Expedited Articles

Mixed Steroidal 1,2,4,5-Tetraoxanes: Antimalarial and Antimycobacterial Activity

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Mixed 1,2,4,5-tetraoxanes possessing simple spirocycloalkane and spirocholic acid-derived substituents were prepared and shown to have significantly higher in vitro antimalarial activity than bis-substituted tetraoxanes. Out of 41 synthesized tetraoxanes, 12 were in vitro more potent against *Plasmodium falciparum* chloroquine-resistant W2 clone than artemisinin, and the most potent one was 2.4 times as active as arteether. In addition, 9 compounds exhibit higher activity than chloroquine against *P. falciparum* chloroquine-susceptible D6 clone. Cytotoxicity was assessed for most active compounds against the Vero cell line, showing a cytotoxicity/antimalarial potency ratio of 1/(1400−9500). For the first time, tetraoxanes were screened against *Mycobacterium tuberculosis* with MICs as low as 4.73 μM against H37Rv strain. Mixed tetraoxanes were synthesized in a simple procedure from cholic acid methyl esters by direct coupling of steroidal gem-dihydroperoxide to simple ketones and further transformed into corresponding acids and amides.

Introduction

Present resurgence of malaria and lack of proper treatment affect 300−500 million people annually, causing several million deaths.[1] Increased resistance of certain malaria types (e.g., *Plasmodium falciparum*) to common drugs, such as chloroquine, additionally justifies further search for new drugs. However, antimalarial properties of artemisinin and its derivatives opened new possibilities in malaria treatment.[2]

Tuberculosis (TB) affects 1.7 billion people worldwide, with 3 million deaths per year.[3] In areas of the world where the disease is prevalent, people with suppressed immune systems, such as those who are HIV positive, are especially prone to infection. It is estimated that about 8 million new cases emerge annually, mostly in sub-Saharan Africa, and the disease is slowly but steadily spreading in the developed countries as well. Multidrug-resistant TB strains have developed because of poor compliance with treatment regulations.[4] The current lack of new leads, with only one compound in clinical development and a handful in the early discovery phase,[5] additionally warrants the development of new antitubercular drugs.

1,2,4,5-Tetraoxacycloalkane is an important pharmacophore, since it renders antimalarial activity[6] similar to that 1,2,4-trioxanes. A part of our own research is directed toward introducing the steroid molecule as a tetraoxane carrier,[7,8] and recent results revealed that amide derivatives of steroidal tetraoxanes are potent antimalarials with low cytotoxic effect on healthy cells.[8] The cholic acid derived carrier is envisaged to render solubility under physiological conditions and to enhance cell membrane permeability because of its amphiphilic character. Unlike carboxylic acids, the primary and secondary amide derivatives are not ionized under physiological conditions; thus, the possibility of cell membrane lysis is not expected. The morphological examination of HeLa cells treated for 24 h with a bis-steroidal tetraoxane amide (at concentrations of 66 μM, the apoptosis has been observed)[8] revealed no degradation of the cell membrane, indicating that we are dealing with the antimalarials of low toxicity.

Bearing in mind that tetraoxanes with identical substituents at C(3) and C(6) afford little opportunity for selective manipulation of other functionality in the molecule, we decided to devise an approach to mixed steroidal tetraoxanes possessing the steroid moiety at one side of the tetraoxane and the simple cycloalkane at the opposite. This would lead to a reduced molecular weight of the active molecule (compared to bis-steroidal tetraoxanes, refs 7 and 8) and, at the same time, would preserve the effectiveness of steroid carrier. Here, we present the synthesis of second-generation cholic acid

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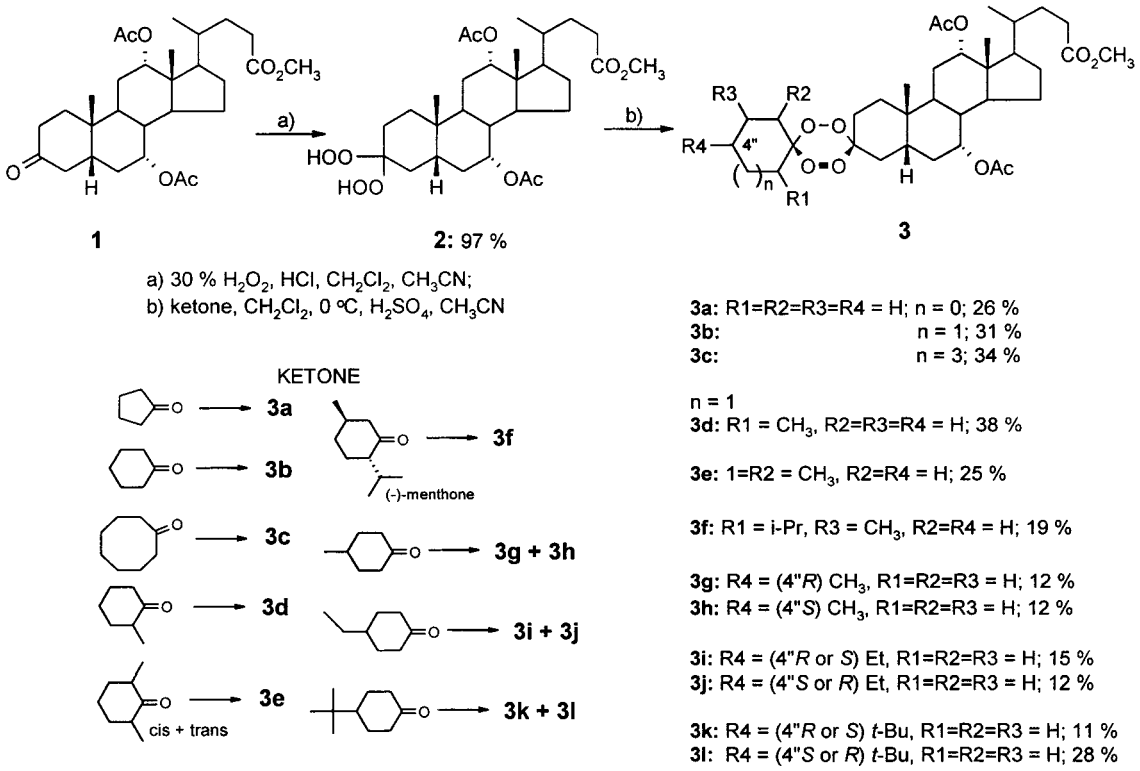
Scheme 1



based, mixed 1,2,4,5-tetraoxanes derived from steroidal gem-dihydroperoxide, and we report on their in vitro antimalarial and antitubercular activity.

Synthesis of Mixed Tetraoxanes

It could be envisaged that the synthesis of mixed tetraoxanes is feasible through direct coupling of ketones with hydrogen peroxide, or alternatively, by coupling of gem-dihydroperoxides with the appropriate ketone. However, the first alternative, even using a single ketone, gives a complex product mixture,[6−9] while the second one was not possible in direct fashion.[10]

Steroidal geminal dihydroperoxides were synthesized from ketones using H_2O_2/dry ether (or *tert*-butyl alcohol) solutions,[11] or using a 30% H_2O_2 procedure.[7] However, both methods were inappropriate for a drug-developing program. The first method, although affording high yield of the product, was probably abandoned because of the requirement of nonaqueous H_2O_2 solutions; the second one afforded gem-dihydroperoxides in 40−50% yield (along with other products).

In this work, the methyl 3,3-dihydroperoxy-7α,12α-diacetoxy-5β-cholan-24-oate (**2**) was prepared in high yield by modification of the previously reported procedure,[7] using a 30% H_2O_2/CH_2Cl_2/CH_3CN reaction medium (Scheme 1).[12] Crude 3,3-dihydroperoxide **2** was then treated with the corresponding ketone in the presence of sulfuric acid as catalyst, at 0 °C, and the overall reaction time was ca. 15 min.

Simple ketones reacted well with gem-dihydroperoxide **2**, affording mixed tetraoxanes **3a**−**l** (Scheme 1). As expected, nonsubstituted cycloalkanones gave single products (**3a**−**c**), while substituted precursors afforded diastereomeric mixtures. 4″-Methyl-, 4″-ethyl-, and 4″-*tert*-butyltetraoxane mixtures were separated into corresponding diastereomers (**3g**, **3h**; **3i**, **3j**; and **3k**, **3l**; respectively). 2-Methylcyclohexanone, 2,6-dimethylcyclohexanone (cis and trans mixture), and (−)-menthone afforded nonresolvable diastereomeric mixtures **3d**, **3e**, and **3f**, respectively.

The configuration at C(4″) in **3h** was assigned by X-ray crystallographic structural analysis of the corresponding acid **4h**, and it appears to be *S* (Figure 1).[13] The six-membered 1,2,4,5-tetraoxane and methylcyclohexane rings adopted conformations very similar to chair forms, the puckering parameters being $Q = 0.646$ Å, $\theta = 3.5°$, $\phi = 315.5°$ for the first and $Q = 0.559$ Å, $\theta = 3.9°$, $\phi = 215.1°$ for the second.[14] The methyl substituent is in the axial position. A hydrogen bond between the carboxylic group and one of the carbonyl functions of a symmetry-related molecule is observed: O25···O29 (x, $y - 1$, z) = 2.700(3) Å. The complete data, atomic parameters, and geometry are given as Supporting Information. Consequently, it is reasonable to propose the 4″*R* configuration of the corresponding carbon in diastereomer **3g** (see Experimental Section in Supporting Information for spectroscopic data).

Our coupling conditions afforded tetraoxanes in good yield (24−39%) except in the case of menthone where

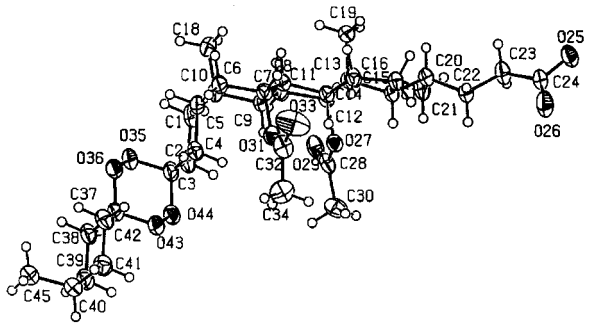
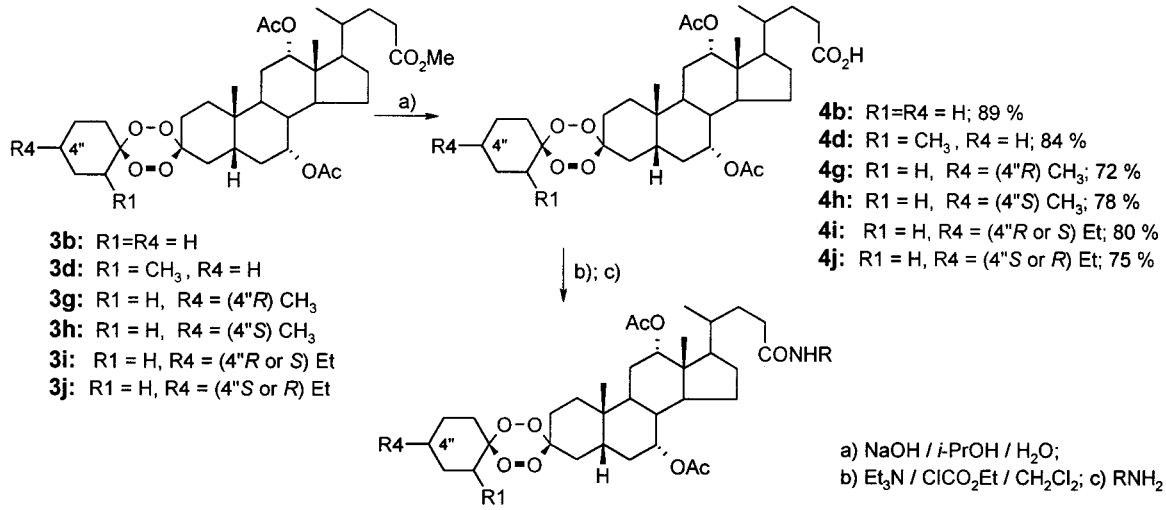


Figure 1. ORTEP plot of **4h**.[13]

Scheme 2



R1=R4 = H **5:** R = H; 89 %. **6:** R = nPr; 70 %. **7:** R = $CH_2CO_2CH_3$; 64 %.
R1 = CH_3, R4 = H **8:** R = H; 68 %. **9:** R = CH_3; 64 %. **10:** R = Et; 96 %. **11:** R = nPr; 70 %.
R1 = H, R4 = (4"*R*) CH_3 **12:** R = H; 74%. **13:** R = CH_3; 82 %. **14:** R = Et; 90 %. **15:** R = nPr; 74 %.
R1 = H, R4 = (4"*S*) CH_3 **16:** R = H; 76 %. **17:** R = CH_3; 70 %. **18:** R = Et; 90 %. **19:** R = nPr; 70 %.
R1 = H, R4 = (4"*R* or *S*) Et **20:** R = H; 85%. **21:** R = CH_3; 76 %. **22:** R = Et; 82 %. **23:** R = nPr; 79 %.
R1 = H, R4 = (4"*S* or *R*) Et **24:** R = H; 79 %. **25:** R = CH_3; 77 %. **26:** R = Et; 75 %. **27:** R = nPr; 77 %.

the α-isopropyl substituent lowers the yield even more than two methyls in 2,6-dimethylcyclohexanone. Attempted preparation of mixed tetraoxanes starting with aromatic ketones (2-furyl methyl ketone, 4-nitroacetophenone, 6-methoxy-1-tetralone, and anisaldehyde) and **2**, or simple gem-dihydroperoxide, e.g., 1,1-dihydroperoxy-4-methylcyclohexane, failed.

It was found that tetraoxanes possessing carboxylic acid termini render poor in vitro antimalarial activity compared to corresponding esters.[6a] Since it is feasible to anticipate that an ester moiety would easily hydrolyze in vivo,[6a] in order to secure a nonacidic protic group to facilitate the solubility in polar solvents, tetraoxanes **3b**, **3d**, and **3g**−**j** were further transformed into amide derivatives **5**−**7**, **8**−**11**, and **12**−**27**, respectively, via mixed anhydrides in 53−81% overall yield (Scheme 2). Applying the mixed anhydride procedure on a compound with tetraoxane functionality, we opened a new approach to complex compounds of this type having significantly higher activity with respect to bis-steroidal tetraoxanes (vide infra and ref 8).

While the reason for derivatization of the single products is obvious, the diastereomeric tetraoxane mixture **3d**, obtained from racemic 2-methylcyclohexanone, was used for derivatization as a probe for testing the generality of the amide moiety effect on the activity observed earlier[8] (vide infra). All corresponding tetraoxane mixtures (**3d** → **4d** → **8**−**11**, Scheme 2) were carefully monitored via their respective specific rotations (see Experimental Section in Supporting Information for details).

Molecular masses of synthesized tetraoxanes were confirmed by using single-stage electrospray ionization (ESI) mass spectrometry in the positive ion mode. All analyzed compounds yield abundant molecular ion peaks by coordinating ammonium, sodium, and potassium ions ($[M + NH_4]^+$, $[M + Na]^+$, and $[M + K]^+$, respectively).

Table 1. In Vitro Antimalarial Activities of Mixed Tetraoxanes **3, 4d**, and **8**−**11**

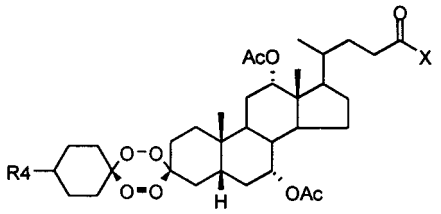
	Plasmodium falciparum IC_{50}[a] (nM)	
compound	D6[b]	W2[c]
3a	39.14	27.67
3b	21.47	16.96
3c	48.44	20.00
3d	27.03	15.64
3e	31.18	357.08
3f	154.36	73.24
3g	9.99	5.05
3h	24.64	13.16
3i	6.73	15.46
3j	17.70	28.31
3k	107.48	52.73
3l	51.87	27.77
4d	67.63	37.51
8	17.31	11.12
9	15.22	10.02
10	12.65	6.97
11	13.15	9.29
artemisinin	8.4[d]	7.3[d]
artelinic acid	9.73[e]	3.30[e]
arteether	2.7[d]	1.4[d]
chloroquine[f]	13.76	185.38
mefloquine[f]	28.29	5.02

[a] IC, inhibition concentration. [b] *P. falciparum* African D6 clone. [c] *P. falciparum* Indochina W2 clone. [d] Taken from ref 6c. [e] Taken from ref 2a. [f] Control drugs.

Antimalarial Activity

The in vitro antimalarial activity of all 41 synthesized mixed tetraoxanes **3a**−**27** against *Plasmodium falciparum* D6 and W2 clones is given in Tables 1 and 2. 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. The W2 clone of the Indochina I isolate is resistant to chloroquine and pyrimethamine but is susceptible to mefloquine. For comparison, in Table 1 are presented the activities of endoperoxides artemisinin, arteether, and artelinic acid.

Table 2. In Vitro Antimalarial Activities of 4-Substituted Amides and Acids



	R4 = H			R4 = Me			R4 = Et		
		IC_{50}[a] (nM)			IC_{50}[a] (nM)			IC_{50}[a] (nM)	
X	compd	D6[b]	W2[c]	compd	D6[b]	W2[c]	compd	D6[b]	W2[c]
OH	**4b**	11.83	19.22	**4g**	1.53	0.98	**4i**	5.76	7.26
				4h	31.10	17.74	**4j**	9.83	14.43
NH_2	**5**	11.83	4.74	**12**	1.17	0.58	**20**	8.40	10.71
				16	20.04	14.10	**24**	31.06	12.52
NHMe				**13**	5.96	3.61	**21**	7.21	7.82
				17	16.66	11.87	**25**	27.95	6.15
NHEt				**14**	5.80	3.93	**22**	19.79	9.56
				18	15.59	9.93	**26**	23.14	18.07
$NHPr^n$	**6**	14.76	6.80	**15**	3.22	3.82	**23**	8.34	4.51
				19	84.94	83.01	**27**	90.15	122.68
Gly-OCH_3	**7**	22.01	10.77						

[a] IC, inhibition concentration. [b] *P. falciparum* African D6 clone. [c] *P. falciparum* Indochina W2 clone.

Inspection of Tables 1 and 2 reveals that most of synthesized mixed tetraoxanes were more potent against W2 than against D6 clone, with the exception of the inactive mixture **3e**, and 4″-ethyl substituted compounds **4i**, **4j**, **20**, **21**, and **27**. The influence of substitution was first examined by changing the spirocycloalkane substituent at the tetraoxane ring (methyl ester series **3a**−**c**). It was found that the spirocyclohexane moiety afforded the most active compound (**3b**: IC_{50}(D6) = 21.47 nM, IC_{50}(W2) = 16.96 nM), although the resistance index (RI = IC_{50}(W2)/IC_{50}(D6)) for **3c** was ca. 2 times better. Further analysis of the methyl ester series reveals that substitution at the C(4″) position of the spirocyclohexane ring affords further SAR information; there is remarkably different activity of epimers on both clones, with an average RI for 4″-methyl and 4″-*tert*-butyl isomers being 0.52, in sharp contrast to 4″-ethyls (RI = 1.95).

On the basis of our previous findings,[8] we also investigated the influence of amide functionality on antimalarial activity within the series. First, the relevant information was obtained from the spirocyclohexane series (**3b** → **5**−**7**, Table 2), and as expected, all amide derivatives were more active on both clones than the parent methyl ester **3b**. Methyl glycocholate **7**, with a stable RI of ca. 0.5, indicated a loss of activity in comparison to primary and *n*-propylamides, **5** and **6**, respectively. On the basis of this information, we turned to the spiro-2″-methylcyclohexane series to systematically investigate the influence of the *N*-alkyl chain within the amides (**3d** → **8**−**11**, Table 1). Again, the amides afforded higher activity than the parent ester **3d**. Securing the C_1−C_3 chain within the *N*-alkyl series, we prepared amides of (4″*R*)-methylspirocyclohexane (**3g** → **12**−**15**), (4″*S*)-methylspirocyclohexane (**3h** → **16**−**19**), (4″*R* or *S*)-ethylspirocyclohexane (**3i** → **20**−**23**), and (4″*S* or *R*)-ethylspirocyclohexane (**3j** → **24**−**27**) series (Table 2). All 4″-substituted spirocyclohexyltetraoxane amides, 4″-methyl- and 4″-ethyl, are significantly more active than the corresponding methyl esters except for the inactive *n*-propylamides **19** and **27**. The most active primary amide, **12** (IC_{50}(D6) = 1.17 nM, IC_{50}(W2) = 0.58 nM; Table 2), is ca. 9 times more active than the parent methyl ester **3g**.

Table 3. In Vitro Antimycobacterial Activity of (4″*R*)-Methyl Derivatives against *Mycobacterium tuberculosis* Strain H37Rv

	inhibition	MIC[a]		cytotoxcity[b] (IC_{50})	
compound	(%)	μg/mL	μM	μg/mL	μM
3g	29	>6.25	>9.63		
4g	21	>6.25	>9.84		
12	99	3.13	4.94	3.49	5.51
13	98	6.25	9.65	5.69	8.78
14	98	3.13	4.73	5.37	8.11
15	94	6.25	9.25	8.34	12.34
isoniazid[c]		0.05	0.36	>1000	>7000
rifampin[c]		0.12	0.15	110.67	134.48

[a] MIC = minimal inhibitory concentration. [b] VERO cell line. [c] Control drugs.

Very encouraging results were obtained in tests on the hemolytic behavior of this tetraoxane class of compounds. Our initial experiments using *n*-propylamide **6** as a test compound revealed no RBC membrane lysis,[15] suggesting that antimalarial activity of our compounds (primarily the amides) is the consequence of interaction specific to infected RBC and is not the result of uncontrolled RBC membrane lysis.

Antimycobacterial Activity and Cytotoxicity

We entered the NIAID's Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) program and had a compound with the peroxide functionality evaluated as potential antimycobacterial. The initiative yielded four tetraoxanes of the (4″*R*)-methyl series (out of six) that passed the initial screening (level 1) against *Mycobacterium tuberculosis*, strain H37Rv, at the single concentration of 6.25 μg/mL exhibiting >90% inhibition (Table 3). MICs were determined at level 2 of the screening protocol, with amides **12** and **14** being the

most active. In analogy with antimalarial activity, it is observed that amides were more active substrates than the corresponding acid or ester. For compounds that entered screening level 2, the cytotoxicity against the Vero cell line was assessed and in comparison to their antimycobacterial activity, relatively high level of cytotoxicity is found.

Discussion

With our new and simple synthesis of dispirocycloalkyl-1,2,4,5-tetraoxacyclohexanes, a new generation of tetraoxanes with cholic acid derivatives as a carrier was prepared, and their in vitro antimalarial activity was evaluated. Screening results given in Tables 1 and 2 clearly indicate that the substitution pattern at the spirocyclohexane ring is a very important structural element. Cyclohexanone itself afforded only one tetraoxane (**3b**), while prochiral 4-substituted cyclohexanones afforded the respective epimeric pairs (**3g**, **3h**; **3i**, **3j**; **3k**, **3l**).

Introducing the methyl to C(4″) position greatly enhanced the activity against both *P. falciparum* clones, while doing so with the ethyl group did not provide improvement (Table 1). One feature merits special comment: a remarkable difference is observed in the activity of each epimeric pair possessing the same functionality at C(24) of the side chain (esters, acids, amides). In general, members of the 4″*R* (and 4″(*R* or *S*)) series[16] of all tetraoxanes are more active than the respective epimers up to an activity ratio of 30 (excluding **3k** and **3l**, which exhibited low activity on both D6 and W2 *P. falciparum* clones presumably because of the bulkiness of their *tert*-butyl groups; Tables 1 and 2). Since subtle differences in conformation (and structure) in each of the diastereomers contribute to the activity of a compound, at present we cannot further elaborate on the activity differences of the 4″-methyl- and 4″-ethyltetraoxane series (e.g., simple MM calculation on the (4″*S*)-methyl compound **4h** showed that all chair conformations of spirocyclohexane and dispirotetraoxacyclohexane rings fall within 1.1 kcal/mol, regardless of the orientation of the methyl group). The extensive QSAR calculations are underway in order to build a pharmacophore model.

Changing ester groups for amides enhanced the activity against both clones, and interesting results are obtained with primary amide **5** and alkylamides **6**, **13**−**15**, **21**, and **23**. All of them show effects similar to artemisinin (or better) against W2 *P. falciparum* clone, and the acid **4g** and the primary amide **12** are among the compounds with the highest known in vitro antimalarial activity on both CQ-susceptible and CQ-resistant *P. falciparum* strains.

The influence of the cholic acid carrier on the antimalarial activity is apparent: (a) tetraoxane **3b** is 1.5−1.8 times (primary amide **5**, 3−6 times) more active than bis(1,1-dioxycyclohexane);[6b] (b) methyl epimer **3g** is 2−4 times (primary amide **12**, 20−36 times) more active than bis(1,1-dioxy-4-methylcyclohexane);[6c] (c) *tert*-butyl epimer **3l** is ca. 3.7 times more active than bis(1,1-dioxy-*4-tert*-butylcyclohexane);[6c] (d) **3d** is ca. 2 times (primary amide **9**, ca. 3.5 times) more active than bis(1,1-dioxy-2-methylcyclohexane);[6c] (e) **3e** is 3−30 times more active than bis(1,1-dioxy-2,6-dimethylcyclohexane).[6b]

The level of antimycobacterial activity of the (4″*R*)-methyl series (**12**−**15**) is high, but the selectivity index (SI; IC_{50}(Vero)/MIC (μg/mL)) for these compounds is rather low (1.7−0.9). However, when the cytotoxicity values (IC_{50} (μM), Table 3) are compared to the antimalarial activity of the same tetraoxanes (**12**−**15**; Table 2), one obtains an excellent SI (IC_{50} (Vero)/IC_{50}(D6 or W2)), with the lowest value being 1400!

Conclusion

We have devised a method for the preparation of steroidal mixed tetraoxanes starting from parent ketones, via the corresponding gem-dihydroperoxide, using simple reagents, mild reaction conditions, and a short reaction time. The usefulness of this new approach and the contribution of the steroid carrier are exemplified with the series of 41 mixed tetraoxanes. It was found that their in vitro antimalarial activity is significantly higher than that of nonsteroidal mixed tetraoxanes,[10] bis-steroidal tetraoxanes,[8] and simple cyclohexane-based tetraoxanes.[6] The synthesis of mixed cholic acid derived tetraoxanes enabled further selective transformations of the carrier molecule, thus furnishing 12 compounds more active in vitro than artemisinin and 7 compounds more active than mefloquine, on chloroquine-resistant *P. falciparum* W2 clone. Most prominent in the series is the essentially nontoxic primary amide **12** being ca. 6 times more active than artelinic acid and 2.4 times as active as arteether (IC_{50}(W2) = 0.58 nM; RI = 0.50; SI = 9500).

For the first time, the tetraoxanes were assessed as antimycobacterials and a high level of their activity was established (MIC_{12-15}: 3.13−6.26 μg/mL).

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Supporting Information Available: Experimental Section, crystal data for acid **4h**, atomic parameters, and geometry, and ^{1}H and ^{13}C NMR spectra of gem-dihydroperoxide **2** and of 1,1-dihydroperoxy-4-methylcyclohexane. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) While the configuration at C(4″) in the 4″-methyl series is determined (see Figure 1 and commentary), the configuration of C(4″) in the 4″-ethyl and 4″-*tert*-butyl series remains unknown. Therefore, the descriptors are arbitrarily assigned as *R* or *S*, and *S* or *R*. All C(4″) epimeric pairs (**3g**, **3h**; **3i**, **3j**; **3k**, **3l**) are listed in Table 1 and Experimental Section according to their elution order.

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