Synthesis and Antimalarial Activity of Sixteen Dispiro-1,2,4,5-tetraoxanes: Alkyl-Substituted 7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadecanes

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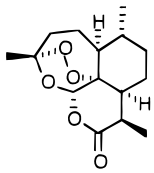
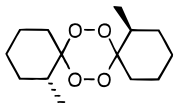
Sixteen alkyl-substituted dispiro-1,2,4,5-tetraoxanes (7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecanes) were synthesized to explore dispiro-1,2,4,5-tetraoxane SAR and to identify tetraoxanes with better oral antimalarial activity than prototype tetraoxane **1 (**WR 148999). The tetraoxanes were prepared either by peroxidation of the corresponding cyclohexanone derivatives in H_2SO_4/CH_3CN or by ozonolysis of the corresponding cyclohexanone methyl oximes. Those tetraoxanes with alkyl substituents at the 1 and 10 positions were formed as single stereoisomers, whereas the five tetraoxanes formed without the stereochemical control provided by alkyl groups at the 1 and 10 positions were isolated as mixtures of diastereomers. Three of the sixteen tetraoxanes were inactive (IC_{50}'s > 1000 nM), but five (**2**, **6**, **10**, **11**, **12**) had IC_{50}'s between 10 and 30 nM against the chloroquine-sensitive D6 and chloroquine-resistant W2 clones of *Plasmodium falciparum* compared to corresponding IC_{50}'s of 55 and 32 nM for **1** and 8.4 and 7.3 nM for artemisinin. We suggest that tetraoxanes **13**, **16**, and **17** were inactive and tetraoxanes **4** and **7** were weakly active due to steric effects preventing or hindering peroxide bond access to parasite heme. Tetraoxanes **1**, **10**, **11**, and **14**, along with artemisinin and arteether as controls, were administered po b.i.d. (128 mg/kg/day) to *P. berghei*-infected mice on days 3, 4, and 5 post-infection. At this dose, tetraoxanes **10**, **11**, and **14** cured between 40% and 60% of the infected animals. In comparison, artemisinin and tetraoxane **1** produced no cures, whereas arteether cured 100% of the infected animals. There was no apparent relationship between tetraoxane structure and in vitro neurotoxicity, nor was there any correlation between antimalarial activity and neurotoxicity for these seventeen tetraoxanes.

Introduction

The discovery of artemisinin (qinghaosu), a naturally occurring endoperoxide sesquiterpene lactone, and subsequent efforts to elucidate its SAR and mechanism of action established the peroxide bond as the critical pharmacophoric functional group.[1] This discovery was the beginning of a significant effort to identify synthetically accessible antimalarial peroxides.[2] Among the most structurally simple class of peroxides to emerge from these studies were the dispiro-1,2,4,5-tetraoxanes, as exemplified by **1 (**WR 148999).[3] Tetraoxanes such as **1** are notable in that they differ considerably in structure from artemisinin, are readily prepared in one step from substituted cyclohexanones, and possess good antimalarial activity, although they do suffer from low oral activity,[4] a defect shared in part by the semisynthetic artemisinins.[5] However, tetraoxanes are probably better known as precursors to macrocyclic lactones via thermolysis[6] and in ozonolysis as undesired side reaction products resulting from dimerization of carbonyl oxide intermediates.[7]



Artemisinin



WR 148999 (1)

Existing data suggest that the low oral activity of tetraoxane **1** is very likely a function of first-pass drug metabolism (possibly by CYP 1A2)[4] rather than solubility-limited dissolution, since various tetraoxane formulation strategies and incorporation of polar functional groups at the 1 and 10 positions in a series of tetraoxane analogues of **1**[8] both failed to enhance oral antimalarial activity. Thus, the overarching objective in this work was to identify tetraoxanes with better oral antimalarial activity. A secondary, but important qualifying, aim was to determine alkyl substitution patterns in the dispiro-1,2,4,5-tetraoxane (7,8,15,16-tetraoxadispiro[5.2.5.2]-

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hexadecane) skeleton associated with maximal antimalarial potency against *Plasmodium falciparum* and minimal neurotoxicty.

Design

Using tetraoxane **1** as a prototype, we designed tetraoxanes **2**−**4** bearing larger alkyl groups at the 1 and 10 positions. Tetraoxanes **5** and **6** are dimethyl-substituted isomers of **2**. Tetraoxane **7** with two *tert*-butyl groups at the 3 and 12 positions is a conformationally rigid analogue, but one without alkyl groups flanking the central tetraoxane heterocycle. Analogues **8**−**13** form a group of isomeric tetramethyl-substituted tetraoxanes. Tetraoxane **14** is derived from (−)-menthone and **15** is its transpositional isomer. Tetraoxanes **16** and **17** are hexamethyl- and octamethyl-substituted tetraoxane analogues, respectively.

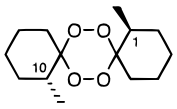
With this set of tetraoxane analogues, we hoped to identify a tetraoxane prototype associated with good oral activity, which we presumed would necessarily possess good metabolic stability. We had previously hypothesized[9] that analogues of tetraoxane **2** with greater steric bulk flanking the peroxide oxygen atoms might increase metabolic stability based on the idea that the peroxide bonds of tetraoxane **1** were the point of metabolic attack. However, the 1,1,10,10- and 1,5,10,14-tetramethyl tetraoxane analogues of **1** were completely inactive,[9] suggesting that the two additional axial methyl groups in these inactive tetraoxanes prevented their activation to presumed parasiticidal carbon radicals by inhibiting electron transfer from heme or other iron(II) species.[10] From this outcome we hypothesize that tetraoxane reductive activation in the parasite and tetraoxane metabolic inactivation by cyp P450s may both be a function of peroxide bond accessibility and, therefore, two sides of the same coin.

Results

Chemistry. As previously reported, three tetraoxanes (**4**, **6**, **7**) were prepared by ozonolysis of their corresponding cyclohexanone methyl oximes[11] and two (**1**, **12**) by acid-catalyzed peroxidation reactions.[3] The remaining twelve tetraoxanes (**2**, **3**, **5**, **8**−**11**, **13**−**17**) were prepared according to the peroxidation method developed by McCullough et al.[12] in which cyclohexanones are treated with hydrogen peroxide in H_2SO_4/CH_3CN. The unoptimized yields ranged from 4% to 68%. The efficiency of this peroxidation reaction is in part a function of the solubility of the tetraoxane reaction product in the reaction solution; higher yield reactions are those where insoluble tetraoxane products drive the reaction forward − the converse is true for less efficient reactions. In some cases, another confounding factor is formation of the undesired hexaoxonanes, an unpredictable outcome that appears to be a function of the cyclohexanone starting material structure.[11]

In ^{1}H NMR spectra of tetraoxanes with alkyl substituents at the 1 and 10 positions (**2**−**4**, **8**−**10**, **14**−**16**), as was also the case for tetraoxane **1**, characteristic peaks for the two methine protons at the 1 and 10 positions fell between 2.75 and 3.35 ppm. In ^{13}C NMR spectra for each of the tetraoxanes, diagnostic signals were present at 108−111 ppm corresponding to the spiro quarternary carbon atoms.

Table 1. Antimalarial Activity of 1,10-Dialkyl-Substituted Analogues of Tetraoxane **1** (WR 148999) against *P. falciparum* in Vitro



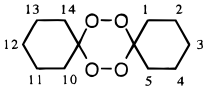
compd	R	IC_{50} (nM)[a] D6	IC_{50} (nM)[a] W2
1	methyl	55	32
2	ethyl	23	19
3	propyl	84	39
4	*tert*-butyl	>300	160
artemisinin		8.4	7.3
arteether		2.7	1.4

[a] Average of $n \geq 2$.

As for **1**, we observed that tetraoxanes **2**−**4** were formed as single stereoisomers, which we assume are the centrosymmetric, or *meso*-(*t*-10)-1,10, stereoisomers.[9] Similarly, tetraoxanes **8**−**10**, **15**, and **16** were formed as single stereoisomers, presumably the centrosymmetric and most thermodynamically stable stereoisomers, an outcome which we attribute to stereocontrol by the 1,10-dimethyl groups. Attempts to establish their relative configuration via *J* values were complicated by overlapping corresponding signals. Tetraoxanes **5**, **6**,[11] **7**,[11] **11**, and **12**, formed without the stereochemical control provided by methyl groups at the 1 and 10 positions, were isolated as mixtures of at least two diastereomers. No isomers exist for tetraoxanes **13** and **17**. Tetraoxane **14**, generated from (−)-menthone, was optically active, and although it was formed as a single stereoisomer, its optical activity indicated that it was not the centrosymmetric stereoisomer as we had anticipated. Its absolute configuration has not been established at this point. Thus, even though some of the tetraoxanes described above exist as diastereomeric mixtures, and for others the stereochemical assignments are incomplete, we thought it worthwhile to screen these compounds and then initiate additional structural studies if their antimalarial activities warranted such an effort.

Antimalarial Activity. The in vitro antimalarial data in Table 1 reveals that replacing the two methyl groups in **1** with ethyl groups (**2**) increased potency slightly, but this trend did not continue with further increases in the size (**3**, **4**) of the alkyl substituent. Our previous data,[8] however, indicates that the size of the substitutuent at the 1 and 10 positions is less critical when the substituent contains unsaturated or polar functional groups. Antimalarial potency did not diminish for the two isomers (**5**, **6**) of **1** but did drop off significantly when the two methyl groups of **6** were replaced with *tert*-butyl groups (**7**) (Table 2). For the six tetramethyl tetraoxane isomers **8**−**13**, potency was maintained in **8**, decreased slightly in **9**, and was lost entirely in **13**; **10**−**12** were 2−4-fold more potent than **1** but were still less potent than artemisinin. Tetraoxanes **14** and **15**, analogues of **10** where two of the methyl groups were replaced with isopropyl groups, were less potent than **10**, although **14** was as potent as tetraoxane prototype **1**. We suggest that, like the 1,1,10,10- and 1,5,10,14-tetramethyl-substituted tetraoxane analogues,[9] the densely substituted **16** and **17**, the bis-geminal

Table 2. Antimalarial Activity of Alkyl-Substituted Tetraoxanes[a] against *P. falciparum* in Vitro[b]



compd	R	IC_{50} (nM)[c] D6	W2
5	2,11-dimethyl	76	51
6	3,12-dimethyl	23	21
7	3,12-di-*tert*-butyl	200	100
8	1,2,10,11-tetramethyl	50	43
9	1,3,10,12-tetramethyl	110	49
10	1,4,10,13-tetramethyl	15	19
11	2,3,11,12-tetramethyl	12	10
12	2,4,11,13-tetramethyl	30	15
13	3,3,12,12-tetramethyl	>1000	>1000
14	1,10-diisopropyl-4,13-dimethyl	47	37
15	4,13-diisopropyl-1,10-dimethyl	140	94
16	1,3,3,10,12,12-hexamethyl	>1000	>1000
17	2,2,4,4,11,11,13,13-octamethyl	>1000	>1000

[a] The 1,1,10,10- and 1,5,10,14-tetramethyl analogues were inactive (ref 9). [b] For controls, see Table 1. [c] Average of $n \geq 2$.

Table 3. Antimalarial Activity of Tetraoxanes Administered sc to Mice Infected with *P. berghei*

compd	mg/kg/day[a]	av % parasitemia (day 6)	no. of mice dead/ day died
1	64	11	1/8 2/10 1/16 1/18
2	64	28	1/8 2/10 1/18 1/20
6	64	22	2/10 1/12 1/13 1/15
8	64	3	1/9 2/10 1/12 1/13
10	64	13	1/8 1/9 1/10 1/12 1/15
11	64	14	1/9 2/10 1/18 1/19
12	64	82	1/7 3/8 1/10
14	64	7	1/8 2/9 1/10 1/13
arteether	64	0	7/C[b]
control		74	1/7 2/8 1/9 1/10

[a] Compounds were administered sc b.i.d. on days 3, 4, and 5 post-infection. [b] Survival beyond 60 days is considered curative (C).

methyl-substituted **13**, and the di-*tert*-butyl-substituted **4** and **7** were either inactive or weakly active due to steric effects preventing or hindering peroxide bond access to parasite heme.[10] Finally we note that with one exception (**10**), these tetraoxanes (Tables 1 and 2), like artemisinin, were more potent against the chloroquine-resistant W2 clone than against the chloroquine-sensitive D6 clone of *P. falciparum*, although in each case the potency difference was unremarkable.

To select tetraoxanes for subsequent po experiments, the eight most potent tetraoxanes, along with arteether as a control, were administered sc b.i.d. (64 mg/kg/day) to *P. berghei*-infected mice on days 3, 4, and 5 post-infection (Table 3). Tetraoxanes **6** and **12** were among this group of eight tetraoxanes, even though **6** and **12** are known to be substantially less effective than **1** in the sc single-dose Rane test. We note that there was no correlation between in vitro IC_{50} data and either reduction of parasitemia or survival data. As measured by suppression of parasitemia, tetraoxanes **10** and **11** were equal to and tetraoxanes **8** and **14** were superior to **1**. On the other hand, survival data indicated that tetraoxanes **6**, **8**, and **10** were nearly as effective and tetraoxanes **2** and **11** were better than **1**. From this sc in vivo data it is also clear that each of the tetraoxanes was much less active than arteether. On the basis of the combined in vitro and sc in vivo data, tetraoxanes

Table 4. Antimalarial Activity of Tetraoxanes Administered Orally to Mice Infected with *P. berghei*

compd	mg/kg/day[a]	no. of mice dead/day died[b]
1	128	3/12 2/13 1/20 1/24
10	128	1/9 1/26 3/C
11	128	1/15 1/17 1/18 2/C
14	128	1/8 2/16 1/32 3/C
artemisinin	128	1/13 3/14 1/26
arteether	128	5/C
control		1/7 5/8 1/9

[a] Compounds were administered po b.i.d. on days 3, 4, and 5 post-infection. [b] Survival beyond 60 days is considered curative (C).

Table 5. In Vitro Neurotoxicity of Tetraoxanes against NB2a Neuroblastoma Cells

compd	neurite length IC_{50} (μM)[a]	compd	neurite length IC_{50} (μM)[a]
1	8.4	**11**	4.4
2	9.3	**12**	6.7
3	4.5	**13**	2.0
4	3.4	**14**	37
5	16	**15**	40
6	1.1	**16**	1.4
7	2.8	**17**	3.6
8	2.1	artemether	351
9	>1	dihydroartemisinin	0.22
10	17		

[a] Average of $n = 3$.

1, **10**, **11**, and **14**, along with artemisinin and arteether as controls, were administered po b.i.d. (128 mg/kg/day) to *P. berghei*-infected mice on days 3, 4, and 5 post-infection (Table 4). At this dose, tetraoxanes **10**, **11**, and **14** cured between 40% and 60% of the infected animals. In comparison, artemisinin and tetraoxane **1** produced no cures, whereas arteether cured 100% of the infected animals.

Neurotoxicity. Even though reported clinical neurotoxicity for the semisynthetic artemisinins is very rare,[13] neurotoxicity may be a potential drawback for antimalarial peroxides. Indeed, in work initiated at the Walter Reed Army Institute of Research, several of the artemisinins have produced demonstrable neurotoxicity in cell culture[14] and in animal models.[15] Thus, we thought it useful to assess the relative neurotoxicity of these tetraoxanes using a neurite outgrowth assay.[16] Data in Table 5 show IC_{50}'s against the NB2a neuroblastoma cell line[14,16] with artemether and dihydroartemisinin as controls. In this screen, we note that artemether is nonneurotoxic, whereas dihydroartemisinin, the presumed metabolite of all of the semisynthetic artemisinins,[5] is quite neurotoxic. While we have previously observed that cell proliferation in NB2a cells is not significantly more sensitive to dihydroartemisinin than a comparable function in a nonneuronal cell line, when NB2a cells are induced to differentiate, their sensitivity to dihydroartemisinin increases.[17] This contributes to our confidence that the neurite outgrowth assay more closely reflects potential selective neurotoxicity. The IC_{50}'s for the tetraoxanes ranged from 1 to 40 μM. Although tetraoxane prototype **1** (WR 148999) had an IC_{50} of 8 μM in this experiment, previous data[14] for **1** revealed that this compound was devoid of neurotoxicity against either the NB2a or NG108-15 neuroblastoma cell lines. There was no apparent relationship between tetraoxane structure and neurotoxicity, nor was

there any correlation between antimalarial activity (Tables 1 and 2) and neurotoxicity for these seventeen tetraoxanes.

Discussion

In this work we identified five tetraoxanes with in vitro potency superior to that of prototype tetraoxane **1**, yet the most potent tetraoxane of the group (**11**) was still 1.4-fold less potent than artemisinin and was significantly less potent than arteether. Given the apparent potency barrier of the alkyl-substituted tetraoxane class, a significant improvement in oral activity now becomes a critical issue if these compounds can be considered as potential replacements for the semisynthetic artemisinins. In this respect, we were partially successful, as three tetraoxanes to emerge from this work had better oral activity than either artemisinin or tetraoxane **1** but had weaker oral activity than arteether. On the basis of data from **1**[4] we suspect that oral activity for these tetraoxanes is more a function of drug metabolism than poor absorption, but we performed no pharmacokinetic experiments to confirm this hypothesis. Another potential criterion to consider for this class of compounds is neurotoxicty,[13−17] and from our in vitro data, it is clear that some of the tetraoxanes were only some 5−10-fold less neurotoxic than dihydroartemisinin. Nonetheless, more detailed studies using in vivo models and subsequent cyto- and histopathological experiments would be necessary to assess whether neurotoxicity would disqualify these compounds from further consideration.

A new synthetic method[18] in which trimethylsilyl derivatives of geminal bis-hydroperoxides are reacted with ketones in the presence of trimethylsilyl trifluoromethanesulfonate now permits the synthesis of nonsymmetrical tetraoxanes. This advance should substantially expand the possibilities for tetraoxane design[19] since this new synthetic method is not symmetry-limited as is the acid-catalyzed peroxidation of ketones. With this new technology[17] available, we hope that the results presented here, combined with our previous data on tetraoxanes substituted at the 1 and 10 positions with unsaturated and polar functional groups, provide a good basis on which to move forward in the search for more orally active[20] tetraoxane antimalarials.

Experimental Section

Melting points are uncorrected. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Varian XL-300 spectrometer using $CDCl_3$ as a solvent. All chemical shifts are reported in parts per million (ppm) and are relative to internal $(CH_3)_4Si$ for ^{1}H and $CDCl_3$ (77.0 ppm) for ^{13}C NMR. Microanalyses were performed by M-H-W Laboratories, Phoenix, AZ.

Alkyl-Substituted Cyclohexanones. 2-Methyl-, 2-ethyl-, 3-methyl-, 2,3-dimethyl-, 3,4-dimethyl-, 2,5-dimethyl-, 3,5-dimethyl-, and 3,3,5,5-tetramethylcyclohexanone and (−)-menthone were available from TCI or Aldrich Chemical Co. 4,4-Dimethyl- and 2,4,4-trimethylcyclohexanone were obtained by hydrogenation (Pd−C)[21] of the commercially available enones. 2-Propyl- and 5-isopropyl-2-methylcyclohexanone were prepared by hydrogenation of 2-allylcyclohexanone and carvone, respectively. 2,4-Dimethylcyclohexanone was obtained by methylation of the morpholinoenamine of 4-methylcyclohexanone as described by Stork et al.[22]

Tetraoxane Synthesis. Tetraoxanes **2**, **3**, **5**, **8**−**11**, and **13**−**17** were prepared on a 10-mmol scale via the H_2SO_4/CH_3CN method developed by McCullough et al.[12] using 50% H_2O_2 as previously described.[8] In each case, product tetraoxanes were isolated as white crystalline solids, stable at room temperature in the solid state for years. Tetraoxanes are rather stable peroxide compounds,[23] and dispiro-tetraoxanes derived from alkyl-substituted cyclohexanones are not friction-sensitive.[24] Although we have encountered no difficulties in working with these tetraoxanes, routine precautions such as the use of shields, fume hoods, and avoidance of metal salts should be observed whenever possible.

1,10-Diethyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane (2): yield, 24%; mp 103−104 °C (CH_3CN); ^{1}H NMR 0.87 (t, J = 7.5 Hz, 6H), 1.12−1.95 (m, 20H), 2.78−3.00 (m, 2H); ^{13}C NMR 12.18, 20.15, 22.30, 24.50, 26.58, 29.74, 45.33, 109.64; VPO MW 282, calcd MW 284. Anal. ($C_{16}H_{28}O_4$) C, H.

1,10-Dipropyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane (3): yield, 7%; mp 82−84 °C (EtOH); ^{1}H NMR 0.89 (t, J = 6.9 Hz, 6H), 1.04−1.83 (m, 24H), 2.75−2.96 (m, 2H); ^{13}C NMR 14.15, 20.71, 22.30, 24.41, 27.14, 29.32, 29.65, 43.21, 109.61; VPO MW 295, calcd MW 312. Anal. ($C_{18}H_{32}O_4$) C, H.

2,11-Dimethyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane (5): yield, 32%; mp 132−134 °C (CH_3CN) (lit.[25] mp 65−66 °C); ^{1}H NMR 0.60−1.22 (m, 10H), 1.23−2.00 (m, 12H), 3.09 (br s, 2H); ^{13}C NMR 21.50 (br s), 22.00, 28.61, 29.16 (br s), 31.43 (br s), 34.14, 37.67 (br s), 39.61 (br s), 108.64; VPO MW 272, calcd MW 256.

1,2,10,11-Tetramethyl-7,8,15,16-tetraoxadispiro[5.2.5.2]-hexadecane (8): yield, 4%; mp 158−160 °C (CH_3CN/H_2O 4:1); ^{1}H NMR 0.90 (d, J = 6.4 Hz, 6H), 1.05 (d, J = 6.8 Hz, 6H), 0.90−1.20 (m, 2H), 1.25−1.80 (m, 12H), 3.12−3.30 (m, 2H); ^{13}C NMR 10.63, 20.09, 21.54, 30.46, 34.74, 34.96, 45,24, 109.30; VPO MW 290, calcd MW 284. Anal. ($C_{16}H_{28}O_4$) C, H.

1,3,10,12-Tetramethyl-7,8,15,16-tetraoxadispiro[5.2.5.2]-hexadecane (9): yield, 20%; mp 145−147 °C (CH_3CN); ^{1}H NMR 0.90 (t, J = 6.2 Hz, 6H), 1.00 (t, J = 6.9 Hz, 6H), 0.82−1.93 (m, 14H), 3.08−3.31 (m, 2H); ^{13}C NMR 13.75, 21.72, 30.26, 30.94, 32.16, 38.85, 39.56, 108.90; VPO MW 283, calcd MW 284. Anal. ($C_{16}H_{28}O_4$) C, H.

1,4,10,13-Tetramethyl-7,8,15,16-tetraoxadispiro[5.2.5.2]-hexadecane (10): yield, 26%; mp 144−146 °C (EtOH); ^{1}H NMR 0.80−1.20 (m, 16H), 1.30−1.85 (m, 10H), 3.05−3.26 (m, 2H); ^{13}C NMR 13.53, 21.93, 28.97, 30.82, 34.30, 38.67, 38.73, 109.06; VPO MW 285, calcd MW 284. Anal. ($C_{16}H_{28}O_4$) C, H.

2,3,11,12-Tetramethyl-7,8,15,16-tetraoxadispiro[5.2.5.2]-hexadecane (11): the precipitate was treated with $HClO_4$/HAc to remove impurities; yield, 6% (mixture of 2 diasteromers 2.2:1); mp 103−104 °C (CH_3CN/H_2O 4:1); ^{1}H NMR major isomer, 0.85 (d, J = 7.0 Hz, 12H), 1.21−2.08 (m, 14H), 2.45−2.85 (m, 2H); minor isomer, 0.93 (d, J = 6.3 Hz, 12H), 1.21−2.08 (m, 14H), 2.98−3.20 (m, 2H); ^{13}C NMR 12.24, 18.13 (br s), 19.18, 19.66, 24.60 (br s), 27.23 (br s), 27.78 (br s), 28.26 (br s), 30.96, 32.40, 34.65 (br s), 38.43, 108.66; VPO MW 285, calcd MW 284. Anal. ($C_{16}H_{28}O_4$) C, H.

3,3,12,12-Tetramethyl-7,8,15,16-tetraoxadispiro[5.2.5.2]-hexadecane (13): yield, 29%; mp 191−192 °C (CH_3CN); ^{1}H NMR 0.72−0.97 (m, 6H), 0.95 (s, 6H), 1.16−1.46 (m, 8H) 1.47−1.73 (m, 4H), 2.13−2.51 (m, 4H); ^{13}C NMR 25.52 (br s), 27.74, 28.17 (br s), 29.91, 34.38 (br s), 34.98 (br s), 108.33; VPO MW 258, calcd MW 284. Anal. ($C_{16}H_{28}O_4$) C, H.

1,10-Diisopropyl-4,13-dimethyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane (14): yield, 20%; mp 97−98 °C (CH_3OH); $[\alpha]^{27}_D$ −84.4° (*c* 0.1, $CHCl_3$); ^{1}H NMR 0.76−1.27 (m, 22H), 1.30−1.92 (m, 10H), 2.23−2.59 (m, 2H), 3.02−3.37 (m, 2H); ^{13}C NMR 18.60, 21.41, 21.94, 23.02, 24.52, 28.98, 34.58, 39.84, 49.89, 110.94; VPO MW 329, calcd MW 340. Anal. ($C_{20}H_{36}O_4$) C, H.

4,13-Diisopropyl-1,10-dimethyl-7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane (15): yield, 26%; mp 140−142 °C (EtOH); ^{1}H NMR 0.91 (d, J = 6.5 Hz, 12H), 1.02 (d, J = 6.8 Hz, 6H), 0.95−1.19 (m, 4H), 1.30−1.82 (m, 12H), 3.15−3.35 (m, 2H); ^{13}C NMR 13.45, 19.54, 19.93, 29.18, 30.83, 32.26, 33.56, 39.10, 40.04, 109.40; VPO MW 344, calcd MW 340. Anal. ($C_{20}H_{36}O_4$) C, H.

1,3,3,10,12,12-Hexamethyl-7,8,15,16-tetraoxadispiro-[5.2.5.2]hexadecane (16): yield, 34%; mp 158−159 °C (EtOH);

^{1}H NMR 0.80−1.05 (m, 18H), 1.10−1.70 (m, 10H), 1.84−2.05 (m, 2H), 2.95−3.13 (m, 2H); ^{13}C NMR 13.68, 24.50, 26.42, 30.40, 32.07, 34.85, 35.26, 44.10, 109.13; VPO MW 315, calcd MW 312. Anal. ($C_{18}H_{32}O_4$) C, H.

2,2,4,4,11,11,13,13-Octamethyl-7,8,15,16-tetraoxadispiro-[5.2.5.2]hexadecane (17): yield, 68%; mp 185−186 °C (CH_3-OH) (lit. mp 184−186 °C); ^{1}H NMR 1.05 (s, 24H), 1.30 (s, 4H), 1.42 (br s, 4H); 2.17 (br s, 4H), ^{13}C NMR 31.55 (br s), 31.73, 31.97 (br s), 40.53, 43.47, 51.97, 110.11.

Antimalarial Screens. In vitro activity against *P. falciparum* was determined using a modification of the semiautomated microdilution technique of Desjardins et al.[26] and Milhous et al.[27] Two *P. falciparum* malaria parasite clones,[28] designated as Sierra Leone (D6) and Indochina (W2), were used in susceptibility testing. The former is resistant to mefloquine and the latter to CQ, pyrimethamine, sulfadoxine, and quinine. Test compounds were dissolved in dimethyl sulfoxide and solutions serially diluted with culture media. Erythrocytes with 0.25−0.5% parasitemia were added to each well of a 96-well microdilution plate to give a final hematocrit of 1.5%. Inhibition of uptake of tritiated hypoxanthine was used as an index of antimalarial activity. Results were initially recorded as IC_{50} (ng/mL) values and then converted to nM values.

In vivo activity against *P. berghei* was obtained against a drug-sensitive KBG 173 strain of *P. berghei*. A modification of the Thompson test[29] was used to measure the sc and po antimalarial activity of selected tetraoxanes. Five-week-old CD-1 mice were inoculated on day 0 with 5×10^6 trophozoites of *P. berghei* obtained from an infected mouse at 60% parasitemia, diluted with uninfected mouse blood, and injected intraperitonealy. On each of days 3, 4, and 5, groups of five or seven mice were treated b.i.d. with 64 (sc) or 128 (po) mg/kg dose of tetraoxanes. Blood films were taken 1 day after completion of drug treatment (day 6) and weekly thereafter until day 60. Parasitemia values were determined from Giemsa-stained blood films. Drug activity was evaluated by suppression of parasitemia, extension of survival time, and curative activity. Untreated mice survive on average 6.2 days. Mice living 60 days post-infection and blood film negative were considered cured. A drug was considered to be toxic if the mice died before the untreated control mice.

Neurotoxicity Screen. As described by Fishwick et al.,[16] neuroblastoma cells were grown in 25-mL culture flasks with 15 mL of medium in a humidified 37 °C incubator with 5% CO_2. Culture medium used for the NB2a cell proliferation consisited of high glucose DMEM with Glutamax-1, supplemented with 5% (v/v) horse serum, 5% (v/v) fetal calf serum and 100 units/mL penicillin plus 100 μg/mL streptomycin, with optional 25 μg/mL gentamicin. NB2a cells were plated onto 48-well microtiter culture plates at a cell density of 15 000 cells/mL. After 24 h the cells were induced to differentiate and generate neurites in the presence of the drugs, by the following method: the proliferation medium was removed and replaced with serum-free medium plus 0.5 mM dibutyryl cyclic AMP containing the drugs at concentrations of 10 and 0.1 μM. The cells were incubated for a further 24 h, after which neurite outgrowth was measured.

Cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, stained for 10 min with Coomassie blue cell stain 0.6% (w/v) (Coomassie brilliant blue G in 10% (v/v) acetic acid, 10% (v/v) methanol and 80% (v/v) PBS), and then washed with PBS and distilled water. The cells were then viewed by a light microscope (Zeiss Axiovert 35M) linked by a video camera to a Kontron Vidas 2.0 image analyzer. Subsequently, 10 different fields of approximately 20 cells were chosen for each drug and control.

A program was written using the available functions of the image analyzer in order to permit the automatic measurement of the total length of neurites (in pixels) for the cells in a given field and to express the results as the average length of neurites/cell.

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