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Diversified Synthesis of *N*-Benzoyl Piperidine Tetraoxane Analogues Using Molybdenum Trioxide as a Newer Catalyst and Its Antiplasmodial Activity

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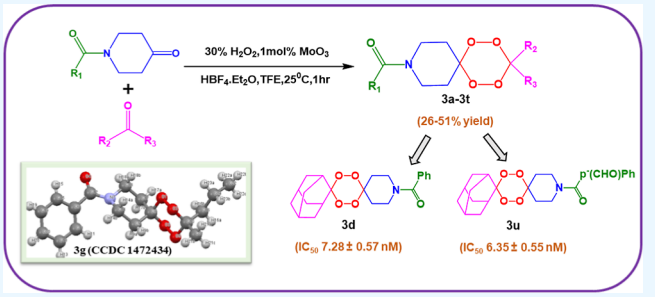
ABSTRACT: Molybdenum trioxide has been proven to be an efficient catalyst in synthesizing mixed *N*-benzoyl piperidine dispiro-1,2,4,5-tetraoxane analogues using a one-pot reaction. This catalyst facilitated the synthesis of 21 tetraoxanes using cyclic, acyclic, and aromatic ketones. The structure and methodology of this reaction have been validated by single-crystal X-ray analysis of compound ″3g″. The nature of dispiro-1,2,4,5-tetraoxane being synthesized has an impact on the overall yield of tetraoxanes such as symmetric dispiro-1,2,4,5-tetraoxanes ranging from 53 to 82%, while yields of *N*-benzoyl piperidine dispiro-1,2,4,5-tetraoxanes ranged from 26 to 51%. Additionally, the *in vitro* antiplasmodial activity of the newly developed tetraoxanes against *Plasmodium falciparum* was assessed, which exhibited significantly higher activity in the nanomolar range, with values ranging from 6.35 to 44.65 nM. Molecular docking studies revealed that newer tetraoxane derivatives bind to the active site of the falcipain-2 enzyme through H-bonding and hydrophobic contacts, which are the primary indicators of the effectiveness of the synthesized compounds. Findings suggest that these peculiar compounds may act as antiplasmodial agents, which spur further study and advancement in the battle against malaria.



INTRODUCTION

The recent *World Malaria Report* 2022 presented devastating facts about malaria infection. Despite tremendous efforts by the WHO and various countries, approximately 247 million cases and 6,19,000 related deaths were recorded in 2021 due to malaria, which occurs preferably among young children and pregnant women in the subtropical regions.[1,2] Protozoan parasites of the genus Plasmodium are the causative agents, with *Plasmodium falciparum* strains causing potentially lethal diseases. In the absence of a reliable vaccine, the use of synthetic small molecules to cure malaria is the only option. In recent times, only artemisinin and its synthetic analogues have been proven effective against all forms of the parasite and have emerged as the most staunch and potent antimalarial drug.[2b] Unfortunately, recent studies suggest that this class of compounds has also fallen prey to drug resistance, as evidenced by the decreased parasite clearance rates on artemisinin treatment.[3] Moreover, scarce supply from the plant Artemisia annua, high cost, and low or poor bioavailability of artemisinin are added disadvantages.[4] To overcome these issues, extensive research has been carried out to develop lead molecules against drug-resistant parasites. The structure of artemisinin makes it explicit that its antimalarial properties result from the endoperoxide moiety, which combines with heme to produce free radicals that are toxic to parasites and eventually lead to parasite death.[5]

Inspired by the success of artemisinin in curing malaria infection, scientists have been continually exploring newer classes of peroxide-based antimalarials, such as 1,2,4-trioxanes and 1,2,4,5-tetraoxanes, as synthetic alternatives of artemisinin.[6] Tetraoxane antimalarials work by a similar mechanism of the bioactivation of the 1,2,4-trioxolane peroxides by interaction with Fe(II).[7] Stability studies on endoperoxides have revealed that 1,2,4,5-tetraoxanes show considerably higher stability and better shelf life than their 1,2,4-trioxane equivalents.[8,9] Hence, greater efforts have been directed toward the development of 1,2,4,5-tetraoxanes which have two endoperoxide bonds, easy to synthesize,[10] inexpensive, and highly active antimalarial agents,[11] with little or no cross-resistance.[12,13] Moreover, various tetraoxanes have shown excellent antimalarial,[12,14] antitumor,[10,15] and anthelminthic activities.[16]

Recently, several newer synthetic derivatives of tetraoxanes have been synthesized, in which 7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecanes showed remarkable in

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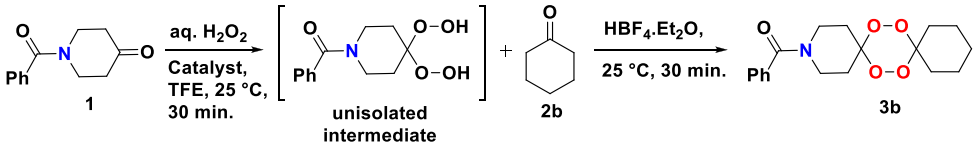
Table 1. Optimization of Reaction Conditions Using Various Mo(VI) Catalysts for Tetraoxane 3b Synthesis from 1-Benzoylpiperdin-4-one and Cyclohexanone 2b in 2,2,2-Trifluoroethanol (TFE) Solvent[a]



entry	catalyst	catalyst loading (mol %)	H_2O_2 (eq.)[b,c]	$HBF_4.Et_2O$ (eq.)	temperature (°C)	time (min.)	yield (%)[d]
1	C1	1.0	1.0[b]	1.0	25	60	29
2	C1	1.0	2.0[b]	1.0	25	60	38
3	C1	1.0	3.0[b]	1.0	25	60	33
4	**C1**	**1.0**	**2.0**[b]	**2.0**	**25**	**60**	**47**
5	C1	1.5	2.0[b]	2.0	25	60	47
6	C1	1.0	2.0[b]	3.0	25	60	41
7	C1	0.5	2.0[b]	2.0	25	60	36
8	C1	0.1	2.0[b]	2.0	25	60	28
9	C1	1.0	1.0[c]	2.0	25	60	36
10	C1	1.0	2.0[c]	2.0	25	60	29
11	C2	1.0	2.0[b]	2.0	25	60	35
12	C3	1.0	2.0[b]	2.0	25	60	37
13	C1	1.0	2.0[b]	2.0	25	120	41
14	C1	1.0	2.0[b]	2.0	25	180	36
15	C1	1.0	2.0[b]	2.0	0	60	21
16	C1	1.0	2.0[b]	2.0	40	60	42
17	MTO	0.1	2.0[b]	2.0	25	60	25
18	MTO	0.1	2.0[b]	2.0	25	120	24

[a]C1 = MoO_3, C2 = $MoOCl_4$, and C3 = $MoO_2(acac)_2$. [b]30% H_2O_2. [c]50% H_2O_2 and [d]Isolated Yield.

vitro antimalarial activity.[17,18] These symmetric derivatives are limited in number, and only some symmetric derivatives have been obtained in good yields.[19−21] Further, the design and synthesis of nonsymmetric tetraoxanes provide more opportunity for diversification using various functional groups in the tetraoxane moiety.[19] Some of the nonsymmetric synthetic tetraoxane derivatives such as WR148999, RKA182, RKA216, E209, and N205 showed comparable antiplasmodial efficacy to a natural antimalarial drug, artemisinin.[22−25]

To date, several multistep procedures have been well documented for the synthesis of tetraoxanes; however, the synthesis of *gem*-dihydroperoxide intermediate and its isolation from the reaction mixture are generally unstable and explosive.[19,26] In previous reports, one-pot and two-step synthesis of nonsymmetrical tetraoxanes has been reported to be catalyzed by various catalysts, such as TMSOTf,[27] Re_2O_7,[10,28] $Bi(OTf)_3$,[26] $MeReO_3$,[19a] and more recently using silica sulfuric acid.[19b] Along with these, Re_2O_7 was also used for the synthesis of chiral tetraoxane-containing antimalarials as its solubility and absorption were improved by the reduction in molecular symmetry.[7] However, these catalysts have some limitations due to their high cost and moisture sensitivity, which prevents them from being widely used in library synthesis. Molybdenum oxide as a catalyst is selective and active in an ample number of reactions, including those involving hydrogen or oxygen, which are often referred to as redox processes, as well as isomerization, addition, and decomposition, which are typically categorized as acid−base processes. The valence state of molybdenum ions, their local environment, and the type of crystal plane to which they are exposed are all factors that influence the catalytic behavior of molybdenum oxides.[29a] It is reported that 2,2,2-trifluoroethanol[19,29b,30] along with MoO_3[29b] can activate H_2O_2 in oxidation reactions, and the use of 2,2,2-trifluoroethanol could suppress byproduct formation during the reaction.[19]

Considering the above-mentioned facts regarding the synthesis of tetraoxanes, we aimed to identify a readily available, affordable, feasible, and effective catalyst for the synthesis of tetraoxanes. Our group has been working on the design and synthesis of diversified compounds against the malaria parasite.[6a,31a] We herein report molybdenum trioxide as an economical, stable at room temperature, and efficient catalyst for one-pot synthesis of *N*-benzoyl dispiro-1,2,4,5-tetraoxanes in the presence of 2,2,2-trifluoroethanol as the solvent along with $HBF_4.Et_2O$ and H_2O_2 for the diverse production of substituted tetraoxanes. We chose three Mo(VI) salts, viz., MoO_3, $MoOCl_4$, and $MoO_2(acac)_2$, and screened them for their catalytic properties in the synthesis of model 1,2,4,5-tetraoxane (3b) synthesis (Table 1) by the reaction of 1-benzoyl-4-piperidinone (1) with different stoichiometric ratios of H_2O_2, $HBF_4.Et_2O$, and cyclohexanone (2b) reagents using 2,2,2-trifluoroethanol as a solvent. After optimization of the synthetic procedure, the remaining tetraoxanes (3a and 3c−3u) were synthesized. The synthesized compounds were screened for their antiplasmodial activity and have been patented.[31b]

RESULTS AND DISCUSSION

The optimization reactions were performed to obtain model tetraoxane compound **3b**. Briefly, first, we tried the reaction using the various amounts of MoO_3 (0.1, 0.5, 1.0, and 1.5 mol %) along with 30% H_2O_2 (1.0, 2.0, and 3.0 equiv) and $HBF_4.Et_2O$ (1.0, 2.0, and 3.0 equiv) in 2,2,2-trifluoroethanol solvent (5 mL) at 25 °C for 1 h. The results indicated that 1 mol % of MoO_3 with 2 eq. of 30%H_2O_2 and 2 eq. of $HBF_4.Et_2O$ was the most effective combination giving an isolated yield of 47% (Table 1; entry 4) as compared to others.

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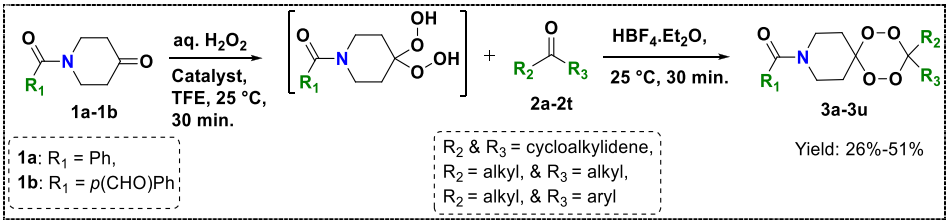
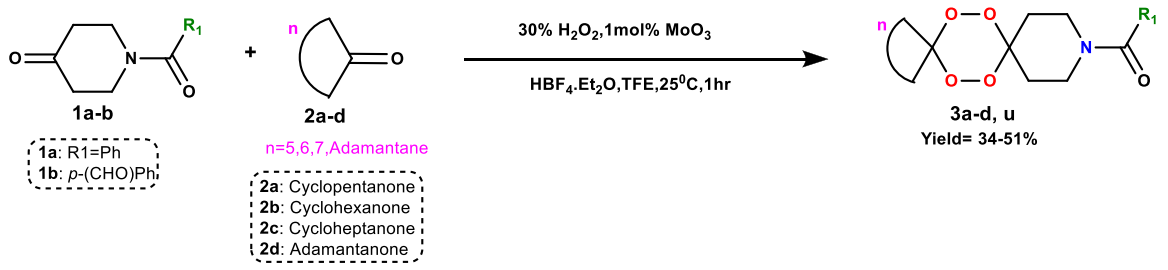
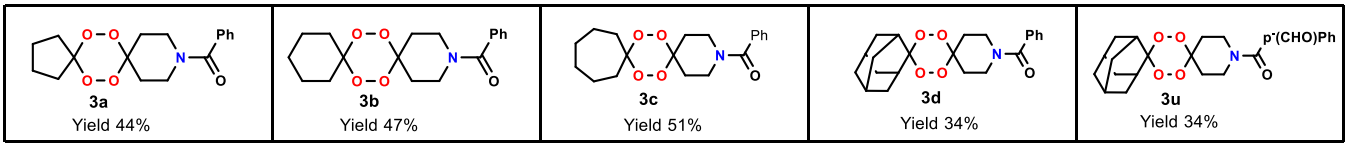
Scheme 1. One-Pot Synthesis of *N*-Benzoyl Piperidine-Linked Dispiro-1,2,4,5-tetraoxanes

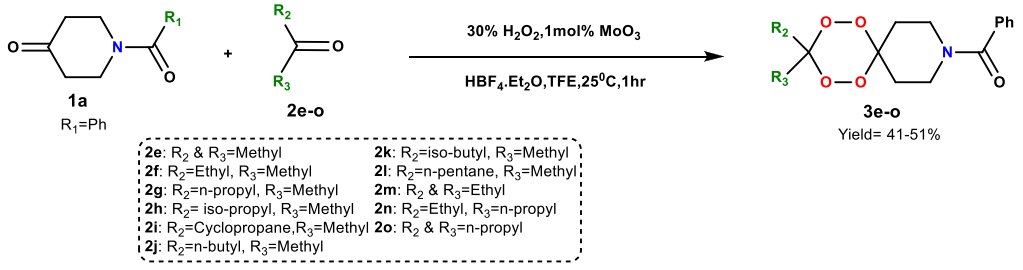


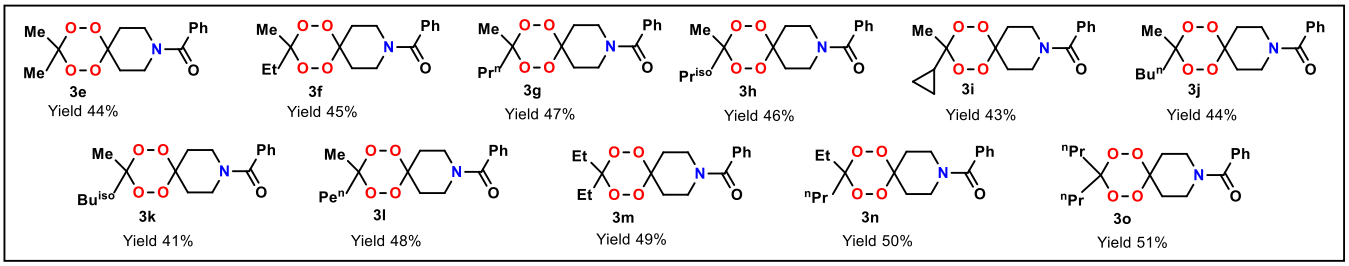
Scheme 2. Synthesis of *N*-Benzoyl Piperidine-Linked Dispiro-1,2,4,5-tetraoxanes Using Cyclic Ketones





Scheme 3. Synthesis of *N*-Benzoyl Piperidine-Linked Dispiro-1,2,4,5-tetraoxanes Using Acyclic Ketones





It was also observed that a further increase in MoO_3 amount did not affect the yield of tetraoxane **3b** (Table 1; entry 5), whereas on decreasing the amount of catalyst loading, a decrease in the yield of the product was obtained (Table 1; entries 7 and 8). Increasing or decreasing the amount of 30% H_2O_2 or $HBF_4.Et_2O$ resulted in a further decrease in the reaction yield (Table 1; entries 1−3, 5, and 6).

Subsequently, we also looked at the effect of the H_2O_2 concentration. We first used 1 equiv and then 2 equiv of 50% H_2O_2 instead of 30%H_2O_2 (Table 1; entries 9 and 10) and observed that the tetraoxane yield was lower as compared to when 30% H_2O_2 was used previously (Table 1; entry 4). Thus, for all practical purposes, we decided to use only 30% H_2O_2 for the synthesis of all newer tetraoxane derivatives.

Additionally, we evaluated the catalytic activity and efficiency of other molybdenum catalysts, viz., $MoOCl_4$ and $MoO_2(acac)_2$, and compared with the MoO_3 catalyst. We performed the same set of reactions using 1 mol % of $MoOCl_4$ and $MoO_2(acac)_2$, with 2 eq. of 30% H_2O_2 and $HBF_4.Et_2O$ in 2,2,2-trifluoroethanol solvent (5 mL) at 25 °C for 1 h (Table 1; entries 11 and 12). The results revealed that the catalytic activity of $MoOCl_4$ and $MoO_2(acac)_2$ catalysts was lower as compared with the MoO_3 catalyst (Table 1; entries 11 and 12).

After the selection of the MoO_3 catalyst with high catalytic activity, the time and temperature for the synthesis of tetraoxane product (**3b**) was optimized. First, by keeping the amount of the MoO_3 catalyst constant (1 mol %), we

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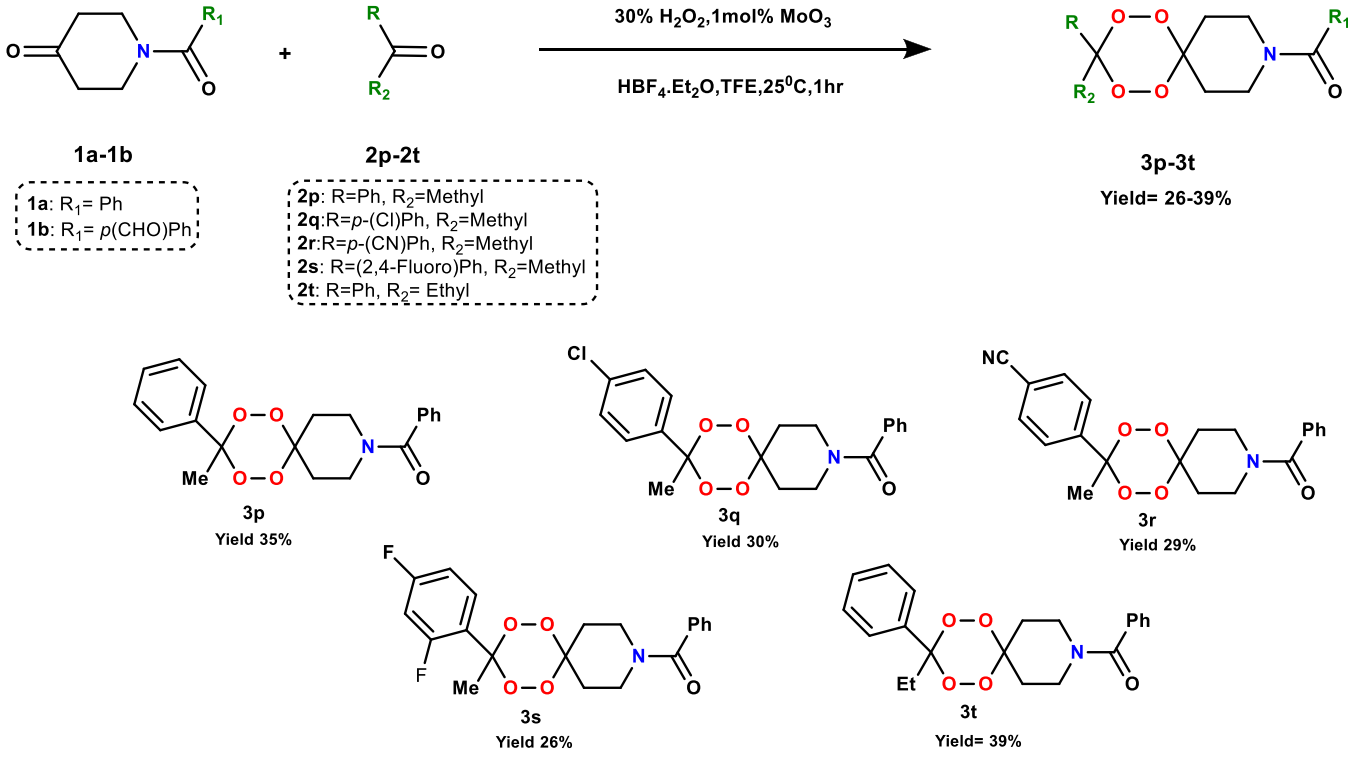
Scheme 4. Synthesis of *N*-Benzoyl Piperidine-Linked Dispiro-1,2,4,5-tetraoxanes Using Aromatic Ketones and Adamantanone



performed the reaction at three different temperatures, i.e., 0, 25, and 40 °C (Table 1, entries 13−16). The yield of tetraoxane product **3b** was 41% at 25 °C for 2 h, whereas a 36% yield was obtained when the reaction was carried out for a longer period, i.e., 3 h (Table 1; entry 14). Further, the reaction performed at 0 °C gave a poor yield of product **3b** (Table 1; entry 15). It was attributed to the formation of byproducts during the reaction, well-reported in the literature.[32] Further, on increasing the temperature of the reaction from 25 to 40 °C, no such substantial increase in the product yield **3b** was obtained (Table 1; entry 16).

Moreover, we also compared the catalytic efficiency between MoO_3 and the widely used highly expensive methyltrioxorhenium (MTO) in tetraoxane synthesis under the same set of reactions, which indicated that MoO_3 showed better catalytic efficiency in terms of product yield as compared to MTO (Table 1; entries 17 and 18). Thus, we concluded that MoO_3 is an inexpensive, efficient, and effective catalyst for the synthesis of tetraoxanes as compared to the rhenium base MTO catalyst. The overall optimized reaction conditions and catalyst loadings are summarized in Table 1.

After optimizing the various reaction parameters and broadening the scope of the present one-pot synthesis of tetraoxane, we have carried out further reactions using different ketones using MoO_3 as a newer catalyst.

Initially, we synthesized precursors of ketone such as (1-benzoylpiperidine-4-one (**1a**) and 4-(4-oxopiperidine-1-carbonyl)benzaldehyde (**1b**) using a previously reported procedure (Scheme 1).[33,34] Further, a variety of ketones **2** (**2a**−**2t**) such as cyclic ketones (**2a**−**2d**), acyclic ketones (**2e**−**2o**), and acetophenone derivatives (**2p**−**2t**) were used to obtain the desired mixed dispiro-1,2,4,5-tetraoxanes (**3a**−**3u**) with 26−51% yields (Schemes 2, 3, and 4). In the case of mixed tetraoxanes (**3a**, **3b**, and **3c**), synthesized from cyclic ketones (**2a**, **2b**, and **2c**), we observed that as the size of the ring increases, the reaction yield also increases (Scheme 2). However, in the case of 2-adamantanone (**2d**), branching at both α carbon and γ carbon resulted in a lower yield of tetraoxane (**3d**) (Scheme 2). In the case of tetraoxanes (**3e**−**3o**) synthesized from acyclic ketones (**2e**−**2o**), the alkyl chain branching at α carbon plays a crucial role in reaction yield (Scheme 3). Additionally, in the case of mixed tetraoxanes (**3p**−**3t**) synthesized from acetophenone and its derivatives (**2p**−**2t**) (Scheme 4), we observed a lower reaction yield in comparison to cyclic and acyclic ketones. Moreover, the electron-withdrawing group on acetophenone decreased the product yield. Synthesis of dispiro-1,2,4,5-tetraoxanes by employing benzophenone and its derivatives (as ketone 2) was unsuccessful. This could probably be due to the presence of a sterically hindered carbonyl group. Materials and methods along with the synthesis procedures of *N*-benzoyl piperidine dispiro-1,2,4,5-tetraoxanes are described in the Supporting Information.

Furthermore, the MoO_3 catalyst is also useful in the synthesis of symmetric tetraoxanes using (cyclic and acyclic) ketones with a higher yield and a lower reaction time (Table 2). Extension of the ring size in cyclic ketones decreases the yield of symmetric tetraoxanes (Table 2; entries 1−2). The lengthening of the alkyl chain in acyclic ketones also affected the yields of the symmetric tetraoxanes. The yield of symmetric tetraoxanes decreases as the lengths of both the alkyl chains in acyclic ketones increase (Table 2; entries 3−5).

In accordance with the literature, symmetric dispiro-1,2,4,5-tetraoxanes were simpler to synthesize than mixed tetraoxanes and had higher yields. However, due to their rarity, we concentrated primarily on synthesizing nonsymmetric or mixed tetraoxanes.

Moreover, our design, synthesis, and structure of the scaffold were further established by analyzing single-crystal X-ray crystallography analysis of compound **3g** (Figure 1). Crystallo-

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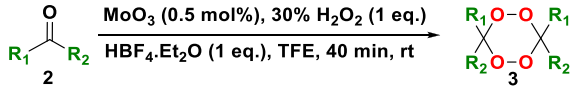
Table 2. Synthesis of Symmetric Dispiro-1,2,4,5-tetraoxanes Using Cyclic and Acyclic Ketones



entry		ketone		TO [3]	yield[a] (%)	ref
		R_1	R_2			
1	2b	cyclohexylidene		3w	82	35
2	2u	4-methylcyclohexylidene		3x	78	32
3	2e	methyl		3y	80	36
4	2m	ethyl		3z	62	37
5	2o	propyl		3za	53	37

[a]Isolated yield.

graphic data of compound **3g** in CIF (CCDC 1472434) is presented in the Supporting Information.

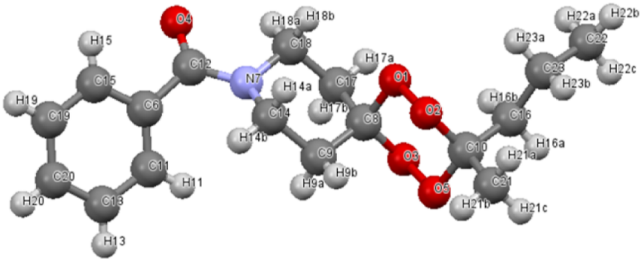


Figure 1. X-ray structure of compound **3g** (CCDC 1472434).

All the 21 newly synthesized tetraoxane compounds (**3a**−**3u**) were screened against the chloroquine-sensitive *P. falciparum* (3D7 strain) of malaria parasite *in vitro*, following the reported culture method.[34] Artemisinin was taken as a standard control drug for comparative study with an IC_{50} value of 5.97 nM. The data in Table 3 show that all tetraoxane compounds (**3a**−**3u**) displayed nanomolar activity ranging from 6.35 to 44.65 nM. The two tetraoxane compounds **3d** and **3u** showed excellent antiplasmodial activity with an IC_{50} of 7.28 and 6.35 nM, respectively, due to the presence of lipophilic character of the adamantylidene group. The antiplasmodial activity of *N*-benzoyl piperidine dispiro-1,2,4,5-tetraoxanes varied with modification in R_1 and R_2 groups and the *N*-benzoyl group. In the case of chosen cyclic ketones (**2**), the antiplasmodial activity of tetraoxane having cyclohexanone (**3b**) was found to be greater, followed by cyclopentanone (**3a**) and then cycloheptanone (**3c**) (Table 3; entries 1−3). The antiplasmodial activity of tetraoxanes derived from aliphatic ketones decreased with the elongation of alkyl chain, i.e., from methyl to n-propyl (Table 3; entries 5−7). Furthermore, the tetraoxanes having an aryl group demonstrated better antiplasmodial activity as compared to tetraoxanes having an alkyl group (Table 3; entries 16−19). The tetraoxane compounds that have an aryl ring with chlorinated and fluorinated groups (**3q** and **3s**) showed good antiplasmodial activity with IC_{50} values of 11.79 and 10.54 nM, respectively. Cytotoxicity (CC_{50}) of the compounds was evaluated in human embryonic kidney cells 293 (HEK-293) using the MTT assay. None of the compounds showed detectable cytotoxicity at the higher level of the test concentration (100 μg/mL; Table 3). The Supporting Information provides a method employed for *in vitro* antiplasmodial efficacy and cytotoxicity testing.

Table 3. *In Vitro* Antiplasmodial Activity of 1,2,4,5-Tetraoxanes versus the 3D7 Strain of *Plasmodium falciparum*

entry	TO code	MEAN IC_{50} ± SE[a](nM)	CC_{50} value (μg/mL)
1	3a	19.35 ± 2.05	>100
2	3b	17.89 ± 1.96	>100
3	3c	21.87 ± 1.79	>100
4	**3d**	**7.28±0.57**	**>100**
5	3e	29.89 ± 1.28	>100
6	3f	42.46 ± 3.04	>100
7	3g	43.44 ± 2.38	>100
8	3h	41.25 ± 2.33	>100
9	3i	44.65 ± 1.44	>100
10	3j	17.48 ± 1.24	>100
11	3k	18.91 ± 2.12	>100
12	3l	16.69 ± 1.93	>100
13	3m	17.47 ± 1.59	>100
14	3n	17.83 ± 1.57	>100
15	3o	17.29 ± 1.78	>100
16	3p	16.26 ± 0.93	>100
17	3q	11.79 ± 0.8	>100
18	3r	15.67 ± 0.78	>100
19	3s	10.54 ± 0.81	>100
20	3t	18.36 ± 1.44	>100
21	**3u**	**6.35±0.55**	**>100**
22	**artemisinin**	**5.97±0.61**	**>100**

[a]The mean IC_{50} was calculated from triplicate results.

To support the experimental data, docking analysis was carried out using the *Plasmodium* cysteine protease falcipain-2 enzyme as it plays a vital role in the degradation of hemoglobin into small peptides in the acidic food vacuole of *P. falciparum*. Recent reports reveal that endoperoxide antimalarials are potent at inhibiting the falcipain-2 enzyme. Thus, targeting this falcipain-2 enzyme could be a striking feature for designing new antimalarial compounds.[38] Docking is commonly used to predict the binding affinity and biological activity of small molecules as this is crucial for drug designing. Therefore, molecular docking was performed using Autodock 4.0 between three synthesized tetraoxanes and drug artemisinin by taking falcipain-2 enzyme as the receptor macromolecule. The three-dimensional crystal structure of falcipain-2 complexed with epoxysuccinate E64 (PDB code: 3BPF) having a resolution of 2.9 Å was retrieved from the protein data bank.[18] The crystal structure of falcipain-2 comprises four chains [(A, B, C, and D)]; however, in the present study, we have taken only chain A. All the water molecules and heteroatoms were deleted from the crystal structure for the sake of clarity. Polar hydrogen atoms were added, and Gasteiger charges were calculated using the AutoDock Tools.[39] Among the tested tetraoxane analogues, compounds **3d**, **3q**, and **3u** displayed the highest *in vitro* activity against the 3D7 strain of P. falciparum. Thus, to further understand interactions between these compounds and falcipain-2 enzyme, docking calculations were carried out. Docking of compounds **3d**, **3q**, and **3u** with falcipain-2 was accomplished using a grid size of 256 Å along all the three (X, Y, and Z) axes and a grid spacing of 0.375 Å. Docking results revealed successful docking of all the three compounds (**3d**, **3q**, and **3u**) into the binding pocket of falcipain-2 enzyme comparable to the control drug artemisinin (Table 4). 50 Docked conformations of each compound were evaluated using docking results, and the conformation with top-ranking

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Table 4. Docking Results Showing Interacting Residues and Amino Acid Residues Involved in H-Bonding along with the Binding Energy Obtained

ligand	receptor	binding energy (kcal/mol)	H-bonding residues	H-bond distance (Å)	interacting residues
3d	3BPF	−7.97	VAL71	2.89	PHE75, LYS76, ASN77, TYR78, GLY79, CYS80, VAL71, ASP72, GLN68, ASN81, PRO111, ALA110, ASN112, SER108, ASP109
			CYS80	3.10	
3q	3BPF	−6.53	CYS80	3.07	VAL71, ASP72, CYS73, SER74, PHE75, ASN77, TYR78, GLY79, CYS80, ASN81, ASP109, ALA110, PRO111, ASN112
			ASP109	3.23	
3u	3BPF	−7.54	VAL71	3.35	VAL71, ASP72, PHE75, ASN77, TYR78, GLY79, CYS80, ASN81, SER108, ASP109, ALA110, PRO111, ASN112
			CYS80	2.91	
artemisinin	3BPF	−7.68	LEU172	2.85	CYS42, TRP43, ASN81, GLY82, GLY83, LEU84, SER149, VAL150, LEU172, ASN173, HIS174
			HIS174	3.29	

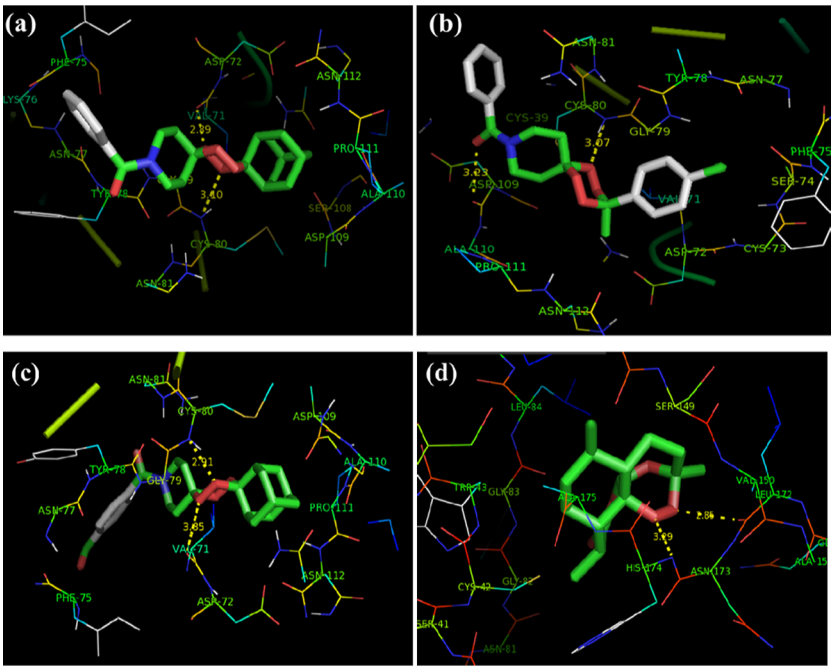

Figure 2. Pictorial representation of the docking results obtained from molecular docking of compounds (a) **3d**, (b) **3q**, (c) **3u**, and (d) artemisinin with falcipain-2 enzyme (PDB code: 3BPF).

having the lowest binding energy was taken for further inspections using PyMol. The results of docking in the active site of the falcipain-2 enzyme are illustrated in Table 4.

According to the findings, compound **3d** displayed the lowest binding energy −7.97 kcal/mol as compared to the other two compounds **3q** and **3u** having binding energies −6.53 and −7.42 kcal/mol (Table 4). The best docked pose of compound **3d** visualized using PyMol revealed hydrogen bonding of O of the tetraoxane ring with N of CYS80 and O of VAL71 amino acid residues of the falcipain-2 enzyme with 3.10 and 2.89 Å H-bonding distance, respectively. Further, it also displayed hydrophobic interactions with PHE75, LYS76, ASN77, TYR78, GLY79, CYS80, VAL71, ASP72, GLN68, ASN81, PRO111, ALA110, ASN112, SER108, and ASP109 amino acid residues of the falcipain-2 enzyme (Figure 2a). Thus, the lower the binding energy of the receptor−ligand complex, the greater the affinity of the ligand for the receptor, and the existence of hydrogen bond and hydrophobic interactions supports the formation of a stable complex.

Figure 2b illustrates the best docked pose of compound **3q** with PDB id 3BPF. The docking studies revealed two hydrogen bonds with N of CYS80 and O of ASP109 amino acid residues of the falcipain-2 enzyme having H-bond distancing of 3.07 and 3.23 Å, respectively. The H-bonding and hydrophobic interactions of 3q with amino acid residues of the falcipain-2 enzyme are responsible for the stability of the docked complex.

The docked conformer of **3u** having the least binding energy was visualized using PyMol, and the results obtained are shown in Figure 2c. The results revealed the formation of two hydrogen bonds: by the O of tetraoxane ring with the O of VAL71 and N of CYS80 amino acid residues of the falcipain-2 enzyme having H-bond distancing values of 3.35 and 2.91 Å, respectively. The molecular docking of all the three tetraoxane analogues with falcipain-2 enzyme was found comparable with the docking of the control drug artemisinin with falcipain-2 enzyme with binding energy −7.68 kcal/mol (Figure 2d and Table 4).

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EXPERIMENTAL SECTION

All reactions were carried out in a well-dried round-bottom flask. Solvents used for reactions and column chromatography were of commercial grade and distilled prior to use. TLC was performed on precoated silica gel aluminum plates with a 60F254 indicator, visualized by irradiation with UV light. Column chromatography was performed using silica gel with 100−200 mesh. ^{1}H NMR and ^{13}C NMR were recorded on 400 and 100 MHz JEOL JNM-ECS 400 spectrometry instruments, respectively, using $CDCl_3$ as a solvent. TMS was used as the reference for proton NMR, and the δ scale used for chemical shift values for ^{1}H NMR and multiplicity is as follows: s (singlet), d (doublet), and m (multiplet). High-resolution mass spectra were obtained by an Agilent ESI-TOF mass spectrometer. Substrates of *N*-benzoyl piperidine dispiro-1,2,4,5-tetraoxane (3a−3u) and substrate of symmetric dispiro-1,2,4,5-tetraoxane (3w−3za) were synthesized according to the experimental procedure described in the Supporting Information. The structure of dispiro-1,2,4,5-tetraoxane was also confirmed by the crystal data of compound **3g** shown in Figure 1. All of the compounds were characterized by ^{1}H NMR, ^{13}C NMR, and mass spectrometry (Supporting Information). The purity of these compounds was checked by TLC analysis and their spectral data.

Determination of Antiplasmodial Activity. *Malaria Parasite Culture.* The culture of erythrocytic stages of chloroquine-sensitive *P. falciparum* strain 3D7 was procured from the International Centre of Genetic Engineering and Biotechnology (ICGEB), New Delhi. It was continuously maintained as stocks in 25 cm^2 tissue culture flasks, on human O$^+$ red blood cells under low-oxygen concentration (3%) and a high carbon dioxide atmosphere (4%) along with nitrogen (93%), at a temperature of 37 °C, in RPMI 1640 (Invitrogen) with 25 mM HEPES, 25 mM $NaHCO_3$, 200 mM L-glutamine, 50 mg/L gentamicin (Gibco), and 5 g/L Albumax II (Life Technologies). The stock cultures were started with 5% hematocrit and parasitemia of less than 1%. Subcultures were made at about 5% parasitemia.

Susceptibility of Parasites to Drugs. An asynchronous erythrocytic culture of P. falciparum at 1−1.5% parasitemia and 4% hematocrit in complete RPMI-1640 medium in multiwell plates was used to determine the sensitivity of the parasite strain to the various synthesized tetraoxane analogues. The inhibitory concentration of an individual compound needed to prevent the growth and multiplication of *P. falciparum* was determined *in vitro* using a dose response assay in 24-well tissue culture plates in triplicates. Culture was challenged with a graded concentration of compound solutions, covering the range from 1 to 100 nM, for 48 h at 37 °C in a CO_2 incubator. The medium was changed in each well after 24 h with or without the compound. After 48 h of incubation, thin blood smear slides were prepared, air-dried, methanol-fixed, and stained in Giemsa solution. Stained slides were examined for counting the number of parasites in random adjacent microscopic fields, equivalent to about 4000 erythrocytes at 1000× magnification. The percentage inhibition of parasitemia in relation to control was then calculated by examining thin smear Giemsa stained slides. The assay results were computed to determine the IC_{50} values of each drug. The reproducibility of counts was checked by two other readers to maintain the quality control.

Method of Cytotoxicity. The cytotoxicity of the compounds was evaluated in human embryonic kidney cells 293 (HEK-293) using the MTT assay, and CC_{50} values were calculated. Assays were performed in 96-well microtiter plates, each well containing 100 μL of DMEM supplemented with 1% penicillin−streptomycin−glutamine solution and 10% fetal bovine serum and 5×10^3 HEK-293 cells. Serial drug dilutions of eight 2-fold dilution steps covering the range from 100 to 0.78 μg/mL were prepared. After 72 h of incubation, the plates were inspected under an inverted microscope to ensure the growth of the controls and sterile conditions. 10 μL of MTT reagent (5 mg of MTT dissolved in 1 mL of PBS) was then added to each well, and the plates were incubated for 2−4 h in the cell culture incubator. 100 μL of detergent reagent (90% isopropanol, 9.999% triton x-100, and 0.001% conc. HCl) was then added to each well, and the plates were incubated for another 2 h in the dark at room temperature. The absorbance in each well was read with a Biotek Synergy HT microplate reader at a wavelength of 570 nm. Data were analyzed using the microplate reader software. Each CC_{50} value obtained is the mean of at least two separate experiments performed in duplicate.

CONCLUSIONS

In summary, we have developed a newer, efficient, and robust catalyst MoO_3 for the synthesis of symmetric and nonsymmetric tetraoxane analogues using a one-pot approach. Using this newly developed catalyst, we synthesized 26 tetraoxane compounds that can be categorized into two types: 5 symmetric dispiro-1,2,4,5-tetraoxanes and 21 mixed *N*-benzoyl piperidene dispiro-1,2,4,5-tetraoxanes. The symmetric tetraoxanes were obtained in 53−82% yields, whereas *N*-benzoylpiperidene dispiro-1,2,4,5-tetraoxanes were obtained in 26−51%. The modest yield of the mixed tetraoxanes is achieved due to the presence of high functionalities in the molecule. This methodology offers a highly effective and diversified approach for both symmetric and nonsymmetric tetraoxane syntheses using diversified cyclic, acyclic, and aromatic ketones. The structure of synthesized tetraoxane **3g** was established by single-crystal X-ray analysis, confirming the feasibility of molybdenum trioxide as an efficient catalyst for tetraoxane synthesis. The newly synthesized 21 mixed tetraoxane compounds were screened against the 3D7 strain of the malaria parasite *in vitro*. One of the synthesized mixed tetraoxane compounds **3u** showed a low nanomolar activity of 6.35 nM comparable to artemisinin (5.97 nM). Molecular docking of three synthesized tetraoxanes displaying promising antiplasmodial activity was also performed by taking falcipain-2 enzyme as the receptor macromolecule. Molecular docking studies revealed that newer tetraoxane derivatives bind the active site of the falcipain-2 enzyme through H-bonding as well as hydrophobic interactions, which are the foremost interpreters for the activity of the synthesized compounds. These findings will facilitate further studies on quantitative structure−activity relationship evaluations of the second generation of tetraoxane compounds and the identification of new antiplasmodial lead molecules that are effective against artemisinin-resistant parasites.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c01628.

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Synthetic procedures, spectral data of compounds, X-ray crystallography data, NMR spectra images of compound **3a−3u**, and mass spectra images of compounds **3a−3u** (PDF)

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Notes

The authors declare no competing financial interest.

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