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Synthesis and evaluation of hybrid mannich base-tetraoxane derivatives as potent anti-malarial agents

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ABSTRACT

In spite of being a promising drug against malaria, artemisinin at present, demands considerable modifications due to increased resistance of malarial parasites towards it. Presence of Mannich base phenol side chain within the tetraoxane scaffold has shown to deliver a new class of hybrid drugs with dual potency against *Plasmodium falciparum*. Taking this into consideration 5 hybrid chemotypes containing Mannich base as a pharmacophoric group with a tetraoxane core were synthesized and evaluated for anti-malarial activity. Synthesized compounds MS-I and MS-IV showed IC_{50} values of 0.391 and 0.383 µg/100 µL respectively while rest showed IC_{50} values of 0.464, 0.433 and 0.762 µg/100 µL respectively. MS-I and MS-IV were subsequently screened against mutant RKL-2 strain yielding IC_{50} values of 0.807 and 1.619 µg/100 µL respectively.

Keywords: Malaria, Plasmodium Falciparum, Mannich Bases, Artemisinin, Tetraoxanes

INTRODUCTION

Malaria is a life-threatening disease caused by the Plasmodium species which is transmitted to humans by the bite of a female Anopheles mosquito. *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* are the species known to cause malaria in humans, of which *P. falciparum* causes most fatalities [1]. Its worldwide numbers border on about two hundred million clinical cases and reportedly half a million deaths [2]. As per the National Vector Borne Disease Control Programme (NVBDCP) several people in India succumbed to this dreaded disease in the past couple of years. Counteraction to most of the standard anti-malarials is increasing at an alarming rate resulting in substituting combinations of sulfadoxine and pyrimethamine with artesunate-mefloquine in many states of India [3]. Clinical evidence for artemisinin resistance in south-east Asia was first reported in 2008 [4]. Emerging resistance to artemisinin combination therapies (ACT) was reported in Southern Laos, central Myanmar and North-Eastern Cambodia in 2014 [5]. Artemisinin and its derivatives showed rapid action against *P. falciparum* when compared to other drugs for malaria [6]. Chemically, it is a sesquiterpene

lactone containing a peroxide bridge which leads to its therapeutic activity [7]. The antimalarial action exerted by the endoperoxide linkage of artemisinin led to exploration of peroxides as potential replacements for traditional antimalarial drugs such as Chloroquine (CQ) and mefloquine. The availability of artemisinin from its natural source is in short supply and synthetic peroxides are not available for clinical application because of limitations like chemical (availability, purity, and cost), biopharmaceutical (poor availability and limited pharmacokinetics) and non-compliance. As a result, extensive research into synthetic endoperoxide antimalarial drugs has been undertaken to produce molecules that are structurally simpler and synthetically accessible with a projected low cost. The structurally simple class of peroxides that emerged from these studies were the 1, 2, 4, 5-tetraoxanes. Tetraoxane-based antimalarials have been reported to show significant promise because of their artemisinin-like activity [8, 9, 10]. The antimalarial property of artemisinin and of other peroxides such as 1,2,4,5-tetraoxacycloalkanes (tetraoxanes) have recently begun to be exploited in the development of new approaches to fight Chloroquine (CQ)-resistant strains of malaria due to the presence of endoperoxide ring system. New tetraoxanes employing steroidal backbones have now been prepared that is highly active, inexpensive and demonstrates low toxicity. Trioxanes and tetraoxanes and their derivatives have been the successful outcomes to the modifications in artemisinin [11]. The stability of the tetraoxane moiety to hydride reduction and acidic conditions (upto pH 1.6) enabled the synthesis of a series of mixed dicyclohexylidene tetraoxanes and a new type of steroidal mixed tetraoxane with better *in-vitro* and *in-vivo* activities than the corresponding carboxylic amides [12]. Dihydroperoxides and tetraoxanes derived from symmetrically substituted bis (arylmethyl) acetones in modest to good yields were earlier synthesized using several methods [13]. The interaction of thapsigargin with OZ277 (Ranbaxy Laboratories, Mumbai), artesunate or artemether was additive, data consistent with previous observations indicating that activity of anti-malarial peroxides does not derive from reversible interactions with parasite targets [14]. Mannich bases have been reported to be highly reactive and possess potent anti-bacterial and anti-malarial actions [15]. Mannich base phenol side chain within the tetraoxane template has shown to deliver a new class of hybrid drug with potency for a dual mechanism of action versus *P. falciparum* [16]. In this study, a hybrid chemotype containing both the Mannich base pharmacophore and the tetraoxane core is proposed to be synthesized and evaluated for anti-malarial activity. The rationale for selecting Mannich side chain is to preclude the known toxicological concerns with quinolines while retaining the ability to inhibit hemozoin biocrystallisation [17]. Based on the recent literature on tetraoxane derivatives with potential for developing potent antimalarial agents, a work plan was prepared.

EXPERIMENTAL

Roswell Park Memorial Institute (RPMI) 1640 powder (glutamine and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) added), gentamycin and amphotericin were procured from HiMedia while the rest of the reagents were obtained from Merck. Melting range of the synthesized compounds was determined by melting point apparatus (*Buchi M-560*). The UV- Spectra (λ_{max}) of the synthesized compounds were recorded by *Shimadzu*, UV- 1800 instrument. The FTIR spectra of the synthesized compounds were recorded on *Bruker FTIR*. The ^{1}H-NMR and ^{13}C-NMR spectra of the synthesized compounds in DMSO were recorded at 400 MHz and 100 MHz respectively by *Bruker Advance-II* 400 NMR spectrometer.

GENERAL SCHEME FOR THE SYNTHESIS OF COMPOUNDS

The initial step involved the conversion of aromatic ketones and aldehydes to *gem*-dihydroperoxides [18]. Aluminium chloride was used as a catalyst for conversion of ketones to corresponding *gem*-dihydroperoxides by aqueous hydrogen peroxides in room temperature with good yields and shorter reaction time. Phenyl carbonyl compound (1 mmol) and aluminium chloride (0.15 mmol) (0.02g) were mixed with acetonitrile (4ml) to which 30% hydrogen peroxide was added and the mixture stirred at room temperature for 8 hours. After completion of the reaction (TLC), the solution was quenched with 15ml of water. The

products were extracted by Ethyl acetate (3x5ml). The mixtures of the organic layers were dried with Magnesium sulphate and concentrated in vacuum to Obtain the final product.

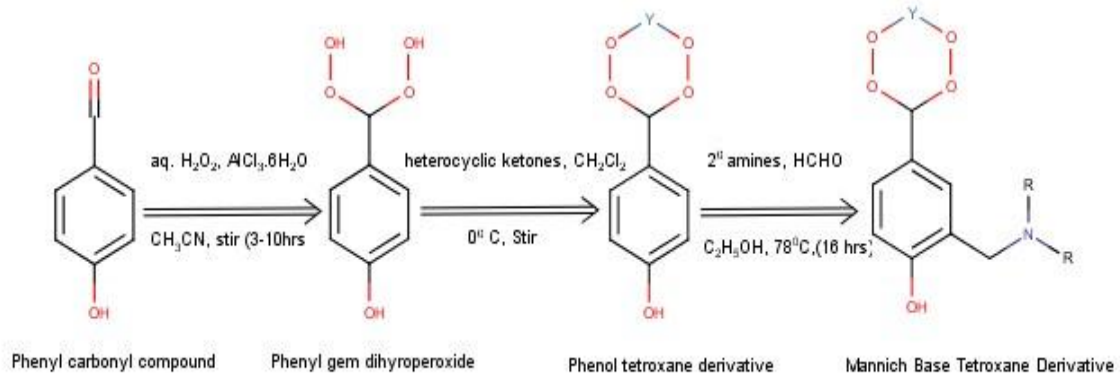


Fig. 1: The complete reaction scheme; R–R' Substituents used on the Phenol tetraoxane moiety (Table I). (a) aq. H_2O_2, $AlCl_3.6H_2O$, CH_3CN, Stir at RT(3-10 hrs); (b) CH_2Cl_2, heterocyclic ketones, stirred for 30 min, RT, cooled, H_2SO_4/CH_3CN mixture (1.66 ml, 1:10v/v) added drop wise, stirred for additional 50 min dried at room temperature; (c) secondary amines (R-R') aq. HCHO in ethanol. Refluxed for 16 hrs at 78°C, extracted with dil. HCl washed with water, dried and recrystallized with suitable solvent. To a cooled solution (ice-bath) of phenyl gem-dihydroperoxide (0.34g, 2.3 mmol) in 20ml dichloromethane, the desired heterocyclic ketone (0.36g, 2.3 mmol) was added and stirred for 30 min at the same temperature, to which a cooled sulphuric acid/acetonitrile mixture (1.66 ml, 1:10 v/v) was added drop wise [12]. After an additional 50 min of stirring, the reaction mixture was dried at room temperature. The phenol tetraoxane derivative (0.05mol) was subjected to Mannich reaction with a secondary amine (0.1 mol) and aqueous formaldehyde (0.1 mol) in ethanol. After refluxing for 16 hrs at 78 °C, the solvent was removed under reduced pressure and the residue was dissolved in 50 ml dichloromethane (Fig. 1). The organic solution was extracted with dilute hydrochloric acid (0.1 M, 2x75 ml). This solution was basified (pH 9-10) and extracted with dichloromethane (3x75 ml). The combined extracts were washed with water (1x100 ml) and dried with anhydrous sodium sulphate and the solvent was evaporated under reduced pressure to give the product and recrystallized with ethyl acetate.

ANTIMALARIAL ACTIVITY EVALUATION

The *in-vitro* antimalarial assay was carried out in 96 well-microtitre plates. The cultures of *P.falciparum* 3D7 and RKL-2 strains were routinely maintained in RPMI-1640 media supplemented with 1 % D-glucose, 5 % sodium bicarbonate, 1.2 ml of gentamycin/L, 1.2 ml amphotericin B and 10 % heat inactivated human serum. The asynchronous parasites of *P. falciparum* were synchronized by treating with 5 % sorbitol to obtain only the ring stage parasitized cells. For carrying out the assay, the initial ring stage parasitaemia of 0.8-1.5 % at 3 % haematocrit in a total volume of 200 µL of RPMI 1640 was uniformly maintained [19]. Stock solutions of 1 mg/ml of each of the test samples were prepared in dimethyl sulfoxide. All the wells of the microtitre plate (from 1-12) were supplied with 100 µL of incomplete media (RPMI-1640, 5 % sodium bicarbonate). The outer wells were taken as control; the inner wells contained 3% haematocrit. In addition to incomplete media and blood, the wells contained the synthesized compounds in different concentrations. A stock solution of 1 mg/ml of each of the test samples was prepared in dimethyl sulfoxide (DMSO). Incomplete media and 100 µL of the stock solution (500 µg/ml concentration) is put in well no.10 and subsequent dilutions are made in the preceding wells until a concentration of 3.91µg/ml is obtained in well no.3 [20]. The culture plates were incubated in a candle jar in a CO_2 atmosphere and stored at 37°C in an incubator.

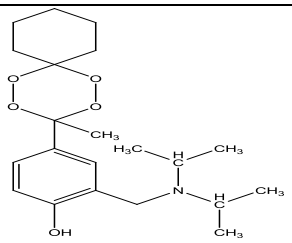
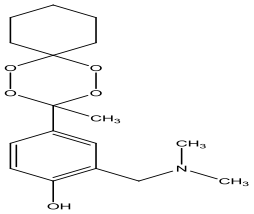
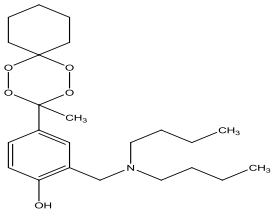
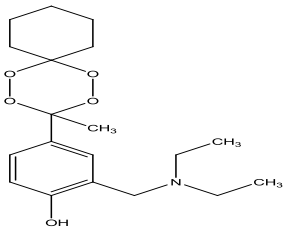
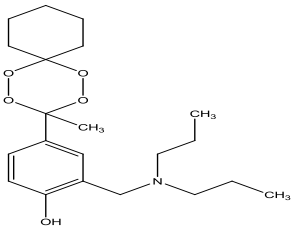
After 36-40h incubation, the blood smears from each well were prepared in triplicates and stained with Giemsa. The slides were microscopically observed to record maturation of the ring stage parasites into trophozoites, schizonts and counting the number of dead parasites in presence of different concentrations of the test compounds. The IC_{50} value of the test drugs were calculated using the software! NonLin_V1.1. CQ was used as the standard reference drug.

RESULTS AND DISCUSSION

Synthetic work

A new series of hybrid Mannich base tetraoxane derivatives have been synthesized and screened for their antimalarial activity. All the designed compounds (MS I to MS V) were synthesized as per the scheme described in Step-I, Step-II & Step-III of the scheme of synthesis (Fig. 4). A total of five compounds (MS-I to MS-V) were synthesized.

Table I- Substituents used on the tetraoxane moiety

Compound	R	R´	Product	Yield
MS-I	- C_3H_7	- C_3H_7		46%
MS-II	- CH_3	- CH_3		68%;
MS-III	- C_4H_9	- C_4H_9		52 %;
MS-IV	- C_2H_5	- C_2H_5		57%;
MS-V	- C_3H_7	- C_3H_7		53%;

Chemical shifts of the synthesized products are expressed as δ values (ppm), downfield from tetramethylsilane (TMS) used as internal standard. Significant ^{1}H-NMR data was written in order: multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet), number of protons, coupling constants in hertz [21].

PHYSICOCHEMICAL CHARACTERIZATION, FTIR AND NMR DATA OF SYNTHESIZED COMPOUNDS

Compound Code: MS-I

2-[(diisopropylamino) methyl]-4-(3-methyl-1, 2, 4, 5 tetraoxaspiro (5.5) undecan-3-yl) phenol; mw= 379.49 Light brown crystal; mp: 180-183 °C; R_f = 0.8 (Chloroform/Water= 4:1); IR (ν_{max}, cm^{-1}) 979.71 (C-O, stretch), 1056.51 (C-C, stretch),1361.54 (C-N, stretch), 1575.45 (C=C, aromatic rings), 3648.26 (O-H, stretch); ^{1}H-NMR (D_2O, 400 MHz, δ in ppm): 1.76-1.77 (d, 12H, J=16 Hz, 4X-CH_3), 2.07 (m, 6H, cyclohexyl, 3X-CH_2), 2.41 (m, 4H, cyclohexyl, 2X-CH_2), 3.10 (s, 3H, -CH_3), 3.14-3.19 (m, 2H, 2X-CH), 3.66 (s, 2H, -CH_2), 4.70 (s, 1H, -OH), 6.64-6.68 (m, 1H, Ar-H), 7.72- 7.76 (m, 2H, Ar-H); ^{13}C-NMR (D_2O, 100 MHz, δ in ppm): 30.20, 116.87, 131.83

Compound Code: MS-II

2-[(dimethylamino) methyl]-4-(3-methyl-1, 2, 4, 5-tetraoxaspiro (5.5) undecan-3-yl) phenol; mw= 323.38 Pale yellow crystal; mp. 250-254 °C; R_f= 0.74 (Chloroform/Water= 4:1); ^{1}H-NMR (D_2O, 400 MHz, δ in ppm): 1.77 (s, 3H,-CH_3), 2.08 (m,6H, cyclohexyl,3X-CH_2), 2.27-2.29 (m,4H,cyclohexyl,2X-CH_2), 2.58-2.66 (d,6H, J=32Hz, 3X-CH_3), 4.02 (s,2H,-CH_3), 4.07 (s,1H,-OH), 6.48-6.50 (m,1H,Ar-H), 7.72-7.75 (m,2H,Ar-H); ^{13}C-NMR (D_2O, 100 MHz, δ in ppm): 19.00, 23.01, 28.02, 42.01, 157.83.

Compound Code: MS-III

2-[(dibutylamino) methyl]-4-(3-methyl-1, 2, 4, 5-tetraoxaspiro (5.5) undecan-3-yl) phenol; mw=407.54 Blackish brown crystals; mp: 197-202°C; R_f= 0.71 (Chloroform/Water = 4:1); ^{1}H-NMR (D_2O, 400 MHz, δ in ppm): 1.85 (m, 6H, 2X-CH_3), 2.16 (m, 8H, 4X-CH_2), 2.44 (s, 3H-CH_3), 4.70 (s, 1H-OH), 6.53-6.56 (m, 1H, Ar-H), 7.75-7.78 (m, 1H, Ar-H); ^{13}C-NMR (D_2O, 100 MHz, δ in ppm): 25.17, 116.91, 122.73, 192.41, 174.59, 201.77

Compound Code: MS-IV

2-[(diethylamino) methyl]-4-(3-methyl-1, 2, 4, 5-tetraoxaspiro (5.5) undecan-3-yl) phenol; mw=351.44 Dark red crystals; mp: 100-103°C; R_f = 0.82 (Chloroform/Water = 4:1); ^{1}H-NMR (D_2O, 400 MHz, δ in ppm): 1.84 (m, 6H, 3X-CH_3), 2.26 (m, 4H, 2X-CH_2), 4.70 (m, 2H-CH_2), 6.50 (s, 1H,-OH), 6.52 (m, 1H, Ar-H), 7.68-7.70

Compound Code: MS-V

2-[(dipropylamino) methyl]-4-(3-methyl-1, 2, 4, 5-tetraoxaspiro (5.5) undecan-3-yl) phenol; mw=379.49 Maroon crystals; mp: 173-177°C; R_f = 0.79 (Chloroform/Water = 4:1); ^{1}H-NMR (D_2O, 400 MHz, δ in ppm): 1.84 (m, 6H, 3X-CH_3), 2.40 (m, 6H, 2X-CH_2), 4.70 (s, 1H,-OH), 6.51-6.52 (m, 1H, Ar-H), 7.71-7.74 (m, 2H, Ar-H); ^{13}C-NMR (D_2O, 100 MHz, δ in ppm): 25.11, 116.61, 122.60, 132.36, 174.30, 201.66

EVALUATION OF *IN-VITRO* ANTIMALARIAL ACTIVITY

All the synthesized compounds were initially tested for *in-vitro* antimalarial activity against the CQ sensitive strain 3D7. Two selected compounds were also subjected to *in-vitro* antimalarial activity screening against the mutant RKL-2 strain of *P. falciparum* in Department of Pharmaceutical Sciences, Dibrugarh University (Dibrugarh, Assam). All the compounds synthesized were tetraoxane derivatives (MS-I to MS-V). The *in-vitro* anti-malarial assay was done by Jaswant Singh Bhattacherji (JSB) staining slide method in 96 well microtitre plates by serial dilution. The stock solution of the test drug contained 1mg/ml which underwent dilution in the subsequent wells to reach the least concentration of 3.91µg/ml. Likewise; the stock solution of CQ contained 0.1mg/ml which had undergone dilution in the subsequent wells to reach the least concentration of 0.391µg/ml. All the synthesized compounds showed anti-malarial activity at different concentration of the test drug. The Inhibitory concentration-50 (IC_{50}) values of the synthesized compounds against the strain 3D7 of the synthesized compounds and the reference drug CQ, have been calculated using the software!

NonLin_V1.1 (Table II). The IC_{50} value of CQ was found to be 0.039µg/100µL. Two compounds MS-I and MS-IV showed comparatively lower IC_{50} values while the other compounds showed much higher IC_{50} values. Two of the synthesized compounds were tested against CQ resistant RKL-2 strain of *P. falciparum* by taking CQ as standard (Table III).

Table II- *IC_{50} values of the synthesized compounds against 3D7 strain

Compound code	IC_{50} value (µg/100µL)
MS-I	0.391
MS-II	0.464
MS-III	0.433
MS-IV	0.383
MS-V	0.762
CQ	0.039

*Data are presented as mean of duplicate observations.

Table III-*IC_{50} values of the two selected compounds against RKL-2 strain

Compound code	IC_{50} value (µg/100µL
MS-I	0.807
MS-IV	1.619
CQ	0.253

*Data are presented as mean of duplicate observations

CONCLUSION

The present study involved the synthesis of some novel hybrid Mannich Base Tetraoxane derivatives with substituted aromatic and heterocyclic ring at side chain and evaluation for antimalarial activity. The structural assignments of new compounds were made on the basis of UV-Visible, IR and NMR analysis. All the synthesized compounds exhibited some degree of antimalarial activity against the CQ sensitive 3D7 strain of *P. falciparum in-vitro*, which, however, was considerably less than that of standard drug CQ. The antimalarial activity data, when after correlation made with IC_{50} values clearly suggest that a six-membered heterocyclic ring substituent at para-position of tetraoxane ring attached to an isopropyl side chain of Mannich Base may be an important requirement for antimalarial activity of the prepared derivatives. Artemisinin and its synthetic analogue and semi-synthetic analogues constitute promising class of antimalarial agents. Some of the tetraoxane based compounds have shown significant antimalarial potential and much of work has been done on this type of compounds in recent years [22]. Apart from being antimalarial agents, they also show antibacterial and antineoplastic property. Hence, there are considerable numbers of reports available that discuss various synthetic methods and structure-activity relationship study among the series of tetraoxane based compounds. On the basis of recent literature and present findings, future work may be carried out involving the molecular or structural modification, especially at the side chain of tetraoxane nucleus with the application of computer assisted Quantitative Structure-Activity Relationship (QSAR) study along with molecular modelling technique for developing novel series of hybrid Mannich base tetraoxane derivatives as potential antimalarial agents.

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