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Synthesis, thermal stability, antimalarial activity of symmetrically and asymmetrically substituted tetraoxanes

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Abstract—Symmetrically and asymmetrically substituted 1,2,4,5-tetraoxanes were synthesized by the oxidative system H_2O_2/TFE in presence of $MeReO_3$ as a catalyst. All of the synthesized compounds were characterized spectroscopically, and evaluated for cytotoxicity, and antimalarial activity. Several of these tetraoxanes exhibited in vitro antimalarial activity without showing any cytotoxicity. Thermal stability of these compounds was studied by differential scanning calorimetry.

Since last two decades endoperoxides have attracted considerable amount of attention of chemist and biologist due to their potent activity against malarial parasite, which affects more than 500 million people, causing deaths of 1–3 million people every year.[1–7] Artemisinin and its semisynthetic derivatives represent the endoperoxide class of compounds, which shows antimalarial activity against chloroquine-resistant strain of *Plasmodium falciparum*.[8] The impact of drug resistance is acute for malaria chemotherapy because of the availability of limited number of clinically useful antimalarial drugs. Thus synthesis of new chemical entities for the antimalarial therapy remains a challenging task for the scientists involved in the malaria research.[6] Structure–activity relationship studies conducted on artemisinin and its semisynthetic derivatives revealed that the peroxide linkage is the most crucial pharmacophore in these molecules.[9]

This discovery was the beginning of a significant effort to identify synthetically accessible antimalarial peroxides. Dispiro-tetraoxane is one of such class of compounds which was found to be equally potent as artemisinin.[10] However, the structural diversity of this important class of compounds is not available.[11,12]

The most common method of synthesis of symmetrical tetraoxane is acid catalyzed cyclocondensation of hydrogen peroxide with ketones or aldehydes,[13–17] ozonolysis of olefins,[18] enol ethers,[19,20] *O*-ether oxime,[21] and cyclocondensation of bis(trimethylsilyl) peroxide with carbonyl compounds catalyzed by trimethylsilyl trifluoromethanesulfonate (TMSOTf).[22,23] A number of other methods have also been reported for the synthesis of these compounds.[24] The unsymmetrical tetraoxanes have been synthesized by cyclocondensation of ketones and aldehydes with steroidal gem-bis-hydroperoxides (H_2SO_4 catalyst),[23] aliphatic and alicyclic gem-hydroperoxides ($MeReO_3$–HBF_4, catalyst),[25] and gem-bis(trimethylsilyldioxy)alkanes (TMSOTf, catalyst).[26] As part of our ongoing efforts toward the synthesis of biologically active compounds,[27,28] and due to the fact that a very limited number of tetraoxanes are known, this study was aimed to synthesize, characterize, and evaluate the antimalarial activity of symmetrically and asymmetrically substituted tetraoxanes.

The tetraoxanes reported in this paper have been synthesized by a previously published method.[25] Symmetrical tetraoxanes were synthesized in moderate to good yield. Reaction of the aldehydes or ketones, H_2O_2 (30% aqueous solution), and HBF_4 (54% freshly prepared ethereal solution), in presence of 0.1 mol% MTO in TFE led to the formation of symmetrical tetraoxanes at room temperature. *p*-Substituted aromatic aldehydes led to the formation of symmetrical tetraoxanes in good yield (Scheme 1), while *o*-substituted benzaldehyde led to a

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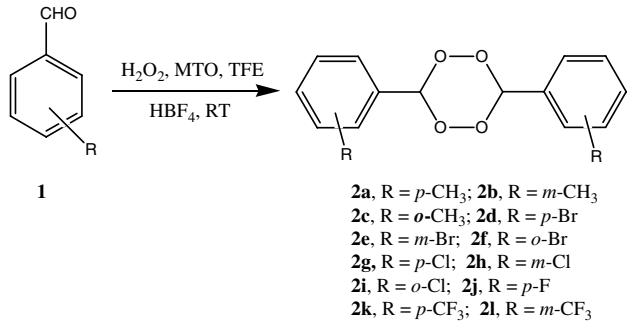


1

2a, R = *p*-CH_3; **2b**, R = *m*-CH_3
2c, R = ***o***-CH_3; **2d**, R = *p*-Br
2e, R = *m*-Br; **2f**, R = *o*-Br
2g, R = *p*-Cl; **2h**, R = *m*-Cl
2i, R = *o*-Cl; **2j**, R = *p*-F
2k, R = *p*-CF_3; **2l**, R = *m*-CF_3

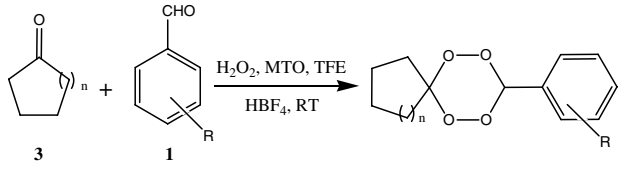
Scheme 1.

poor yield of the product. However, reaction of benzaldehyde substituted by OH, OR, or NH_2 functionality did not give the desired tetraoxanes.

Under identical reaction conditions, unsymmetrical tetraoxanes were prepared (Scheme 2). After completion of the reaction chloroform was added to the reaction mixture and the organic layer was washed with $NaHSO_3$, followed by saturated solution of $NaHCO_3$. Organic layer was dried over Na_2SO_4 and excess of solvent was removed under reduced pressure. The purification of tetraoxanes has been very difficult by column chromatography due to the formation of uncharacterizable side products. The crude product was washed with cold hexanes followed by mixture of cold methanol and hexanes (1:1). Pure tetraoxanes were obtained by washing the reaction mixture two to three times.

This synthetic methodology is being extended to the synthesis of functionalized tetraoxanes, which in turn can be transformed to tetraoxane-aminoquinoloine conjugates. This work is in progress and will be published in due course of time. Compounds **2a**, **2g**, **2j**, and **2k** have been prepared earlier.[29–31] All of the compounds reported in above schemes have been characterized spectroscopically.[32]

Thermal stability: Thermal stability of these tetraoxanes was studied by differential scanning calorimetry (DSC). DSC has been used to determine a number of characteristic properties of a sample such as fusion, crystallization, and glass transition temperatures (T_g). It can also be used to study oxidation, radical formation processes, and many other chemical reactions.[33–37] Recently, Solaja et al. have demonstrated that tetraoxanes exert their antimalarial activity due to their ability to form diradicals.[38] In order to study the thermal stability of these tetraoxanes, we recorded DSC of these compounds on a neat material. DSC traces of the unsymmetrical tetraoxanes clearly show an exothermic peak followed by negative endotherm corresponding to the melting points of the respective tetraoxanes, while in case of symmetrical tetraoxanes, the radical formation temperature was found to be very high (Table 1). As for example, compound **2b** shows an endothermic peak at 157 °C attributed to the melting of the tetraoxane. For same compound an exothermic peak was observed corresponding to the ring-opening polymerization[39] with onset at 190.57 °C and maximum at 210.35 °C (Fig. 1). The amount of heat released during this process is about −2135.4 J/g. The DSC traces clearly demonstrated that these compounds are thermally stable.



3 **1**

4a, R = H, n = 1; **4b,** R = *p*-Br, n = 1
4c, R = *p*-Cl, n = 1; **4d**, R = H, n = 2
4e, R = *p*-Cl, n = 2; **4f**, R = *m*-Cl, n = 2
4g, R = *o*-Cl, n = 2; **4h**, R = *p*-Br, n = 2
4i, R = H, n = 3; **4j**, R = *p*-Br, n = 3
4k, R = *p*-Cl, n = 3

Scheme 2.

Assay for in vitro antimalarial activity: The antimalarial activity was determined by measuring plasmodial LDH activity as described earlier.[40] A suspension of red blood cells infected with D6 or W2 strains of *P. falciparum* (200 μL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 μg/mL Amikacin) was added to the wells of a 96-well plate containing 10 μL of serially diluted test samples. The plate was flushed with a gas mixture of 90% N_2, 5% O_2, and 5% CO_2 and incubated at 37 °C for 72 h in a modular incubation chamber (Billups-Rothenberg, CA). Parasitic LDH activity was determined by using Malstat™ reagent (Flow Inc., Portland, OR) according to the procedure of Makler and Hinrichs.[41] Briefly, 20 μL of the incubation mixture was mixed with 100 μL of the Malstat™ reagent and incubated at room temperature for 30 min. Twenty microliters of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) was then added and the plate was further incubated in the dark for 1 h. The reaction was then stopped by the addition of 100 μL of a 5% acetic acid solution. The plate was read at 650 nm. Artemisinin and chloroquine were included in each assay as the drug controls.

Table 1. DSC temperature of tetraoxanes

Compound	DSC exothermic (°C)	
	On set temp. (°C)	Peak max. (°C)
2a	237.87	240.97
2b	190.57	210.35
2c	163.76	170.23
2d	221.18	224.46
2e	160.60	165.52
2g	174.34	186.67
2h	118.93	124.95
2i	233.69	236.98
4a	57.02	64.90
4b	65.02	67.94
4d	83.21	88.30
4e	97.20	100.19
4f	90.75	97.67
4g	108.40	117.30
4h	117.12	120.87
4i	57.02	64.90
4j	52.75	58.09

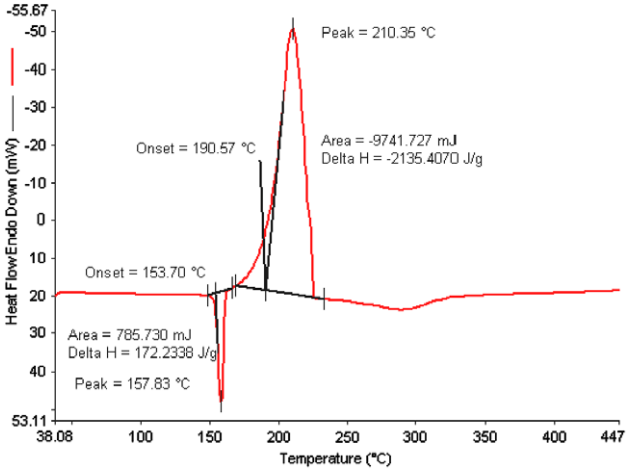


Figure 1. DSC curve of compound **2b**.

IC_{50} values were computed from the dose–response curves. To determine the selectivity index of antimalarial activity of compounds, their in vitro cytotoxicity to mammalian cells was also determined. The assay was performed in 96-well tissue culture-treated plates as described earlier.[42] Vero cells (monkey kidney fibroblasts) were seeded to the wells of 96-well plate at a density of 25,000 cells/well and incubated for 24 h. Samples at different concentrations were added and plates were again incubated for 48 h. The number of viable cells was determined by Neutral Red assay. IC_{50} values were obtained from dose–response curves. Doxorubicin was used as a positive control.

In vitro antimalarial activity of these tetraoxanes was determined against chloroquine sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum*. Among the series, compounds **2a**, **2b**, **2c**, **2d**, **2h**, **2j**, **4d**, **4e**, and **4f** exhibited the most potent antimalarial activity with IC_{50} values ranging from 0.95 to 2.66 μM for D6 and from 0.67 to 2.59 μM for W2 strain as shown in Table 2. Other compounds also showed moderate to mild antimalarial activities (IC_{50} 3.81–16.72 μM). None of these tetraoxanes showed any cytotoxicity (Table 2) to mammalian kidney fibroblast (Vero cells). A higher selectivity index (SI > 10) for antimalarial activity was observed for compounds **2a**, **2b**, **2c**, **2d**, **2h**, **2j**, and **4d** in either D6 or W2 strain. It is interesting to note that most of the active compounds like **2a**, **2b**, **2c**, **2d**, **2h**, **2j**, and **4d** have shown equal or better activity in chloroquine-resistant (W2) strain in comparison to chloroquine sensitive (D6) strain. The activity profile of these compounds clearly indicates that the tetraoxanes with substituent present at *meta* or *para* position of the benzene ring show better antimalarial activity (entry **2b**, **2h**, **4d**, **4e**, and **4f**) than the tetraoxanes having substituent at *ortho* position (entry **2i** and **4g**), which is in good agreement with the observation made by Iskra et al.[43] Furthermore, smaller or larger ring size than cyclohexane in the tetraoxane has negative effect on the antimalarial activity of these tetraoxanes (entry **4a**, **4b**, **4i**, **4j**, and **4k**). Interestingly, symmetrical tetraoxanes having methyl substituent at *ortho*, *para*, and *meta* position exhibited very good antimalarial activity. Among these, compound with methyl group at *meta* position of the tetraoxane (entry **2b**) was the most potent against both strains of *P. falciparum* (D6 and W2). However, when methyl group of compounds **2a** and **2b** was substituted by CF_3 group, the resulting tetraoxane (entry **2k** and **2l**) lost antimalarial activity.

Table 2. Antimalarial activity and cytotoxicity of substituted tetraoxanes

Compound	*P. falciparum* (D6 Clone)		*P. falciparum* (W2 Clone)		Cytotoxicity (Vero cells)
	IC_{50} (μM)	SI	IC_{50} (μM)	SI	
2a	1.47	>11.9	1.18	>14.9	NC
2b	0.96	>18.2	0.77	>22.7	NC
2c	2.46	>7.1	1.29	>13.6	NC
2d	1.39	>8.5	0.67	>13.6	NC
2e	NA		NA		
2f	NA		NA		
2g	4.47	>3.4	3.19	>4.8	NC
2h	1.37	>11.1	0.70	>21.6	NC
2i	NA		NA		
2j	1.64	>10.4	1.14	>14.9	NC
2k	NA		NA		
2l	NA		NA		
4a	8.09	>2.6	7.65	>2.8	NC
4b	NA		NA		
4c	16.72	>1.0	16.72	>1.0	NC
4d	2.20	>9.2	1.82	>11.1	NC
4e	2.66	>6.6	2.59	> 6.8	NC
4f	2.14	>8.2	2.47	>7.1	NC
4g	14.04	>1.3	9.24	>1.9	NC
4h	3.81	>4.0	5.39	>2.8	NC
4i	9.99	>1.9	7.59	>2.5	NC
4j	NA		NA		
4k	16.72	>1.0	16.72	>1.0	NC
CQ	0.05	>298	0.41	>42	NC
ART	0.035	>476	0.015	>1400	NC

NC, no cytotoxicity up to 16.72 μM.
NA, no activity up to 16.72 μM.
SI, selectivity index (IC_{50} for cytotoxicity/IC_{50} for antimalarial activity).

Caution: We have not encountered any difficulties in working with these compounds, routine precautions such as shields, fume hoods, and avoidance of transition metal salts should be observed whenever possible, as organic peroxides are explosive in nature.

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32. *Synthesis of symmetrical tetraoxanes: General method.* A solution of 2 mmol of aldehydes, 4 mmol of H_2O_2, and 0.002 mmol of MTO in 4 mL of TFE was stirred for 2 h at room temperature. Into the solution, 2 mmol of same aldehyde was added, followed by the addition of 2 mmol of a 54% ethereal solution of HBF_4. The reaction mixture was left under stirring for an additional hour. Chloroform was added and the organic phase was washed with dilute $NaHSO_3$ and then with saturated solution of $NaHCO_3$. The organic layer was dried over Na_2SO_4 and excess of solvent was evaporated under reduced pressure. Products were purified by washing with hexanes followed by mixture of hexane and methanol (1:1).
Synthesis of unsymmetrical tetraoxanes: General method. A solution of 2 mmol of cycloalkanone, 4 mmol of H_2O_2, and 0.002 mmol of MTO in 4 mL of TFE was stirred for 2 h at room temperature. Into the solution, 4 mmol of desired aldehyde was added, followed by the addition of 2 mmol of a 54% ethereal solution of HBF_4. The reaction mixture was left under stirring for an additional hour. Chloroform was added to the reaction mixture and the organic phase was washed with dilute $NaHSO_3$ followed by saturated solution of $NaHCO_3$. Chloroform layer was dried over Na_2SO_4 and excess of solvent was evaporated under reduced pressure. Products were isolated by SiO_2 column chromatography using hexane/ethyl acetate as an eluent (10:1). Identities of all of the synthesized compounds were confirmed by IR, NMR, and MS data.
*3,6-Di-o-tolyl-[1,2,4,5]tetroxane (***2c***).* Yield 19%; mp: 180–182 °C; IR (KBr, cm^{-1}): 2951, 1369, 1192, 1039, 1016, 922; ^1H NMR (300 MHz, $CDCl_3$): 2.54 (s, 6H), 7.11 (s, 2H), 7.22 (m, 4H), 7.36 (m, 2H), 7.48 (d, $J = 6$ Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): 19.24, 106.23, 126.27, 127.62, 129.44, 130.93, 131.07, 137.43; HRMS Calcd for $C_{16}H_{16}O_4$: 272.2958. Found: 295.2829 [M^++Na].
*3-(2-Chloro-phenyl)-1,2,4,5-tetraoxa-spiro[5.5] undecane (***4g***):* Yield: 11%; mp: 122–124 °C; IR (KBr, cm^{-1}): 2944, 1349, 1275, 1156, 1063, 1010, 990; ^1H NMR (300 MHz, $CDCl_3$): 1.46–1.68 (m, 6H), 2.34–2.40 (m, 4H), 7.00 (s, 1H), 7.19–7.46 (m, 4H); ^{13}C NMR (75.5 MHz, $CDCl_3$): 21.83 (CH_2), 22.19 (CH_2), 25.31 (CH_2), 29.67 (CH_2), 30.21 (CH_2), 31.72 (CH_2), 104.61 (CH), 108.92 (Cquart), 127.02 (CH) 128.79 (CH), 129.88 (CH), 132.05 (CH), 134.12 (Cquart); HRMS Calcd for $C_{13}H_{15}ClO_4$: 270.7085. Found: 270.7089 (M^+).
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