

Cholic Acid Derivatives as 1,2,4,5-Tetraoxane Carriers: Structure and Antimalarial and Antiproliferative Activity^{1,†}

Dejan Opsenica,[#] Gabriela Pocsfalvi,[▽] Zorica Juranić,[⊥] Bernard Tinant,[§] Jean-Paul Declercq,[§] Dennis E. Kyle,^{||} Wilbur K. Milhous,^{*,||} and Bogdan A. Šolaja^{*,‡}

Institute of Chemistry, Technology and Metallurgy, Belgrade, Yugoslavia, Centro Internazionale di Servizi di Spettrometria di Massa del CNR di Napoli, Naples, Italy, National Cancer Research Institute, Belgrade, Yugoslavia, Laboratoire de Chimie Physique et de Cristallographie, Université Catholique de Louvain, Louvain-la-Neuve, Belgium, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100, and Faculty of Chemistry, University of Belgrade, 11001 Belgrade, Yugoslavia

Received March 24, 2000

Cholic acid-derived 1,2,4,5-tetraoxanes were synthesized in order to explore the influence of steroid carrier on its antimalarial and antiproliferative activity in vitro. Starting with chiral ketones, cis and trans series of diastereomeric tetraoxanes were obtained, and the cis series was found to be ~2 times as active as the trans against *Plasmodium falciparum* D6 and W2 clones. The same tendency was observed against human melanoma (Fem-X) and human cervix carcinoma (HeLa) cell lines. The amide C(24) termini, for the first time introduced into the carrier molecule of a tetraoxane pharmacophore, significantly enhanced both antimalarial and antiproliferative activity, as compared to the corresponding methyl esters, with *cis*-bis(*N*-propylamide) being most efficient against the chloroquine-susceptible D6 clone (IC₅₀ = 9.29 nM). *cis*- and *trans*-bis(*N*-propylamides) were also screened against PBMC, and PHA-stimulated PBMC, showing a cytotoxicity/antimalarial potency ratio of 1/10 000.

Introduction

Malaria is still one of the most deadly diseases affecting more than 500 million people with over 1 million deaths per year.² Combating malaria is an important objective of WHO that established the Roll Back Malaria as one of its highest priority programs.³

The 1,2,4,5-tetraoxacyclohexane (tetraoxane) moiety became an increasingly interesting pharmacophore since its antimalarial activity⁴ was found to be very similar to that of 1,2,4-trioxanes such as naturally occurring artemisinin,⁵ its semisynthetic derivatives,⁶ and related compounds.⁷ Therefore, substantial efforts have been directed to the synthesis of 1,2,4,5-tetraoxacycloalkanes and association of the antimalarial activity thereof, both in vitro and in vivo.⁸ Further development of the tetraoxane carrier is additionally justified by indications of reduced sensitivity of certain *Plasmodium falciparum* strains to artemisinin.⁹

The design of an effective 1,2,4,5-tetraoxane-based antimalarial drug meets several problems, among others: (a) hydrophilic/lipophilic ratio of the carrier molecule; (b) stability under physiological conditions; (c) cytotoxic/antimalarial activity ratio of the investigated tetraoxane and of possible metabolites.

In continuation of our investigation on the synthesis of steroidal tetraoxanes and the evaluation of their antimalarial activity against *P. falciparum* African D6 and Indochina W2 clones,^{8a} we prepared several cholic acid-derived tetraoxanes differing in the C(24) substituent. The idea was to synthesize tetraoxanes with protected polar groups (easily to hydrolyze in vivo)^{8b} thus possibly rendering solubility under physiological conditions, to screen the activity of the compounds derived from the natural carrier differing in solubility within the series, and to investigate the influence of steric effect of two predicted diastereomeric series on their activity. In addition, the extensive in vitro antiproliferative activity screening of the synthesized compounds was envisaged to provide the cytotoxicity information.

Chemistry

Few methods proved effective for the 1,2,4,5-tetraoxacyclohexane (tetraoxane) ring synthesis. The hydrogen peroxide approach (30%,^{4,8a,10} 50%,^{8b} and 70–90%¹¹ H₂O₂) is the most widely used (along with various acid catalysts), while the (TMSO)₂/TMSOTf anhydrous procedure proved a good alternative to the above method.¹² Recent methods^{8d,13} did not prove better (20–50%). At present, all known procedures for tetraoxane synthesis afford symmetrically 3,6-disubstituted 1,2,4,5-tetraoxacyclohexane ring systems (only recently “mixed” tetraoxanes became available).¹⁴ Thus, when a single enantiomer of a chiral ketone is used as starting material, two diastereomers will be obtained.¹⁵ Therefore, it is reasonable to anticipate different activity of the diastereomers, providing additional information on the structure–activity relationship (SAR). In this work

[†] Part of the projected dissertation of D.O., University of Belgrade.

* Corresponding author: Bogdan Šolaja, Faculty of Chemistry, University of Belgrade, Studentski trg 16, P.O. Box 158, YU-11001 Belgrade, Yugoslavia. Phone: +381-11-63-86-06. Fax: +381-11-63-60-61. E-mail: bsolaja@chem.bg.ac.yu.

[#] Institute of Chemistry, Technology and Metallurgy.

[▽] Centro Internazionale di Servizi di Spettrometria di Massa del CNR di Napoli.

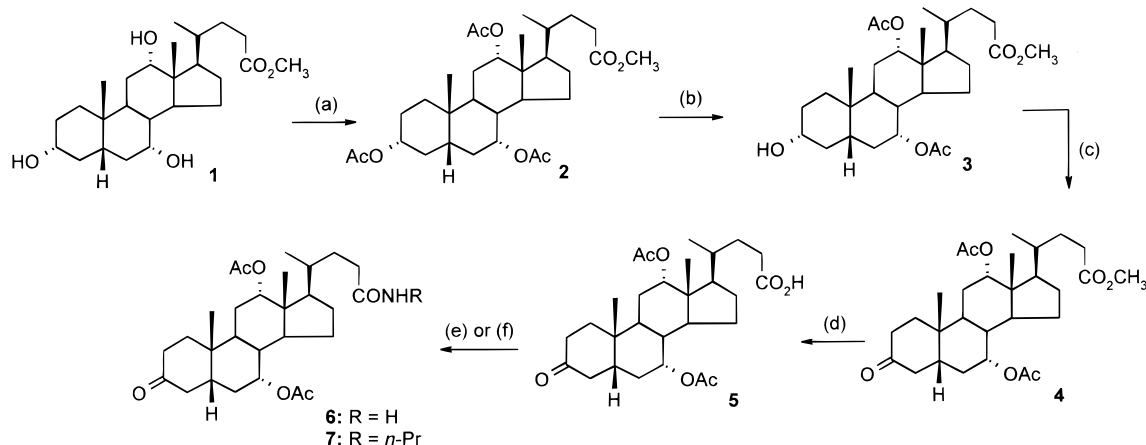
[⊥] National Cancer Research Institute.

[§] Université Catholique de Louvain.

^{||} Walter Reed Army Institute of Research.

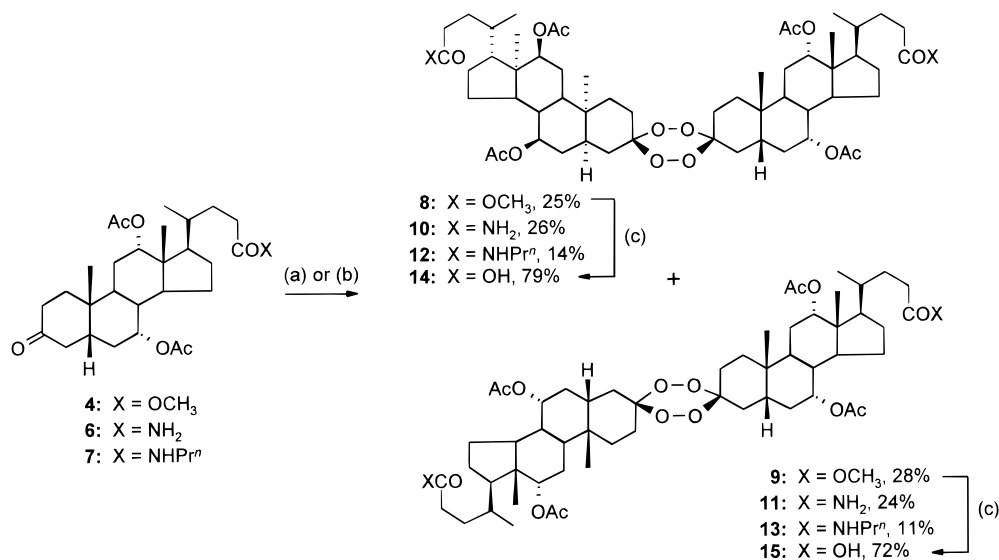
[‡] University of Belgrade.

Scheme 1^a



^a (a) $\text{Ac}_2\text{O/TMSOTf/CH}_2\text{Cl}_2$, 86%; (b) $\text{K}_2\text{CO}_3/\text{MeOH}$, 96%; (c) Jones/acetone, 81%; (d) $\text{NaOH/CH}_2\text{Cl}_2/\text{MeOH}$, 93%; (e) benzene/ SOCl_2 , $\text{NH}_4\text{Cl/Et}_3\text{N/CH}_2\text{Cl}_2$, 61%; (f) benzene/ SOCl_2 , $n\text{-PrNH}_2$, 68%.

Scheme 2^a



^a (a) Toluene/H₂O₂/H₂SO₄, 0–5 °C; (b) CH₂Cl₂/(Me₃Si)₂O₂/TMSOTf; (c) NaOH/CH₂Cl₂/MeOH.

we used the $\text{H}_2\text{O}_2/\text{H}^+$ and $(\text{TMSO})_2/\text{TMSOTf}$ procedures and ketones **4**, **6**, and **7** (Scheme 2) as starting materials.

The required ketones were synthesized as shown in Scheme 1. Methyl cholate was in three steps transformed into ketone **4** of analytical purity (67% from **1**). Further steps include high-yield selective hydrolysis of the methyl ester moiety (**4** \rightarrow **5**, 93%) and preparation of amides **6** (61%) and **7** (68%).

Tetraoxanes **8** and **9**, possessing a methyl ester functionality, were synthesized from ketone **5** using 32% H₂O₂/H₂SO₄/H₂O/EtOH/organic solvent mixtures at 0–5 °C (Scheme 2). The peroxyacetalization reaction was examined in various organic solvents (and their mixtures) such as dichloromethane, dioxane, acetonitrile, benzene, and toluene with the latter being the solvent of choice. In other solvents a complex mixture of easily decomposable products was obtained. Lower temperatures (up to –78 °C), where applicable, did not improve the tetraoxane yield, as well as the extension of the reaction time to more than 2 h. Tetraoxanes with a primary amide and *n*-propylamide moiety, **10/11** and **12/13**, were synthesized using the (TMSO)₂/TMSOTf method¹² from **6** and **7**, respectively. It is interesting to

note that while the $\text{H}_2\text{O}_2/\text{H}^+$ procedure worked well with the C(24) ester functionality, it failed with primary and secondary amides **6** and **7**. On the contrary, the (TMSO)₂/TMSOTf method was sluggish with the C(24) esters. The acids **14** and **15** were obtained by selective hydrolysis of corresponding methyl esters **8** and **9**, respectively.

The tetraoxane structure of synthesized compounds was deduced from their NMR spectra: each compound exhibited the characteristic signal at ca. 108 ppm (OO–C–OO moiety), Table 1. Liquid secondary ionization (LSI) and electrospray ionization (ESI) mass spectra of the above compounds yielded molecular ion peaks in all cases. These experiments along with the LSIMS high-resolution probe accurate mass measurements confirmed that tetraoxanes, and not hexaoxonanes (Table 1), were obtained.¹⁶

As expected, both diastereomeric tetraoxane series were formed, and although the ^1H and ^{13}C NMR spectra (CDCl_3) of each pair are almost identical (at the level of 200-MHz spectrometer), the clear distinction between tetraoxanes within each pair can be made on the basis of the appearance of the corresponding acetate methyl group signals. In one series, the acetate methyls appear

Table 1. Physical and Selected Spectral Data of Tetraoxanes **8**–**15**

compd	mp (°C)	$[\alpha]_D^{20}$	NMR (CDCl ₃)		MS [method] ^a
			¹ H acetate methyls	¹³ C C(3) carbons	
8	251–252	+74.60	2.10, 1.60 ^b	108.59, 108.91 ^b , 108.67 ^b	1063.8 (8) [M + Na] ⁺ , 1041.7 (10) [M + H] ⁺ [ESI]
9	167–170	+50.75	2.12, 2.07, 1.64 ^b	108.62, 108.77 ^b	1063.5 (1) [M + Na] ⁺ , 1041.6 (12) [M + H] ⁺ [ESI]
10	211–217	+72.06	2.10	108.62	1011.6 (100) [M + H] ⁺ [LSI]
11	196–199	+44.80	2.12, 2.07	108.66	1011.6 (80) [M + H] ⁺ [LSI]
12	240–243	+73.90	2.10	108.62	1095.7 (28) [M + H] ⁺ [LSI]
13	171–174	+41.66	2.12, 2.07	108.66	1095.8 (100) [M + H] ⁺ [LSI]
14	228–232	+71.04	2.00 ^c , 2.04 ^c	108.24 ^c	1035.6 (43) [M + Na] ⁺ [LSI]
15	199–202	+49.07	2.12, 2.08	108.68	1035.5 (100) [M + Na] ⁺ , 1013.5 (21) [M + H] ⁺ [LSI]

^a See Experimental Section for conditions. ^b Recorded in C₆D₆. ^c Recorded in DMSO-*d*₆.

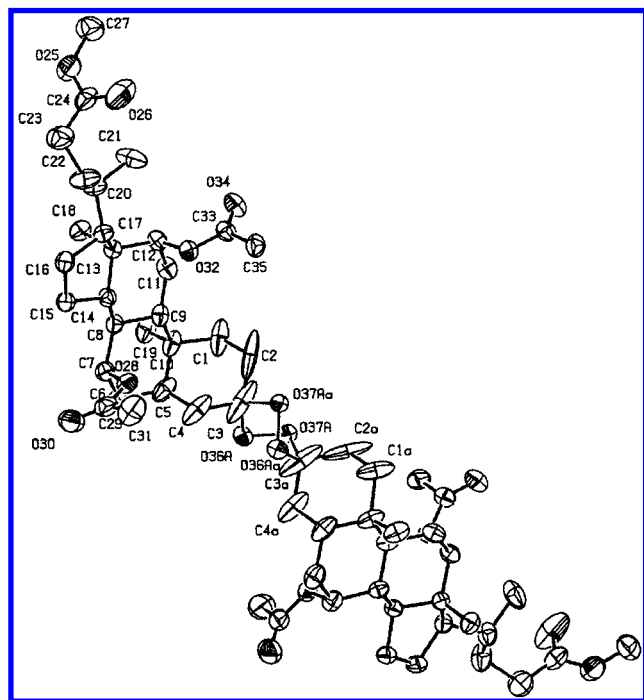


Figure 1. ORTEP plot of tetraoxane **8** (only position A for the O–O bridge is shown). For more clarity, hydrogen atoms are not represented. Cis substitution is apparent: C(2), C(2a) same side of the tetraoxane ring.

as a broad singlet (at ca. 2.10 ppm), in the another as a broad singlet (at ca. 2.12 ppm) followed by a smaller broad singlet (at ca. 2.07 ppm) (Table 1), indicating that the first series is *C*₂ symmetrical. In addition, the existence of two tetraoxane series is demonstrated by respective $[\alpha]_D$ values ($[\alpha]_D$ (**8**, **10**, **12**, **14**) = 71–74; $[\alpha]_D$ (**9**, **11**, **13**, **15**) = 42–50) and melting points (Table 1).

The substitution pattern of the tetraoxane ring (cis or trans) at C(3) and C(6) atoms (steroid numbering: C(3) and C(3'), respectively), i.e., the structure of each diastereomer, could not be determined on the basis of NMR spectral data; however, the successful preparation of the single crystal of tetraoxane **8** enabled us to solve the problem by X-ray crystallographic structural analysis. The tetraoxane **8** with *C*₂ symmetry is shown in Figure 1. Two positions (A and B) were found for the O(36)–O(37) atoms (crystallographic numbering). The six-membered 1,2,4,5-tetraoxane ring adopts a twisted conformation, unlike the simple tetraoxanes.¹⁷ The endocyclic torsion angles are: 82°, –44°, –40°, 82°, –44°, –40°, $\Delta C_2 = 4^\circ$ for position A (beginning with C(3)–O(36)–O(37)–C(3*)) and –77°, 40°, 43°, –77°, 40°, 43°, $\Delta C_2 = 3^\circ$ ¹⁸ for position B. The complete data,

atomic parameters, and geometry are given as Supporting Information.

On the basis of the above data, we propose that compounds **8**, **10**, **12** (and **14**) belong to the cis series (see Figure 1: *cis*-C(2),C(2a)), while **9**, **11**, **13** (and **15**) belong to the trans series. However, the conformation of the presented compounds in solution remains unknown at present.

Biochemistry

Antimalarial Activity. Antimalarial activity of compounds **8**–**15** against *P. falciparum* D6 and W2 clones was investigated (Table 2) showing that esters **8** and **9**, as well as the corresponding acids **14** and **15**, were inactive against both clones. The amide moiety in compounds **10**, **11** and **12**, **13**, for the first time introduced here as the auxiliary functional group in a tetraoxane molecule, increases the antimalarial activity of tetraoxanes **10**–**13**, with primary amides being somewhat more active against the *P. falciparum* chloroquine-resistant W2 clone, while *N*-propylamides were more active against the chloroquine-susceptible D6 clone. Most active was tetraoxane **12** with IC₅₀ = 9.29 nM against the D6 clone.

Antiproliferative Activity. The antiproliferative activity of all given compounds was tested against human melanoma Fem-X and human cervix carcinoma HeLa cell lines, and four compounds (**8**, **10**, **13**, and **14**) were chosen by NIH-NCI for in vitro screening (growth inhibitory activities, GI) using a diverse panel of 60 human cancer cell lines.²⁰ The tetraoxanes with the *N*-propylamide moiety were also screened for cytotoxic activity against normal human peripheral blood mononuclear cells (PBMC) nonstimulated and stimulated with phytohemagglutinine (PHA).

Tested tetraoxanes exhibit the antiproliferative activity at micromolar concentrations on various cell lines (Table 2). IC₅₀ values for Fem-X and HeLa cells were determined after 72 h of continuous agent's action by MTT test, while GI₅₀ values for 60 human cancer cell lines were obtained applying a 48-h continuous drug exposure protocol using SRB (sulforhodamine B) protein assay.²⁰

Methyl esters **8** and **9**, as well as *N*-propylamide **13**, were the least active of all screened compounds with IC₅₀ not below ca. 20 μM on Fem-X and HeLa cells. GI₅₀ MG_MID (meangraph midpoint) values derived for **8** and **13** are 81.3 and 57.5 μM, respectively, with 60 human cancer cell lines. The activity of tetraoxane **13** against renal cancers is quite interesting since it targets only UO-31 (GI₅₀ = 0.10 μM) and 786-0 cell lines (GI₅₀ = 4.36 μM). GI₅₀ MG_MID for this compound is 57.5

Table 2. In Vitro Antimalarial and Antiproliferative Activities of Tetraoxanes **8**–**15**

compd	<i>P. falciparum</i> IC ₅₀ (nM) ^a		antiproliferative activity		
	D6 ^c	W2 ^d	IC ₅₀ (μM) ^a 72 h		GI ₅₀ (μM) ^b 48 h
			Fem-X ^e	HeLa ^f	cell line (MG_MID)
8	>96	>96	>29	>29	CCRF-CEM ^g 14.8 (81.3)
9	>96	>96	19.5	20.5	
10	23.74	18.79	3.1	3.7	HCC-2998 ^h 0.97 (1.62)
11	128.58	59.35	6.2	6.0	
12	9.29	60.43	23	33	
13	20.08	30.12	94	166	UO-31 ⁱ 0.10 (57.5)
14	>99	>99	4.2	5.2	DU-145 ^j 1.2 (2.0)
15	>99	>99	7.6	9.2	
artemisinin	8.6 ^k	7.3 ^k			
chloroquine	EC ₅₀ (FCR-3) ^l	7.8	EC ₅₀ (FM3A) ^m	10	
<i>cis</i> -platinum	EC ₅₀ (FCR-3) ^l	18	EC ₅₀ (FM3A) ^m	32	
				3.5	

^a IC, inhibition concentration. ^b GI, growth inhibition. Presented are only the most sensitive cell lines to the corresponding drug out of 60. ^c *P. falciparum* African D6 clone. ^d *P. falciparum* Indochina W2 clone. ^e Human melanoma. ^f Human cervix carcinoma. ^g Leukemia. ^h Colon cancer. ⁱ Renal cancer. ^j Prostate cancer. ^k Taken from ref 17. ^l Chloroquine-sensitive FCR-3 strain.¹⁹ ^m Mouse mammary tumor FM3A.¹⁹

μM with GI₅₀ for 35 cell lines exceeding a concentration of 100 μM, meaning that the actual mean of GI₅₀ is >57.5 μM. In general, primary amides **10** and **11** exhibited more potent antiproliferative activity on Fem-X and HeLa cells as compared to *N*-propylamide congeners **12** and **13**. Moreover, the in vitro activity of tetraoxane **10** (as well as that of **14**) is quite similar to that of *cis*-platinum (Table 2). The mode of HeLa cells death induced by tetraoxane **12** was analyzed using fluorescence microscopy, after cell staining with acridine orange and ethidium bromide.²¹ Morphological examination of cells treated for 24 h with 2 × IC₅₀ (66 μM) of the above compound revealed that high concentration of tetraoxane **12** induced fragmentation of nuclei into many small clumps, typical for apoptosis.

Discussion

In this work steroidal tetraoxanes using a cholic acid carrier were synthesized and their antimalarial and antiproliferative activities evaluated. It was found that an amide functionality renders enhanced antimalarial activity in vitro as compared to methyl ester and carboxylic acid moieties within the series of the congeners. The antimalarial activity of tetraoxane **12** is high (IC₅₀ = 9.29 nM), and **12** is almost as active as artemisinin (Table 2). In addition, the *N*-propylamide moiety significantly lowers the cytotoxicity within the series.

Having in mind the relatively low cytotoxicity of *N*-propylamides **12** and **13** on cancer cell lines and the pronounced antimalarial activity of **12**, they were further screened for cytotoxic activity against normal human peripheral blood mononuclear cells (PBMC) nonstimulated and stimulated with phytohemagglutinine (PHA) in 5 μg/mL concentration of nutrient medium. PBMC were obtained from three healthy volunteers. Determined IC₅₀ values for nonstimulated PBMC were >100 and >320 μM for tetraoxanes **12** and **13**, respectively. PHA-stimulated PBMC were somewhat less resistant to cytotoxic action of the investigated compounds with IC₅₀ values of 98, 90, and 100 μM for compound **12** (first, second, and third person's PBMC) and 270, 320, and 164 μM for compound **13**. Being affected by tetraoxanes **12** and **13** only at very high concentrations, the PBMC can be considered as resistant to their cytotoxic action.

Table 3. Comparison of Antimalarial Activity and Cytotoxicity of Tetraoxanes **12** and **13**

compd	antimalarial activity	cytotoxicity IC ₅₀ (μM) 72 h		
	IC ₅₀ (nM)	PHA-stimulated		selectivity
	D6 clone (A)	PBMC (B)	PBMC (C)	B/A C/A
12	9.29	>100	96 ^a	>10700 10333
13	20.08	>320	251 ^a	>15900 12500

^a Mean value of three people's PHA-stimulated PBMC inhibition is given in the text.

In Table 3 the inhibitory activity of **12** and **13** on the *P. falciparum* D6 clone, as well as on PBMC and PHA-stimulated PBMC, is compared. The high selectivity values combined with pronounced antimalarial activity of **12** indicate that tetraoxanes bearing the *N*-propylamide moiety are good lead compounds for further investigation.

Inspection of Tables 1 and 2 reveals also that the antiproliferative activity of the *cis* series (*cis*: **10**, **12**, **14**) is >2 times as great as that of the second diastereomeric series (*trans*: **11**, **13**, **15**). The antimalarial activity of the amides follows the same trend, with the exception of **12/13** ratio against the W2 clone.

Conclusion

In this work the two diastereomeric series of steroidal tetraoxanes with different bile acid side chain termini were synthesized and characterized, and their biological activity was evaluated. The antimalarial activity of bis-(*N*-propylamide) **12** on the *P. falciparum* D6 clone is very close to that of artemisinin, so considering its low toxicity on PBMC (and on cancer cell lines tested) it represents a good lead for further research in this area (selectivity > 10 000). The antiproliferative activity of some tetraoxanes is very close to that of *cis*-platinum, while *N*-propylamide **13** targets almost only renal cancer UO-31 out of the 60 cell lines tested, with GI₅₀ = 0.10 μM, TGI = 0.23 μM, and LC₅₀ = 0.54 μM.

Experimental Section

General. Melting points were determined on a Boetius PMHK apparatus and were not corrected. Specific rotations were determined on a Perkin-Elmer 141-MC at the given temperatures. IR spectra were recorded on a Perkin-Elmer spectrophotometer FT-IR 1725X. ¹H and ¹³C NMR spectra were

recorded on a Varian Gemini-200 spectrometer (at 200 and 50 MHz, respectively) in the indicated solvent using TMS as internal standard. Chemical shifts are expressed in ppm (δ) values and coupling constants (J) in Hz.

Mass spectra were taken on the following spectrometers: CI spectra were recorded on a MS Finnigan-MAT 8230 spectrometer with double focusing reverse geometry, using isobutane. LSI MS (FAB) spectra were recorded on a VG-ZAB-T instrument equipped with a Cs ion gun. Accelerating voltage was set to 8 kV using MNBA as matrix. Probe accurate mass measurements were performed in the presence of PEG internal calibrant at 5000 resolution. ESI MS spectra were recorded on an Autospec TOF instrument in positive ion mode using $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (1:1) with 1% AcOH as carrying solvent solution. The stock solutions of the samples were diluted in order to obtain 10 pmol/ μL solution using the carrying solvent. The source temperature was 75 °C, and the cone voltage was set to 40 V.

Thin-layer chromatography (TLC) was performed on pre-coated Merck silica gel 60 F_{254} plates, using *N,N*-dimethyl-*p*-phenylenediammonium dichloride peroxide reagent for peroxide moiety detection,²² and Lobar LichroPrep Si 60 (40–63 μm) columns coupled to a Waters RI 401 detector were used for column chromatography.

Methyl 3 α ,7 α ,12 α -Triacetoxy-5 β -cholan-24-oate (2). Methyl cholate (**1**) (1.00 g, 2.37 mmol, mp = 157–158 °C) was dissolved in the previously prepared solution of Ac_2O (1.00 mL) and TMSOTf (26 μL , 0.14 mmol) at room temperature. After stirring for 5 min, the reaction was quenched with saturated NaHCO_3 and worked-up as given in ref 23 to obtain 1.12 g (86%) of **2** as light yellow oil. Crystallization from acetone/hexane afforded pure **2**. Mp = 93–96 °C (colorless powder) (lit.²⁴ mp = 90.5–91 °C). IR (film): 3021m, 2948s, 2871s, 1734s, 1468m, 1438s, 1378s, 1245s, 1063s, 1022s, 756s cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 5.09 (bs, H–C(12)), 4.91 (d, J = 2.6 Hz, H–C(7)), 4.65–4.50 (m, H–C(3)), 3.66 (s, $\text{CH}_3\text{O}_2\text{C}(24)$), 2.14 (s, $\text{CH}_3\text{COO}-$), 2.09 (s, $\text{CH}_3\text{COO}-$), 2.05 (s, $\text{CH}_3\text{COO}-$), 0.92 (s, $\text{H}_3\text{C}-\text{C}(10)$), 0.81 (d, J = 6.2 Hz, $\text{H}_3\text{C}-\text{C}(20)$), 0.73 (s, $\text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, CDCl_3): 174.6, 170.5, 170.4, 75.36, 74.05, 70.6, 51.5, 47.3, 45.0, 43.4, 40.9, 37.7, 34.6, 34.3, 31.2, 30.8, 30.7, 28.8, 27.1, 26.8, 25.5, 22.7, 22.5, 21.6, 21.5, 17.4, 12.1. MS (CI, isobutane, m/z): 549 ($\text{M}^+ + \text{H}$), 489 ($\text{M}^+ - \text{AcOH}$), 429 ($\text{M}^+ - 2 \times \text{AcOH}$), 369 ($\text{M}^+ - 3 \times \text{AcOH}$).

Methyl 3 α -Hydroxy-7 α ,12 α -diacetoxy-5 β -cholan-24-oate (3). Triacetate **2** (5.30 g, 9.66 mmol) was dissolved in dry methanol (80 mL), 2.40 g (17.4 mmol) of anhydrous K_2CO_3 was added and the suspension was stirred at room temperature for 2 h. The reaction was quenched by addition of acetic acid (3 mL), after CO_2 was liberated resulting solution was evaporated under reduced pressure, and the residue was transferred as EtOAc suspension to the separatory funnel. The organic layer was well washed with brine and dried over anhydrous Na_2SO_4 . Crystallization of the crude product from EtOAc/hexane mixture afforded methyl 3 α -hydroxy-7 α ,12 α -triacetoxy-5 β -cholan-24-oate (**3**; 4.72 g, 96%). Mp = 59–62 °C (colorless plates) (lit.²⁵ mp = 57–59 °C). IR (KBr): 3446m, 2953s, 2870s, 1735s, 1654m, 1467m, 1379s, 1245s, 1101s, 1075s, 1022s cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 5.08 (bs, H–C(12)), 4.90 (d, J = 2.6 Hz, H–C(7)), 3.66 (s, $\text{CH}_3\text{O}_2\text{C}(24)$), 3.60–3.40 (m, H–C(3)), 2.13 (s, $\text{CH}_3\text{COO}-$), 2.09 (s, $\text{CH}_3\text{COO}-$), 0.91 (s, $\text{H}_3\text{C}-\text{C}(10)$), 0.81 (d, J = 6.0 Hz, $\text{H}_3\text{C}-\text{C}(20)$), 0.73 (s, $\text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, CDCl_3): 173.2, 169.4, 73.97, 69.9, 69.4, 50.0, 47.7, 43.5, 41.8, 39.5, 36.99, 36.2, 33.4, 33.0, 32.8, 29.8, 29.3, 29.2, 28.8, 27.4, 25.6, 23.99, 21.2, 21.06, 20.12, 19.9, 15.9, 10.6. MS (CI, isobutane, m/z): 507 ($\text{M}^+ + \text{H}$), 447 ($\text{M}^+ + \text{H} - \text{AcOH}$), 387 ($\text{M}^+ + \text{H} - 2 \times \text{AcOH}$), 369 ($\text{M}^+ + \text{H} - 2 \times \text{AcOH} - \text{H}_2\text{O}$).

Methyl 3-Oxo-7 α ,12 α -diacetoxy-5 β -cholan-24-oate (4). 3-Hydroxy compound **3** (2.00 g, 3.95 mmol) was oxidized by Jones reagent under standard reaction conditions to afford after repeated crystallization of the crude product ketone **4** (1.61 g, 81%). Mp = 196–198 °C (colorless prisms; benzene/hexane). $[\alpha]_D^{20} = +57.08$ (c = 0.97, CHCl_3) (lit.²⁵ mp = 190–191 °C; lit.²⁵ $[\alpha]_D^{20} = +61.60$ (c = 2.0, CHCl_3)). IR (KBr):

3441m, 2965m, 2923m, 2875m, 1745s, 1713s, 1435m, 1382m, 1258s, 1238s, 1215m, 1072m cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 5.13 (bs, H–C(12)), 5.00 (d, J = 2.8 Hz, H–C(7)), 3.66 (s, $\text{CH}_3\text{O}_2\text{C}$), 2.99 (dd, J = 13.2, 13.2 Hz, H α -C(4)), 2.12 (s, $\text{CH}_3\text{COO}-$), 2.07 (s, $\text{CH}_3\text{COO}-$), 1.02 (s, $\text{H}_3\text{C}-\text{C}(10)$), 0.82 (d, J = 6.2 Hz, $\text{H}_3\text{C}-\text{C}(20)$), 0.77 (s, $\text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, CDCl_3): 210.86, 173.20, 169.14, 168.86, 73.85, 69.20, 50.14, 45.97, 43.80, 43.15, 41.87, 40.75, 36.32, 35.21, 34.71, 33.17, 32.99, 29.44, 29.31, 28.38, 25.72, 24.40, 21.37, 20.22, 20.06, 19.90, 16.08, 10.82. MS (CI, isobutane, m/z): 506 ($\text{M}^+ + \text{H}$), 445 ($\text{M}^+ - \text{AcOH}$), 385 ($\text{M}^+ - 2 \times \text{AcOH}$), 367 ($\text{M}^+ - 2 \times \text{AcOH} - \text{H}_2\text{O}$).

3-Oxo-7 α ,12 α -diacetoxy-5 β -cholan-24-oic Acid (5). Methyl ester **4** (5.00 g, 9.9 mmol) was hydrolyzed at 80 °C with NaOH (600 mg, 15 mmol) in *i*-PrOH/ H_2O mixture (80 mL, 3:1 v/v). Upon completion of the hydrolysis (TLC) the reaction mixture was poured onto ice-cooled HCl acidified water. After filtration and crystallization 4.53 g (93%) of acid **5** was obtained. Mp = 200–201.5 °C (colorless powder, ethanol) (lit.^{26b} mp = 197 °C). $[\alpha]_D^{20} = +56.70$ (c = 0.97, CHCl_3). IR (KBr): 3500m, 2965m, 2878m, 1734s, 1713s, 1643w, 1437m, 1381s, 1254s, 1032m cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 5.14 (bs, H–C(12)), 5.01 (d, J = 2.4 Hz, H–C(7)), 3.00 (dd, J = 15.0, 13.8 Hz, H α -C(4)), 2.12 (s, $\text{CH}_3\text{COO}-$), 2.08 (s, $\text{CH}_3\text{COO}-$), 1.02 (s, $\text{H}_3\text{C}-\text{C}(10)$), 0.84 (d, J = 6.2 Hz, $\text{H}_3\text{C}-\text{C}(20)$), 0.77 (s, $\text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, CDCl_3): 212.49, 179.56, 170.54, 170.27, 75.18, 70.56, 47.27, 45.03, 44.46, 43.19, 42.04, 37.64, 36.53, 36.02, 34.45, 34.31, 30.79, 30.72, 30.41, 29.70, 27.02, 25.73, 22.71, 21.56, 21.42, 21.27, 17.41, 12.18. MS (CI, isobutane, m/z): 491 ($\text{M}^+ + \text{H}$), 431 ($\text{M}^+ - \text{AcOH}$), 371 ($\text{M}^+ - 2 \times \text{AcOH}$), 353 ($\text{M}^+ - 2 \times \text{AcOH} - \text{H}_2\text{O}$).

3-Oxo-7 α ,12 α -diacetoxy-5 β -cholan-24-amide (6). The acid **5** (3.00 g, 6.11 mmol) was dissolved in dry benzene (50 mL) under inert atmosphere, cooled to 0 °C followed by addition of SOCl_2 (534 μL , 7.35 mmol). The reaction mixture was then stirred at reflux for 3 h, cooled to room temperature, and solvent evaporated under reduced pressure. Such obtained crude acid chloride was dissolved in dry CH_2Cl_2 (25 mL), cooled to 0 °C, and the suspension of NH_4Cl (3.27 g, 61.1 mmol) and Et_3N (8.47 mL, 61.1 mmol) in dry CH_2Cl_2 (50 mL) was added. The stirring was continued for 15 min at 0 °C and at room temperature overnight. The reaction mixture was poured onto water, extracted with CH_2Cl_2 (3 \times 30 mL), organic extracts were washed with water and brine, and dried over anhydrous Na_2SO_4 . Crude product was purified by dry-flash chromatography (SiO_2) and after crystallization the pure amide **6** was obtained (1.97 g, 61%). Mp = 214 °C (colorless prisms, benzene/hexane). $[\alpha]_D^{20} = +57.59$ (c = 1.01, CHCl_3). IR (KBr): 3426m, 2964m, 2878m, 1714s, 1688s, 1626m, 1380m, 1254s, 1032m cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 5.46 (bs, NH_2 , exchangeable with D_2O), 5.14 (bs, H–C(12)), 5.00 (d, J = 2.6 Hz, H–C(7)), 2.99 (dd, J = 13.4, 13.4 Hz, H α -C(4)), 2.12 (s, $\text{CH}_3\text{COO}-$), 2.07 (s, $\text{CH}_3\text{COO}-$), 1.02 (s, $\text{H}_3\text{C}-\text{C}(10)$), 0.84 (d, J = 6.2 Hz, $\text{H}_3\text{C}-\text{C}(20)$), 0.77 (s, $\text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, CDCl_3): 212.18, 175.63, 170.47, 170.18, 75.22, 70.54, 47.47, 45.08, 44.52, 43.21, 42.098, 37.67, 36.58, 36.07, 34.67, 34.34, 32.65, 31.28, 30.85, 29.74, 27.13, 25.78, 22.74, 21.60, 21.45, 21.31, 17.56, 12.23. MS (CI, isobutane, m/z): 490 ($\text{M}^+ + \text{H}$), 430 ($\text{M}^+ + \text{H} - \text{AcOH}$), 370 ($\text{M}^+ + \text{H} - 2 \times \text{AcOH}$), 352 ($\text{M}^+ + \text{H} - 2 \times \text{AcOH} - \text{H}_2\text{O}$). Anal. ($\text{C}_{28}\text{H}_{43}\text{NO}_6 \cdot 0.5\text{H}_2\text{O}$) C, H.

***N*-(*n*-Propyl)-3-oxo-7 α ,12 α -diacetoxy-5 β -cholan-24-amide (7).** Starting with acid **5** (2.95 g, 6.01 mmol) the same procedure was applied as for **6**, only replacing the $\text{NH}_4\text{Cl}/\text{Et}_3\text{N}$ mixture by 2 equiv of *n*-Pr NH_2 . After crystallization of the crude product from benzene/hexane mixture, *N*-propylamide **7** was obtained (2.17 g, 68%). Mp = 216 °C (colorless needles, benzene/hexane). $[\alpha]_D^{20} = +50.10$ (c = 1.00, CHCl_3). IR (KBr): 3407m, 2954m, 2935m, 2871m, 1735s, 1718s, 1664s, 1534m, 1376m, 1258s, 1244s, 1028m cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 5.59–5.43 (m, H–N, exchangeable with D_2O), 5.13 (bs, H–C(12)), 5.00 (d, J = 2.8 Hz, H–C(7)), 3.30–3.17 (m, $\text{CH}_3\text{CH}_2\text{CH}_2\text{N}$), 2.99 (dd, J = 13.4, 13.4 Hz, H α -C(4)), 2.11 (s, $\text{CH}_3\text{COO}-$), 2.07 (s, $\text{CH}_3\text{COO}-$), 1.57–1.43 (m, $\text{CH}_3\text{CH}_2\text{CH}_2\text{N}$), 1.02 (s, $\text{H}_3\text{C}-\text{C}(10)$), 0.92 (t, J = 7.4 Hz, $\text{CH}_3\text{CH}_2\text{CH}_2\text{N}$), 0.83

(d, $J = 6.2$ Hz, $\text{H}_3\text{C}-\text{C}(20)$), 0.77 (s, $\text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, CDCl_3): 212.18, 175.63, 170.47, 170.18, 75.22, 70.54, 47.47, 45.08, 44.52, 43.21, 42.10, 37.67, 36.58, 36.07, 34.67, 34.34, 32.65, 31.28, 30.85, 29.74, 27.13, 25.785, 22.74, 21.60, 21.45, 21.31, 17.56, 12.23. MS (CI, isobutane, m/z): 532 ($\text{M}^+ + \text{H}$), 472 ($\text{M}^+ + \text{H} - \text{AcOH}$), 412 ($\text{M}^+ + \text{H} - 2 \times \text{AcOH}$). Anal. ($\text{C}_{31}\text{H}_{49}\text{NO}_6$) C, H.

Bis(methyl 3-dioxy-7 α ,12 α -diacetoxy-5 β -cholan-24-oate) (8 and 9). A solution of methyl ester **4** (400 mg, 0.79 mmol) in toluene (8.3 mL) was added to previously cooled (0 °C) solution of EtOH (1.20 mL), H_2O (1.12 mL) and H_2SO_4 (2.16 mL). After 15 min, 32% H_2O_2 (200 μL) was added and intensive stirring was continued next 2 h at 0 °C, when the reaction mixture was diluted with H_2O (20 mL) and toluene (30 mL). The organic layer was washed with water (2×10 mL), saturated NaHCO_3 (2×10 mL), brine, and dried over anhydrous Na_2SO_4 . Column chromatography (Lobar B, LichroPrep Si 60, eluent: heptane/EtOAc (7:3)) of the obtained complex mixture afforded two main fractions, which upon crystallization afforded **8** (104 mg, 25%) and **9** (116 mg, 28%). **8**: mp = 251–252 °C (colorless prisms, hexane/acetone). $[\alpha]_D^{20} = +74.60$ ($c = 1.10$, CHCl_3). IR (KBr): 2995m, 1737s, 1638w, 1440m, 1378m, 1250s, 1027m cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 5.09 (bs, $2 \times \text{H}-\text{C}(12)$), 4.92 (bs, $2 \times \text{H}-\text{C}(7)$), 3.66 (s, $2 \times \text{CH}_3\text{O}_2\text{C}(24)$), 2.10 (bs, $4 \times \text{CH}_3\text{COO}-$), 0.94 (s, $2 \times \text{H}_3\text{C}-\text{C}(10)$), 0.81 (d, $J = 5.8$ Hz, $2 \times \text{H}_3\text{C}-\text{C}(20)$), 0.73 (s, $2 \times \text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, CDCl_3): 174.48, 170.45, 108.59, 75.16, 70.52, 51.42, 47.196, 44.92, 43.19, 37.55, 34.52, 34.47, 32.05, 30.72, 30.61, 28.32, 27.04, 25.57, 22.67, 22.03, 21.54, 21.23, 17.37, 12.09. ^1H NMR (200 MHz, C_6D_6): 5.16 (bs, $2 \times \text{H}-\text{C}(12)$), 4.94 (bs, $2 \times \text{H}-\text{C}(7)$), 3.40 (s, $2 \times \text{CH}_3\text{O}_2\text{C}-$), 1.60 (bs, $2 \times \text{CH}_3\text{COO}-$), 0.80 (d, $J = 6.0$ Hz, $2 \times \text{H}_3\text{C}-\text{C}(20)$), 0.60 (s, $2 \times \text{H}_3\text{C}-\text{C}(10)$), 0.44 (s, $2 \times \text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, C_6D_6): 173.64, 169.53, 108.91, 108.67, 75.00, 70.43, 50.93, 47.65, 45.23, 43.81, 37.71, 34.80, 34.65, 31.01, 30.80, 30.6, 28.72, 27.30, 25.99, 22.95, 22.02, 22.09, 20.56, 17.56, 12.17. MS (ESI, m/z): 1063.8 ($[\text{M} + \text{Na}]^+$, 8), 1041.7 ($[\text{M} + \text{H}]^+$, 10), 981.6 (4), 921.7 (14), 597.0 (13), 579.0 (20), 537.4 (61), 477.3 (50), 417.3 (18), 238.0 (60), 197.0 (100), 178.2 (54), 149.0 (35). Anal. ($\text{C}_{58}\text{H}_{88}\text{O}_{16}$) C, H. **9**: mp = 167–170 °C (colorless prisms, hexane/acetone). $[\alpha]_D^{20} = +50.75$ ($c = 1.10$, CHCl_3). IR (KBr): 2960m, 1738s, 1439m, 1378m, 1238s, 1026m cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 5.09 (bs, $2 \times \text{H}-\text{C}(12)$), 4.92 (bs, $2 \times \text{H}-\text{C}(7)$), 3.66 (s, $2 \times \text{CH}_3\text{O}_2\text{C}(24)$), 2.12 (bs, ca. $2 \times \text{CH}_3\text{COO}-$), 2.07 (bs, ca. $2 \times \text{CH}_3\text{COO}-$), 0.94 (s, $2 \times \text{H}_3\text{C}-\text{C}(10)$), 0.81 (d, $J = 5.8$ Hz, $2 \times \text{H}_3\text{C}-\text{C}(20)$), 0.73 (s, $2 \times \text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, CDCl_3): 174.51, 170.47, 108.62, 75.23, 70.59, 51.47, 47.27, 44.98, 43.24, 37.56, 34.58, 34.52, 30.78, 30.66, 28.33, 27.08, 25.62, 22.71, 22.02, 21.47, 21.31, 17.41, 12.13. ^1H NMR (200 MHz, C_6D_6): 5.15 (bs, $2 \times \text{H}-\text{C}(12)$), 4.95 (bs, $2 \times \text{H}-\text{C}(7)$), 3.38 (s, $2 \times \text{CH}_3\text{O}_2\text{C}(24)$), 1.64 (bs, $4 \times \text{CH}_3\text{COO}-$), 0.80 (d, $J = 5.2$ Hz, $2 \times \text{H}_3\text{C}-\text{C}(20)$), 0.66 (s, $2 \times \text{H}_3\text{C}-\text{C}(10)$), 0.45 (s, $2 \times \text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, C_6D_6): 173.64, 169.55, 108.77, 75.02, 70.48, 50.93, 47.67, 45.25, 43.89, 38.08, 37.71, 34.78, 34.67, 32.56, 31.03, 30.80, 30.60, 28.85, 27.28, 26.02, 25.06, 22.93, 22.06, 20.82, 20.53, 17.54, 12.19. MS (ESI, m/z): 1063.5 ($[\text{M} + \text{Na}]^+$, 1), 1041.7 ($[\text{M} + \text{H}]^+$, 12), 981.6 (1), 921.5 (6), 537.3 (51), 477.3 (75), 417.3 (47), 385.3 (45), 351.2 (17), 307.0 (100). Anal. ($\text{C}_{58}\text{H}_{88}\text{O}_{16}$) C, H.

X-ray Analysis of 8. A first data set was collected at room temperature but the structure could not be refined properly because of disorder and high thermal agitation in the lateral chain and for the atoms in the vicinity of C(3). With the hope to solve these disorder problems, it was decided to collect a new data set at low temperature (100 K).

The crystal (0.3 \times 0.3 \times 0.2 mm) was mounted in inert oil and transferred to the cold gas stream of a MAR345 image plate equipped with Mo K α graphite monochromatized radiation ($\lambda = 0.71069$ Å). Crystal data for $\text{C}_{58}\text{H}_{88}\text{O}_{16} \cdot \text{C}_3\text{H}_6\text{O}$ are as follows: $M_r = 1097.35$, orthorhombic, space group $P2_12_12_1$; $a = 23.525(8)$, $b = 8.606(3)$, $c = 15.982(5)$ Å; $V = 3236(2)$ Å 3 , $Z = 2$. A total of 36415 reflections were measured; 3356 were independent ($R_{\text{int}} = 0.051$). The structure was solved by direct

methods 27 and refined by full-matrix least-squares on F^2 . 28 Final R values: $R_1 = 0.099$ for 3193 reflections with $I > 2\sigma(I)$, $R_1 = 0.101$ for all data, $wR_2 = 0.295$. The oxygen atoms of the tetraoxane O(36) and O(37) (Figure 1, crystallographic numbering) occupy two positions (A and B) with site occupation factors of 0.52 and 0.48, respectively. In the final Fourier difference synthesis, four peaks of electron density ranging from 1.2 to 0.8 e Å $^{-3}$ could not be interpreted; this explains probably the relatively high R indices. The cocrystallized acetone molecule is located on the 2-fold axis.

Bis(3-dioxy-7 α ,12 α -diacetoxy-5 β -cholan-24-oic acid) (14). Methyl ester **8** (250 mg, 0.24 mmol) was dissolved in $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1) mixture (100 mL), 1.25 M NaOH (29.95 mL) solution was added at room temperature and the clear reaction mixture was stirred for 3 days. (1.25 M solution of NaOH was prepared by dissolving 2.50 g NaOH in 50 mL of MeOH:H $_2\text{O} = 95:5$ (v/v) mixture.) The reaction was quenched by addition of glacial AcOH (until pH 5), CH_2Cl_2 (30 mL) and water (50 mL) were added, and separated water layer was extracted with CH_2Cl_2 (5×30 mL). Combined organic layers were washed with water and brine, dried over anhydrous Na_2SO_4 , and evaporated to dryness. Crystallization from acetone/hexane gave 192 mg (79%) of **14** as colorless powder. Mp = 228–232 °C. $[\alpha]_D^{20} = +71.04$ ($c = 1.10$, DMSO). IR (KBr): 3450s, 2953s, 1737s, 1441w, 1380s, 1253s, 1126w, 1081m cm^{-1} . ^1H NMR (200 MHz, DMSO- d_6): 11.94 (bs, $2 \times \text{HO}_2\text{C}(24)$ exchangeable with D_2O), 4.98 (bs, $2 \times \text{H}-\text{C}(12)$), 4.82 (bs, $2 \times \text{H}-\text{C}(7)$), 2.04 (s, ca. $2 \times \text{CH}_3\text{COO}-$), 2.00 (s, ca. $2 \times \text{CH}_3\text{COO}-$), 0.91 (s, $2 \times \text{H}_3\text{C}-\text{C}(10)$), 0.74 (d, $J = 6.0$ Hz, $2 \times \text{H}_3\text{C}-\text{C}(20)$), 0.70 (s, $2 \times \text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, DMSO- d_6): 175.04, 169.97, 169.84, 108.24, 74.70, 70.28, 47.17, 44.77, 43.19, 36.88, 34.35, 30.84, 30.59, 28.00, 26.83, 25.37, 22.395, 22.12, 21.43, 21.08, 17.38, 12.09. MS (LSI, m/z): 1035.6 ($[\text{M} + \text{Na}]^+$, 43), 1012.6 (4), 993.6 (23), 969.6 (19), 951.6 (34), 891.5 (100), 849.5 (29), 794.0 (26), 780.0 (45), 766.1 (27), 749.1 (29), 735.0 (66%), 719.0 (34), 691.0 (36), 674.0 (47), 658.0 (42), 643.0 (53), 631.0 (58). Anal. ($\text{C}_{58}\text{H}_{84}\text{O}_{16} \cdot 2\text{H}_2\text{O}$) C, H.

Bis(3-dioxy-7 α ,12 α -diacetoxy-5 β -cholan-24-oic acid) (15). Starting with methyl ester **9** (130 mg, 0.12 mmol), the same procedure was repeated as with **8**. After crystallization from $\text{CH}_2\text{Cl}_2/i\text{-Pr}_2\text{O}$ the acid **15** was obtained as colorless powder (91 mg, 72%). Mp = 199–202 °C. $[\alpha]_D^{20} = +49.07$ ($c = 1.02$, CHCl_3). IR (KBr): 3473m, 3456m, 2953s, 1737s, 1441m, 1380s, 1250s, 1125w, 1081w, 1027m cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 5.09 (bs, $2 \times \text{H}-\text{C}(12)$), 4.93 (bs, $2 \times \text{H}-\text{C}(7)$), 2.12 (bs, ca. $2 \times \text{CH}_3\text{COO}-$), 2.08 (bs, ca. $2 \times \text{CH}_3\text{COO}-$), 0.94 (s, $2 \times \text{H}_3\text{C}-\text{C}(10)$), 0.82 (d, $J = 5.6$ Hz, $2 \times \text{H}_3\text{C}-\text{C}(20)$), 0.73 (s, $2 \times \text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, CDCl_3): 179.48, 170.56, 170.38, 108.68, 75.29, 70.66, 47.29, 45.03, 43.28, 37.64, 34.62, 34.51, 30.68, 30.46, 28.39, 27.11, 25.66, 22.74, 22.05, 21.52, 21.36, 17.45, 12.18. MS (LSI, m/z): 1035.6 ($[\text{M} + \text{Na}]^+$, 100), 1013.5 ($[\text{M} + \text{H}]^+$, 21), 1011.4 (17), 993.5 (27), 951.4 (41), 909.4 (11), 893.4 (51), 849.5 (12), 833.4 (17), 779.9 (14), 734.9 (17), 699.5 (15), 673.9 (13), 644.9 (16), 628.9 (15). Anal. ($\text{C}_{58}\text{H}_{84}\text{O}_{16}$) C, H.

Bis(3-dioxy-7 α ,12 α -diacetoxy-5 β -cholan-24-amide) (10 and 11). Using procedure as given in ref 12, **6** (500 mg, 1.02 mmol) was treated with TMSOTf/TMS $_2\text{O}_2$ solution in CH_3CN . The reaction mixture was poured into well-stirred benzene/ NaHCO_3 /ice/water mixture, extracted with benzene, washed with brine and dried over anhydrous Na_2SO_4 . Column chromatography (Lobar B, LichroPrep Si 60, eluent: EtOAc/THF (7:3)) of the obtained complex mixture afforded two main fractions, which upon crystallization afforded **10** (133 mg, 26%) and **11** (124 mg, 24%). **10**: mp = 211–217 °C (colorless powder, $\text{CH}_2\text{Cl}_2/i\text{-Pr}_2\text{O}$). $[\alpha]_D^{20} = +72.06$ ($c = 1.30$, CHCl_3). IR (KBr): 3452s, 2955s, 2873m, 1718s, 1672s, 1443m, 1379s, 1256s, 1166w, 1126m, 1104m, 1080w, 1029w, 967w cm^{-1} . ^1H NMR (200 MHz, CDCl_3): 5.45 (bs, $2 \times \text{NH}_2$), 5.09 (bs, $2 \times \text{H}-\text{C}(12)$), 4.92 (bs, $2 \times \text{H}-\text{C}(7)$), 2.10 (bs, $4 \times \text{CH}_3\text{COO}-$), 0.94 (s, $2 \times \text{H}_3\text{C}-\text{C}(10)$), 0.83 (d, $J = 5.8$ Hz, $2 \times \text{H}_3\text{C}-\text{C}(20)$), 0.73 (s, $2 \times \text{H}_3\text{C}-\text{C}(13)$). ^{13}C NMR (50 MHz, CDCl_3): 175.70, 170.54, 108.62, 75.23, 70.59, 47.41, 45.01, 43.24, 37.62, 34.60, 32.65, 31.30, 30.59, 28.39, 27.15, 25.64, 22.74, 22.09, 21.62, 21.32,

17.54, 12.20. MS (LSI, m/z): 1011.6 ($[M + H]^+$, 100), 967.7 (17), 909.6 (16), 891.6 (29), 851.6 (15), 793.6 (17), 766.4 (20), 735.5 (20), 677.5 (24), 647.5 (15), 613.2 (95), 577.4 (14), 561.4 (20). Anal. ($C_{56}H_{86}N_2O_{14} \cdot 3H_2O$) C, H, N: mp = 196–199 °C (colorless powder, $CH_2Cl_2/i-Pr_2O$). $[\alpha]_D^{20} = +44.80$ ($c = 1.20$, $CHCl_3$). IR (KBr): 3452s, 2955s, 2877m, 1738s, 1670s, 1441m, 1379s, 1255s, 1166w, 1125m, 1081m, 1027w, 966w cm^{-1} . 1H NMR (200 MHz, $CDCl_3$): 5.42 (bs, $2 \times NH_2$), 5.10 (bs, $2 \times H-C(12)$), 4.92 (bs, $2 \times H-C(7)$), 2.12 (bs, $ca. 2 \times CH_3COO-$), 2.07 (bs, $ca. 2 \times CH_3COO-$), 0.94 (s, $2 \times H_3C-C(10)$), 0.83 (d, $J = 6.0$ Hz, $2 \times H_3C-C(20)$), 0.73 (s, $2 \times H_3C-C(13)$). ^{13}C NMR (50 MHz, $CDCl_3$): 175.64, 170.51, 170.31, 108.66, 75.27, 70.63, 47.43, 45.03, 43.26, 37.60, 34.615, 34.22, 32.67, 32.03, 31.30, 30.50, 28.35, 27.15, 25.66, 22.73, 22.03, 21.51, 21.36, 17.54, 12.20. MS (LSI, m/z): 1011.6 ($[M + H]^+$, 80), 967.6 (6), 919.3 (10), 851.6 (6), 766.2 (14), 735.5 (10), 677.5 (11), 613.2 (100), 581.2 (9), 566.2 (11). Anal. ($C_{56}H_{86}N_2O_{14}$) C, H.

Bis(*N*-(*n*-propyl)-3-dioxy-7 α ,12 α -diacetoxy-5 β -cholan-24-amide) (12 and 13). Using the same procedure as above, **7** (532 mg, 1.00 mmol) was transformed into **12** (74 mg, 14%) and **13** (62 mg, 11%) after chromatography of the crude product (Lobar B, LichroPrep Si 60, eluent: EtOAc). **12**: mp = 240–243 °C (colorless powder, $CH_2Cl_2/i-Pr_2O$). $[\alpha]_D^{20} = +73.90$ ($c = 1.40$, $CHCl_3$). IR (KBr): 3422m, 2960s, 2874m, 1738s, 1650s, 1551m, 1441m, 1378s, 1250s, 1027m cm^{-1} . 1H NMR (200 MHz, $CDCl_3$): 5.59–5.43 (m, $2 \times H-N$), 5.09 (bs, $2 \times H-C(12)$), 4.93 (bs, $2 \times H-C(7)$), 3.28–3.12 (m, $2 \times CH_3CH_2CH_2N$), 2.10 (bs, $4 \times CH_3COO-$), 1.60–1.40 (m, $2 \times CH_3CH_2CH_2N$), 0.96–0.87 (m, $2 \times H_3C-C(10)$), $2 \times CH_3CH_2CH_2N$, 0.82 (d, $J = 5.8$ Hz, $H_3C-C(20)$), 0.73 (s, $H_3C-C(13)$). ^{13}C NMR (50 MHz, $CDCl_3$): 173.19, 170.51, 108.62, 75.24, 70.57, 47.44, 44.98, 43.21, 41.11, 37.59, 34.67, 34.56, 33.53, 31.52, 30.59, 28.35, 27.11, 25.60, 22.81, 22.04, 21.55, 21.28, 17.52, 12.15, 11.27. MS (LSI, m/z): 1095.7 ($[M + H]^+$, 28), 1035.7 (3), 613.1 (3), 532.4 (5), 504.4 (4), 460.2 (14), 412.3 (10), 307.0 (100). Anal. ($C_{62}H_{98}N_2O_{14}$) C, H, N: mp = 171–174 °C (colorless powder, $CH_2Cl_2/i-Pr_2O$). $[\alpha]_D^{20} = +41.66$ ($c = 1.40$, $CHCl_3$). IR (KBr): 3951m, 3424m, 2960m, 2875m, 1738s, 1651m, 1547m, 1441m, 1379s, 1250s, 1165w, 1125w, 1026m, 966w cm^{-1} . 1H NMR (200 MHz, $CDCl_3$): 5.38–5.49 (m, $2 \times H-N$), 5.09 (bs, $2 \times H-C(12)$), 4.92 (bs, $2 \times H-C(7)$), 3.28–3.13 (m, $2 \times CH_3CH_2CH_2N$), 2.12 (bs, $ca. 2 \times CH_3COO-$), 2.07 (bs, $ca. 2 \times CH_3COO-$), 1.60–1.40 (m, $2 \times CH_3CH_2CH_2N$), 0.94 (s, $2 \times H_3C-C(10)$), 0.96–0.86 (m, $2 \times H_3C-C(10)$), $2 \times CH_3CH_2CH_2N$, 0.82 (d, $J = 6.0$ Hz, $2 \times H_3C-C(10)$), 0.73 (s, $2 \times H_3C-C(13)$). ^{13}C NMR (50 MHz, $CDCl_3$): 173.18, 170.54, 108.66, 75.29, 70.63, 47.49, 45.01, 43.26, 41.13, 37.60, 34.71, 34.60, 33.60, 31.56, 28.35, 27.15, 25.66, 22.85, 22.73, 22.03, 21.525, 21.38, 17.54, 12.18, 11.31. MS (LSI, m/z): 1095.8 ($[M + H]^+$, 100), 1035.8 (5), 967.7 (2), 909.6 (2), 851.7 (2), 793.6 (3), 766.2 (4), 677.5 (3), 613.2 (16). Anal. ($C_{62}H_{98}N_2O_{14} \cdot 2H_2O$) C, H.

Antimalarial Activity. The in vitro antimalarial drug susceptibility screen was a modification of the procedures first published by Desjardins et al.,²⁹ with modifications developed by Milhous et al.³⁰ In brief, the assay relies on the incorporation of radiolabeled hypoxanthine by the parasites and inhibition of isotope incorporation is attributed to activity of known or candidate antimalarial drugs. For each assay, proven antimalarials were used as controls. The incubation period was 66 h and the starting parasitemia was 0.2% with a 1% hematocrit. The medium used was RPMI-1640 culture medium 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 were normally used, W2 and D6.³¹ 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 the Sierra I/UNC isolates and is susceptible to chloroquine and pyrimethamine but has reduced susceptibilities to mefloquine and halofantrine.

Drugs were dissolved directly in dimethyl sulfoxide (DMSO) and diluted 400-fold with complete culture media. The compounds were then diluted 2-fold, 11 times, to give a concentration range of 1048-fold. These dilutions were performed

automatically by a Biomek 1000 or 2000 liquid handling system into 96-well microtiter plates. The diluted drugs were then transferred (25 μ L) to test plates, 200 μ L of parasitized erythrocytes (0.2% parasitemia and 1% hematocrit) was added, and the whole was incubated at 37 °C in a controlled environment of 5% CO_2 , 5% O_2 and 90% N_2 . After 42 h, 25 μ L of [3H]-hypoxanthine was added and the plates were incubated for an additional 24 h. At the end of the 66-h incubation period, the plates were frozen at –70 °C to lyse the red cells and later thawed and harvested onto glass fiber filter mats by using a 96-well cell harvester. The filter mats were then counted in a scintillation counter and the data downloaded with the custom, automated analysis software developed at WRAIR. For each drug, the concentration–response profile was determined and 50% inhibitory concentrations (IC_{50}) were determined by using a nonlinear, logistic dose–response analysis program.

Antiproliferative Activity. Materials and methods:

Stock solutions of investigated compounds were prepared in DMSO at a concentration of 8 mM and afterward diluted by nutritive medium (RPMI-1640 medium supplemented with L-glutamine (3 mmol/L), streptomycin (100 μ g/mL), and penicillin (100 IU/mL), 10% heat-inactivated FBS and 25 mM Hepes, adjusted to pH 7.2 by bicarbonate solution) to various final concentrations. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemicals) was dissolved, 5 mg/mL in phosphate buffer saline, pH 7.2, and filtered through a Millipore filter, 0.22 μ m, before use. RPMI-1640 cell culture medium and FBS were products of Sigma Chemicals.

Cell culture: Human malignant melanoma Fem-X cells and human cervix carcinoma HeLa cells were maintained as a monolayer culture in the same nutrient medium. The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere by twice weekly subculture.

Treatment of Fem-X and HeLa cells: Target cells were seeded, in triplicate (2000 cells/well), into 96-well microtiter flat bottomed plates and 20 h later, five different concentrations of investigated compounds were added to the wells to various final concentrations, except to the control wells where a nutrient medium with correspondent concentration of DMSO only was added to the cells. All analyses were done in triplicate. Nutrient medium with corresponding concentrations of compounds, but void of cells was used as blank, in triplicate too.

Determination of HeLa and Fem-X cell survival: Cell survival was determined as that reported earlier³² by MTT test 72 h after the drug addition. In brief, 20 μ L of MTT solution (5 mg/mL PBS) was added to each well. Samples were incubated for a further 4 h at 37 °C in 5% CO_2 and humidified air atmosphere. Then, 100 μ L of 10% SDS in 0.01 M HCl was added to the wells. Optical density (OD) at 570 nm was read the next day. To obtain cell survival (%), optical density at 570 nm of a sample with cells grown in the presence of various concentration of investigated agent (OD) was divided with control optical density OD_c (the OD of cells grown only in nutritive medium) $\times 100$. (It was implied that the OD of the blank was always subtracted from the OD of a corresponding sample with target cells.) Concentration IC_{50} was defined as the concentration of a drug needed to inhibit cell survival by 50%, compared with vehicle-treated control. The NIH-NCI screening results are given in growth inhibitory concentrations (GI). The GI_{50} is the interpolated value representing the concentration at which the percentage growth is +50.

Preparation of peripheral blood mononuclear cells (PBMC): PBMC were separated from whole-heparinized blood of three healthy volunteers by Lymphoprep (Oslo, Norway) gradient centrifugation. Interface cells, washed three times with nutrient medium, were counted and resuspended in the same medium. Then, 100 000 PBMC were seeded in 100 μ L of nutrient medium with or without PHA, and 2 h later 50 μ L of investigated tetraoxanes in various dilutions was put into the wells in triplicate; in the control, sample nutrient medium was added only. Final concentration of PHA was 5 μ g/mL of

nutrient medium. Determination of PBMC survival and IC₅₀ was done in the same way as reported for HeLa and Fem-X cells.

Acknowledgment. We thank the NIH-NCI's Developmental and Therapeutics program for evaluation of our tetraoxanes.

Supporting Information Available: Complete crystal data for compound **8**, atomic parameters, and geometry; also bar graphs indicating GI₅₀, TGI, and LC₅₀ for tetraoxane **13** in the NCI 60-human cell line assay. This material is free of charge via the Internet at <http://pubs.acs.org>.

References

- Presented in part at the ECSOC-3, The Third Electronic Conference on Synthetic Organic Chemistry, MDPI, <http://www.mdpi.org/ecsoc-3.htm>, Sep 1–30, 1999.
- (a) Malaria Foundation International, <http://www.malaria.org/>, and the sites given therein. (b) Wahlgren, M.; Bejarano, M. T. A blueprint of 'bad air'. *Nature* **1999**, *400*, 506–507.
- Malaria Foundation International, Roll Back Malaria, <http://www.malaria.org/RBM.html>, and the sites given therein.
- Vennerstrom, J. L.; Fu, H.-N.; Ellis, W. Y.; Ager, A. L., Jr.; Wood, J. K.; 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.
- (a) Klayman, D. L. Qinghaosu (Artemisinin): An Antimalarial Drug from China. *Science* **1985**, *228*, 1049–1055. (b) Luo, X.-D.; Shen, C.-C. The Chemistry, Pharmacology, and Clinical Applications of Qinghaosu (Artemisinin) and its Derivatives. *Med. Res. Rev.* **1987**, *7*, 29–52. (c) Butler, A. R.; Wu, Y. L. Artemisinin (Qinghaosu): A New Type of Antimalarial Drug. *Chem. Soc. Rev.* **1992**, *21*, 85–90.
- Selected references: (a) Lin, A. J.; Klayman, D. L.; Milhous, W. K. Antimalarial Activity of New Water-Soluble Dihydroartemisinin Derivatives. *J. Med. Chem.* **1987**, *30*, 2147–2150. (b) Cumming, J. N.; Wang, D.; Park, S. B.; Shapiro, T. A.; Posner, G. H. Design, Synthesis, Derivatization and Structure–Activity Relationships of Simplified, Tricyclic, 1,2,4-Trioxane Alcohol Analogues of the Antimalarial Artemisinin. *J. Med. Chem.* **1998**, *41*, 952–964. (c) O'Neill, P. M.; Searie, N. L.; Raynes, K. J.; Maggs, J. L.; Ward, S. A.; Storr, R. C.; Park, B. K.; Posner, G. H. A Carbonyl Oxide Route to Antimalarial Yingzhao A Analogues: Synthesis and Antimalarial Activity. *Tetrahedron Lett.* **1998**, *39*, 6065–6068. (d) Nga, T. T.; Menage, C.; Beque, J.-P.; Bonnet-Delpon, D.; Gantier, J.-C.; Pradines, B.; Doury, J.-C.; Thac, T. D. Synthesis and Antimalarial Activities of Fluoroalkyl Derivatives of Dihydroartemisinin. *J. Med. Chem.* **1998**, *41*, 4101–4108. (e) Posner, G. H.; Parker, M. H.; Northrop, J.; Elias, J. S.; Ploypradith, P.; Xie, S.; Shapiro, T. A. Orally Active, Hydrolytically Stable, Semisynthetic, Antimalarial Trioxanes in the Artemisinin Family. *J. Med. Chem.* **1999**, *42*, 300–304. (f) Posner, G. H.; Ploypradith, P.; Parker, M. H.; O'Dowd, H.; Woo, S.-W.; Northrop, J.; Krasavin, M.; Dolan, P.; Kensler, T. W.; Xie, S.; Shapiro, T. A. Antimalarial, Antiproliferative, and Antitumor Activities of Artemisinin-Derived, Chemically Robust, Trioxane Dimers. *J. Med. Chem.* **1999**, *42*, 4275–4280. (g) O'Neill, P. M.; Searle, N. L.; Kan, K.-W.; Storr, R. C.; Maggs, J. L.; Ward, S. A.; Raynes, K.; Park, B. K. Novel, Potent, Semisynthetic Antimalarial Carba Analogues of the First-Generation 1,2,4-Trioxane Artemether. *J. Med. Chem.* **1999**, *42*, 5487–5493.
- (a) Takaya, Y.; Kurumada, K.-i.; Takeuji, Y.; Kim, H.-S.; Shibata, Y.; Ikemoto, N.; Wataya, Y.; Oshima, Y. Novel Antimalarial Guaiane-type Sesquiterpenoids from *Nardostachys chinensis* Roots. *Tetrahedron Lett.* **1998**, *39*, 1361–1364. (b) Posner, G. H.; O'Dowd, H.; Ploypradith, P.; Cumming, J. N.; Xie, S.; Shapiro, T. A. Antimalarial Cyclic Peroxy Ketals. *J. Med. Chem.* **1998**, *41*, 2164–2167. (c) D'Ambrosio, M.; Guerriero, A.; Deharo, E.; Debitus, C.; Munoz, V.; Pietra, F. New Types of Potentially Antimalarial Agents: Epidoxy-Substituted Norditerpene and Norsesterpenes from the Marine Sponge *Diacarnus levii*. *Helv. Chim. Acta* **1998**, *81*, 1285–1292. (d) Oh, C. H.; Kim, H. J.; Wu, H. S.; Won, H. S. A new type of 1,2,4-trioxanes structurally related to antimalarial artemisinin. *Tetrahedron Lett.* **1999**, *40*, 8391–8394.
- (a) Todorović, N. M.; Stefanović, 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. (b) Dong, Y.; Matile, H.; Chollet, J.; Kaminsky, R.; Wood, J. K.; Vennerstrom, J. L. Synthesis and Antimalarial Activity of 11 Dispiro-1,2,4,5-tetraoxane Analogues of WR 148999. 7,8,15,16-Tetraoxadispiro-[5.2.5.2]hexadecanes Substituted at the 1 and 10 Positions with Unsaturated and Polar Functional Groups. *J. Med. Chem.* **1999**, *42*, 1477–1480. (c) Tsuchiya, K.; Hamada, Y.; Masuyama, A.; Nojima, M.; McCullough, K. J.; Kim, H.-S.; Shibata, Y.; Wataya, Y. Synthesis, Crystal Structure and Anti-Malarial Activity of Novel Spiro-1,2,4,5-Tetraoxacycloalkanes. *Tetrahedron Lett.* **1999**, *40*, 4077–4080. (d) Kim, H.-S.; Shibata, Y.; Wataya, Y.; Tsuchiya, K.; Masuyama, A.; Nojima, M. Synthesis and Antimalarial Activity of Cyclic Peroxides, 1,2,4,5,7-Pentoxocanes and 1,2,4,5-Tetroxanes. *J. Med. Chem.* **1999**, *42*, 2604–2609.
- Reed, M. B.; Saliba, K. J.; Caruana, S. R.; Kirk, K.; Cowman, A. F. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* **2000**, *403*, 906–909.
- (a) Kharasch, M. S.; Sosnovsky, G. Structure of Peroxides Derived from Cyclohexanone and Hydrogen Peroxide. *J. Org. Chem.* **1958**, *23*, 1322–1326. (b) Ledaal, T. A Simple Laboratory Method for the Preparation of (Dimeric) Ketone Peroxides. *Acta Chem. Scand.* **1967**, *21*, 1656–1657. (c) Sanderson, J. R.; Paul, K.; Story, P. R.; Denson, D. D.; Alford, J. A. Macrocycles. The Synthesis and Thermal Decomposition of Some Disubstituted Dicyclohexylidene Diperoxides. *Synthesis* **1975**, 159–161.
- (a) Sanderson, J. R.; Zeiler, A. G.; Wilterdink, R. J. An Improved Synthesis of Dicyclohexylidene Diperoxide. *J. Org. Chem.* **1975**, *40*, 2239–2241. (b) Sanderson, J. R.; Zeiler, A. G. A Simple Synthesis of Dicyclohexylidene Diperoxide and Tricyclohexylidene Triperoxide. *Synthesis* **1975**, 125–127. (c) Sanderson, J. R.; Wilterdink, R. J.; Zeiler, A. G. The Preparation of Dicyclohexylidene Diperoxides by a Two-Step Procedure. *Synthesis* **1977**, 479–481. (d) McCullough, K. J.; Morgan, A. R.; Nonhebel, D. C.; Pauson, P. L.; White, G. J. Ketone-derived Peroxides. Part I. Synthetic Methods. *J. Chem. Res. (S)* **1980**, 34.
- Jefford, C. W.; Jaber, A.; Boukouvalas, J. Efficient Preparation of 1,2,4,5-Tetroxanes from Bis(trimethylsilyl) Peroxide and Carbonyl Compounds. *Synthesis* **1988**, 391–393.
- Dong, Y.; Vennerstrom, J. L. Dispiro-1,2,4,5-tetraoxanes via Ozonolysis of Cycloalkane *O*-Methyl Oximes: A Comparison with the Peroxidation of Cycloalkanes in Acetonitrile-Sulfuric Acid Media. *J. Org. Chem.* **1998**, *63*, 8582–8585.
- Kim, H.-S.; Tsuchiya, K.; Shibata, Y.; Wataya, Y.; Ushigoe, Y.; Masuyama, A.; Nojima, M.; McCullough, K. J. Synthetic Methods for Unsymmetrically substituted 1,2,4,5-tetroxanes and of 1,2,4,5,7-pentoxocanes. *J. Chem. Soc., Perkin Trans. 1* **1999**, 1867–1870.
- Until now, and except for ref 8a, only in a few cases have chiral ketones (as racemic mixture) been used for tetraoxane synthesis, and no single diastereomer was screened (see refs 4, 8b, 10c).
- For problems arising with mass determination of tetraoxanes and corresponding hexaoxanes, see ref 13.
- McCullough, K. J.; Wood, J. K.; Bhattacharjee, A. K.; Dong, Y.; Kyle, D. E.; Milhous, W. K.; Vennerstrom, J. L. Methyl-Substituted Dispiro-1,2,4,5-tetraoxanes: Correlations of Structural Studies with Antimalarial Activity. *J. Med. Chem.* **2000**, *43*, 1246–1249.
- Duax, W. L.; Weeks, C. M.; Rohrer, D. C. *Topics in Stereochemistry*; John Wiley and Sons: New York, 1976; Vol. 9, pp 271–383.
- Takaya, Y.; Tasaka, H.; Chiba, T.; Uwai, K.; Tanitsu, M.; Kim, H.-S.; Wataya, Y.; Miura, M.; Takeshita, M.; Oshima, Y. New Type of Febrifugine Analogues, Bearing a Quinolizidine Moiety, Show Potent Antimalarial Activity against *Plasmodium Malaria* Parasite. *J. Med. Chem.* **1999**, *42*, 3163–3166.
- Drug discovery and development program, National Cancer Institute, Bethesda, MD, <http://dtp.nci.nih.gov/>.
- Duke, R. C.; Cohen, J. J. Morphological and Biochemical Assays of Apoptosis. In *Current Protocols in Immunology*; Coligan, J. E., Kruisbeck, A. M., Margulies, D. H., Shevach, E. M., Strober, W., Eds.; John Wiley and Sons: New York, 1992; pp 3.17.1–3.17.16.
- Knappe, E.; Peteri, D. Dunnschichtchromatographische Identifizierung organischer Peroxide. *Z. Anal. Chem.* **1962**, *190*, 386–389.
- Procopiu, P. A.; Baugh, S. P. D.; Flack, S. S.; Inglis, G. G. A. An extremely fast and efficient acylation reaction of alcohols with acid anhydrides in the presence of trimethylsilyl trifluoromethanesulfonate as catalyst. *Chem. Commun.* **1996**, 2625–2626.
- Plattner, P. A.; Heuser, H. Über Beziehungen zwischen Konstitution und optischer Drehung in der Cholsäure-Reihe. *Helv. Chim. Acta* **1944**, *27*, 748–757.
- Jones, A. S.; Webs, M.; Smith, F. Basic Derivatives of Steroids. 3-Amino-7,12-dihydroxy- and 3-Amino-12-hydroxy-cholanic acid. *J. Chem. Soc.* **1949**, 2164–2168.
- (a) *Beilstein Handbuch Der Organischen Chemie*; Springer-Verlag: Berlin/Göttingen/Heidelberg, 1972; E-III, 10, p 4583. (b) *Ibid.* p 4582.
- Sheldrick, G. M. Phase annealing in SHELX-90: Direct Methods for Larger Structures. *Acta Crystallogr.* **1990**, *A46*, 467–473.

- (28) Sheldrick, G. M. *SHELXL-93. Program for the Refinement of Crystal Structures*; University of Göttingen: Germany, 1993.
- (29) 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.
- (30) 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.
- (31) Oduola, A. M. J.; Milhous, W. K.; Weatherly, N. F.; Bowdre, J. H.; Desjardins, R. E. *Plasmodium falciparum*: induction of resistance to mefloquine in cloned strains by continuous drug exposure in vitro. *Exp. Parasitol.* **1988**, *67*, 354–360.
- (32) Juranić, Z.; Radulović, S.; Joksimović, J.; Juranić, I. The Mechanism of 8-Cl-cAMP Action. *J. Exp. Clin. Cancer Res.* **1998**, *17*, 269–275.

JM000952F