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2-*N*-Methyl modifications and SAR studies of manzamine A

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ABSTRACT

Quaternary carbolinium salts have been reported to show improved antimalarial activity and reduced cytotoxicity as compared to electronically neutral β-carbolines. In this study, mono- and di-methylated quaternary carbolinium cations of manzamine A were synthesized and evaluated for their in vitro antimalarial and antimicrobial activity, cytotoxicity, and also their potential for glycogen synthase kinase (GSK-3β) inhibition using molecular docking studies. Among the analogs, 2-*N*-methylmanzamine A (**2**) exhibited antimalarial activity (IC_{50} 0.7–1.0 μM) but was less potent than manzamine A. However the compound was significantly less cytotoxic to mammalian kidney fibroblasts and the selectivity index was in the same range as manzamine A.

1. Introduction

Malaria, a major tropical infectious disease caused primarily by the protozoan parasite *Plasmodium falciparum*, is one of the most serious health problems worldwide and is responsible for the death of over 1.12 million individuals every year with more than 40 percent of the global population at risk.[1] β-Carboline alkaloids are widely distributed in a number of plant and animal species, as well as marine invertebrates. Some of these alkaloids such as manzamine A (**1**), akagerine, 10-hydroxycanthin-6-one, and 4-methoxy-1-vinyl-β-carboline (MVC) (Fig. 1) display a diverse array of biological activities including antiplasmodial efficacy.[2–4] Manzamine A, as a first representative of the manzamine alkaloids, bearing a unique multi-heterocyclic ring system coupled to a β-carboline moiety, was isolated from the sponge *Haliclona* sp. collected near Manzamo Island by Higa and co-workers in 1986.[5] Despite its potent in vitro activity as an antimalarial agent against *P. falciparum* the moderate toxicity of manzamine A hampered its development as a drug candidate.[4,6–8]

Recently, Ihara and co-workers have reported that the rhodacyanine family of compounds and the β-carboline system of MVC, owing in both cases to a π-delocalized lipophilic cationic (DLC) structure, display reasonable antimalarial and antileishmanial activities against *P. falciparum* and *Leishmania major*, respectively, with low cytotoxicity against mammalian cells.[9–13] The conceptual term, DLC, was originally proposed by Chen in their studies related to anticancer agents.[14] It has been reported that several DLC compounds exhibit selective antitumor activity by accumulating in the

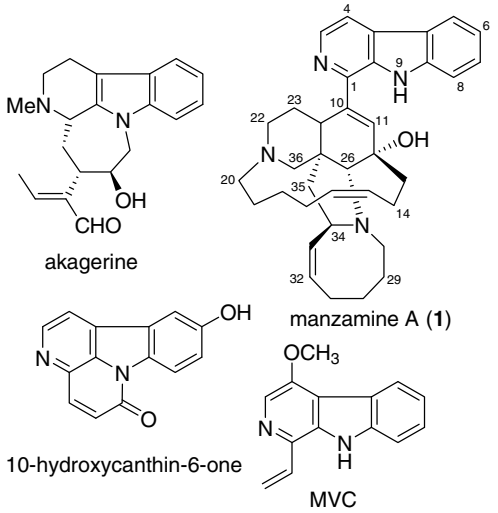

Figure 1. β-Carboline alkaloids with potential biological activities.

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mitochondria of carcinoma cells.[15–17] Vaidya and co-workers found that mitochondrial membrane potential collapse is an antimalarial mechanism of some drugs,[18] and many researchers concluded that DLCs could therefore represent a new antiplasmodial class of drugs.

Our goal was to synthesize manzamine A analogs with decreased toxicity and improved therapeutic index for antimalarial efficacy and continuing structure–activity relationship studies. The rationale for the modification of the β-carboline moiety through quarternization of the pyridine nitrogen to form β-carbolinium salts is based on previous work that showed reduced toxicity and increased antimalarial activity of DLC's formed upon transformation with either alkyl tosylates or alkyl halides as a correlation to the π-delocalization of the cationic species.[10,11,19]

Recently, we found that manzamine A inhibits GSK-3β with an IC_{50} of 10.2 μM so inhibition of this multifunctional serine/threonine kinase may in part be the reason for various therapeutic activities of manzamine A analogs.[20] Since, the crystal structure of GSK-3β is available, we initiated structure-based drug design paradigm of manzamine A analogs using molecular docking coupled with molecular dynamics. Quaternization of the neutral pyridine nitrogen to the desired quaternary ammonium cation was challenging but could finally be accomplished by methylation of manzamine A in the presence of methyl trifluoromethanesulfonate to afford three methylated products (**2**–**4**) in varying yields depending upon reaction conditions. The structures of **2**–**4** were confirmed using high-resolution ESI-MS, as well as ^{1}H, ^{13}C NMR, HMQC, and HMBC. The in vitro antimalarial and antimicrobial activity in addition to cytotoxicity versus mammalian cells of analogs **2**–**4** has also been evaluated.

2-*N*-Methylmanzamine A (**2**), 2-*N*,12-*O*-dimethylmanzamine A (**3**) trifluoromethanesulfonate, and 9-methylmanzamine A (**4**) (Scheme 1) are generated from the treatment of the β-carboline moiety with methyl trifluoromethanesulfonate to afford the mono- and di-methylated products.[11,19]

According to our previous unpublished work showing that N-methylation of the secondary amine of the β-carboline moiety drastically decreases antimalarial potency, along with the low yield of **4**, we were not interested to pursue further evaluation of this compound. Using methyl iodide as the alkylating agent furnished the nonpreferential analog **4** in higher yield.

The structures of analogs **2** and **3** were confirmed with spectroscopic and MS techniques. The high-resolution ESI-MS of analog **2** showed the $[M]^+$ ion peak at *m*/*z* 563.36747 amu in positive mode, in accordance with its molecular formula ($C_{37}H_{47}N_4O$). The ^{1}H NMR spectrum of **3** in MeOD in 400 MHz clearly indicated the presence of a new singlet methyl group at 4.57 (s, 3H, NCH_3) ppm. This finding was confirmed by a new methyl singlet at 45.3 ppm in the ^{13}C NMR spectrum. The HMBC spectrum of **2** supported the methylation of the pyridinium nitrogen as shown by a correlation between the CH_3 singlet at 4.57 ppm and both adjacent aromatic carbon signals at 134.6 ppm (C-3) and 138.8 ppm (C-1) (Fig. 2).

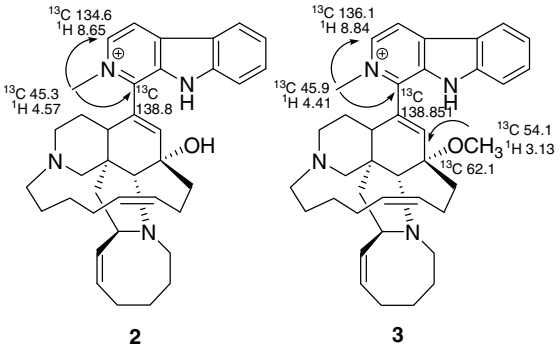


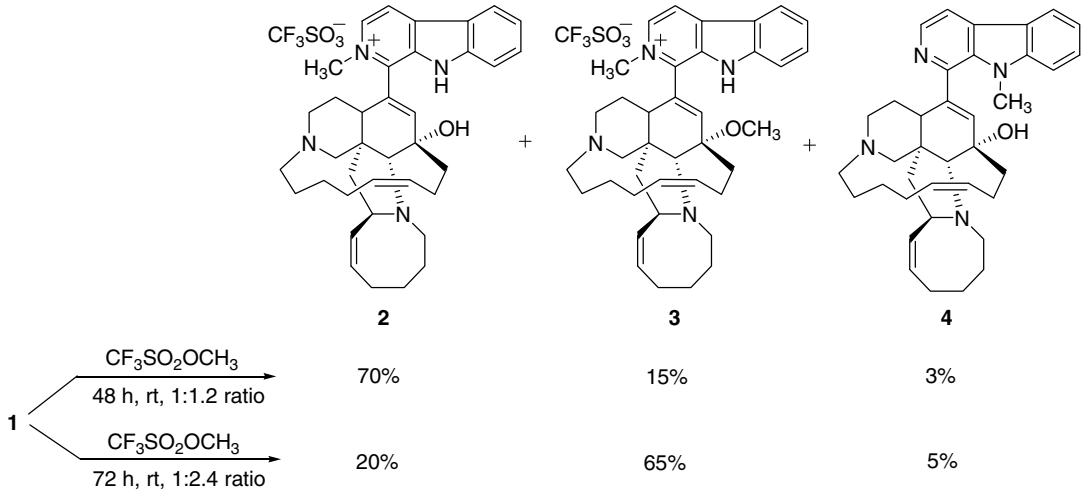
Figure 2. Selected HMBC correlations of analogs **2** and **3**.

High-resolution TOF-ESI-MS of analog **3** provided a molecular mass of $[M]^+$ at *m*/*z* 577.4245, corresponding to the addition of two methyl mass units, in comparison with the parent molecule. The ^{1}H and ^{13}C NMR of compound **3** in DMSO-d_6 contained two methyl proton signals at 4.41 (s, 3H, NCH_3) and 3.13 ppm (s, 3H, OCH_3) along with corresponding carbon resonances at 45.9 and 54.1 ppm, respectively, in agreement with the structure of analog **3** (Fig. 2). In addition, HMBC experiment revealed an unambiguous correlation between the methoxy protons at 3.13 ppm and neighboring quaternary C-12 carbon at 62.1 ppm. These findings are consistent with the fact that we observed the NH group at position 9 of the β-carboline moiety still intact at 11.4 (s, 1H, NH), which in turn indicates that secondary amine group is not involved in the methylation.

2. Biological evaluation

All synthetic analogs were evaluated for in vitro antimalarial and antimicrobial activity as well as cytotoxicity against mammalian cells.

Antimalarial activity was determined against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum*. As shown in Table 1, compound **2** showed antimalarial activity



Scheme 1.

Table 1
In vitro antimalarial activity, selectivity index, cytotoxicity to Vero cells, and docking scores

Compound	*P. falciparum* D6		*P. falciparum* W2		Cytotoxicity to Vero cells	Docking Score
	IC_{50} (μM)	S.I.	IC_{50} (μM)	S.I.	IC_{50} (μM)	ChemScore[a]
1	0.017	29.4	0.020	25.23	0.501	24.9 (±1.0)
2	0.736	>24	1.011	>17.5	NC	22.3 (±0.6)
3	NA	—	NA	—	NC	20.6 (±0.8)
4	NA	—	NA	—	NC	—
Chloroquine	0.013	>1000	0.135	>100	NC	—
Artemisinin	0.0063	>2700	0.0045	>3700	NC	—

NA, no antimalarial activity up to 8 μM; NC, no cytotoxicity up to 17 μM: selectivity index (S.I.), IC_{50} (Vero cells)/IC_{50} (*P. falciparum*).
[a] GOLD ChemScore was an average over 3 runs. Standard deviation in parentheses.

with an IC_{50} of 0.7 and 1.0 μM for D6 and W2 strains, respectively. Compounds **3** and **4** were inactive. None of the synthesized analogs (**2**–**4**) showed any cytotoxicity to mammalian kidney fibroblasts (Vero cells) while manzamine A was cytotoxic (IC_{50} 0.5 μM). Although the antimalarial activity of **2** was much less potent than manzamine A, the selectivity index of **2** remained comparable to manzamine A due to the loss of cytotoxicity. These results indicated that quarternization of the β-carboline moiety resulted in reduced toxicity but still retained antimalarial efficacy in the case of **2**. However, further methylation of **2** (to generate **3**) resulted in loss of antimalarial activity. Methylation of the indoline nitrogen of manzamine A (**4**) resulted in the loss of both antimalarial activity and cytotoxicity.

Manzamine A and some of its analogs are known to inhibit *Homo sapiens* glycogen synthase kinase-3β (GSK-3β).[20] Based on the assumption that the antimalarial activity of these analogs may be due to kinase inhibition of *P. falciparum*, we used the crystal structure of HsGSK-3β (PDB: 1gng)[21] for docking studies to predict the optimal binding positions and relative binding propensities of **1**–**3**. Based on the evidence that **1** was found to be an ATP-noncompetitive inhibitor of GSK-3β,[20] we attempted to dock **1**–**3** into a pocket located in the vicinity of the activation pocket formed by three basic residues, Arg96, Arg180, and Lys205. We used the GOLD 3.1.1 docking program[22] with the scoring function ChemScore,[23] to compare the difference in binding affinity between the ligands and to compare with the experimental in vitro antimalarial activity. The co-crystallized ligands in 1gng, namely 2-amino-2-hydroxymethyl-propane-1,3-diol and sulfate ions, were removed before docking. The active site was defined as any atom that lay within a 15 Å radius of the δN of Arg96. The geometry of **1** was optimized using MMFF94 in Sybyl 7.2, starting from the published X-ray crystal structure of manzamine A.[5] **2** and **3** were built from the crystal structure of **1** followed by geometry optimization using the same force field. ChemScore is a dimensionless fitness function which is a measure of the free energy of a ligand binding to a protein. Since GOLD uses a genetic algorithm to dock ligands, consecutive docking runs do not give exactly the same fitness scores. To address this issue of inherent randomness of GOLD docking, we performed 3 consecutive docking runs to obtain average ChemScores for each ligand, which are given in Table 1 for **1**–**3**.

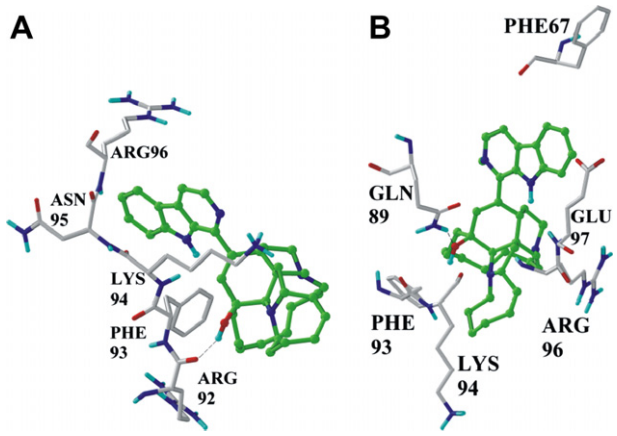


Figure 4. Interactions of **1** (A) and **2** (B) with the GSK-3β ATP-noncompetitive binding pocket residues.

Docking scores agree with the in vitro antimalarial activity data and show that **1** > **2** > **3**. This is consistent with the experimental findings that the first methylation on the tertiary nitrogen of the β-carboline ring (**2**) and the second methylation on the 12-hydroxy group (**3**) each decrease the antimalarial activity a step compared to having free tertiary nitrogen on the β-carboline ring and a 12-hydroxyl in manzamine A (**1**) (Table 1).

The predicted binding positions of **1**–**3** within the ATP-noncompetitive binding pocket (as identified by the Lee and Richards solvent accessible molecular surface)[24] are shown in Figure 3.

Compound **1** and **3** have the same binding mode, whereas the binding position of **2** is very different. The polar and hydrophobic interactions of **1** are shown in Figure 4. Manzamine A (**1**) shows a strong hydrogen bonding of its 12-hydroxyl group with the backbone carbonyl of Arg92. Also, the hydrophobic β-carboline ring interacts with the side chains of Lys94, Asn95, and Arg96. The NH of the β-carboline ring is pointing toward Phe93 indicating a probable steric clash if any substitution is made at this position. In comparison to **1**, **2** adopts a binding mode in a different region of the ATP-noncompetitive binding pocket, leading to less favorable hydrogen bonding and hydrophobic interactions. The crucial

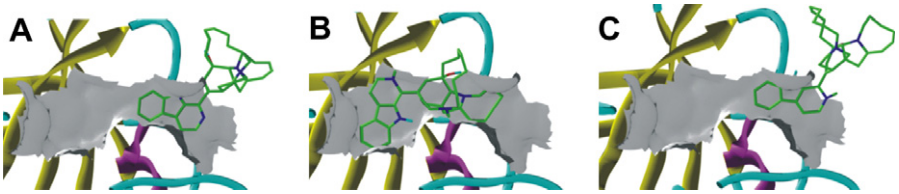


Figure 3. Binding positions of **1**–**3** within the ATP-noncompetitive binding pocket of GSK-3β (A: **1**, B: **2**, C: **3**). Ligands **1**–**3** are shown with green carbon; the protein is in ribbon format colored according to secondary structures; the binding pocket interior surface (as detected with the Lee/Richards molecular surface) is in white.

Table 2
In vitro antimicrobial activity (all values in μM)

Compound	*C. albicans*			*C. neoformans*			MRSA			*M. intracellulare*		
	IC_{50}	MIC	MFC	IC_{50}	MIC	MFC	IC_{50}	MIC	MBC	IC_{50}	MIC	MBC
1	16.4	—	—	2.7	5.7	23	0.18	—	—	0.18	0.36	2.84
2	—	—	—	71.1	—	—	44.5	89	89	17.8	44.5	44.5
3	—	—	—	52	87	87	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—	—	—	—	—
Amphotericin B	0.3	0.68	1.35	0.8	1.35	1.35	NT	NT	NT	NT	NT	NT
Ciprofloxacin	NT	NT	NT	NT	NT	NT	0.3	1.5	—	1.1	1.5	3

IC_{50}, the concentration that affords 50% inhibition of growth; minimum inhibitory concentration (MIC) is the lowest test concentration that allows no detectable growth; minimum fungicidal/bactericidal concentration (MFC/MBC) is the lowest test concentration that kills the organism.
'—', not active at the highest test concentration of 91, 89, 87, 89, 5.4, and 3.0 for samples **1**, **2**, **3**, **4**, Amphotericin B, and ciprofloxacin, respectively; NT, not tested.
Amphotericin B and ciprofloxacin are used as positive antifungal and antibacterial controls, respectively.

Table 3
Anti-HCV activity in the Huh-7 replicon assay

Compound	ΔCt HCV	ΔCt rRNA	% Inhibition	
			HCV	rRNA
2	9.56	15.74	99.87	100.00
RS-446[a]	7.48	−0.25	99.43	−19.22

[a] RS-446 (2-Me-C) was used as the control. The compound and the control are tested at 10 μM in triplicate immediately after seeding.

interactions for **2** are also shown in Figure 4. The 12-hydroxyl of **2** forms a hydrogen bond, but it is with Gln89, unlike that of **1** with Arg92. The hydrogen bonding between the NH_2 of Gln89 and the 12-hydroxyl of **2** is weaker (more distant) than the interaction seen in **1**. Also, **2** shows hydrophobic interactions with the side chain of Phe67. **3** adopts a binding position similar to **1**, and **3** does not show any hydrogen bonding interaction, due to the second methylation at the 12-hydroxyl group. Thus, the docking scores are in good agreement with the antimalarial results indicating their utility in the rational design of more active analogs from this class.

Antimicrobial activity was determined against a panel of human pathogenic bacteria and fungi (Table 2). Compounds **1** to **4** were inactive against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, in addition to the opportunistic filamentous fungus *Aspergillus fumigatus* (data not shown). Moreover, compound **4** was inactive against all species tested, indicating that quaternarization of the secondary amine decreases bioactivity in general. Among compounds **1**–**3**, manzamine A (**1**) remains the most potent with strong activity against the opportunistic yeast *Cryptococcus neoformans*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Mycobacterium intracellulare*, with bioactivity against the latter microorganism rivaling the positive control Ciprofloxacin. Results indicated that the conversion of hydroxyl group to a methoxy in compound **3** also decreases in vitro antimicrobial activity. In summary, derivatization of either the pyridine or secondary amine of the β-carboline moiety, along with methylation of the C-12 hydroxyl group of manzamine A decrease in vitro activity against several pathogenic microorganisms. Also anti-HCV activity for compound **2** has been evaluated (Table 3).

In conclusion, the preliminary structure–activity relationship study in regards to the first and second methylation showed that the introduction of the first methyl at the pyridine nitrogen in the β-carboline moiety of manzamine A significantly decreases the cytotoxicity but also reduced the antimalarial activity of manzamine A. Introduction of a second methyl group at C-12-OH position completely eliminated the antimalarial activity indicating that OH group is an essential pharmacophore for the antimalarial activity of the manzamine class of alkaloids. The docking studies of these analogs on GSK-3β delineated the ability of manzamine A (**1**) to form a stronger hydrogen bond than **2** and the inability of **3** to interact favorably with the ATP-noncompetitive site amino acids of GSK-3β, because **3** lacks a complementary donor atom at its C-12-OH position.

3. Experimental

3.1. General preparation of compounds 3–4

Treatment of manzamine A (54.8 mg, 0.1 mmol) with methyl trifluoromethanesulfonate (135.5 μL, 1.2 mmol) in a molar ratio of 1:1.2 in dry methylene chloride while stirring for 48 h at ambient temperature resulted in the formation of major product **2** in 70% yield in addition to two minor products **3** in 15% and **4** in 3% yield. Purification was completed via HPLC using a reverse-phase C8 column with a mobile-phase gradient of water/acetonitrile and a flow rate of 10 mL/min. The major compound appears at a retention time of 40 min.

By increasing the molar ratio of methyl trifluoromethanesulfonate from 1.2 to 2.4 and stirring for 72 h, compound **3** was generated in 65% yield alongside the minor products **2** in 20% and **4** in 5% yield. The resultant yellow solid was washed with diethyl ether and purified using the method above affording compound **3** at a retention time of 30 min.

3.1.1. 2-*N*-Methylmanzamine A trifluoromethanesulfonate (2)

$[\alpha]_D^{25}$ +57.5 (*c* 0.08, MeOH); UV λ_{max} (MeOH) 260, 310, 375 nm; IR ν_{max} ($CHCl_3$) 3207 (NH), 3073, 3011, 2928, 2855, 1671, 1628, 1577, 1520, 1415, 1296, 1198, 1030 cm^{-1}; ^1H NMR (MeOD) δ 8.65 (2H, m), 8.43 (1H, d), 7.87 (2H, m), 7.52 (1H, t), 6.27 (1H, s), 5.68 (2H, m), 5.53 (1H, t), 4.71 (1H, m), 4.57 (3H, s, NCH_3), 4.43 (1H, m), 3.89 (1H, s), 3.65 (1H, m), 3.48 (1H, t) and 3.35–0.90 (complex); ^{13}C NMR (MeOD) δ 144.8, 141.8, 138.8, 135.4, 135.2, 134.6, 132.6, 132.2, 132.0, 126.9, 122.7,122.1, 119.9, 116.3, 113.1, 74.8, 69.7, 68.7, 66.6, 56.9, 53.1, 48.7, 48.4, 46.8, 45.3, 43.2, 40.1, 39.7, 33.0, 28.4, 26.4, 25.4, 24.9, 24.3 and 20.8; HRESIMS *m*/*z* calcd for $C_{37}H_{47}N_4O$ $[M]^+$ 563.3750, found 563.3674.

3.1.2. 2-*N*,12-*O*-Dimethylmanzamine A trifluoromethanesulfonate (3)

$[\alpha]_D^{25}$ +25.7 (*c* 0.14, MeOH); UV λ_{max} (MeOH) 225, 260, 314, 380 nm; IR ν_{max} ($CHCl_3$) 3443 (NH), 3331, 3258, 2981, 2935, 1679, 1606, 1527, 1457, 1391, 1365, 1250, 1157, 1051 cm^{-1}; ^1H NMR (DMSO-d_6) δ 11.40 (1H, s, NH), 8.84 (2H, m), 8.48 (1H, d), 8.23 (1H, d), 7.72 (1H, t), 7.42 (1H, t), 5.88 (1H, s), 5.52 (1H, m), 5.40 (2H, m), 5.22 (1H, m), 4.41 (3H, s, NC H_3), 4.01 (2H, m), 3.81 (1H, d), 3.56 (2H, m), 3.32 (5H, m), 3.20 (2H, m), 3.13 (3H, s, OCH_3), 2.89 (3H, m), 2.74 (1H, m) and 1.43–2.19 (complex); ^{13}C NMR (DMSO-d_6) δ 145.4, 145.1, 141.8, 138.8, 137.2, 136.1, 135.3, 133.4, 131.9, 129.6, 129.3, 125.8, 123.6, 121.9, 119.8, 117.2, 114.6, 74.1, 62.9, 62.1, 61.5, 59.1, 58.2, 54.1, 50.4, 45.9, 45.0,

43.8, 43.0, 36.7, 27.6, 26.4, 25.8, 25.4, 25.0, 24.2, 23.2 and 18.7; HRESIMS m/z calcd for $C_{38}H_{49}N_4O$ $[M]^+$ 577.3906, found 577.4044.

3.1.3. 9*N*-Methylmanzamine A (4)

$[\alpha]_D^{25}$ +18.6 (c 0.07, MeOH); UV λ_{max} (MeOH) 260, 310, 375 nm; IR ν_{max} ($CHCl_3$) 3648 (NH), 3294, 3011, 2924, 2838, 1675, 1628, 1519, 1443, 1329, 1199, 1017 cm^{-1}; ^1H NMR (MeOD) δ 8.63, (1H, br s), 8.40 (1H, d), 7.78 (1H, d), 7.53 (1H, d), 7.40 (1H, dd), 6.40 (1H, s), 5.91 (1H, br s), 5.62 (1H, q), 5.52 (1H, td), 5.26 (1H, t), 4.31 (1H, br s), 3.92 (3H, s, NCH_3), 3.50 (1H, d), 3.08 (2H, m), 2.78 (1H, m), 2.56 (2H, m), and 1.2–2.5 (complex); ^{13}C NMR (MeOD) δ 154.2, 143.5, 139.9, 138.2, 137.4, 135.1, 134.5, 134.2, 132.3, 129.9, 129.2, 128.5, 122.2, 118.3, 117.2, 113.3, 112.5, 103.5, 75.1, 70.0, 68.7, 56.0, 55.0, 53.5, 50.9, 49.6, 47.1, 44.7, 40.9, 40.3, 32.7, 31.7, 28.1, 26.8, 26.0, 25.7, and 21.7; HRESIMS m/z calcd for $C_{37}H_{47}N_4O$ $[M+H]^+$ 563.3750, found 563.3764.

3.2. Assay for in vitro antimalarial activity and cytotoxicity

A 200 μL suspension of red blood cells infected with D6 or W2 strain of *P. falciparum* (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 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.[25] Twenty microliters of the incubation mixture were mixed with 100 μL of the Malstat™ reagent and incubated at room temperature for 30 min followed by addition of 20 μL of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) and further incubation in the dark for 1 h. The reaction was stopped by the addition of 100 μL of 5% acetic acid. The plate was read at 650 nm. Artemisinin and chloroquine were included as the drug controls. 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 as described earlier.[26] 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.

3.3. Assay for antimicrobial activity

All organisms are obtained from the American Type Culture Collection (Manassas, VA) and include the fungi *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906 and the bacteria methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing is performed using a modified version of the CLSI (formerly NCCLS) methods.[27–30] *M. intracellulare* is tested using a modified method of Franzblau et al.[31] Samples are serially diluted in 20% DMSO/saline and transferred in duplicate to 96-well flat bottom microplates. Microbial inocula are prepared by correcting the OD630 of microbe suspensions in incubation broth to afford final target inocula. Drug controls [Ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and Amphotericin B (ICN Biomedicals, Ohio) for fungi] are included in each assay. All organisms are read at either 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) or 544ex/590em, (*M. intracellulare*, *A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany) prior to and after incubation. Minimum fungicidal or bactericidal concentrations are determined by removing 5 μL from each clear well, transferring to agar and incubating. The MFC/MBC is defined as the lowest test concentration that kills the organism (allows no growth on agar).

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