Malaria-Infected Mice Are Cured by a Single Dose of Novel Artemisinin Derivatives

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We disclose here for the first time the curative activity of a new generation of trioxane dimers, designed logically and prepared easily from the natural trioxane artemisinin in only four or five chemical steps that would be easily accomplished also on a manufacturing scale. Four of these trioxane dimers cure malaria-infected mice after only a single subcutaneous dose, and two other dimers cure after three oral doses.

Introduction

The public health crisis posed by drug-resistant malaria and the urgent need for safe and effective new therapies are widely recognized.[1−3] The malaria genome sequence promises to provide new drug targets in the future, but another route of drug development (i.e., starting with natural products) has already yielded exciting results during the past several years. Long before the discovery of its protozoal etiology, the distinctive clinical manifestations of malaria made it recognizable as a separate entity within the febrile illnesses. This led to the use of herbal remedies specifically targeted against malaria, and modern scientific methods have now shown that several of these plants contain potent antimalarial compounds. Best known is quinine, the active principle of cinchona bark that, some 300 years after its introduction, remains the drug of choice for treatment of severe falciparum malaria.[4,5] More recently identified is artemisinin (qinghaosu, **1**), the active ingredient in decoctions or teas of *Artemisia annua* that Chinese herbalists have traditionally used to treat malaria.[6,7] Since its identification in the 1970s, artemisinin as well as semi-synthetic derivatives and synthetic trioxanes have been utilized clinically. Natural product **1** and its C10-oxygenated semi-synthetic derivatives artemether (**1b**) and artesunate (**1c**) share an unusual endoperoxide moiety that is essential for antimalarial activity. The principal metabolite for both of these monomers **1b** and **1c** is dihydroartemisinin (**1a**), which provides both antimalarial activity and a major route of elimination. As a class, these compounds have proven unusually valuable for (1) their brisk and potent antimalarial activity, (2) their lack of resistance and cross-resistance with other antimalarials, and (3) their action against the gametocyte forms of the parasite responsible for infecting mosquitoes and thus for transmitting this infectious disease (artemisinins reviewed in[8−12]). For these reasons, trioxanes are now considered an essential component of artemisinin combination therapy (ACT) against drug-resistant malaria.[13,14]

Despite their obvious value, however, these first-generation monomeric trioxane drugs share a number of disadvantages, including a short plasma half-life of typically less than 1 h and predictable recrudescence of parasitemia when used as monotherapy.[15] When combined with other antimalarials, the trioxanes contribute a rapid and profound reduction in parasite burden. However, they are quickly eliminated, and by just 1 day after treatment, the more persistent components of the combination (which typically have half-lives of days to weeks) are left to function alone. This pharmacokinetic discrepancy compromises the ability of the trioxanes to prevent the emergence of drug-resistant parasites.

During the past 15 years, we have designed, synthesized, and evaluated in vitro the antimalarial activity of nearly 1000 synthetic and semi-synthetic trioxanes.[16,17] We disclose here for the first time the in vivo curative activity of a new generation of trioxane dimers, designed logically and prepared easily from plant-derived artemisinin in only four or five chemical steps that would be easily accomplished also on a manufacturing scale. Four of these designer trioxane dimers cure malaria-infected mice after only a single subcutaneous dose, and two other dimers cure malaria-infected mice after three oral doses (Table 1).

Table 1. Antimalarial Activity of Trioxanes in *P. Berghei*-Infected Mice

trioxanes	survival[a] number of days after infection			
	single subcutaneous dose			three oral doses
	30 mg/kg	10 mg/kg	3 mg/kg	30 mg/kg
	Dimers			
5	15.0	13.3	9.7	>30.0[b]
6	27.0	10.0	8.0	>30.0[b]
7	>30.0	12.3	8.0	
8	>30.0	15.0	12.3	12.7
9	>30.0	25.3	14.0	14.7
10	>30.0	10.0	9.0	7.0
	Monomer Control			
1c	9.1 ± 3.7	8.6 ± 3.5	7.3 ± 1.1	7.5 ± 0.4

[a] Three animals per dimer treatment group. All mice that survived 30 days were sacrificed, no parasites were detected, and the mice were considered cured. In four experiments over 6 months, survival of vehicle-treated controls ($N = 20$) was 6.8 ± 0.4 (M ± SD) days after infection. Artesunate control values are M ± SD for nine animals. No artesunate-treated mice were cured. For both sc and po administration, the vehicle is Tween 80 (70%) and ethanol (30%), followed by 10× dilution with water. [b] An average survival of >30 days indicates that all three mice per treatment group survived to at least day 30 post-infection.

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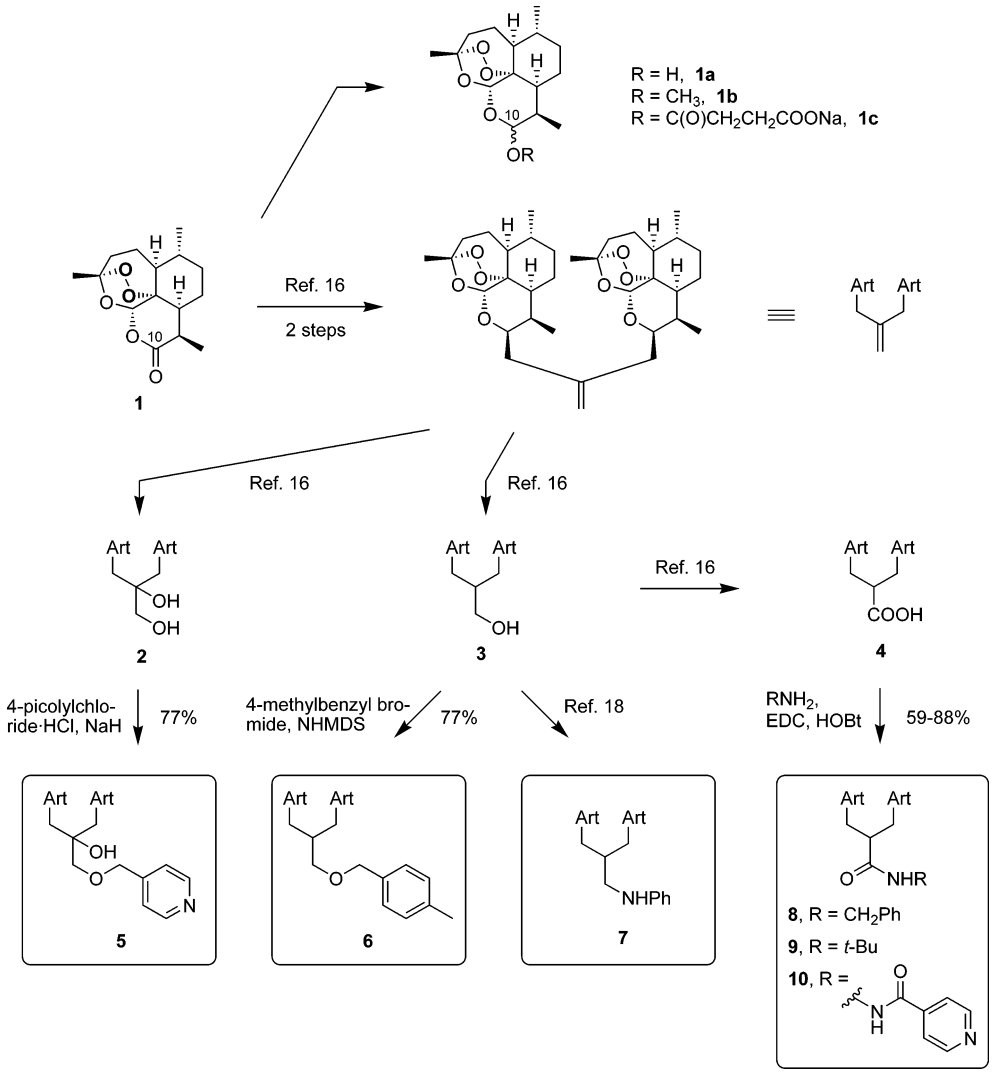
Scheme 1. First Generation Artemisinins and Scheme for Synthesis of Artemisinin-Based Dimers



Chemistry

In Scheme 1, the chemical conversions of artemisinin into previously described[16] dimeric trioxane diol **2** (log $P = 4.68$), primary alcohol **3** (log $P = 5.76$), and carboxylic acid **4** (log $P = 3.0$) are outlined. We considered that more lipophilic versions of these dimer alcohols and carboxylic acid would be more desirable for high in vivo efficacy. Therefore, trioxane dimers WC-isobudiol-OCH_2Pyr (**5**, log $P = 6.25$), WC-isobu-OCH_2-Tol (**6**, log $P = 8.9$), KB07 (**7**, log $P = 7.95$),[18] IP-IV-22y (**8**, log $P = 6.95$), SS-isobuC(O)NHTB (**9**, log $P = 6.38$), and ASR-isobuC(O)-isoniaz (**10**, log $P = 5.19$) were prepared also as outlined in Scheme 1 using standard Williamson ether syntheses (**5** and **6**), reductive amination (**7**), and amide bond formation (**8**−**10**). Importantly, none of these chemical transformations destroyed the critical peroxide pharmacophore within these 1,2,4-trioxanes.

Biology

Following a standard protocol,[19] trioxane dimers **5**−**10** were formulated for subcutaneous or oral administration to NMRI mice that were infected on day 0 with the GFP strain of *P. berghei*. Average survival data after various dosing regimens are summarized in the table; the clinically used trioxane drug artesunate is included as a standard. Parasitemia levels were determined using standard flow cytometry techniques.[20] In a regimen that utilized just a single subcutaneous dose on day 1 after infection, trioxane dimer ether **5** at 30 mg/kg prolonged average survival of the mice to 15 days (well beyond the 6.8 days of untreated controls); in desirable contrast, ether dimer **6** at 30 mg/kg prolonged average survival to 27 days and cured two out of three mice. At 30 mg/kg, trioxane dimer amine **7** and the three trioxane dimer amides **8**−**10** cured all of the malaria-infected mice. Furthermore, even at a single subcutaneous dose of only 3 mg/kg, dimer amides **8** and **9** prolonged mouse survival considerably. In particular, dimer *t*-butyl amide **9** at one dose of 10 mg/kg prolonged average survival to 25 days and cured two out of three mice; these results far exceed the current benchmark set by the clinically used trioxane drug artesunate (Table 1). At doses considerably higher than 30 mg/kg, artesunate is more efficacious.[13] In a regimen of three consecutive daily oral doses of 30 mg/kg starting on day 1 after infection, pyridyl dimer **5** and tolyl dimer **6** are curative, in sharp contrast to artesunate, which increased survival versus control by less than 1 day. Importantly for safety considerations, neither overt toxicity nor behavioral change attributable to trioxane dimer administration was observed visually in any of the cured animals.

Conclusions

Given the enormous number of people who need antimalarial therapy and the limited resources available for health care in

areas where malaria is endemic, the usual requirements for efficacy, safety, ease of administration, and low cost are especially stringent for antimalarials. The semi-synthetic artemisinin-derived dimers reported here successfully address several shortcomings of their first generation predecessors. Most important is the curative activity after a single low parenteral dose, which is distinctly unusual in literature reports for this class. Several factors could contribute to this finding, including an increase in antimalarial potency over that attained with first generation compounds, which may reduce parasitemia sufficiently to permit complete clearance of parasites in vivo. These dimers may also have a longer plasma half-life based on the intentionally designed carbon-to-carbon nonacetal linkage at the C10 position, rather than the hydrolytically more vulnerable carbon-to-oxygen acetal linkage of previous C10 derivatives **1b** and **1c**.

Although inexpensive by western standards, the widespread use of artemisinins is limited in part by availability and by cost (largely attributable to the isolation of artemisinin from its plant source).[21] Increased cultivation of high-yielding strains of *Artemisia annua* and better extraction methods are being implemented, as are efforts to engineer the overproduction of artemisinin or its biosynthetic precursors.[22,23] The potency and curative activity of our novel artemisinin-derived dimers reported here provide a substantially more efficient and economical use of the price-setting natural product.

Ongoing studies are focused on identifying the best of these and related trioxane dimer candidates as lead compounds to enter advanced preclinical evaluation and ultimately studies in humans.

Experimental Section[16]

Each of the new trioxane dimers is stable in the absence of solvent at 60 °C for at least 24 h and is hydrolytically stable in a solution of pH 7.4 H_2O/DMSO (1/4) at 25 °C for at least 12 h. Log *P* values were calculated using ACD/Labs version 10.0.

Ether 5. To a solution of 4-picolylchloride hydrochloride (33 mg, 0.20 mmol) in DMF (2 mL) at 0 °C was added sodium hydride (60% dispersion in mineral oil, 40 mg, 1.00 mmol), and the heterogeneous mixture was stirred at rt for 30 min. To the mixture was added a solution of **2** (62 mg, 0.10 mmol) in DMF (1 mL) dropwise. It was warmed to rt and stirred for 5 h. The reaction was cooled to 0 °C and quenched with water (3 mL) and saturated aq NH_4Cl (1 mL). Ether (4 mL) was added, and the layers were separated. The aqueous layer was extracted with ether (5 × 2 mL). The combined organic solution was washed with saturated aq $CuSO_4$ solution (2 × 1 mL), dried ($MgSO_4$), and concentrated. The crude oil was purified by flash column chromatography (elution with EtOAc/hexanes = 2:1) on silica gel that had been treated with Et_3N (1 mL per 100 mL gel) in hexanes before use. Ether **5** (55 mg, 77%) was formed as a white solid: $[\alpha]^{24}_D = +37$ (*c* 0.34, $CHCl_3$); mp 85−86 °C; IR (thin film) 3500, 2924, 1716, 1102, 1053, 1011; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53 (d, J = 5.2 Hz, 2H), 7.33 (d, J = 6.0 Hz, 2H), 5.33 (s, 1H), 5.29 (s, 1H), 4.67 (d, J = 13.6 Hz, 1H), 4.60 (m, 2H), 4.58 (d, J = 13.6 Hz, 1H), 4.02 (br s, 1H), 3.80 (d, J = 9.2 Hz, 1H), 3.66 (d, J = 9.2 Hz, 1H), 2.66 (dq, J = 13.6, 6.8 Hz, 1H), 2.58 (dq, J = 13.6, 6.8 Hz, 1H), 2.29 (m, 2H), 2.03−1.73 (m, 9H), 1.68−1.53 (m, 5H), 1.40−1.18 (m, 14H including s at 1.38 and 1.32), 0.97−0.82 (m, 14H including d at 0.94 with J = 5.2 Hz, and d at 0.87 with J = 7.6 Hz and 0.85 with J = 7.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 149.1, 148.8, 122.2, 103.1, 102.9, 89.4, 88.7, 81.1, 74.3, 73.9, 71.4, 71.1, 70.9, 52.3, 52.0, 44.3, 43.8, 37.4, 37.4, 36.5, 36.5, 36.2, 35.1, 34.4, 34.3, 30.8, 30.7, 26.1, 26.0, 24.8, 24.7, 24.7, 24.7, 20.1, 20.0, 13.1, 12,7; HRMS (FAB) calcd for $C_{40}H_{60}NO_{10}$ [(M + H)$^+$], 714.4217; found, 714.4199. Anal. Calcd for $C_{40}H_{59}NO_{10}$: C, 67.30; H, 8.33; N, 1.96. Found: C, 67.16; H, 8.42; N, 1.92.

Ether 6. To a solution of **3** (97 mg, 0.16 mmol) in THF (1 mL) at 0 °C was added sodium bis(trimethylsilyl)amide (NHMDS) in THF (1.0 M, 0.48 mL, 0.48 mmol) and 4-methylbenzyl bromide (59 mg, 0.32 mmol) in THF (0.5 mL). The reaction was warmed to rt and stirred for 16 h. It was quenched with saturated aq NH_4Cl (1 mL) and layers were separated. The aqueous layer was extracted with EtOAc (3 × 2 mL). The combined organic solution was dried ($MgSO_4$) and concentrated. The purification of the crude product by column chromatography (elution with EtOAc/hexanes = 1:5) gave ether **6** (87 mg, 77%) as a white solid: $[\alpha]^{24}_D = +77$ (*c* 0.30, $CHCl_3$); mp 51−52 °C; IR (thin film) 2938, 1451, 1376, 1101, 1008 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 (d, J = 8.0 Hz, 2H), 7.12 (d, J = 8.0 Hz, 2H), 5.33 (s, 1H), 5.31 (s, 1H), 4.49 (d, J = 11.6 Hz, 1H), 4.42 (d, J = 11.6 Hz, 1H), 4.30 (m, 1H), 4.20 (m, 1H), 3.66 (dd, J = 9.2, 5.2 Hz, 1H), 3.60 (dd, J = 9.2, 4.8 Hz, 1H), 2.72 (dq, J = 15.2, 7.6 Hz, 1H), 2.65 (dq, J = 14.4, 7.2 Hz, 1H), 2.38−2.26 (m, 5H including s at 2.33), 2.10 (m, 1H), 2.03 (m, 1H), 1.99 (m, 1H), 1.90−1.20 (m, 26H including s at 1.41 and 1.38), 0.98−0.82 (m, 14H including d at 0.85 with J = 7.2 Hz and 0.84 with J = 7.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.8, 136.0, 128.8, 127.8, 103.2, 103.0, 88.9, 88.5, 81.2, 81.2, 74.9, 72.8, 72.7, 71.8, 52.5, 52.4, 44.7, 44.5, 37.3, 37.3, 36.6, 36.6, 35.6, 34.5, 34.5, 30.6, 30.5, 30.0, 29.6, 26.2, 26.1, 24.8, 24.7, 24.6, 21.1, 20.2, 20.2, 13.4, 13.1; HRMS (FAB) calcd for $C_{42}H_{63}O_9$ [(M + H)$^+$], 711.4472; found, 711.4445.

Amide 8. To a solution of **4** (15 mg, 0.024 mmol) in anhydrous dichloromethane (1.0 mL) was added EDC[a] (18 mg, 0.094 mmol, 4.0 equiv) and HOBt (3.5 mg, 0.026 mmol, 1.1 equiv). A further 0.5 mL of anhydrous dichloromethane was added to wash down the flask walls, and then the reaction mixture was treated with benzyl amine (0.010 mL, 0.094 mmol, 4.0 equiv) and triethylamine (0.013 mL, 0.094 mmol, 4.0 equiv). It was stirred at room temperature for 18 h, at which time TLC analysis showed full consumption of starting material. Hydrochloric acid (1%, 5 mL) and methylene chloride (10 mL) were added, and organics were extracted with methylene chloride (3 × 20 mL), dried ($MgSO_4$), and concentrated in vacuo to give a sticky solid. Flash column chromatography on silica eluting with 30% ethyl acetate/hexanes isolated amide **8** as a white solid (14 mg, 82%): $[\alpha]^{23}_D$ +110 ($CHCl_3$, *c* 0.43); mp = 75−78 °C; IR (thin film) 2938, 2874, 1671, 1522, 1453, 1376, 1187, 1093, 1052, 1012, 941, 878, 826, 732, 700 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.40−7.20 (m, 5H), 6.27 (t, br, J = 5.6 Hz, 1H), 5.28 (s, 1H), 5.22 (s, 1H), 4.45 (d, br, J = 5.2 Hz, 2H), 4.15−4.05 (m, 2H), 2.80−2.64 (m, 2H), 2.61−2.54 (m, 1H), 2.38−2.14 (m, 3H), 2.05−1.96 (m, 2H), 1.85−1.16 (m, 25H, including singlets at 1.38 and 1.27), 1.00−0.81 (m, 14H, including apparent triplet at 0.94 with J = 5.6 Hz and two doublets at 0.86 with J = 7.6 Hz and 0.83 with J = 7.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 175.73, 138.40, 128.46, 128.12, 127.14, 103.43, 103.35, 88.58, 88.33, 81.19, 81.04, 76.41, 73.88, 52.57, 52.38, 44.73, 44.55, 44.39, 44.05, 37.42, 37.17, 36.53, 36.49, 34.50, 34.46, 33.13, 32.83, 30.17, 29.95, 26.20, 26.04, 24.85, 24.77, 24.64, 24.51, 20.20, 13.56, 13.06; HRMS (ESI) calcd for $C_{41}H_{59}NO_9Na$ [(M + H)$^+$], 732.4082; found, 732.4080.

Amide 9. A flame-dried 20 mL recovery flask equipped with a magnetic stir bar and a septum along with an Ar balloon was charged with **4** (50 mg, 0.10 mmol) and dissolved in 2.0 mL of freshly distilled CH_2Cl_2. The flask was cooled down to 0 °C, and EDC (23 mg, 0.12 mmol, 1.5 equiv) and HOBT (16 mg, 0.12 mmol, 1.5 equiv) were added, respectively. The mixture was allowed to stir for 2 h. The *tert*-butylamine (0.020 mL, 0.19 mmol, 2.5 equiv) and triethylamine (0.040 mL, 0.58 mmol) were added to the reaction at 0 °C, and it was left stirring overnight, warming up to room temperature. The reaction was quenched by the addition of 10 mL of distilled water, and the mixture was placed into a separatory funnel with additional methylene chloride (5 mL). The mixture was extracted with methylene chloride (3 × 30 mL). The combined extracts were washed with water (5 mL) and brine (5 mL), dried

[a] Abbreviations: EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole.

over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo to give the crude product that was purified by flash column chromatography, which was eluted with 50% ethyl acetate in hexanes to afford amide **9** (58 mg, 88%) as an amorphous solid: $[\alpha]^{25}_D$ +85.3 (*c* 1.00, $CHCl_3$); IR (thin film) 3404, 2954, 2875, 1668, 1512, 1453, 1377, 1225, 1126, 1094, 1051, 1011, 941, 878, 754 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 5.82 (s, 1H), 5.26 (s, 2H), 4.14−4.10 (m, 1H), 4.09−4.00 (m, 2H), 3.18−3.13 (m, 1H), 2.93−2.95 (m, 1H), 2.77−2.66 (m, 2H), 2.36−2.24 (m, 3H), 2.12−1.94 (m, 3H), 1.89−1.71 (m, 5H), 1.65−1.56 (m, 3H), 1.53−1.41 (m, 5H), 1.39−1.30 (m, 18H, including three singlets at 1.37, 1.35 and 1.32), 0.94−0.90 (m, 8H), 0.84−0.79 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.4, 103.4, 103.3, 88.23, 88.22, 81.1, 81.0, 76.6, 74.6, 60.3, 52.6, 52.5, 51.1, 45.2, 44.70, 44.68, 37.3, 37.1, 36.5, 36.4, 34.5, 33.2, 32.4, 30.2, 29.9, 28.6, 26.2, 26.1, 24.74, 24.65, 24.6, 24.5, 20.18, 20.16, 13.6, 13.2; HRMS (FAB) calcd for $C_{38}H_{61}NO_9H$ [$(M + H)^+$], 676.4425; found, 676.4411.

Amide 10. Acid **4** (100 mg, 0.16 mmol), EDC (37 mg, 0.19 mmol), and HOBt (26 mg, 0.19 mmol) were added to dichloromethane (7 mL) under argon. After stirring at room temperature for 3 h, isoniazid (44 mg, 0.32 mmol) and triethylamine (90 μL, 0.64 mmol) were added. The reaction was allowed to stir at room temperature overnight at which point the colorless solution turned pale yellow. The reaction was quenched with 1% HCl (5 mL). The organic layer was extracted with dichloromethane (3 × 20 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash silica gel column chromatography (100% EtOAc) to yield amide **10** as a white solid (70 mg, 0.094 mmol, 59%): $[\alpha]^{22.0}_D$ +68 (*c* = 0.30, $CHCl_3$); mp = 146−149 °C; IR (thin film) 3519, 3230, 2940, 2875, 1668, 1453, 1378, 1252, 1187, 1125, 1095, 1032, 1013, 940, 877, 826, 733 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ 8.77−8.75 (m, 2H), 7.80−7.78 (m, 2H), 5.38 (s, 1H), 5.33 (s, 1H), 4.23−4.20 (m, 1H), 4.15−4.10 (m, 1H), 2.68−2.55 (m, 2H), 2.29−2.09 (m, 3H), 1.93−1.76 (m, 7H), 1.71−1.60 (m, 4H), 1.59−1.35 (m, 10H), 1.32 (s, 3H), 1.28 (s, 3H), 1.23−1.14 (m, 4H), 0.97−0.93 (m, 9H), 0.89−0.85 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.5, 162.4, 150.0, 121.5, 103.7, 103.4, 88.9, 88.4, 81.1, 80.9, 76.5, 73.7, 52.4, 52.3, 44.7, 44.4, 43.1, 37.5, 37.2, 36.4, 34.4, 33.2, 32.6, 30.1, 30.0, 29.8, 26.0, 24.8, 24.6, 20.2, 13.5, 12.9; HRMS (FAB) calcd for $C_{40}H_{58}N_3O_{10}$ [$(M + H)^+$], 740.4122; found, 740.4121.

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Supporting Information Available: ^1H and ^{13}C NMR spectra, as well as HPLC traces for all the new trioxane dimers. This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added in Proof. The importance of high lipophilicity for a trioxane to have high antimalarial oral activity has been noted: Singh, C.; Kanchan, R.; Sharma, U.; Puri, S. K. New Adamantane-Based Spiro 1,2,4-Trioxanes Orally Effective against Rodent and Simian Malaria. *J. Med. Chem.* **2007**, *50*, 521−527.

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