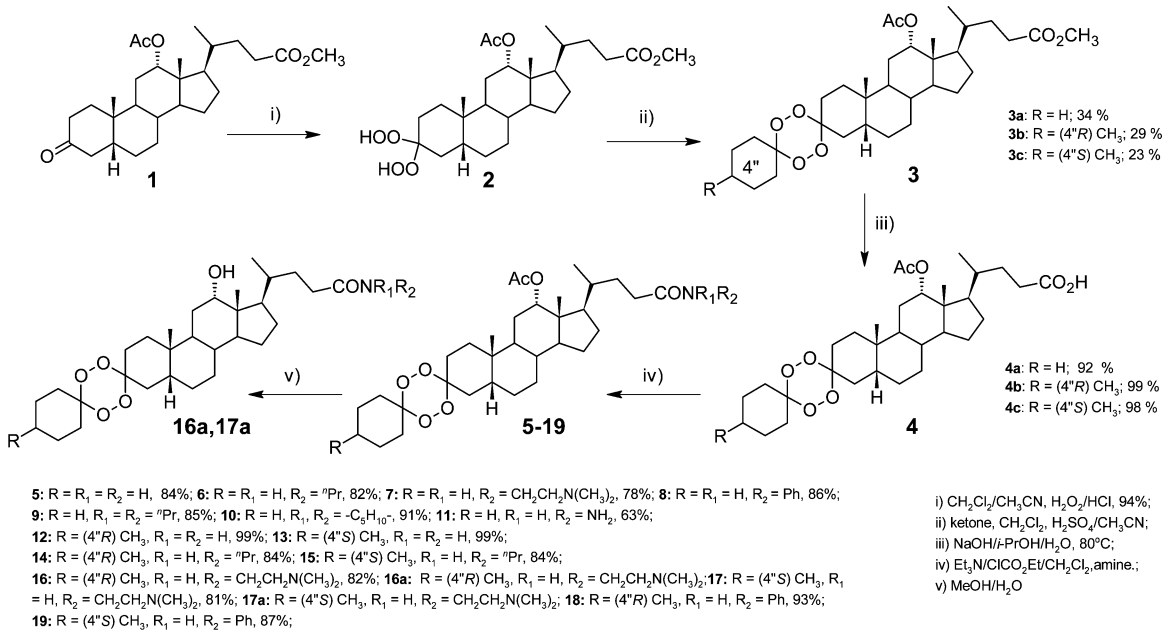
Scheme 1



5: R = R_1 = R_2 = H, 84%; **6:** R = R_1 = H, R_2 = ^{n}Pr, 82%; **7:** R = R_1 = H, R_2 = $CH_2CH_2N(CH_3)_2$, 78%; **8:** R = R_1 = H, R_2 = Ph, 86%; **9:** R = H, R_1 = R_2 = ^{n}Pr, 85%; **10:** R = H, R_1, R_2 = -C_5H_{10}-, 91%; **11:** R = H, R_1 = H, R_2 = NH_2, 63%; **12:** R = (4″*R*) CH_3, R_1 = R_2 = H, 99%; **13:** R = (4″*S*) CH_3, R_1 = R_2 = H, 99%; **14:** R = (4″*R*) CH_3, R_1 = H, R_2 = ^{n}Pr, 84%; **15:** R = (4″*S*) CH_3, R_1 = H, R_2 = ^{n}Pr, 84%; **16:** R = (4″*R*) CH_3, R_1 = H, R_2 = $CH_2CH_2N(CH_3)_2$, 82%; **16a:** R = (4″*R*) CH_3, R_1 = H, R_2 = $CH_2CH_2N(CH_3)_2$;**17:** R = (4″*S*) CH_3, R_1 = H, R_2 = $CH_2CH_2N(CH_3)_2$, 81%; **17a:** R = (4″*S*) CH_3, R_1 = H, R_2 = $CH_2CH_2N(CH_3)_2$; **18:** R = (4″*R*) CH_3, R_1 = H, R_2 = Ph, 93%; **19:** R = (4″*S*) CH_3, R_1 = H, R_2 = Ph, 87%;

i) CH_2Cl_2/CH_3CN, H_2O_2/HCl, 94%;
ii) ketone, CH_2Cl_2, H_2SO_4/CH_3CN;
iii) NaOH/*i*-PrOH/H_2O, 80°C;
iv) $Et_3N/ClCO_2Et/CH_2Cl_2$,amine.;
v) MeOH/H_2O

Accordingly, in this work, we present the results of our study on the preparation, antimalarial and antiproliferative activity, and in vitro metabolism of deoxycholic acid (DCA)- and CA-derived tetraoxanes. We explore the differences in in vitro antimalarial activity and cytotoxicity between the two series and analyze the obtained in vivo results.

Chemistry

Tetraoxanes **3a**−**c** were prepared according to our procedure described earlier[5b−d,10] using HCl and an H_2SO_4/CH_3CN system for bishydroperoxyacetalization and peroxyacetalization reactions, respectively (Scheme 1). *Gem*-dihydroperoxide **2** was obtained in high yield from ketone **1** and was further coupled to cyclohexanones to give **3a** and **3b** + **3c** in the yield of 34% and 52%, respectively. Epimeric tetraoxanes, obtained from 4-methylcyclohexanone, were separated as methyl esters **3b** and **3c.** Esters **3** were further transformed into corresponding amides via acids **4** according to a well-established procedure[5b−d,10] utilizing mixed anhydride intermediates: **3** → **4** → **5**−**19**. The overall yield of amides in each series starting from *gem*-dihydroperoxide **2** was 19−39%. The obtained compounds have been fully characterized using standard spectroscopic methods.

The configuration at C(4″) in coupled products could not be determined on the basis of NMR spectral data; however, it was assigned by X-ray crystallographic structural analysis of the corresponding amide **14**, and it appears to be *R* (Figure 1). Consequently, tetraoxanes **3b**, **4b**, **12**, **14**, **16**, **16a**, and **18** were assigned to the same 4″*R* series, while the diastereomeric 4″*S* series consists of compounds **3c**, **4c**, **13**, **15**, **17**, **17a**, and **19**.

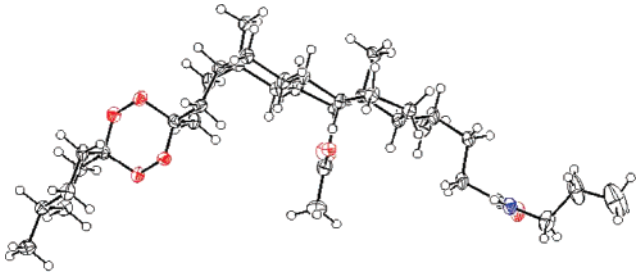


Figure 1. ORTEP plot of tetraoxane **14**.[11] Carbons are represented as partially filled elipsoids; hydrogens are unfilled circles; oxygens are in red; nitrogens are in blue.

The asymmetric part of the unit cell of **14** consists of two independent tetraoxane molecules; there are no cocrystallized solvent molecules. The two tetraoxane molecules have a very similar geometry; only differences in the orientation of the extremities of the C(17) side chain are observed (N(25)−C(26)−C(27)−C(28) = −168° in molecule 1 and −62° in molecule 2). One observed intermolecular hydrogen bonds involving the amide bond. In molecule 1: N(25)−H(25)···O(29) (1 − *x*, 0.5 + *y*, 2 − *z*), H···O 2.11(2) Å, N···O = 2.978(4) Å, N−H···O = 168°. In molecule 2: N(25)−H(25)···O(29) (−*x*, 0.5 + *y*, 2 − *z*), H···O 2.13(2) Å, N···O = 2.984(4) Å, N−H···O = 165°.

The cholic acid-based tetraoxanes (Chart 1) were obtained by an analogous procedure starting from known acids [5b] and *N*,*N*-dimethylethan-1,2,-diamine (see Experimental Section).

Antimalarial Activity

The synthesized tetraoxanes **3**−**27** were screened in vitro against three *P. falciparum* strains: D6 (chloroquine-susceptible), W2 (chloroquine-resistant, susceptible to mefloquine), and TM91C235 (Thailand), a multidrug-resistant strain, following the protocol given in ref 5a. In general, spirocyclohexylidene tetraoxanes of the DCA series (Table 1, R = H, compounds **3a**, **4a**, **5**−**11**) appear to be less active than their C(4″) methyl analogues (Table 1, R = CH_3). Of the two epimeric C(4″) methyl series, 4″*R* is significantly more active. The above findings both hold true for the CA derivatives as well (Tables 1 and 2, and ref 5b).

Members of the DCA series (Tables 1 and 2, R = H, and R = CH_3 (4″*S*)) are moderately active against D6, W2, and TM91C235 *P. falciparum* strains; however, they are noticeably less active than the corresponding 4″*R* derivatives. In the 4″*R* series, tetraoxane **16** was identified as the most potent in vitro of all of the DCA derivatives. Compound **16** possesses an *N*-(2-dimethylamino)ethyl group that should be protonated under the acidic conditions in parasite's food vacuole and was introduced to increase the concentration of the active compound at the location of action. A few features of its in vitro activity should be noted. First, the compound exerts approximately the same

Table 1. In Vitro Antimalarial Activities of Tetraoxanes **3a**−**27** against *P. Falciparum* D6[a] and W2[b] Strains

R = H					R = CH_3 (4″***R***)					R = CH_3 (4″***S***)				
	IC_{50} (nM)		IC_{90} (nM)			IC_{50} (nM)		IC_{90} (nM)			IC_{50} (nM)		IC_{90} (nM)	
compd	D6	W2	D6	W2	compd	D6	W2	D6	W2	compd	D6	W2	D6	W2
3a	201.43	111.69	258.37	170.27	**3b**	39.20	34.21	26.04	49.29	**3c**	37.95	38.30	62.88	80.30
4a	106.73	93.86	310.14	178.18	**4b**	11.55	12.05	17.08	84.75	**4c**	19.21	42.24	31.69	89.66
5	32.72	19.08	50.41	35.58	**12**	13.62	18.76	18.98	29.72	**13**	22.89	19.43	31.35	118.00
6	74.28	40.76	115.17	110.94	**14**	9.81	10.10	20.21	30.49	**15**	29.29	33.83	39.26	53.70
7	221.21	104.87	ND[c]	276.13	**16**	12.83	16.83	18.66	19.77	**17**	104.14	135.93	148.09	155.03
8	133.24	81.62	195.48	126.25	**18**	23.32	23.10	33.69	38.90	**19**	49.99	50.82	77.45	76.86
9	178.86	150.14	197.37	168.75										
10	105.56	55.49	131.40	78.62										
11	346.75	346.75	ND	ND										
24	6.70	11.24	11.70	25.93	**20**	5.48	7.86	8.07	10.11	**21**	19.78	23.10	29.20	54.61
25	28.18	14.49	45.14	51.13	**22**	18.63	16.67	27.78	24.80	**23**	43.82	43.54	71.16	94.02
26[d]	11.83	4.74	ND	ND										
27	3547	1413												
ART[e]	9.0	6.7	12.8	11.5	**MFQ**	10.52	3.09	21.65	8.41	**CQ**	13.72	349.35	17.63	491.53

[a] *P. falciparum* African D6 clone. [b] *P. falciparum* Indochina W2 clone. [c] ND, not determined. [d] Taken from ref 1b. [e] Average of greater than eight replicates.

Table 2. In Vitro Antimalarial Activities of Tetraoxanes **3a**−**27** against *P. Falciparum* TM91C235[a] Strain

R = H			R = CH_3 (4*R*)			R = CH_3 (4″***S***)		
compound	IC_{50} (nM)	IC_{90} (nM)	compound	IC_{50} (nM)	IC_{90} (nM)	compound	IC_{50} (nM)	IC_{90} (nM)
3a	ND[b]	ND	**3b**	42.40	74.87	**3c**	50.78	77.27
4a	ND	ND	**4b**	19.90	47.02	**4c**	31.76	93.05
5	40.94	76.72	**12**	15.74	30.79	**13**	27.96	53.46
6	ND	ND	**14**	12.35	23.37	**15**	34.12	50.74
7	221.21	ND	**16**	14.72	24.25	**17**	135.85	153.01
8	ND	ND	**18**	32.87	42.71	**19**	66.75	108.96
9	168.70	202.58						
10	84.74	124.68						
11	346.75							
24	10.26	27.78	**20**	6.24	11.02	**21**	27.17	69.71
25	19.65	37.85	**22**	12.24	18.47	**23**	39.17	102.04
27	3547							
ART[c]	13.04	17.40	**MFQ**	16.44	65.71	**CQ**	144.90	268.76

[a] *P. falciparum* multidrug resistant TM91C235 strain (Thailand). [b] Not determined. [c] Average of greater than eight replicates.

activity against both D6 and W2 (Table 1, $IC_{90} \sim 19$ nM), and at the same time, it shows a small IC_{90}/IC_{50} (W2) ratio of 1.23 (as compared with 1.72 (W2) of artemisinin and compared with 2.15 (W2) of mefloquine). Second, compound **16** was 10 times more active than CQ against the multidrug-resistant strain TM91C235, with an $IC_{90} = 24.25$ nM, and its activity was comparable to that of artemisinin ($IC_{90} = 17.40$ nM, Table 2). In addition, of the three *N*-(2-dimethylamino)ethyl CA derivatives **22**, **23**, and **25** that have been prepared, the 4″*R* derivative **22** was again the most potent in the series (Tables 1 and 2, Chart 1). The anilides of CA series, **20**, **21**, and **24**, exert even higher activity when compared with the related *N*-(2-dimethylamino)ethyl derivatives, as well as to the corresponding DCA-derived tetraoxanes **8**, **18**, and **19**. Poor activity of tetraoxanes **9** and **10** clearly supports our earlier finding[12] that tertiary amides do not confer any appreciable antimalarial activity, while tetraoxane **27** is totally inactive in the in vitro antimalarial screen. The observed inactivity of fullerene derivative **27** indicates that the fullerene part acts as a radical sponge.[13] It is feasible to envisage that radicals generated upon reaction of tetraoxane moiety with Fe(II) were trapped by the fullerene part of the molecule within the cell.

It became obvious that the additional C(7) acetyloxy group of cholic acid confers significant in vitro antimalarial activity to the analogues and also plays an important role in our pharmacophore model.[14] In order to better comprehend the activity difference between the CA and the DCA series, we submitted amides **16**, **22**, **23**, and **25** to mice infected with *P. berghei* using a modified Thompson test. In addition, in vitro metabolism studies were performed for the same compounds to assess the bioavailability of drug candidates upon oral administration. Metabolic stability assays and metabolite identification were done using human and mouse liver microsomes to help gauge the first-pass metabolism of the drug candidates in relevant species.[5d]

The mice were infected on day 0, and the tested compounds were administered orally on days 3, 4, and 5 postinfection (Table 3). The data showed that cyclohexylidene CA-derived tetraoxane **25** (Chart 1, R = H, X = $NHCH_2CH_2N(CH_3)_2$) cured all mice at 320 mg/kg/day without any parasitemia on day 31. Compound **25** also cured 3/5 and 2/5 mice at the lower doses of 80 and 40 mg/kg/day, respectively. Having the quite active *N*-(2-dimethylamino)ethyl derivative **25** and being limited by the quantity of substances, we tested **16**, **22**, and **23**, at doses ≤320 mg/kg/day. DCA derivative **16** cured 3/5 mice at 160 mg/kg/day; however, the activity sharply declined when the lower dose of 40 mg/kg/day was administered (0/5 cured and no delay in patency). Of the two epimeric CA-based tetraoxanes **22** and **23**, the former 4″*R* is more active with a minimal curative dose of 40 mg/kg/day.

Antiproliferative Activity

Fifteen tetraoxanes were chosen by NIH−NCI for in vitro screening using a diverse panel of ∼60 human cancer cell lines starting at a concentration of 10^{-4} M.[15] The results, summarized in Table 4, reveal that most compounds tested in antiproliferative screen demonstrated considerable growth inhibition ($GI_{50} < 100$ μM; 50% growth inhibitory activity) against nearly all of the

Table 3. Activity of Tetraoxanes **16**, **22**, **23**, and **25** against *P. Berghei* in Vivo (po)[a]

compound	mg/kg/day	mice dead/day died	mice alive day 31/ total	survival time (days)[b]	met. stab., $t_{1/2}$ (min)	metabolite identity
16 (4″*R*)	160	2/27	3/5	29	human, 110	hydroxylation (1)
	40	2/8, 2/9, 1/10	0/5	9		dihydroxylation (1)
					mouse, >120	hydroxylation (1)
						dihydroxylation (1)
22 (4″*R*))	40	1/9, 1/13, 1/19, 1/24	1/5	19	human, 42	dehydration (2)
		3/8, 1/9, 1/10			mouse, 77	hydroxylation (1)
	20		0/5	9		dihydroxylation (1)
						dehydration
23 (4″*S*))	320	1/12, 1/14, 2/15	1/5	17	human, >120	none detected
	80	1/8. 1/9, 2/10, 1/16	0/5	11	mouse, >120	none detected
25	320		5/5	31	human, 40	hydroxylation (2)
	80	1/13, 1/20	3/5	25		dihydroxylation (3)
	40	1/11, 1/13, 1/17	2/5	21	mouse, 40	hydroxylation (1)
	10	1/7, 4/8	0/5	8		dihydroxylation (2)
infected controls[c]	0	7−9	0/5			

[a] Groups of five *P. berghei* (KBG 173 strain) infected CD-1 mice were treated on days 3, 4, and 5 postinfection with tetraoxanes suspended in 0.5% hydroxyethylcellulose−0.1% Tween 80. Mice alive on day 31 with no parasites in a blood film are considered cured. [b] Including cured mice. [c] All non-infected age controls survived (5/5).

Table 4. Summary of the NCI−DTP 60-Cell-Line Screening for Tetraoxanes **6**, **7**, **12**, **15**−**17a**, **19**−**23**, **25**, and **26**

compd	no. of cell lines[a]	no. of cell lines with GI_{50} < 100 μ**M**[b]	range of GI_{50} (μ**M**)
6	55	52	66.10 to 0.11
7	53	53	23.40 to <0.01
12	54	53	2.34 to 0.11
15	51	51	35.48 to 0.062
16	51	51	2.63 to <0.0050
16a	59	59	13.18 to 0.015
17	51	51	9.33 to <0.0050
17a	58	58	2.69 to 0.13
19	53	14	39.81 to <0.0050
20	44	29	54.95 to 0.16
21	49	35	67.60 to 0.18
22	49	49	3.02 to 0.18
23	48	45	12.88 to 0.057
25	53	53	19.95 to <0.01
26	54	54	12.30 to <0.01

[a] Cell lines for which results were reported by NIH−NCI. [b] GI_{50} refers to 50% growth inhibitory activity.

cancer cell lines reported by NIH−NCI. It is of particular importance that each of the 15 tested tetraoxanes inhibited at least 1 of the cancer cell lines on a submicromolar scale, occasionally at 10 nM, and 3 times even at <5 nM. As expected, the screened compounds exhibited variable growth inhibition and cytotoxicity against different cancer cell lines. A more stringent analysis is shown in Table 5, in which only the results satisfying the following criteria are presented: GI_{50} < 100 nM or/and TGI (total growth inhibition) < 1 μM or/and LC_{50} < 6 μM. The assessed antiproliferative activity, expressed as GI_{50}, TGI, LC_{50} (concentration of the compound at which 50% of the cells are killed) were obtained applying the 48 h continuous drug exposure protocol using the SRB (sulforhodamine B) protein assay.[15] The most susceptible cell lines were melanoma (LOX IMVI), renal (UO-31), and non-small cell lung cancer (HOP-92/62). Out of 80 entries, eight possess GI_{50} values below 10 nM, and in 23 instances, GI_{50} values <100 nM were observed (Table 5). TGI at concentrations <800 nM was observed on 44 instances, and on 7 instances ≤20 nM. The LC_{50} values confirm the potency of our compounds with 26 instances of activity <1 μM. The obtained results indicate that our compounds exhibit very high antiproliferative activity in a dose-dependent manner.

The two most active compounds, **16** and **25**, possessed an *N*-(2-dimethylamino)ethyl terminus (Table 5). Deoxycholic derivative **16** totally arrests cancer cell growth (TGI) between 0.4 and 0.007 μM, and it is most active against colon cancer and melanoma cell lines with LC_{50} = 47 nM and 22 nM, respectively (Figure 2). Its high general toxicity against cancer cells is reflected by an LC_{50} MG-MID value of −5.39 (mean graph midpoint, see Supporting Information for details).

Cholic acid-derived tetraoxane **25**, which also possesses an *N*-(2-dimethylamino)ethyl terminus, most efficiently inhibits melanoma, NSCL, colon, CNS, and renal cancer cells in the submicromolar range (Table 5). The compound was most active against NSCL HOP-62 and melanoma M14 cancer cells with LC_{50} values of 83 nM and 69 nM, respectively (Figure 3a). Highly specific compounds are the *N*-(2-dimethylamino)ethyl-containing compound **17** and the primary amide **26**. Tetraoxane **17** totally arrests melanoma LOX IMVI cell growth at ∼5.2 nM (TGI) and kills them with LC_{50} ∼56 nM (log LC_{50} = −7.25; MG-MID = −4.65); **26** is highly specific against renal UO-31 cancer cells (Figure 3b,c) with LC_{50} = 40 nM (log LC_{50} = −7.40; MG-MID = −4.88).

Discussion

In this report, we present the results of an extensive antimalarial and antiproliferative study of the cholic and deoxycholic acid-derived tetraoxanes seen in Scheme 1 and Chart 1. When tested in vitro against the three *P. falciparum* strains, D6, W2, and TM91C235, the compounds showed moderate to pronounced IC_{90} activity, with several of them being equally or more active than artemisinin. In general, CA derivatives were more active than related DCA compounds in both the in vitro (**7**, **16**, **17** vs **25**, **22**, **23**, Tables 1 and 2) and the in vivo (**16** vs **22**, Table 3) antimalarial screens. Tetraoxane **22** cured one mouse of five at a dose of 40 mg/kg/day, as compared with none cured using the same dose of **16**. In addition, although all compounds were relatively stable in the presence of human and mouse liver microsomes, with in vitro $t_{1/2}$ > 30 min, the more active CA derivative **22** appears considerably less stable metabolically than derivative **16**. Half-lives for **22** were 42 min (human) and 77 min (mouse), in comparison with 110 and >120 min for **16**, respectively (Table 3). In addition, the observed greater in vivo activity of the (4″*R*)-methyl compound over the (4″*S*)-CA analogue (**22** vs **23**) is in accordance with the respective in vitro and in vivo results (Tables 1, 2, and 3), and as above, the less active compound **23** is considerably more stable metabolically than its epimer

Table 5. In Vitro Antiproliferative Activities of Fifteen Tetraoxanes against Selected Cell Lines

			activity after 48 h (μM)		
compound (MG_MID_{TGI})	cell line		GI_{50}[a]	TGI[b]	LC_{50}[c]
6 (−4.64)	leukemia	RMPI-8226	0.11	0.65	6.82
7 (−5.01)	leukemia	MOLT-4	<0.010	>100	
	non-small cell lung cancer	NCI-H460	0.536	1.90	4.70
	renal cancer	786−0	0.159	0.298	0.559
12 (−5.43)	leukemia	HL-60(TB)	0.769	1.84	4.41
	non-small cell lung cancer	NCI-H23	0.660	1.71	3.39
	CNS cancer	SF-295	0.524	1.69	3.46
		SF-539	0.933	1.84	3.61
	melanoma	LOX IMVI	0.111	0.259	-
	renal cancer	A498	0.923	1.92	4.00
		SN12C	0.833	1.58	3.00
	prostate cancer	DU-145	0.699	1.73	3.34
	breast cancer	MDA-MB-231/ATCC	0.821	1.76	3.77
		MDA-MB-435	0.951	1.94	3.96
15 (−5.06)	non-small cell lung cancer	HOP-92	0.134	0.873	4.53
	melanoma	LOX IMVI	0.116	0.391	1.68
	renal cancer	CAKI-1	0.0622	0.163	0.429
		UO-31	0.122	0.329	-
	prostate cancer	DU-145	0.663	1.61	3.91
16 (−6.0)	leukemia	CCRF-CEM	0.056	0.17	0.50
	non-small cell lung cancer	HOP-62	0.054	0.23	1.16
	colon cancer	COLO 205	0.083	0.02	0.49
		HCC-2998	0.077	0.21	0.98
		HCT-116	0.0090	0.021	0.047
		HCT-15	0.094		
	CNS cancer	U251	0.026	0.087	
	melanoma	LOX IMVI	<0.005	0.0074	0.022
		M14	0.078	0.019	0.46
	renal cancer	786−0	0.060	0.15	0.39
	breast cancer	T-47D	0.13	0.41	
16a (−5.14)	leukemia	CCRF-CEM	0.257	0.539	>10
		HL-60(TB)	0.377	1.01	6.09
	non-small cell lung cancer	HOP-92	0.228	0.471	0.973
	melanoma	LOX IMVI	0.184	0.378	0.774
	renal cancer	786−0	0.626	2.04	5.43
		UO-31	0.206	0.421	0.864
17 (−5.62)	melanoma	LOX IMVI	<0.0050	0.00518	0.0564
	renal cancer	786−0	0.0813	0.149	
		ACHN	0.439	1.05	2.30
		CAKI-1	0.602	1.40	3.25
17a (−5.9)	leukemia	CCRF-CEM	0.186	0.384	0.792
		HL-60(TB)	0.264	0.477	0.863
		RPMI-8226	0.292	0.826	4.80
	non-small cell lung cancer	HOP-92	0.171	0.368	0.794
	melanoma	LOX IMVI	0.180	0.352	0.689
	ovarian cancer	IGROV1	0.160	0.338	0.712
	renal cancer	786−0	0.335	1.05	3.65
		UO-31	0.165	0.318	0.613
	prostate cancer	PC-3	0.367	1.62	4.45
		DU-145	0.641	1.91	4.49
19 (−4.37)	non-small cell lung cancer	HOP-92	<0.005	0.13	15.20
20 (−4.29)	melanoma	LOX IMVI	0.161	0.417	1.89
22 (−5.45)	leukemia	CCRF-CEM	0.258	0.725	
	non-small cell lung cancer	NCI-H322M	1.46	2.79	5.34
	melanoma	LOX IMVI	0.183		
	ovarian cancer	OVCAR-3	1.19	2.47	5.11
	renal cancer	CAKI-1	1.37	2.69	5.29
	breast cancer	MDA-MB-435	1.53	2.91	5.52
21 (−4.46)	melanoma	LOX IMVI	0.181	0.370	0.758
	non-small cell lung cancer	A549/ATCC	0.255	0.664	
		HOP-92	0.266	0.766	8.84
	renal cancer	UO-31	0.222	0.487	>10
23 (−5.55)	leukemia	CCRF-CEM	0.067	0.151	0.340
	non-small cell lung cancer	HOP-92	0.070	0.695	2.86
		NCI-H23	0.318	1.69	3.11
	melanoma	LOX IMVI	0.057	0.130	0.295
	renal cancer	ACHN	0.592	1.25	2.64
		SN12C	0.606	1.28	2.72
23 (−5.55)	breast cancer	MDA-MB-435	0.650	1.34	2.78
25 (−5.31)	non-small cell lung cancer	HOP-62	<0.01	0.0166	0.083
	colon cancer	HCT-116	0.0831	1.69	4.62
	CNS cancer	SF-268	0.785	2.25	5.35
	melanoma	LOX IMVI	<0.01	0.0224	0.0688
		M14	0.0440	0.613	2.92

Table 5. Continued

compound (MG_MID (TGI))	cell line		activity after 48 h (μM) GI$_{50}$[a]	TGI[b]	LC$_{50}$[c]
	renal cancer	786−0	0.783	2.09	4.57
26 (−5.39)	non-small cell lung cancer	NCI-H23	0.55	2.07	5.28
		NCI-H522	0.23	0.77	3.02
	ovarian cancer	IGROV1	0.032	0.12	0.43
	renal cancer	CAKI-1	0.20	1.12	3.59
		UO-31	<0.01	0.014	0.040

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

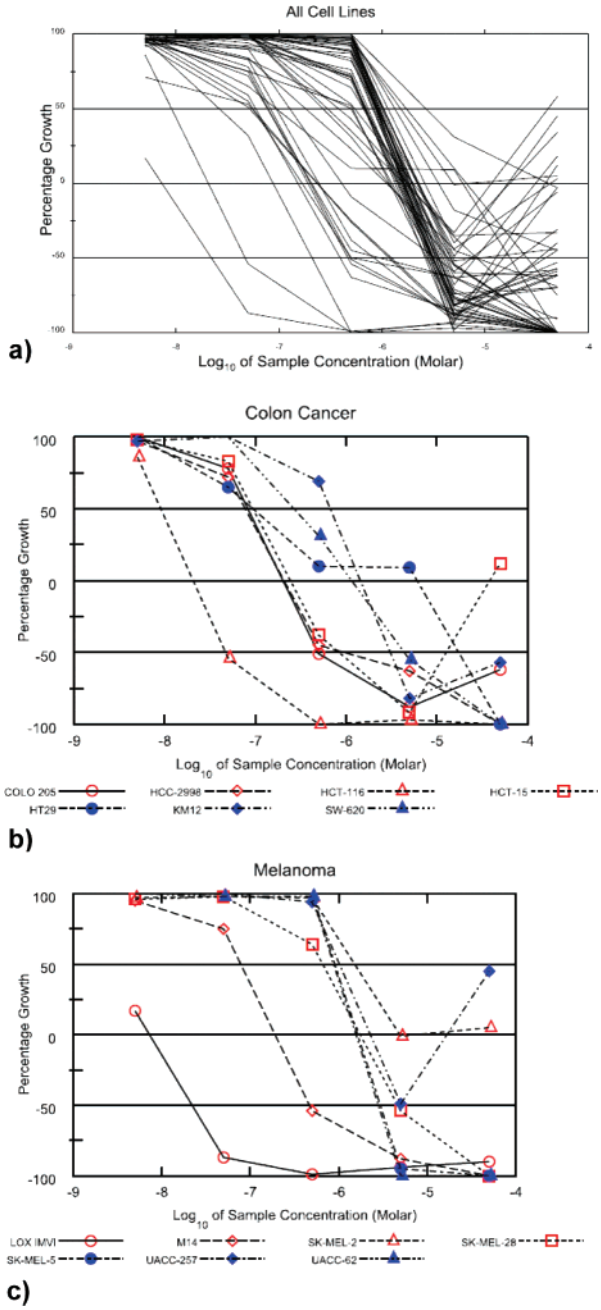


Figure 2. Inhibition of cancer cell growth 48 h after being exposed to tetraoxane deoxycholic derivative **16**. (a) All cancer cell lines, (b) colon cancer cells, and (c) melanoma cells.

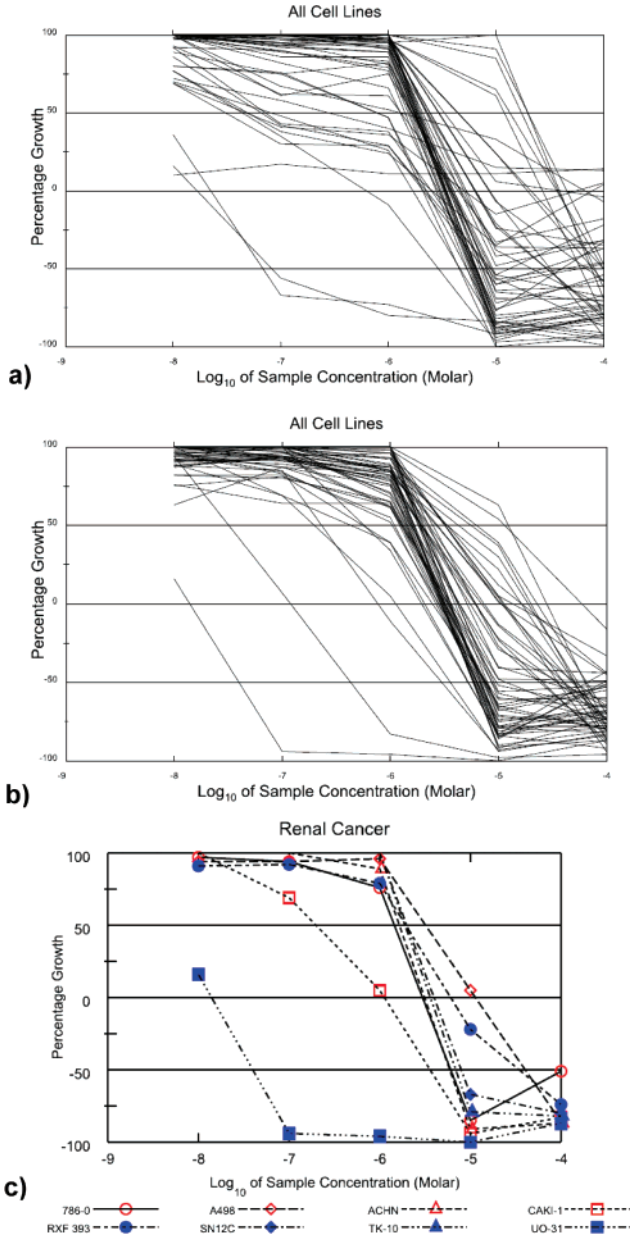


Figure 3. Inhibition of cancer cell growth 48 h after being exposed to tetraoxane cholic acid derivatives **25** and **26**. (a) All cancer cell lines as inhibited by compound **25**, (b) all cancer cell lines as inhibited by compound **26**, and (c) renal cancer cell lines inhibited by tetraoxane **26**.

22. These results collectively suggest that CA is favored over DCA as a potential tetraoxane antimalarial carrier.

The most active compound in vivo of all tested *N*-(2-dimethylamino)ethyl derivatives was cyclohexylidene derivative **25** (Chart 1, R = H, X = $NHCH_2CH_2N(CH_3)_2$), which has an MCD ≤ 40 mg/kg/day. Although **25** is the most metabolically labile of the tested analogues ($t_{1/2}$ = 40 min, human, mouse) and more than three metabolites were generated in human and mouse microsomal preparations, it is still a relatively stable compound, particularly in comparison with artemisinins. Furthermore, no toxicity was observed during in vivo screening,

and the tetraoxane moiety itself is metabolically stable (no peroxide bond scission was observed in in vitro ADME studies).[16] This indicates that the given tetraoxanes, their metabolic products, and their reductive scission products (upon exerting lethal effect on the parasite) were nontoxic to experimental animals (mice), unlike artesunate and some artemisinin-derived trioxane dimers.[17] This is of particular importance since the investigated compounds were shown to have dual activity, both antimalarial and antiproliferative.[18] Details are given in Table 3 and in the text above; however, it is important to add that the essentially nontoxic tetraoxane **25** (MTD > 960 mg/kg; MCD ≤ 40 mg/kg/day) kills two cancer cell lines (NSCL cancer HOP-62 and melanoma LOX IMVI) at LC_{50} ~70−80 nM and totally inhibits the cancer growth of the same cells at concentrations as low as 17−22 nM. Along the same lines is DCA derivative **16** (MTD > 480 mg/kg, 3/5 mice cured): it totally inhibits the cancer growth (TGI) of melanoma LOX IMVI cells at 7.4 nM, kills them at LC_{50} = 22 nM, and at the same time, it is toxic to colon cancer HCT-116 cells (LC_{50} = 47 nM). The current portfolio of potent antiproliferatives is complemented with primary amide **26,** which is specifically very potent against renal UO-31 cancer cells (LC_{50} = 40 nM) as well as tetraoxane **17**, which, like its C(4″) epimer **16**, is specifically active against LOX IMVI cells. It is difficult to and it would be speculative to compare the anticancer potential of our tetraoxanes to compounds tested on different cell lines;[19] however, one may assume on the basis of the same test that most of our compounds shown here and elsewhere[18] are considerably more potent than artemisinin (NSC 369397),[15] artemether (NSC 665970),[15] and certain deoxoartemisinin derivatives.[20] For example, all 15 compounds given in Table 5 have average $MG\_MID_{TGI} = -5.22$ while corresponding values for artemisinin and artemether are −4.00087 and −4.0044, respectively. In terms of the mode of action, we speculate that tetraoxanes may exert their antitumor activity by acting as an Fe(II)-sensitized radical source that induces apoptosis,[12] in a similar fashion to the artemisinins.[19,21,22]

To conclude, we presented the results of a study that demonstrated that DCA and CA tetraoxane derivatives are potent nontoxic antimalarials and antiproliferatives. We showed that the antimalarial potency of a mixed (deoxy)cholic acid-based tetraoxane both in vitro and in vivo depends on the stereochemistry of the substitution as well as the pattern at C(4″), which is in line with previous docking calculations.[14] In addition, on the basis of in vitro and in vivo results, as well as on our pharmacophore model,[14] the conclusion may be drawn that a CA-derived carrier is more effective than a DCA one.

Experimental Section[5b,5d]

Methyl 3,3-Dihydroperoxy-12α-acetoxy-5β-cholan-24-oate (2). Ketone **1** (5 g, 9.91 mmol) was dissolved at room temperature (r.t.) in a CH_3CN/CH_2Cl_2 mixture (200 mL, 3:1) followed by 30% H_2O_2 (10.3 mL, 0.1 mol) and a few drops of concd HCl. The reaction mixture was stirred for 2 h at r.t., quenched with water (20 mL), and was worked-up in the usual manner. The obtained crude product (5.15 g, 94%; colorless foam) was used in the following step. An analytical sample was obtained after column chromatography (Lobar B, LichroPrep Si 60, eluent heptane/EtOAc (7:3)). Colorless foam, softness at 68−70 °C. $[\alpha]_D^{20} = +73.8$ (c = 0.168, $CHCl_3$). Anal. ($C_{27}H_{44}O_8$) C, H.

General Procedure for Preparation of Mixed Tetraoxanes 3. To a cold (0 °C) solution of dihydroperoxide **2** (443.9 mg, 0.9 mmol) and ketone (1.80 mmol) in toluene (12.5 mL), 650 μL of an ice-bath cooled H_2SO_4:CH_3CN mixture (1:10, v/v) was added dropwise. The reaction mixture was stirred at 0 °C for 15 min, and after the usual workup, the crude product was purified by column chromatography to afford tetraoxane **3**.

Methyl 12α-Acetoxy-5β-cholan-24-oate-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (3a). Chromatography: dry-flash SiO_2, eluent heptane/EtOAc (8.5:2.5) and Lobar LichroPrep RP-18, eluent MeOH/H_2O (97.5/2.5)). Yield 178 mg (34%). Colorless foam softens at 74−76 °C. $[\alpha]_D^{20} = +65.7$ (c = 0.07, $CHCl_3$). Anal. ($C_{33}H_{52}O_8 \times H_2O$) C, H.

Methyl 12α-acetoxy-5β-cholan-24-oate-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*R*)- and (4″*S*)-methyl)cyclohexane (3b and 3c). According to the general procedure for mixed tetraoxane **3**, *gem*-dyhidroperoxide **2** (8.46 g, 17.0 mmol) was transformed into **3b** and **3c**. The crude reaction mixture was purified by column chromatography (dry-flash SiO_2, eluent heptane: EtOAc = 8.5/1.5 and Lobar B, LichroPrep RP-18, eluent MeOH/H_2O = 97/3). Isomers were separated by column chromatography on Lobar B, LichroPrep Si 60, eluent heptane: EtOAc = 95/5. 3b (4″*R*): Yield 2.96 g (29%). Colorless foam softens at 78−80 °C. $[\alpha]_D^{20} = +79.2$ (c = 0.048, $CHCl_3$). Anal. ($C_{34}H_{54}O_8$) C, H. 3c (4″*S*): Yield 2.36 g (23%). Colorless foam softens at 80−83 °C. $[\alpha]_D^{20} = +73.8$ (c = 0.084, $CHCl_3$). Anal. ($C_{34}H_{54}O_8 \times 0.5\ H_2O$) C, H.

12α-Acetoxy-5β-cholan-24-oic Acid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (4a). Methyl ester **3a** (3.65 g, 6.33 mmol) was hydrolyzed at 80 °C with NaOH (380 mg, 9.5 mmol) in *i*-PrOH/H_2O mixture (160 mL, 3:1 v/v). After 15 min, the reaction was cooled and diluted with 50 mL H_2O and 100 mL CH_2Cl_2. The water layer was acidified to pH 2 with diluted HCl, and layers were separated. The water layer was further extracted with CH_2Cl_2 (3 × 30 mL), the combined organic layers were washed with water and brine, dried over anhyd Na_2SO_4, and evaporated to dryness. Yield 3.28 g (92%). Colorless foam softens at 99−102 °C. $[\alpha]_D^{20} = +71.25$ (c = 0.08, $CHCl_3$). Anal. ($C_{32}H_{50}O_8 \times 0.5\ H_2O$) C, H.

12α-Acetoxy-5β-cholan-24-oic Acid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*R*)-methyl)cyclohexane (4b). Using the same procedure as described for **4a**, we hydrolyzed methyl-ester **3b** (1.5 g, 2.54 mmol) to 1.45 g (99%) **8b**. Colorless foam softens at 115−117 °C. $[\alpha]_D^{20} = +80.8$ (c = 0.078, $CHCl_3$). Anal. ($C_{32}H_{52}O_8 \times 0.5\ H_2O$) C, H.

12α-Acetoxy-5β-cholan-24-oic Acid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*S*)-methyl)cyclohexane (4c). Using the same procedure as described for **4a**, we hydrolyzed methyl-ester **3c** (1.5 g, 2.54 mmol) to **4c** 1.45 g **(**98%) **8c**. Colorless foam softens at 119−122 °C. $[\alpha]_D^{20} = +67.3$ (c = 0.11, $CHCl_3$). Anal. ($C_{32}H_{52}O_8 \times 0.5\ H_2O$) C, H.

General Procedure for Preparation of Amides 5−19. A solution of **4a** (341.4 mg, 0.6 mmol) in dry CH_2Cl_2 (20 mL), with added Et_3N (84 μL, 0.6 mmol) and $ClCO_2Et$ (58 μL, 0.6 mmol), was stirred for 60 min at 0 °C. The given amount of amine was added, and after 30 min of stirring, the reaction mixture was warmed to r.t. After 90 min, it was diluted with H_2O; the layers were separated, and the reaction mixture was worked-up in a usual manner. Crude product was purified by column chromatography.

12α-Acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (5). Using a suspension of 10 equiv of NH_4Cl and 10 equiv of Et_3N in dry CH_2Cl_2 (20 mL), we obtained 287 mg (84%) of **5**. Column chromatography: Lobar B, LichroPrep Si 60, eluent EtOAc. Colorless foam softens at 106−108 °C. $[\alpha]_D^{20} = +77.5$ (c = 0.08, $CHCl_3$). Anal. ($C_{32}H_{51}NO_7$) C, H.

***N*-(*n*-Propyl)-12α-acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (6).** Using a 6 equiv of *n*-$PrNH_2$ (0.27 mL, 3.30 mmol) in dry CH_2Cl_2 (20 mL), we obtained 270.3 mg (82%) of **6**. Column chromatography: Lobar B, LichroPrep, eluent EtOAc/heptane = 7/3. Colorless foam softens at 87−89 °C. $[\alpha]_D^{20} = +76.4$ (c = 0.072, $CHCl_3$). Anal. ($C_{35}H_{57}NO_7 \times H_2O$) C, H.

***N*-(2-Dimethylamino)ethyl-12α-acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (7).** Using 6 equiv of *N*,*N*-dimethyl-ethylenediamine (0.34 mL,

3,16 mmol) in dry CH_2Cl_2 (20 mL), we obtained 262.9 mg (78%) of **7**. Preparative TLC on SiO_2: eluent $CHCl_3$/MeOH/NH_3 = 9/1/1. Colorless foam softens at 64−66 °C. $[\alpha]_D^{20} = +68.7$ (c = 0.064, $CHCl_3$). HRMS: m/z 633.4440 corresponding to a molecular formula $C_{36}H_{61}O_7N_2$ (error in ppm: 6.1).

***N*-Phenyl-12α-acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (8).** Using 6 equiv of $PhNH_2$ (0.31 mL, 3.37 mmol) in dry CH_2Cl_2 (20 mL), we obtained 307 mg (86%) of **8**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 2.5/7.5. Colorless foam softens at 122−124 °C. $[\alpha]_D^{20} = +67.1$ (c = 0.076, $CHCl_3$). Anal. ($C_{38}H_{55}NO_7$) C, H.

***N*,*N*-Di-(*n*-propyl)-12α-acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (9).** Using 6 equiv of $(n\text{-Pr})_2NH$ (0.45 mL, 3.27 mmol) in dry CH_2Cl_2 (20 mL), we obtained 298 mg (85%) of **9**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 4/6. Colorless foam softens at 139−140 °C. $[\alpha]_D^{20} = +74.3$ (c = 0.07, $CHCl_3$). Anal. ($C_{38}H_{63}NO_7 \times 0.5H_2O$) C, H.

12α-Acetoxy-5β-cholan-24-piperidine-24-on-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (10). Using 6 equiv of piperidine (0.32 mL, 3.22 mmol) in dry CH_2Cl_2 (20 mL), we obtained 295 mg (91%) of **10**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 4/6. Colorless foam softens at 72−74 °C. $[\alpha]_D^{20} = +69.4$ (c = 0.062, $CHCl_3$). Anal. ($C_{37}H_{59}NO_7$) C, H.

12α-Acetoxy-5β-cholan-24-hydrazide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (11). Using a suspension of 6 equiv of $NH_2NH_2 \times 2HCl$ and 12 equiv of Et_3N in dry CH_2Cl_2 (20 mL), we obtained 209.4 mg (63%) of **11**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 3/7. Colorless foam softens at 178−180 °C. $[\alpha]_D^{20} = +70.3$ (c = 0.064, $CHCl_3$).

12α-Acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*R*)-methyl)cyclohexane (12). Using a suspension of 10 equiv of NH_4Cl and 10 equiv of Et_3N in dry CH_2Cl_2 (20 mL), we obtained 274 mg (99%) of **12**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 7/3. Mp = 150−153 °C (colorless prisms, Et_2O). $[\alpha]_D^{20} = +75.0$ (c = 0.096, $CHCl_3$). Anal. ($C_{33}H_{53}NO_7 \times 0.5\ H_2O$) C, H, N.

12α-Acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*S*)-methyl)cyclohexane (13). Using a suspension of 10 equiv of NH_4Cl and 10 equiv of Et_3N in dry CH_2Cl_2 (20 mL), we obtained 294.2 mg (99%) of **13**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 7/3. Mp = 104−106 °C (colorless powder Et_2O/hexane). $[\alpha]_D^{20} = +73.3$ (c = 0.086, $CHCl_3$). Anal. ($C_{33}H_{53}NO_7 \times 0.5\ H_2O$) C, H, N.

***N*-(*n*-Propyl)-12α-acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*R*)-methyl)cyclohexane (14).** Using 3 equiv of $n\text{-PrNH}_2$ (0.12 mL, 1.46 mmol) in dry CH_2Cl_2 (20 mL), we obtained 253.1 mg (84%) of **14**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 4/6. Mp = 113−115 °C (colorless prisms, Et_2O/hexane). $[\alpha]_D^{20} = +70.3$ (c = 0.074, $CHCl_3$). Anal. ($C_{36}H_{59}NO_7 \times 0.5\ H_2O$) C, H, N.

X-ray Analysis of 14. The X-ray intensity data were collected at 100 K with a MAR345 image plate using Mo Kα (λ = 0.71069 Å) radiation. A crystal of approximate dimensions 0.30 × 0.18 × 0.12 mm was chosen, mounted in inert oil, and transferred to the cold gas stream for flash cooling. The unit cell parameters were refined using all of the collected spots after the integration process. Molecular formula = $C_{36}\ H_{59}\ N\ O_7$, Mr = 617.84, monoclinic, $P2_1$, a = 13.039(4), b = 9.963(3), c = 25.874(8) Å, β = 93.76(2)°, V = 3354(2) Å^3, Z = 4, D_x = 1.22 g cm^{-3}, μ = 0.083 mm^{-1}, F(000) = 1352, T = 100 K.

A total of 13 698 reflections were collected from 131 images taken at a crystal-to-detector distance of 160 mm. There are 7190 independent reflections (R_{int} = 0.075). The structure was solved by direct methods with SHELXS97[23] and refined by full-matrix block least-squares on F^2 using SHELXL97. All of the nonhydrogen atoms were refined anisotropically. The hydrogen atoms were calculated with AFIX and included in the refinement with a common isotropic temperature factor. Final R values are R = 0.061 for 5951 observed reflections, R (all data) = 0.075, wR = 0.163, S = 1.03. The data have been deposited with the Cambridge Crystallographic Data Centre (Nr CCDC 649757).

***N*-(*n*-Propyl)-12α-acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*S*)-methyl)cyclohexane (15).** Using 3 equiv of $n\text{-PrNH}_2$ (0.12 mL, 1.46 mmol) in dry CH_2Cl_2 (20 mL), we obtained 245.7 mg (84%) of **15**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 4/6. Mp = 153−155 °C (colorless powder, Et_2O/hexane). $[\alpha]_D^{20} = +69.2$ (c = 0.078, $CHCl_3$). Anal. ($C_{36}H_{59}NO_7 \times 0.5\ H_2O$) C, H, N.

***N*-(2-Dimethylamino)ethyl-12α-acetoxy-5β-cholan-24-amid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*R*)-methyl)cyclohexane (16).** Using 2 equiv of *N*,*N*-dimethyl-ethylenediamine (97 μL, 0.92 mmol) in dry CH_2Cl_2 (20 mL), we obtained 246.3 mg (82%) of **16**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/MeOH/NH_3 = 70/2/1. Colorless foam softens at 96−97 °C. $[\alpha]_D^{20} = +70.4$ (c = 0.054, $CHCl_3$). HRMS-ESI: m/z 647.4654 corresponding to a molecular formula $C_{37}H_{63}O_7N_2$ (error in ppm: 2.9).

***N*-(2-Dimethylamino)ethyl-12α-acetoxy-5β-cholan-24-amid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*S*)-methyl)cyclohexane (17).** Using 2 equiv of *N*,*N*-dimethyl-ethylenediamine (0.102 mL, 0.96 mmol) in dry CH_2Cl_2 (20 mL), we obtained 251.6 mg (81%) of **17**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/MeOH/NH_3 = 70/2/1. Colorless foam softens at 72−74 °C. $[\alpha]_D^{20} = +57.5$ (c = 0.04, $CHCl_3$). HRMS-ESI: m/z 647.4604 corresponding to a molecular formula $C_{37}H_{63}O_7N_2$ (error in ppm: 4.8).

***N*-Phenyl-12α-acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*R*)-methyl)cyclohexane (18).** Using 3 equiv of $PhNH_2$ (0.13 mL, 1.40 mmol) in dry CH_2Cl_2 (20 mL), we obtained 283 mg (93%) of **18**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 15/85. Colorless foam softens at 109−112 °C. $[\alpha]_D^{20} = +59.8$ (c = 0.082, $CHCl_3$). Anal. ($C_{39}H_{57}NO_7 \times 0.5\ H_2O$) C, H, N.

***N*-Phenyl-12α-acetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*S*)-methyl)cyclohexane (19).** Using 3 equiv of $PhNH_2$ (0.13 mL, 1.40 mmol) in dry CH_2Cl_2 (20 mL), we obtained 264.8 mg (87%) of **19**. Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 15/85. Colorless foam softens at 110−113 °C. $[\alpha]_D^{20} = +56.8$ (c = 0.074, $CHCl_3$). Anal. ($C_{39}H_{57}NO_7 \times 0.5\ H_2O$) C, H, N.

***N*-Phenyl-7α, 12α-diacetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*R*)-methyl)cyclohexane (20).** Using the above general procedure for preparation of amides, we reacted 7α,12α-diacetoxy-5β-cholan-24-oic acid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*R*)-methyl)-cyclohexane[5b] (372.5 mg, 0.52 mmol) and $PhNH_2$ (188 μL, 2.06 mmol) to give amide **20** (366.8 mg, 83%). Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 2.5/7.5. Colorless foam softens at 134−136 °C. $[\alpha]_D^{20} = +45.3$ (c = 0.064, $CHCl_3$). Anal. ($C_{41}H_{59}NO_9 \times 2H_2O$) C, H, N.

***N*-Phenyl-7α,12α-diacetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*S*)-methyl)cyclohexane (21).** Using the above general procedure for preparation of amides, we reacted 7α,12α-diacetoxy-5β-cholan-24-oic acid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*S*)-methyl)-cyclohexane[5b] (316.2 mg, 0.50 mmol) and $PhNH_2$ (182 μL, 1.99 mmol) to give amide **21** (316.2 mg, 89%). Column chromatography: Lobar B, LichroPrep, eluent EtOAc : heptane = 2.5/7.5. Colorless foam softens at 133−136 °C. $[\alpha]_D^{20} = +47.6$ (c = 0.082, $CHCl_3$). Anal. ($C_{41}H_{59}NO_9 \times 2H_2O$) C, H, N.

***N*-(2-Dimethylamino)ethyl-7α,12α-diacetoxy-5β-cholan-24-amid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*R*)-methyl)cyclohexane (22).** Using the above general procedure for preparation of amides, we reacted 7α,12α-diacetoxy-5β-cholan-24-oic acid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-

((4″*R*)-methyl)cyclohexane[5b] (276.0 mg, 0.44 mmol) and *N*,*N*-dimethylethan-1,2,-diamine (185 μL, 1.74 mmol) to give amide **22** (259 mg, 84%). Dry-flash chromatography SiO_2: eluent EtOAc/MeOH/NH_3 = 25/1/2. Colorless foam softens at 92−94 °C. $[\alpha]_D^{20}$ = +41.9 (*c* = 0.074, $CHCl_3$). Anal. ($C_{39}H_{64}N_2O_9 \times H_2O \times NH_3$) C, H, N.

***N*-(2-Dimethylamino)ethyl-7α,12α-diacetoxy-5β-cholan-24-amid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*S*)-methyl)cyclohexane (23).** Using the above general procedure for preparation of amides, we reacted 7α,12α-diacetoxy-5β-cholan-24-oic acid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spiro-1″-((4″*S*)-methyl)cyclohexane[5b] (237.7 mg, 0.37 mmol) and *N*,*N*-dimethylethyl-1,2,-diamine (159 μL, 1.50 mmol) to give amide **23** (237.1 mg, 81%). Dry-flash chromatography SiO_2: eluent EtOAc/MeOH/NH_3 = 25/1/2. Colorless foam softens at 102−104 °C. $[\alpha]_D^{20}$ = +50.0 (*c* = 0.076, $CHCl_3$). Anal. ($C_{39}H_{64}N_2O_9 \times 1.5\ H_2O$) C, H, N.

***N*-Phenyl-7α,12α-diacetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (24).** Using the above general procedure for preparation of amides, we reacted 7α,12α-diacetoxy-5β-cholan-24-oic acid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane[5b] (266.6 mg, 0.43 mmol) and $PhNH_2$ (157 μL, 1.72 mmol) to give amide **24** (226.2 mg, 76%). Column chromatography SiO_2: Lobar B, LichroPrep, eluent EtOAc/heptane = 2.5/7.5. Colorless foam softens at 123−125 °C. $[\alpha]_D^{20}$ = +42.9 (*c* = 0.084, $CHCl_3$). Anal. ($C_{41}H_{57}NO_9 \times H_2O$) C, H, N.

***N*-(2-dimethylamino)ethyl-7α,12α-diacetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (25).** 7α,12α-Diacetoxy-5β-cholan-24-oic acid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane[5b] (300 mg, 0.483 mmol) was transformed into amide **25** (139.1 mg, 42%) using $Me_2NCH_2CH_2NH_2$ (318.4 μL, 2.9 mmol) in dry CH_2Cl_2 (25 mL). Crude product was purified using column chromatography Lobar B, LichroPrep RP-18; eluent MeOH/H_2O (95/5). Colorless foam softens at 79−85 °C. Anal. ($C_{38}H_{62}N_2O_9 \times 2\ H_2O$) C, H, N.

***N*-(2-Pyrrolidino-[3″,4″:1,9](C$_{60}$-*I*$_h$)^{5,6}fullerene-1-yl-ethyl)-7α,-12α-diacetoxy-5β-cholan-24-amide-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane (27).** To a suspension of *N*-(2-pyrrolidino-[3′,4′:1,9](C$_{60}$-*I*$_h$)^{5,6}fullerene)-1-yl-ethanaminium trifluoroacetate[24] (50.0 mg, 54.3 μmol) in dichloromethane (DCM, 10 mL), triethylamine (5.5 mg, 7.5 μL, 54.3 μmol) was added, and the reaction mixture was stirred at room temperature for 15 min. In another flask, an ice bath cooled solution of 7α,12α-diacetoxy-5β-cholan-24-oic acid-3-spiro-6′-(1′,2′,4′,5′-tetraoxacyclohexane)-3′-spirocyclohexane[5b] (33.4 mg, 54.3 μmol) in DCM (4 mL) was treated with ethylchloroformate (5.8 mg, 5.2 μL, 54.3 μmol) and triethylamine (5.5 mg, 7.5 μL, 54.3 μmol), and the reaction mixture was stirred at 0 °C for 5 min. Then, the suspension of fulleroamine was slowly added to the solution of the formed mixed anhydride, and the obtained mixture was stirred for 24 h at ambient temperature. After evaporation to dryness, the residue was subjected to dry flash column chromatography. Elution with PhMe/EtOAc 7/3 and subsequent precipitation from a DCM highly concentrated solution with Et_2O gave amide **27** (24.3 mg, 32%) as a brown powder. MALDI-TOF MS Anal. Calcd for $C_{98}H_{60}N_2O_9$: 1408. Found: 1408 $[M]^+$.

In Vitro Antimalarial Activity. The in vitro antimalarial drug susceptibility screen is a modification of the procedures first published by Desjardins et al.,[25] with modifications developed by Milhous et al.,[26] 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. Basically, groups of five mice were inoculated intraperitoneally with erythrocytes infected with a drug-sensitive strain of *P. berghei* on day 0. Drugs were suspended in 0.5% hydroxyethylcellulose−0.1% Tween 80 and administered orally once a day beginning on day 3 postinfection. Dosings are given in Table 3. Cure was defined as survival until day 31 posttreatment. Untreated control mice die on day 6−8 postinfection.

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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.

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