Antimalarial and Antitumor Evaluation of Novel C-10 Non-Acetal Dimers of 10*β*-(2-Hydroxyethyl)deoxoartemisinin

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Received July 28, 2003

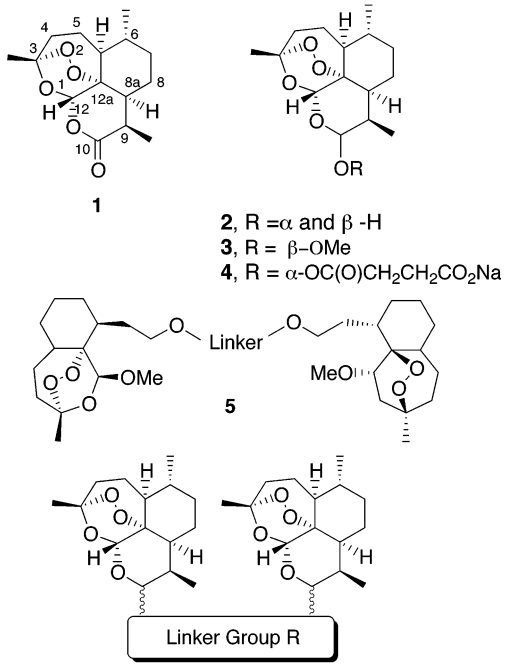
Four series of C-10 non-acetal dimers were prepared from key trioxane alcohol 10*β*-(2-hydroxyethyl)deoxoartemisinin (**9b)**. All of the dimers prepared displayed potent low nanomolar antimalarial activity versus the K1 and HB3 strains of *Plasmodium falciparum*. The most potent compound assayed was phosphate dimer **14a**, which was greater than 50 times more potent than the parent drug artemisinin and about 15 times more potent than the clinically used acetal artemether. In contrast to their potent activity versus malaria parasites, virtually all of the dimers expressed poor anticancer activity apart from the trioxane phosphate ester dimers **14a** and **14b**, which expressed nanomolar growth inhibitory (GI_{50}) values versus a range of cancer cell lines in the NCI 60 human cell line screen. Further detailed studies on these dimers in vitro in HL60 cells demonstrate that both phosphate ester dimers (**14a** and **14b**) are more potent than the anticancer agent doxorubicin. Interestingly, phosphate ester monomers **9c** and **9d**, antimalarially active in the low nanomolar region versus *P. falciparum*, are inactive as anticancer agents even at concentrations in the millimolar region. This observation emphasizes the importance of two trioxane units for high antiproliferative activity, and we propose that the nature of the linker in dimers of this type plays a crucial role in imparting potent anticancer activity.

Introduction

The compound artemisinin, also known as qinghaosu (**1**), is a tetracyclic 1,2,4-trioxane occurring in *Artemisia annua*. Artemisinin and its derivatives dihydroartemisinin (**2**), artemether (**3**), and sodium artesunate (**4**) are routinely used for the treatment of malaria and are particularly effective against cerebral malaria.[1]

Different modes of action have been proposed by various groups to account for the action of artemisinin and its derivatives in treating malaria.[2] While the mode of action of artemisinin as an antimalarial has not been unequivocally established, it has been demonstrated that the peroxide linkage is essential for expression of activity.

Certain artemisinin derivatives, which contain a peroxide moiety, have also been tested for biological activity other than antimalarial activity. For instance, the cytoxicity of artemisinin, dihydroartemisinin, and an ether dimer of artemisinin to Ehrlich ascites tumor cells has been reported. [3] Haynes and co-workers disclosed in a patent the cytotoxicity of a range of



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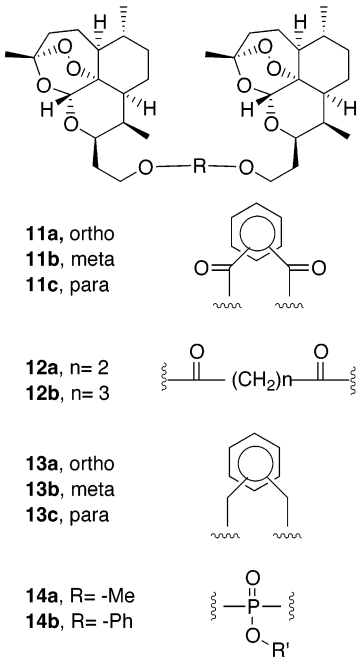
10.1021/jm030974c CCC: $27.50
Published on Web 01/31/2004

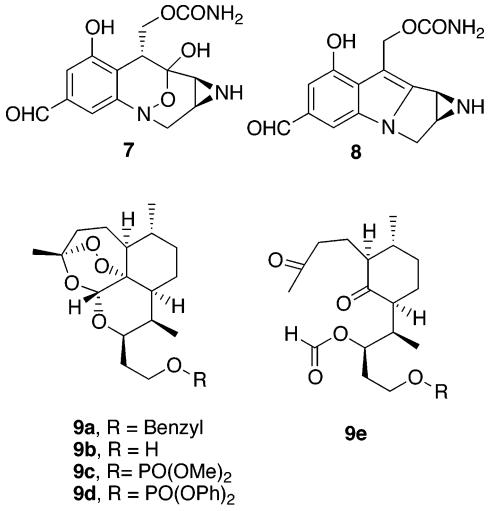
Chart 1



semisynthetic monomers of dihydroartemisinin.[4] The most potent derivatives were shown to be capable of causing extensive DNA damage, as assessed by the DNA comet assay. It was proposed that the observed antitumor effect of these peroxide-based monomers was due to nuclear DNA damage.[4] More recently, Wu and co-workers have described the cytotoxicity of some cyanoarylmethyl analogues of dihydroartmisinin. Some of these peroxides had activities in the nanomolar range against P388 murine leukaemia cell lines.[5]

Alkylating agents remain an effective class of anticancer drugs whose cytotoxic and therapeutic effects are derived primarily from their ability to form intra- and interstrand DNA cross-links. Some dimeric chemical structures capable of DNA cross-linking have especially high biological activities. These include bismustard lexitropsins,[6] dimeric cephalostatin analogues,[7] bisenediynes,[8] and dimers of the saponin antitumor agent OSW-1.[9] Recently, there have been several reports of the potent antimalarial and antitumor activities of a series of synthetic (**5**)[10] and semisynthetic artemisinin-derived dimers (**6a**).[11] Dimers of this type (**6a**) were found to possess potent antitumor activity in the NCI 60 cell line assay with activities comparable to that of paclitaxel. The potential drawback in these derivatives is the presence of the metabolically susceptible C-10 acetal linkage, and Posner and co-workers subsequently prepared a set of metabolically more robust dimers (**6b**).[12a] These compounds were found to possess both potent antimalarial and tumor growth inhibitory properties. More recently Posner has reported on the concise synthesis and antimalarial and antitumor activity of highly stable three-carbon-linked dimers.[12b] In parallel, Jung and co-workers disclosed data on amide- and sulfone-linked C-10 carba dimers. Some of these compounds demonstrated comparable activity to taxol and adriamycin against murine P388 cell lines and human breast cancer MCF7 cell lines.[12c]

The mechanism of antitumor and antimalarial activity of these trioxane dimers may involve either DNA-directed alkylation or protein alkylation, after reductive activation. Other antitumor agents, such as FR-900482 (**7**), upon reductive N−O scission, rearrange to produce cytotoxic mitosene-like intermediates, **8**, which can efficiently cross-link DNA.[13] In a similar manner, we have recently shown that C-10 carba trioxane analogues of dihydroartemisinin (**9a**), upon reductive activation, generate potentially toxic dicarbonyl products (**9e**). [14] In terms of the antimalarial mechanism of action, it was suggested that selective intraparasitic generation of **9e** and subsequent protein alkylation in tandem with free radical mediated damage may have a role to play in the mechanism of action of these analogues.



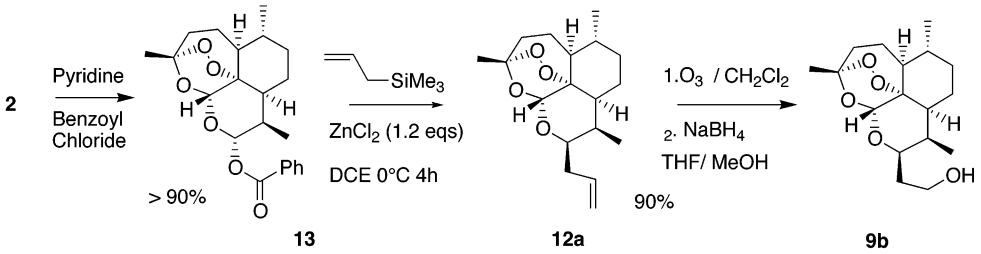
The aim of the present study was to examine the antimalarial and antitumor activities of a new series of non-acetal C-10 carba dimers (Chart 1). It has been noted that the replacement of oxygen at the C-10 position with carbon produces compounds not only with greater hydrolytic stability but also with a longer half-life and potentially lower toxicity.[14−17] Consequently, several groups have developed synthetic and semisynthetic approaches to C-10 carba analogues. In the approach described here, we have employed trioxane alcohol (**9b**) as a key intermediate for the preparation of new dimeric artemisinin analogues.

Chemistry

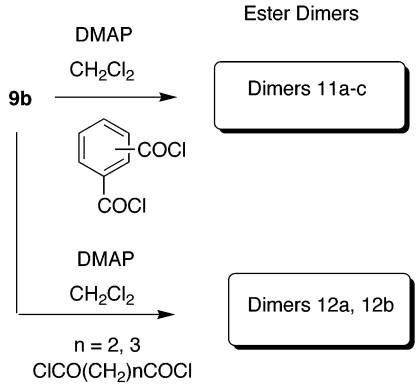
Our optimized procedure was used for the synthesis of **9b**.[17] Thus, the benzoate **10** was prepared in high yield by treatment of DHA with benzoyl chloride in pyridine. NMR studies on the white crystalline solid indicated α stereochemistry with respect to the C−O linkage at C-10 (C-10 H appears as a doublet with a J_{9-10} value of 9.9 Hz, indicating a dihedral angle of about 180° between itself and C-9 H). Reaction of the benzoate with allyltrimethylsilane (5 equiv) in the presence of $ZnCl_2$ catalyst (1.2 equiv) at 0 °C for 4 h gave the desired product, **10a**, in 90% yield (Scheme 1). Ozonolysis of **10a** and in situ reduction of the ozonide provided the key alcohol in 55% yield required for dimerization.

Trioxane dimers **11a**−**c** and **12a**,**b** were prepared by treating 2 equiv of vacuum-dried alcohol **9b** and 1 equiv of the requisite acid chloride in the presence of a catalytic quantity of 4-(dimethylamino)pyridine. Reac-

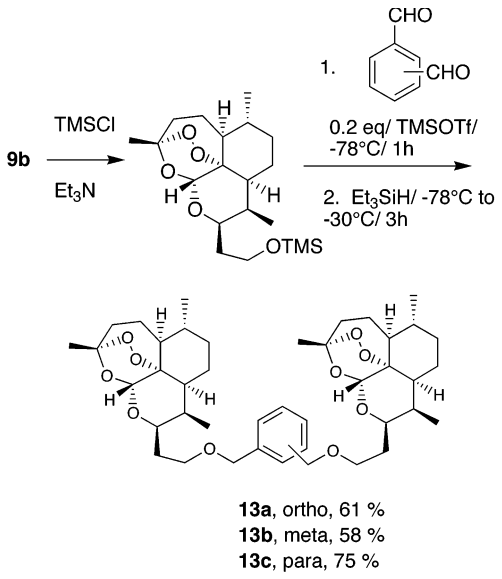
Scheme 1. Synthesis of Trioxane Alcohol **9b**



Scheme 2. Synthesis of Aliphatic and Aromatic Diester Dimers



Scheme 3. Synthesis of Ether-Linked Dimers by Reductive Etherification

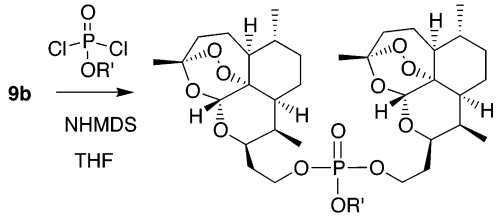


13a, ortho, 61 %
13b, meta, 58 %
13c, para, 75 %

tions were complete after 16 h, and the products were isolated by standard workup and silica gel chromatography (Scheme 2).

For the preparation of ether derivatives, we decided to employ the mild reductive etherification procedure developed by Hatakeyama and co-workers.[18] The trioxane alcohol was converted into the corresponding TMS ether, which was then coupled with an aromatic bis-aldehyde at −78 °C in the presence of TMSOTf. After 1 h at −78 °C, the reducing agent Et_3SiH was added and the reaction was allowed to warm to −30 °C over 3 h. Standard workup gave the required bis-ether dimers **13a**−**c** in good yield (Scheme 3). The phosphate ester dimers were prepared from **9b** by deprotonation with sodium hexamethyldisilazide (NaHMDS) followed by addition of the appropriate phosphate dichloride (Scheme 4). For comparison, two phosphate monomers **9c** and **9d** were prepared in a similar manner using trioxane alcohol **9b** and the appropriate chlorophosphate.

Scheme 4. Synthesis of Phosphate Ester Dimers



14a, R= -Me, (58%)
14b, R = -Ph, (49%)

Table 1. In Vitro Antimalarial Activity of Dimers versus the K1 and HB3 Strains of *Plasmodium falciparum* in Vitro[a]

analogue	K1 strain IC_{50} (nM)	SD	HB3 strain IC_{50} (nM)	SD
9c	2.2	±0.4	ND	
9d	3.1	±0.9	ND	
11a	2.6	±0.9	1.6	±0.4
11b	42.2	±1.9	ND	
11c	4.6	±1.4	ND	
12a	1.8	±1.4	1.4	±1.1
12b	2.4	±0.8	1.1	±0.8
13a	2.9	±1.9	1.3	±1.1
13b	2.7	±1.6	2.1	±1.3
13c	2.4	±0.8	ND	
14a	0.2	±0.3	0.09	0.1
14b	0.5	±0.1	0.18	0.20
artemisinin	12.3	±1.4	14.5	1.5
chloroquine	190	±8	20.	3.1

[a] ND: not determined.

Antimalarial Assessment of New C-10 Carba Dimers

The antimalarial activities for the new dimers, against the chloroquine-resistant K1 strain of *Plasmodium falciparum*, are recorded in Table 1. Several of these dimers have remarkable activity against this strain. For example, phosphate dimer **14a** is greater than 50× more potent than the parent drug artemisinin and about 15× more potent than the clinically used acetal artemether. Even more dramatic is the comparison with the 4-aminoquinoline chloroquine in this strain, where trioxanes **14a** and **14b** are between 400× and 900× more potent. The table clearly demonstrates that trioxane dimers in this class are among the most potent antimalarials to have been tested against the K1 strain of *Plasmodium falciparum*. Against the chloroquine-sensitive HB3 strain, the phosphate dimers were again the most potent of the dimers tested with subnanomolar antimalarial activity.

Table 2. Growth Inhibitory Activities of Trioxane Dimers **14a** and **14b**

	dimer **14a**			dimer **14b**		
trioxane panel and cell line	GI_{50} [a] (nM)	TGI [b] (μM)	LC_{50} [c] (μM)	GI_{50} [a] (nM)	TGI [b] (μM)	LC_{50} [c] (μM)
leukemia						
CCRF-CEM	769	30	100	25.9	56	>100
HL-60(TB)	<10	1.0	20	11.5	2.23	>100
K-562	<10	1.17	34.2	<10	100	>100
MOLT-4	<10	9.80	33.5	<10	11.9	>100
RPMI-8226	<10	13.7	>100	<10	0.01	>100
SR	<10	2.84	>100	<10	0.263	>100
colon cancer						
COLO 205	<10	0.21	2.59	<10	0.17	>100
HCC-2998	1250	16.4	52.9	64.8	2.85	>100
HCT-116	1850	32.6	>100		>100	>100
HCT-15	<10	14.4	38.7	<10	>100	>100
HT29	<10	12.6	40	<10	23.2	>100
KM12	<10	12.6	40	<10	17.9	>100
SW-620	<10	12.6	40	<10	100	>100
melanoma						
LOX IMVI	<10	16.4	52.9	19.1	15.6	>100
MALME-3M	47.4	32.6	>100	194	30.8	>100
SK-MEL-5	199	15.2	38.9	13.0	2.25	>100
UACC-62	486	18.4	42.9	75.3	21.8	>100
prostate						
PC-3	<10	18.8	70.3	<10	65.5	>100
breast cancer						
MCF7	50.3	18.3	72.2	106	>100	>100
NCI/ ADR-RES	994	22.1	75.5	500	45.2	>100
T-47D	<10	<10	25.6	<10	74.6	>100

[a] GI_{50} = concentration of drug required to achieve 50% growth inhibition. [b] TGI = concentration of drug required to achieve total growth inhibition. [c] LC_{50} = concentration of drug required to achieve 50% cell kill (i.e., cytotoxicity).

Antitumor Activities

To determine the growth inhibitory effects of the new dimers, screening assays were performed by the National Cancer Institute (NCI) using a 60 cell line panel using concentrations of drug from the micromolar to nanomolar range.[19] The two most potent compounds in these tests were the phosphate dimers **14a** and **14b**. Data for phosphate ester dimers are recorded in Table 2. GI_{50} is the concentration of drug that inhibits percentage growth by 50%, and a drug effect of this intensity represents primary growth inhibition. In many cases, for **14a**, this value is lower than 10 nM. TGI (total growth inhibition) is the concentration of drug required to achieve cytostasis. TGI values are in the range of 1−10 μM against leukaemia, colon, and certain melanoma and breast cancer cell lines, indicating that this compound has activity in the region recorded for paclitaxel in the recent patent disclosed by Hauser.[11] Out of the different panels of cell lines examined, dimer **14b** is also particularly effective against leukaemia, colon, and melanoma cell lines with TGI values from nanomolar to micromolar concentrations. Notably for both dimers, there was little activity against lung, central nervous system (CNS), and renal cancer cell lines (data not shown). In contrast to the phosphate ester dimers, the rest of the dimeric compounds tested had poor antitumor activity in NCI assays, suggesting that the presence of two trioxane units alone is not sufficient to impart potent growth inhibitory activity. More importantly, it appears that the separation between the two trioxane units is crucial. The dimers **14a** and **14b** have a single phosphorus atom between the two representative trioxane carba alcohols **9b**. The other dimers have significicantly larger linker groups, and this observation suggests a specific cellular target for **14a** and **14b**.

For purposes of comparison, two phosphate ester monomers **9c** and **9d** were prepared. Although these compounds were active in the subnanomolar region versus K1 *Plasmodium falciparum*, both compounds proved to have no antiproliferative activity in the cell lines examined, even at concentrations approaching 1 mM. This result is important because it rules out any role of the phosphate ester function in mediating the observed cytotoxic effects of the dimers and emphasizes the necessity for a bivalent unit.

Having obtained the NCI data shown in Table 2, the two phosphate dimers **14a** and **14b** were studied in more detail in HL60 leukaemia and Jurkat cell lines using dihydroartemisinin and doxorubicin as a positive control. Doxorubicin provides a useful comparison of therapeutic utility because it is used to treat acute leukemias and clinically the maximal initial plasma concentration achieved with this drug after bolus administration is 5 μM, with the lowest concentration achieved at 0.3 μM. Generally, initial plasma concentrations are in the range of 1−3 μM, declining rapidly within 1 h to 25−250 μM. This illustrates that the GI_{50} value obtained in this in vitro study for DOX is comparable to the plasma concentrations achieved clinically.[20a]

Figure 1 summarizes typical curves obtained for these drugs against the two cell lines studied, and the results are recorded in Table 3. Against the HL60 cell line, both phosphate esters demonstrated excellent activity with IC_{50} values superior to that of doxorubicin. The Jurkat cell line in contrast was more resistant to the peroxides studied, and doxorubicin proved to be superior in this cell line. Both phosphate dimers outperformed DHA by almost 10 orders of magnitude. In terms of general toxicity to normal cell lines, we observed that these trioxane dimers were not toxic to lymphocytes at doses approaching 100 μM.

It is fascinating to note that the most potent compound prepared in this study has a similar linker chain length and hydrogen bond acceptor in the side chain to two of the lead compounds prepared by Jung and coworkers. As in this study, Jung has noted that longer dimer linkers result in poor antiproliferative activity (Figure 2).

It is also noteworthy that the cell lines that are particularly sensitive to trioxane dimers **14a** and **14b** all express transferrin.[20−25] Transferrin is an endogenous protein that transports iron from the circulation into cells. Most cancer cell lines express higher surface cell concentrations of transferrin receptors than normal cells and have higher rates of iron influx.[26a] For example, human hepatoma cells can express 800 000 transferrin receptors per cell on the cell surface.[26b] We have noted that all the cell lines that are sensitive to the peroxides **14a** and **14b** overexpress transferrin receptors. On the basis of this knowledge, we propose that the tumoricidal mechanism of action of these peroxides may involve nuclear iron-dependent activation to free radical species that damage DNA.

In preliminary studies using a Jurkat (human leukemic T-cell lymphoblast) cell line, we observed induction of apoptotic cell death on exposure to **14b** ($\geq$30 μM, Table 4).[27] The mechanism underlying the apoptosis

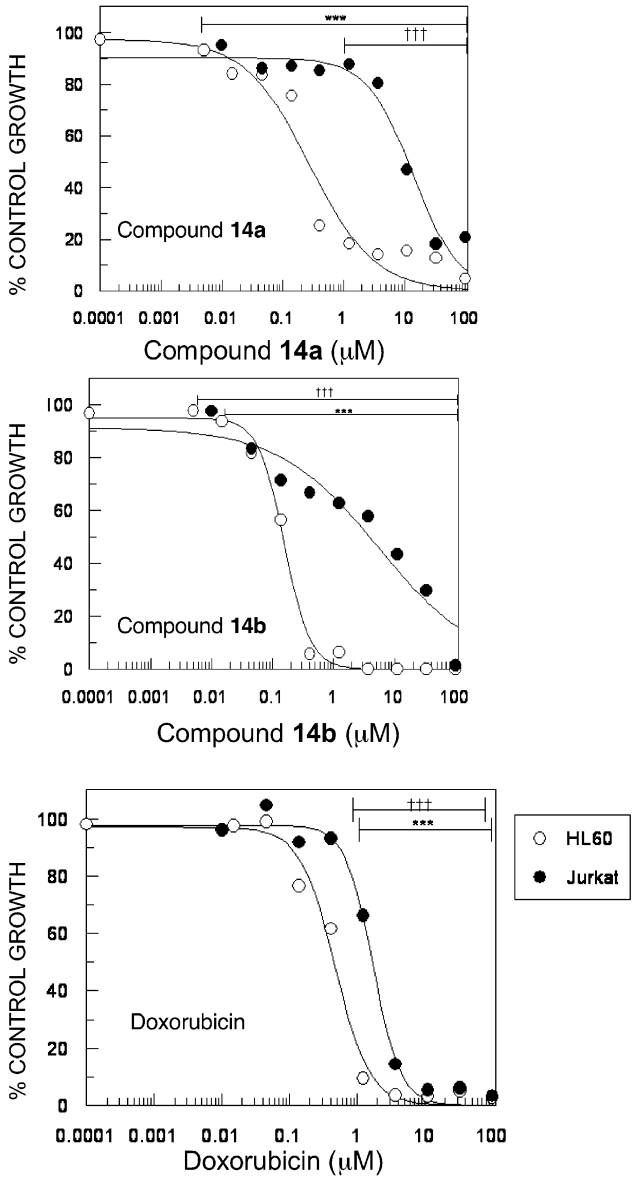


Figure 1. GI_{50} curves of compounds **14a**, **14b**, and doxorubicin (Dox) in HL60 and Jurkat cells: $n = 3$; (***) $p < 0.001$ in HL60.

Table 3. Anticancer Activities of Dimers **14a**, **14b**, DHA (**2**), and Doxorubicin (Dox)

compound	IC_{50} (HL60) (μM)	IC_{50} (Jurkat) (μM)
14a	0.143 ± 0.02	7.7 ± 0.02
14b	0.241 ± 0.08	13.2 ± 3.1
DHA	1.21 ± 0.67	101 ± 0.9
doxorubicin	0.51 ± 0.09	1.7 ± 0.1

observed in Jurkat cells on exposure to compound **14b** will be the focus of future work in this area.

A recent paper has disclosed the combined effect of orally administering ferrous sulfate in combination with dihydroartemisinin.[28] In this in vivo study, the authors claim that this combination has the effect of significantly retarding fibrosarcoma growth in the rat. Given that compounds **14a** and **14b** are significantly more potent than dihydroartemisinin, the examination of a similar approach with dimeric trioxanes in combination with ferrous salts may provide a means for improving the selective toxicity of these potent lead compounds even further.

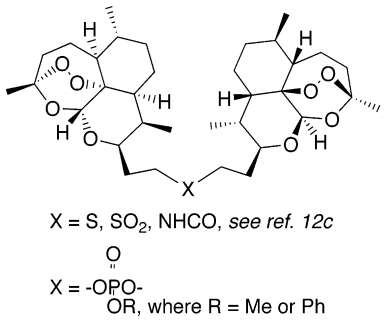


Figure 2. Structures of potent antitumor C-10 carba dimer analogues prepared here and by Jung and co-workers.[12c] Each of the dimers depicted have potent activity against leukaemia, colon and breast cancer lines as measured by the MTT assay. An increase in linker length results in compounds that retain nanomolar antimalarial activity but are poor antitumor compounds.

Table 4. Dose-Dependent Induction of Apoptosis by Dimer **14b** in a Jurkat Cell Line

[**14b**] (μM)	% apoptosis ± SD
0	7.5 ± 1.4
0.1	8.7 ± 2.1
0.3	7.3 ± 1.5
1	8.9 ± 1.9
3	8.7 ± 1.9
10	11.2 ± 1.1
30	17.3 ± 2.5[a]
100	20.1 ± 1.6[a]

[a] $p < 0.05$ from Kruskall−Wallis test.

Experimental Section

Chemistry. Merck Kieselgel 60 F 254 precoated silica plates for TLC were obtained from BDH, Poole, Dorset, U.K. Column chromatography was carried out on Merck 938S silica gel. Infrared (IR) spectra were recorded in the range 4000−600 cm^{-1} using a Perkin-Elmer 298 infrared spectrometer. Spectra of liquids were taken as films. Sodium chloride plates (Nujol mull), solution cells (dichloromethane), and KBr disks were used as indicated.

^1H NMR spectra were recorded using Perkin-Elmer R34 (220 MHz) and Bruker (400, 300, and 200 MHz) spectrometers. Solvents are indicated in the text, and tetramethylsilane was used as the internal reference. Mass spectra were recorded at 70 eV using a VG7070E mass spectrometer. The samples were introduced using a direct-insertion probe. In the text, the parent ion (M^+) is given, followed by peaks corresponding to major fragment losses with intensities in parentheses.

General Procedure 1: Synthesis of Phosphate Ester Monomers. Sodium hexamethyldisilazide [NaHMDS, 1.0 M in THF] (0.64 mL, 0.64 mmol) was added to a solution of alcohol **9b** (100 mg, 0.32 mmol) in anhydrous THF (5 mL) at −78 °C. After the reaction mixture was stirred for 10 min, it was treated with the appropriate chlorophosphate (0.32 mmol) and slowly warmed to room temperature. The reaction mixture was stirred at room temperature for 2 h before being cooled to 0 °C and quenched with water (5 mL). The organic layer was extracted with ether (2 × 10 mL), washed (brine), and dried ($MgSO_4$). The solvent was removed under reduced pressure to give the crude product that was purified by column chromatography on florisil.

General Procedure 2: Synthesis of Bis-ester Dimers. 4-(Dimethylamino)pyridine (0.05 g, 0.4 mmol) and anhydrous dichloromethane (8 mL) were added to a flask charged with vacuum-dried trioxane alcohol (0.11 g, 0.35 mmol). This mixture was cooled to 0 °C, and a bis acid chloride was added. The reaction was stirred overnight, and the crude mixture was

directly absorbed onto flash gel and loaded onto a column. Chromatography (ethyl acetate/*n*-hexane) gave the desired dimer.

General Procedure 3: Synthesis of Bisether Dimers. TMSOTf (10 μL) was added to a mixture of the appropriate dialdehyde and trimethylsilyloxytrioxane alcohol (0.1 g, 0.3 mmol) in anhydrous dichloromethane (3 mL) at −78 °C. After the mixture was stirred at −78 °C for 1 h, triethylsilane (0.04 mL, 0.3 mmol) was added and the mixture was stirred at −78 to −30 °C for 4 h. The reaction mixture was diluted with diethyl ether (30 mL), washed with saturated $NaHCO_3$, and dried over $MgSO_4$. The organic extracts were removed under reduced pressure, and the product was purified by chromatography.

General Procedure 4: Synthesis of Bisphosphate Ester Dimers. Sodium hexamethyldisilazide [NaHMDS, 1.0 M in THF] (0.33 mL, 0.32 mmol) was added via syringe to a solution of trioxane alcohol (0.1 g, 0.32 mmol) in THF (7 mL) at 0 °C. The resulting mixture was stirred for 10 min at 0 °C, and the appropriate dichlorophosphate (0.024 mL, 0.16 mmol) was added. The reaction mixture was kept at 0 °C for 2 h and was then warmed to room temperature and stirred for 40 min. The mixture was then cooled back to 0 °C, quenched with water. The organic layer was extracted three times with ether, and the combined organic portions were washed with saturated NaCl and dried over $MgSO_4$. The organic extracts were removed under reduced pressure, and the product was purified by chromatography.

Dihydroartemisinin 10α-Benzoate (10). Benzoyl chloride (3.17 mL, 27.29 mmol) was added to a solution of dihydroartemisinin (5.0 g, 17.61 mmol) in anhydrous dichloromethane (54 mL) and anhydrous pyridine (9 mL) at 0 °C. The mixture was stirred at room temperature for 16 h. The reaction mixture was then partitioned between 7% citric acid (50 mL) and ethyl acetate (2 × 50 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by silica gel chromatography using ethyl acetate/*n*-hexane (10/90) as the eluent gave the product as a white crystalline solid (91%): mp = 111−112 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.13 (m, 2 H, aromatic), 7.57 (m,1 H, aromatic), 7.45 (m, 2 H, aromatic), 6.02 (d, 1 H, J = 9.90 Hz), 5.53 (s, 1 H), 2.76 (m,1 H), 2.40 (dt, 1 H, J = 14.0, 4.30 Hz), 2.07−1.20 (m, 10 H), 1.43 (s, 3 H), 0.99 (d, 3 H, J = 5.90 Hz), and 0.93 (d, 3 H, J = 7.14 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.41, 133.34, 130.20, 129.79, 128.36, 104.49, 92.62, 91.65, 80.23, 51.73, 45.42, 37.32, 36.32, 34.18, 32.03, 25.94, 24.62, 22.09, 20.22, 15.24, and 12.20; IR (Nujol)/cm^{-1} 2924, 1738, 1491, 1452, 1377, 1272, 1114, 1100, 1037, 877 (O−O), and 831 (O−O); HRMS (CI) $C_{23}H_{32}NO_6$ [M + NH_4]$^+$ requires 406.222 96, found 406.222 70. Anal. ($C_{22}H_{28}O_6$) C, H.

10β-Allyldeoxoartemisinin (10a). A solution of dihydroartemisinin 10α-benzoate (1.1 g, 2.8 mmol) in anhydrous 1,2-dichloroethane (11 mL) was added via cannula to a mixture of allyltrimethylsilane (2.2 mL,13.8 mmol) and $ZnCl_2$ (0.5 g, 3.67 mmol) in anhydrous 1,2-dichloroethane (11 mL), which was stirred over activated 4 Å molecular sieves under nitrogen at 0 °C for 2 h and then allowed to warm to room temperature. The mixture was diluted with ethyl acetate and was washed with 5% citric acid, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate/*n*-hexane (10/90) as eluent to give the product as a white solid (90%): mp = 76−78 °C;[16] ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.92 (m, 1H), 5.32 (s,1H), 5.08 (m, 2H), 4.30 (m, 1H), 2.67 (sex., 1H), 2.44−2.17 (m, 3H), 1.40 (s, 3H), 0.95 (d, 3H, J = 6.0 Hz,), 0.88 (d, 3H, J = 7.5 Hz,); ^{13}C NMR(75 MHz, $CDCl_3$) δ 136.6, 116.1, 103.1, 89.2, 81.1, 74.1, 52.1, 44.1, 37.1, 36.6, 34.4, 34.2, 30.16, 25.98, 24.8, 24.6, 20.1, 12.8; MS (CI, NH_3) 326 ([M + NH_4]$^+$, 20), 309 ([M + H]$^+$, 10), 263 (100), 221 (70), 205 (100), 162 (80), 95 (15), 58 (20); HRMS (CI, NH_3) $C_{18}H_{29}O_4$ [M + H]$^+$ requires 309.206 59, found 309.206 20. Anal. ($C_{18}H_{28}O_4$) C, H.

10β-(2-Hydroxyethyl)deoxoartemisinin (9b). A solution of 10β-allyldeoxoartemisinin (0.6 g, 1.94 mmol) in anhydrous dichloromethane (150 mL) was flushed with N_2 and then subjected to a steady stream of O_3 at −78 °C until the solution became saturated with O_3 and appeared blue. Residual O_3 was flushed from the mixture with N_2, and the solvent was removed under reduced pressure. THF/MeOH (150 mL, 9/1) was added, and the solution was treated with excess sodium borohydride (4.00 g) at 0 °C for 4 h. The resultant mixture was concentrated under reduced pressure, and water and $CHCl_3$ were added. The organic layer was dried over $MgSO_4$ and filtered, and the solvent was removed under reduced pressure. Purification by silica gel chromatography using ethyl acetate/*n*-hexane (40/60) as the eluent gave the product as a white solid (55%): mp = 104−106 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.35 (s, 1H), 4.44 (m, 1H) 3.83 (q, 2H), 2.65 (sex., 1H), 2.54 (m, 1H), 2.32 (dt, 1H, J = 13.5, 4.0 Hz), 1.40 (s, 3H), 0.96 (d, 3H J = 5.90 Hz), 0.86 (d, 3H, J = 7.55 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 103.2, 89.3, 81, 74.9, 62.6, 52.2, 44.1, 37.4, 3.4, 31.6, 30.3, 25.9, 24.6, 20, 12.7; MS (CI, NH_3) 330 ([M + NH_4]$^+$, 30), 313 ([M + H]$^+$, 10), 295 (100), 267 (52), 253 (100), 237 (40), 209 (35), 183 (27); HRMS (CI, NH_3) $C_{17}H_{29}O_5$ [M + H]$^+$ requires 313.201 50, found 313.201 85. Anal. ($C_{17}H_{28}O_5$).

Phosphate Ester Monomer (9c). This compound was prepared from 10β-(2-hydroxyethyl)deoxoartemisinin using general procedure 1 and was purified by column chromatography using ethyl acetate/*n*-hexane (40/60) to give the product as a white solid (89%): mp = 87−88 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (4H, t, aromatic), 7.24 (d, 4H, aromatic), 7.17 (t, 2H, aromatic), 5.28 (s, 1H, H-12), 4.48 (m, 1H, H-10), 4.38 (m, 2H, CH_2O), 2.64 (sex., 1H, J = 7.3 Hz, H-9), 2.32 (td, 1H, J = 13.4, 4.0 Hz, H-4α), 1.38 (s, 3H, 3Me), 0.94 (d, 3H, J = 5.9 Hz, 6Me), 0.80 (d, 3H, J = 7.6 Hz, 9Me); ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.5, 137.7, 131.1, 130.1, 126.6, 125.6, 120.7, 120.5, 103.6, 89.4, 81.3, 71.4, 53.7, 52.7, 44.6, 37.8, 36.9, 34.8, 32.8, 30.9, 30.3, 26.4, 25.1, 20.5, 13.1, and 12.2; IR (Nujol)/cm^{-1} 1601, 1589, 1489, 1302 (P═O), 1225, 1188 (P−O−aryl), 1038, 1008, 995, 956, 879 (O−O), and 822 (O−O); HRMS (ES$^+$) $C_{29}H_{37}NaO_8P$ [M + Na]$^+$ requires 567.2124, found 567.2150. Anal. ($C_{29}H_{37}O_8P$) C, H.

Phosphate Ester Monomer (9d). This compound was prepared from 10β-(2-hydroxyethyl)deoxoartemisinin using general procedure 1 and was purified by column chromatography using ethyl acetate/*n*-hexane (50/50) to give the product as a yellow oil (63%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30 (s, 1H, H-12), 4.34 (m, 1H, H-10), 4.23 (m, 2H, CH_2O), 3.77 (d, 3H, J = 10.9 Hz, OMe), 3.77 (d, 3H, J = 11.1 Hz, OMe), 2.68 (sex., 1H, J = 7.3 Hz, H-9), 2.32 (td, 1H, J = 13.4, 4.0 Hz, H-4α), 1.40 (s, 3H, 3Me), 0.96 (d, 3H, J = 5.9 Hz, 6Me), 0.87 (d, 3H, J = 7.6 Hz, 9Me); ^{13}C NMR (100 MHz, $CDCl_3$) δ 103.5, 89.5, 81.4, 71.4, 66.3, 66.2, 54.7, 54.6, 52.7, 44.6, 37.9, 36.9, 34.8, 31.0, 30.4, 26.3, 25.1, 20.5, and 13.2; IR (neat)/cm^{-1}) 2958, 2879, 1455, 1378, 1280 (P═O), 1222, 1188, 1102, 1038 (P−O−alkyl), 977, 946, 912, 879 (O−O), and 847; HRMS (ES) $C_{19}H_{33}NaO_8P$ [M + Na]$^+$ requires 443.1811, found 443.1802. Anal. ($C_{19}H_{33}O_8P$) C, H.

10β-(2-Hydroxyethyl)deoxoartemisinin Bis-ester (1,2)-Dimer (11a). Using general procedure 2, trioxane dimer (**11a**) was prepared and purified by silica gel chromatography using ethyl acetate/*n*-hexane (40/60) as the eluent to give the product as a white crystalline solid (61%): mp = 63−65 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (dd, 2H, J = 5.66, 3.32 Hz), 7.50 (dd, 2H, J = 5.66, 3.31 Hz), 5.33 (s, 2H), 4.56 (m, 2H), 4.40 (m, 4H), 2.70 (sex., 2H, J = 6.7 Hz), 2.31 (dt, 2H, J = 14.0, 4.05 Hz), 1.36 (s, 6H), 0.95 (d, 6H, J = 6.13 Hz), 0.88 (d, 6H, J = 7.54 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) 167.8, 132.7, 131.2, 129.3, 103.5, 89.4, 81.4, 72.0, 64.0, 52.7, 44.6, 37.8, 36.9, 34.8, 30.5, 29.2, 26.4, 25.18, 25.13, 20.5, 13.2; MS (FAB) 777 ([M + Na]$^+$, 25), 755 ([M + H]$^+$, 721 (100), 704 (62), 664 (65), 483 (42), 443 (48), 77 (35), 55 (55). Anal. ($C_{42}H_{58}O_{12}$) C, H.

10β-(2-Hydroxyethyl)deoxoartemisinin Bis-ester (1,3)-Dimer (11b). Using general procedure 2, trioxane dimer **11a** was prepared and purified by silica gel chromatography using ethyl acetate/*n*-hexane (50/50) as the eluent to give the desired product as a white solid (58%): mp = 168−169 °C; ^{1}H NMR

(300 MHz, $CDCl_3$) δ 8.69 (d, 1H, d, Ar−H), 8.23 (dd, 2H, J = 7.83, 1.65 Hz, Ar−H), 7.51 (t, 1H, J = 7.83 Hz, Ar−H), 5.36 (s, 2H), 4.59 (m, 2H), 4.45 (m, 4H), 2.74 (sex., 2H), 2.33 (dt, 2H, J = 13.0, 3.98 Hz), 1.36 (s, 6H), 0.95 (d, 6H, J = 6.05 Hz), 0.91 (d, 6H, J = 7.56 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.8, 133.8, 130.9, 130.7, 128.5, 103.2, 89.0, 81.0, 71.6, 63.5, 52.3, 44.3, 37.5, 36.5, 34.5, 30.1, 29.0, 26.0, 24.8, 24.7, 20.1, 12.8; MS (FAB) 777 ([M + Na]$^+$, 25), 755 ([M + H]$^+$, 22), 737 (45), 721 (100), 709 (60), 703 (100), 664 (95), 443 (87), 425 (81), 295 (100), 76 (35), 54 (62). Anal. ($C_{42}H_{58}O_{12}$) C, H.

10β-(2-Hydroxyethyl)deoxoartemisinin Bis-ester (1,4)-Dimer (11c). Using general procedure 2, trioxane dimer **11c** was prepared and purified by column chromatography using ethyl acetate/*n*-hexane (50/50) as the eluent to give the product as a white solid (75%): mp = 66−68 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (s, 4H, Ar−H), 5.35 (s, 2H), 4.60 (m, 2H), 4.46 (m, 4H), 2.68 (sex., 2H), 2.30 (dt, 2H), 1.34 (s, 6H), 0.96 (d, 6H, J = 5.92 Hz), 0.90 (d, 6H, J = 7.51 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166, 13.5, 129.9, 103, 89.6, 81.4, 71.9, 71.7, 52.6, 44.5, 37.8, 36.9, 34.8, 30.5, 29.4, 26.3, 25.2, 20.5, 13. Anal. ($C_{42}H_{58}O_{12}$) C, H.

Dimer 12a. Using general procedure 2, trioxane dimer **12a** was prepared and purified by column chromatography using ethyl acetate/*n*-hexane (50/50) as the eluent to give a white solid (66%): mp = 107 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.30 (s, 2H), 4.30 (m, 4H), 4.19 (m, 2H), 2.67 (sex., 2H), 2.62 (s, 4H), 2.32 (dt, 2H, J = 13.5, 3.9 Hz), 1.39 (s, 6H), 0.94 (d, 6H, J = 6 Hz), 0.86 (d, 6H, J = 7.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.3, 103.2, 89, 81, 71.8, 62.9, 52.3, 44.3, 37.4, 36.5, 34.4, 30, 29.2, 28.7, 26, 24.8, 24.7, 20.1, 12.8; MS (FAB) 707 ([M + H]$^+$ (6), 729 ([M + Na]$^+$ (8), 673 (23), 616 (18), 423 (16), 379 (15), 293 (77), 277 (82), 249 (100), 209 (75). Anal. ($C_{38}H_{58}O_{12}$) C, H.

Dimer 12b. Using general procedure 2, trioxane dimer **12b** was prepared and purified by column chromatography (ethyl acetate/*n*-hexane (50/50)) to produce a white solid (60%): mp = 41−43 °C ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30 (s, 2H), 4.30 (m, 4H), 4.19 (m, 2H), 2.60 (sex., 2H), 2.36 (t, 4H), 1.40 (s, 3H), 0.96 (d, 6H, J = 5.9 Hz), 0.86 (d, 6H, J = 7.52 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.2, 103.6, 89.3, 81.4, 72.3, 63, 52.6, 44.6, 37.8, 36.8, 34.8, 33.7, 30.4, 29, 26.4, 25.1, 25.1, 20.5, 20.4, 13.3.

10β-(2-Trimethylsilyloxyethyl)deoxoartemisinin. Triethylamine (0.07 mL, 0.5 mmol) and trimethylsilyl chloride (0.08 mL, 0.63 mmol) was added to a solution of trioxane alcohol (0.1 g, 0.32 mmol) in anhydrous dichloromethane (5 mL) under N_2. The mixture was stirred at 30 °C for 45 min and then filtered through Celite. Purification by column chromatography using ethyl acetate/dichloromethane (10/90) as the eluent gave the product as a colorless syrup (96%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.19 (s, 1H), 4.05 (m, 1H), 3.70 (m, 1H), 3.51 (m, 1H), 2.67 (sex., 1H), 2.26 (dt, 1H, J = 13.1, 3.97 Hz), 1.40 (s, 3H), 0.94 (d, 3H, J = 6.0 Hz), 0.84 (d, 3H, J = 7.53 Hz), 0.10 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 103.9, 89.0, 81.5, 73.5, 61.5, 53.0, 45.0, 37.8, 36.8, 34.7, 32.8, 30.4, 26.6, 25.24, 25.13, 20.7, 13.8, 0.06.

Dimer 13a. Using general procedure 3, **13a** was prepared and purified by silica gel chromatography using ethyl acetate/*n*-hexane (30/70) as the eluent to give the product as a clear oil (53%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (dd, 2H, J = 3.4, 1.98 Hz), 7.26 (dd, 2H, J = 3.49, 2.0 Hz, Ar−H), 5.31 (s, 2H), 4.58 (s, 4H), 4.24 (m, 2H), 3.73 (m, 2), 3.59 (q, 2H), 2.72 (sex., 2H), 2.33 (dt, 2H, J = 15.0, 3.90 Hz), 1.40 (s, 6H), 0.95 (d, 6H, J = 6.0 Hz), 0.87 (d, 6H, J = 4.59 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.9, 128.8, 127.9, 103.6, 89, 81.4, 73.3, 71, 69, 52.8, 45, 37.8, 36, 35, 30.4, 30.1, 26.5, 25, 20.5, 13; MS (FAB) 727 ([M + H]$^+$, 4), 429 (5), 191 (10), 121 (20), 105 (90), 77 (40), 43 (100). Anal. ($C_{42}H_{62}O_{10}$) C, H

Dimer 13b. Using general procedure 3, **13b** was prepared and purified by silica gel chromatography using ethyl acetate/dichloromethane (15/85) as the eluent to give a clear oil (58%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (m, 4H, Ar−H), 5.31 (s, 2H), 4.51 (s, 4H), 4.26 (m, 2H), 3.72 (m, 2H), 3.60 (q, 2H), 2.72 (sex., 2H), 2.31 (dt, 2H, J = 15.0, 3.98 Hz), 1.40 (s, 6H), 0.95 (d, 6H, J = 6.0 Hz), 0.86 (d, 6H, J = 7.48 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 139, 128.7, 127.4, 127, 103.6, 89.3, 81.4, 73.5, 73.2, 69.3, 52.8, 45, 37.8, 37, 34.8, 30.4, 30, 25, 20.5, 13.5. (Anal. $C_{42}H_{62}O_{10}$) C, H.

Dimer 13c. Using general procedure 3, ether dimer **13c** was prepared and purified by silica gel chromatography using ethyl acetate/dichloromethane (10/90) as eluent (74%): mp = 46−48 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33(s, 4H, Ar−H), 5.31-(s, 2H), 4.50 (s, 4H), 4.22 (m, 2H), 3.70 (m, 2H), 3.69 (m, 2H), 2.70 (sex., 2H), 2.30 (dt, 2H, J = 13.1, 3.98 Hz), 1.40 (s, 6H), 0.94 (d, 6H, J = 5.95 Hz), 0.86 (d, 6H, J = 7.53 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138, 128, 103.7, 89, 81, 73.4, 73.27, 69, 52.8, 45, 37.8, 36.9,34.9, 30.4, 30.1, 26.5, 25, 20.5, 13.5; MS (FAB) 1452 ([2M + H]$^+$, (35)), 725 ([M − H]$^+$, (14)), 693 (10), 381 (30), 295 (51), 209 (100), 277 (50), 237 (64), 136 (33), 105 (52), 77 (35). Anal. ($C_{42}H_{62}O_{10}$) C, H.

Dimer 14a. Using general procedure 4, dimer **14a** was prepared and purified by column chromatography using ethyl acetate/*n*-hexane (70/30) as the eluent to give a white solid (58%): mp = 48−50 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30 (s, 2H), 4.29 (m, 4H), 4.16 (m, 2H), 3.76 (d, 3H, J = 11.1 Hz), 2.68 (sex., 2H), 2.28 (dt, 1H, J = 13.1, 4.0 Hz), 1.40 (s, 6H), 0.96 (d, 6H, J = 6.0 Hz), 0.87 (d, 6H, J = 7.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 103.5, 89.3, 81.3, 71.6, 67, 52.7, 44.6, 37.8, 36.9, 34.8, 30.9, 30.3, 26.3, 25.1, 20.5, and 13.2; MS (FAB) 701 ([M + H]$^+$, 10), 307 (8), 237 (8), 191 (25), 154 (72), 136 (68), 107 (46), 77 (52). Anal. ($C_{35}H_{57}O_{12}P$) C, H.

Dimer 14b. Using general procedure 4, dimer **14b** was prepared and purified by column chromatography using ethyl acetate/*n*-hexane (70/30) as the eluent to give a clear oil (49%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (t, 2H, Ar−H), 7.24 (t, 2H, Ar−H), 7.14 (t, 1H, Ar−H), 5.28 (s, 2H), 4.38 (m, 2H), 4.28 (m, 4H), 2.67 (sex., 2H), 2.32 (dt, 2H, J = 13.0, 4.0 Hz), 1.39 (s, 6H), 0.95 (d, 6H, J = 5.9 Hz), 0.84 (d, 3H, J = 7.36 Hz), 0.82 (d, 3H, J = 7.64 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 130, 125, 120.5, 103.5, 89.3, 81.0, 71.5, 67.0, 52.7, 44.6, 37.8, 36.9, 34.8, 30.9, 30.3, 26.4, 25, 20.5, and 13.2. Anal. ($C_{40}H_{59}O_{12}P$) C, H.

Antimalarial Activity. A single chloroquine-resistant strain of *P. falciparum* from Thailand was used in this study, i.e., uncloned K1 strain, which is known to be CQ-resistant. Parasites were maintained in continuous culture using the method of Trager.[29] Cultures were grown in flasks containing human erythrocytes (2−5%) with parasitemia in the range 1−10% suspended in RPMI 1640 medium supplemented with 25 mM HEPES and 32 mM $NaHCO_3$ and 10% human serum (complete medium). Cultures were gassed with a mixture of 3% O_2, 4% CO_2, and 93% N_2.

In Vitro Testing. Antimalarial activity was assessed with an adaption of the 48 h sensitivity assay of Desjardins et al.[30] using [^{3}H]-hypoxanthine incorporation as an assessment of parasite growth. Stock drug solutions were prepared in 100% dimethylsulfoxide (DMSO) and diluted to the appropriate concentration using the complete medium. Assays were performed in sterile 96-well microtiter plates; each plate contained 200 μL of parasite culture (2% parasitemia, 0.5% hematocrit) with or without 10 μL of drug dilutions. Each drug was tested in triplicate, and parasite growth was compared to control wells (which constituted 100% parasite growth). After 24 h of incubation at 37 °C, 0.5 μCi hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto filter mats, dried for 1 h at 55 °C, and counted using a Wallac 1450 Microbeta Trilux liquid scintillation and luminescence counter. IC_{50} values were calculated by interpolation of the probit transformation of the log(dose)−response curve.

Antitumor Activity. The National Cancer Institute, using a protocol previously reported, evaluated the primary growth inhibition, total growth inhibition, and cytotoxicity of dimers **11a**−**14b**.[19] Data are recorded for the two most potent trioxanes, phosphate esters **14a** and **14b**.

Cell Culture. The cell lines (Jurkat and HL60) were maintained in RPMI-1640 media supplemented with 10% v/v FBS and 1% v/v L-glutamine. The cells were incubated under

humidified air containing 5% CO_2 at 37 °C. Cell density was kept below 1×10^6 cells/mL to ensure exponential growth. Cell viability was above 95% for all experiments and was calculated using the trypan blue dye exclusion test.

Cytotoxicity Studies. HL60 cells (2.5×10^4/well) and Jurkat cells (1×10^5/well) were plated in U-bottomed 96-well plates and were exposed to $10^{-10}-10^{-4}$ M of each drug for 72 h. All drug stock solutions were made up in DMSO, and the final solvent concentration was below 0.5% v/v in each incubation. Thymidine (0.5 μCi per well, specific activity 2 Ci/mmol) was added after 48 h for the final 24 h of the incubation. The cells were harvested onto glass-fiber filter mats that were then dried and sealed in plastic sample bags containing a meltex scintillation sheet. [^{3}H]-thymidine incorporation was measured using a β-scintillation counter (1450 Microbeta, Wallac, Oy, Finland). The results are expressed as a percent of vehicle-only cells. The IC_{50} was estimated from individual inhibition curves plotted by Grafit software.

Statistical Analysis. The results were analyzed for non-normality using the Shapiro−Wilk test. Student *t*-tests were used when normality was indicated. A Mann−Whitney U test was used for nonparametric data. All calculations were performed using Arcus Quickstat statistical software. Results were considered to be significant when $P < 0.05$.

Flow Cytometric Analysis. Before cells were incubated, they underwent assessment for viability and background apoptosis by trypan blue dye exclusion (>98% viability) and flow cytometric assessment of propidium iodide DNA intercalation (<2% background apoptosis; see below), respectively. Jurkat cells (1×10^6 cells mL^{-1}) were incubated with various concentrations of **14b** (0−100 μM, dissolved in DMSO) for 24 h at 37 °C in a humidified atmosphere with 5% CO_2.

Flow cytometric analysis of apoptotic nuclei was assessed according to a modification of a method originally described by Nicoletti.[31] Jurkat cells were pelleted by centrifugation (200*g*, 8 min), resuspended in Hanks balanced salt solution (HBSS) containing Triton-X (0.1%) and propidium iodide (50 μM), and incubated for 10 min at 37 °C. The cell suspensions were pelleted and washed in HBSS buffer (1 mL). Cells were stored in the dark at 4 °C prior to analysis. For flow cytometry, the forward and side scatter of cell nuclei was measured simultaneously on a Coulter Epics flow cytometer (Coulter Epics, XL software, Beckman Coulter, Luton, U.K.). The PI fluorescence of individual nuclei with an acquisition of FL_3 was plotted against forward scatter, and the data were registered on a log scale. A minimum of 5000 events were collected and analyzed. Apoptotic nuclei were distinguished from normal nuclei by their hypodiploid DNA. The forward threshold was raised to exclude cell debris. Acquisition and analysis took place using XL software (Beckman Coulter, Luton, U.K.). Data are presented as the mean ± SD from triplicate incubations within a single experiment. Statistical analysis (Kruskal−Wallis test) was performed by comparing incubations containing different concentrations of the drug with the DMSO control, accepting $p < 0.05$ as significant.

Acknowledgment. We thank the U.S. National Cancer Institute (NCI), Wellcome Trust (S.A.W., B.K.P.), NIH (Grant AI 34885, G.H.P.), and UFC Ltd. for financial support (J.P.J., J.C.). J.P.J. was supported by an Overseas Research Students (ORS) Award. The authors also thank the Wellcome Trust for a grant for a mass spectrometer and the EPSRC for an NMR facility (Grant GR/M90801**)**.

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JM030974C