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Approaches towards the stabilization of hemiaminal function at ornithine unit of mulundocandin☆

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Abstract—Semisynthetic modifications at position-12 (ornithine-5-position, hemiaminal function) of mulundocandin were carried out to improve its chemical stability. New carbon–carbon (C–C) and carbon–hydrogen (C–H) linkage at hemiaminal function -12 has been achieved. Lewis acid catalyzed introduction of electron rich aryl group at position-12 of mulundocandin is developed. Synthesized mulundocandin analogues were evaluated for their chemical stability and antifungal activity against *C. albicans* and *A. fumigatus*.

1. Introduction

The incidence of virulent fungal infection, especially systemic mycoses in immuno deficient host are on the increase during the last decades.[1] The majority of life threatening fungal infections is caused by opportunistic pathogens such as *Candida* spp., *Aspergillus* spp., *Pneumocystis carinii* and *Cryptococcus neoformans*.[2] The increase is attributed to indiscriminate use of broad spectrum antibiotics in organ transplantation. Polyenes and azoles are two classes of compounds used for the treatment of fungal infections. Amphotercin B is used alone or in combination with 5-flucytosine. Polyene antifungals are broad spectrum antifungals, however their utility is limited due to nephorotoxicity. Azoles are fungistatic agents, they have tendency to develop drug resistance.[3] Both Polyene and azoles target fungal cell wall membrane, a structure shared by both mammalian and fungal cells, hence these drugs have possibility of toxicity.

Therefore, there is a considerable need for the development of new antifungal agents with improved properties. Echinocandin class of natural products is potent lipopeptide antifungal agents, which inhibit the synthesis of β-(1,3)-D-glucan, an integral component of the cell wall of certain fungi.[4] The most of the echinocandins are unstable and have limited water solubility, hence their development as drug candidates has been hampered. Two new echinocandin antifungals, caspofungin[5] and micafungin[6] have been introduced in the market for the treatment of the invasive Aspergillus infection.[6a] A few more echinocandines as antifungals are under development with an aim to provide improved therapy for fungal infections.[7]

Mulundocandin (MCN or **1**),[8] belongs to an echinocandins class of antifungal lipopeptides. They inhibit the synthesis of β-(1,3)-D-glucan, an essential component of the fungal cell wall that is absent in mammalian cell. Thus, the inhibition of β-(1,3)-D-glucan synthesis represents a fungal specific, potentially non-toxic target. Naturally occurring echinocandins have very reactive hemiaminal function at ornithine unit, which undergoes ring opening and rearrangement, leading to biologically less active linear peptides in acidic or basic medium.[9] Various approaches are reported in the literature for modifications at ornithine-5-position of echinocandins to obtain chemically stable and water-soluble analogues.[10] We have previously described semisynthetic modifications at hemiaminal function of the fungicidal lipopeptide, mulundocandin (**1**) that resulted in improved chemical stability without significant loss of anti-*Candida* activity.[11] In this work, we wish to report the formation of C–C and C–H linkage at position-12 (= ornithine-5) position of mulundocandin with an aim

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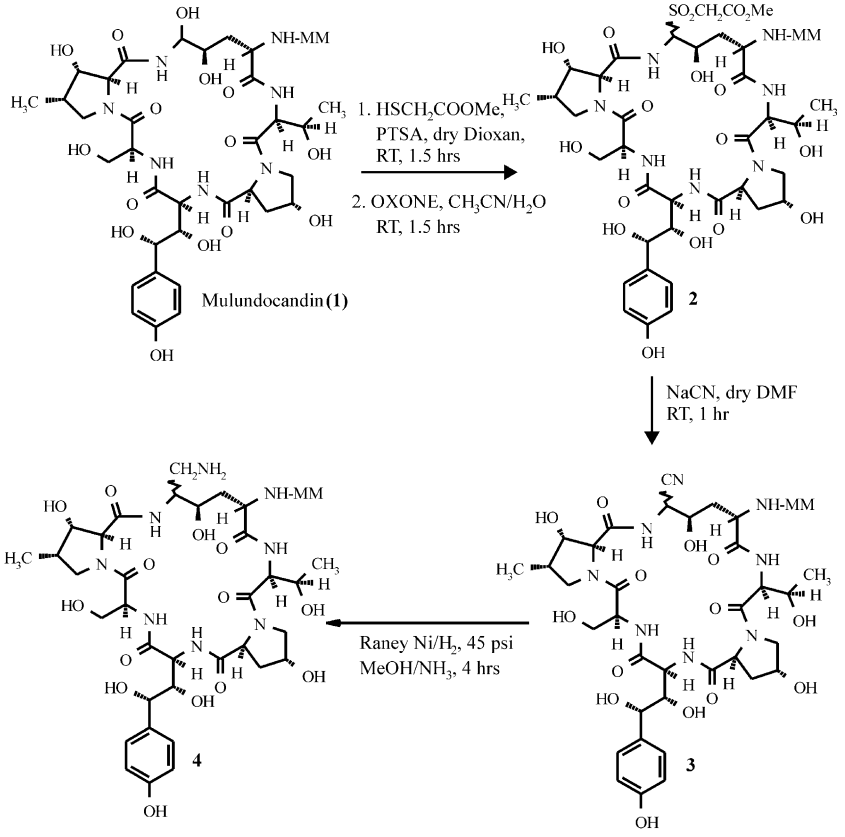
to improve further stability and modify the biological activity.

2. Chemistry

The Orn-5-sulfone mululndocandin (**2**) was used as starting material for this reaction. The sulfone **2** was synthesized by reacting **1** with methylthioglycolate in presence of catalytic amount of *p*-toulene sulfonic acid, followed by oxidation of the corresponding thioether with OXONE.[12] Sulfone-**2** on reaction with sodium cyanide in anhydrous DMF at ambient temperature gave Orn-5-cyanomulundocandin (**3**) in 36% yield as shown in Scheme 1. ^{1}H NMR spectrum of **3** had all the peaks of mulundocandin, except that B_7-H appeared as a broad singlet at δ 5.17. A band at 2320 cm^{-1} (CN) in IR spectrum and peak at 119.69 ppm in ^{13}C NMR spectrum confirmed the presence of cyano group in the molecule.

Compound **3** on reduction with cobalt chloride hexahydrate and sodium borohydride mixture in methanol gave the required amino compound in low yield. Catalytic hydrogenation using Pd–C or Raney-Ni in methanol solvent failed. However, hydrogenation using Raney Ni catalyst in methanolic ammonia gave the desired aminomethyl analogue **4** in 53% yield (Scheme 1). The compound was purified on RP18 column using 50–90% acetonitrile/water, the compound displayed $(M+Na)^+$ peak at 1043.5 (mw: 1021.21) in ESI-MS spectrum. In ^{1}H NMR spectrum had an additional multiplet at δ 2.1, assigned to the methylene protons (–$\underline{CH_2}NH_2$). Compound **4** displayed increased chemical stability as well as improved water solubility on being converting into a hydrochloride salt.

Another approach to synthesize carbon derivatives at Orn-5-position of muludocandin was through introduction of an aryl group. The crucial step during this approach involved the utilization of the very reactive N-acyliminium ion derived intermediate from hemiaminal at position-12. The intermolecular and intramolecular amidoalkylations with π-nucleophiles have been reported in the literature.[13] The Orn-5-methoxymulundocandin (**5**) was chosen as a starting material for this reaction, this in turn was prepared by treating mulundocandin with excess methanol and catalytic amount of PTSA in dry dioxane. We were aware of the fact that it will be very hard to stabilize the N-acylinium ion of mulundcandin, due to inherited unstable nature of this center, yet we decided to go ahead with an aim to introduce an aryl group. We had to select an aryl component, which will be electron rich so that it can capture acylinium ion fast. We chose 3,4-dimethoxy benzene as the electron rich component. (*This type of reaction has not been attempted on any of the echinocandins so far*.) Compound **5** was treated with catalytic amount of titanium tetrachloride in 1,2-dimethoxyethane at −70 °C under nitrogen atmosphere for 5 min and then reacted with 3,4-dimethoxy benzene (veratrole) at −78 °C to −50 °C for 1.5 h. LC-MS analysis of the reaction mixture showed the presence of acid degraded product as the major component and the required compound **6** with



Scheme 1. Formation of C–C linkage at Orn-5 position of mulundocandin.

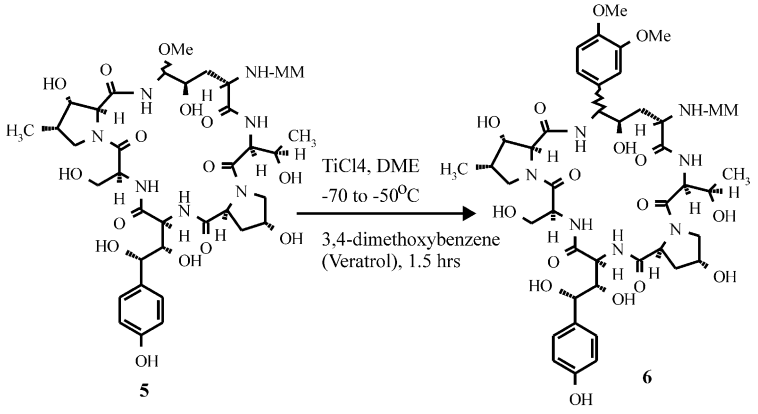
some unidentified minor products. Purification of the reaction mixture on a RP-18 column using acetonitrile in water as the eluent gave Orn-5-(3,4-methoxyphenyl)-mulundocandin (**6**)[17] in 8% yield (Scheme 2). Attempts to improve the yield of **6** by changing the reaction conditions like reaction solvent and Lewis acid catalyst was less successful.

In ^{1}H NMR spectrum of **6**, B_7-H disappeared from its characteristic low field position, two doublets for D_7-H, $D_{7'}$-H and D_8-H, $D_{8'}$-H appeared at δ 7.18 and 6.78, respectively. The three aromatic protons from 3,4-dimethoxybenzene appeared as doublet at δ 7.38, $J = 1.47$ Hz, doublets of doublet at δ 7.10, $J = 8.55$ and 1.47 Hz, and another doublet at δ 6.9, $J = 8.55$ Hz. The two methoxy group of veratrol appeared as two singlets at δ 3.4–3.6 respectively. Its structure was also confirmed by (M + Na) peak at 1150.6 (mw = 1128.31) in its ESI-MS spectrum.

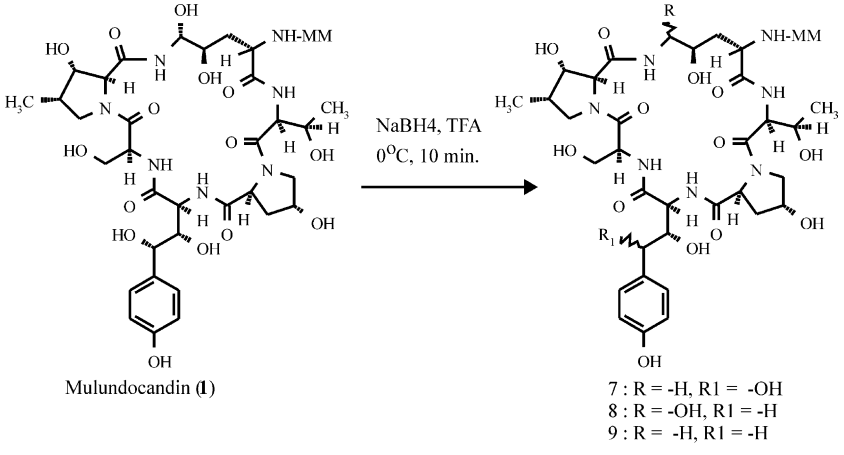
Several reports have appeared in the literature regarding synthesis and SAR studies of deoxy-echinocandins and pneumocandins.[13] In the present study, selective removal of Orn-5-hydroxyl and/or HTyr-4 hydroxyl of mulundocandin was attempted. Initial experiments of reduction studies with varied concentration of TFA and $NaBH_4$ or $NaCNBH_4$ as reducing agent in dry dioxane as well as in acetic acid as solvent gave low yield of either of the deoxymulundocandins. The major product isolated was identified as degraded mulundocandin, presumably, as more time was required for completion of the reaction in these solvents. When we used TFA as a solvent, reaction got completed within 10 min giving desired, Orn-5-deoxymulundocandin (**7**, 38%), HTyr-4-deoxymulundocandin (**8**, 19%) and dideoxymulundocandin (**9**, 14%), along with a small amount of acid degraded MCN (Scheme 3). When reaction time and temperature was increased, more of dideoxymulundocandin was formed.

Comparison of ^{1}H NMR spectrum of **7** with mulundocandin showed two methylene protons of iminol carbon (–NH$\underline{CH_2}$). The two doublets of homotyrosine aromatic ring protons appeared at δ 7.06 and 6.74. In mass spectra the required $(M + Na)^+$ peak appeared at 1014.5 in ESI-MS spectrum (mw: 992.177). The structure of **8** was confirmed by comparing with authentic compound.[14] Dideoxymulundocandin (**9**) showed two gem coupled doublet of doublets at δ 2.95–3.05 for two benzylic protons and multiplet at δ 2.55–2.7 for iminol protons (NH$\underline{CH_2}$) in ^{1}H NMR spectrum. In ESI-MS spectrum $(M + Na)^+$ appeared at 998.7 as a base peak and 976.6 as a molecular ion peak.

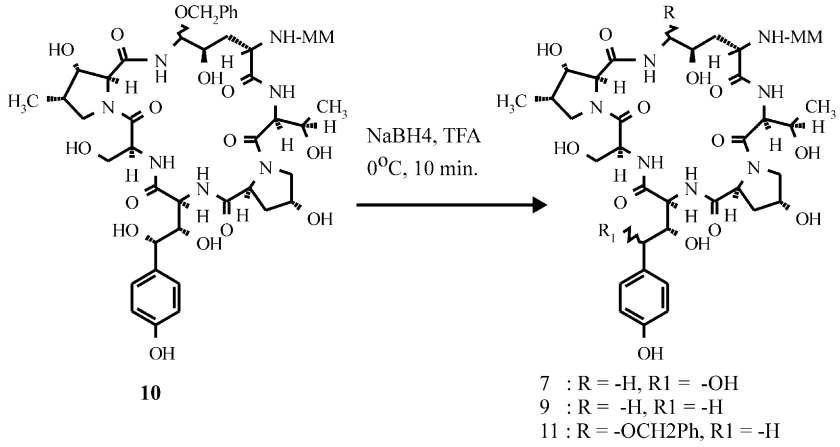
Protection of Orn-5-hydroxyl with benzyl ether increased chemical stability with retention of in vivo



Scheme 2. Introduction of aryl group at Orn-5-position of mulundocandin.



Scheme 3. Reduction studies at mulundocandin.



Scheme 4. Reduction studies on Orn-5-benzyloxy mulundocandin.

anti-*Candida* activity. Benzylic deoxymulundocandin is reported to have the same in vitro *and* in vivo activities against most *Candida* species; and in fact, it shows better in vivo activity against some *candida* species.[15] Thus, it was hoped that selective removal of HTyr-4-hydroxyl group of the Orn-5-benzyloxy mulundocandin (**10**) will give analogue, which will be more stable than mulundocandin due to absence of hemiaminal function, and with deoxy at benzylic position of homotyrosine unit may improve activity. Sodium cyanoborohydride or sodium borohydride in acidic medium are known to cleave benzyl ether linkages and also to remove benzylic hydroxyls giving corresponding deoxygenated compounds. Thus, the reduction of **10**, in a manner similar to described for mulundocandin, with sodium borohydride in TFA at 0 °C gave Orn-5-benzyloxy-HTyr-4-deoxymulundocandin (**11**) in 14%, Orn-5-deoxymulundocandin (**7**) in 11% and dideoxymulundocandin (**9**) in 15% yield (Scheme 4). In ESI MS spectrum, the compound **11** (mw: 1082.293) showed $(M+Na)^+$ peak at 1104.7, which indicates the loss of only one oxygen functionality than in the starting compound. ^1H NMR showed the presence of benzyloxy protons suggesting that the hemiaminal position is protected in the form of benzyl ether. Proton NMR was consistent with the structure.

We had expected that this compound, with stabilized hemiaminal function and reduced at HTyr-4 position, may show better activity, but the result we obtained was not promising. All these Orn-5 modified analogues were qualitatively studied for their chemical stability by keeping in aqueous methanolic solution at pH 5 and 9 for 24 hrs at room temp, followed by TLC and HPLC analysis and were found to be more stable than MCN.

2.1. Biological studies of mulundocandin analogues

2.1.1. In vitro. The synthesized mulundocandin analogues were screened for their antifungal activity in vitro by standard agar well method against *C. albicans* (IV) and *A. fumigatus* (AF-1), in Sabouraud agar bioassay plates.[16] The zones produced by the mulundocandin analogues were compared with the zone of mulundocandin and the results of the assay are given in Table 1.

From the Table 1 it is apparent that, the analogues **3**–**11** in vitro activity is comparable to that of mulundocandin, but with much improved chemical stability in both acidic and basic conditions. The in vitro activities of analogues **4** and **6** are comparable to that of mulundocandin. Compound **4** has the added advantage of improved stability and aqueous solubility in its salt form.

Reduction of mulundocandin generated compounds **7**–**9**, of which **7** retains the in vitro activity (25 mm and 23^{hd} mm zone size against *C. albicans* and *A. fumigatus*,

Table 1. In vitro activities of mulundocandin analogues against *C. albicans* (IV) and *A. fumigatus* (AF-1)

Compd	Concentration (mg/mL) in MeOH	Zone size (mm)	
		C. albicans	*A. fumigatus*
2	1	20	20^{hd}
	2	22	24^{vh}
	5	26	29^{hd}
3	1	19	18^{hd}
	2	22	20^{hd}
	5	25	27^{hd}
4	1	22	$9/30^{hd}$
	2	22	23^{hd}
	5	25	25^{hd}
5	1	25	25^{hd}
	2	26^{h}	$24^{h}/37^{h}$
	5	27	35^{vhd}
6	1	24	30^{hd}
	2	25	30^{hd}
	5	29	32^{hd}
7	1	25	23^{hd}
	2	26	23^{hd}
	5	29	25^{hd}
8	1	30	29^{hd}
	2	33	30^{hd}
9	1	24^{h}	25^{h}
	2	33	30^{hd}
	5	38	35^{hd}
10	2	12	24^{h}
	5	16	26^{h}
11	2	15	15^{hd}
	5	17	18^{hd}
1	0.1	24	24^{hd}
Mulundocandin	1.1	29	29^{hd}
	2.0	32	35^{vh}

h = hazy, vh = very hazy, hd = hazy diffused, vhd = very hazy diffused.

Table 2. Anti-*Candida* activity of mulundocandin derivatives by intraperitoneal injection in Swiss mice

	Approx.CFU count (per gm kidney)/Dose (ip, mg/kg×6; B.i.d)
Activity of analogue **8**	1.8×10^8 (20 mg)
Activity of mulundocandin	1.5×10^8 (20 mg)
Activity of fluconozole	3.8×10^5 (20 mg)
Control (no treatment is given)	2.5×10^8
Activity of analogue **9**	7.7×10^6 (20 mg)
Activity of mulundocandin	1.25×10^7 (20 mg)
Activity of fluconozole	1.0×10^4 (20 mg)
Control (No treatment is given)	6.7×10^7

respectively) of MCN with much improved aqueous stability in both acidic and basic solutions. The benzylic deoxy mulundocandin **8** shows better in vitro activity than mulundocandin, in accordance with literature report.[15]

2.2. In vivo testing of MCN analogues against *C. albicans* using Swiss mice model[16]

Antifungal activity of analogues **8** and **9** was evaluated in vivo in *C. albicans* infected Swiss mice after intraperitoneal administration. The colony formation unit (CFU) in kidney homogenate after administrating MCN derivatives were determined and compared with MCN, a standard antifungal in clinical use (Fluconazole) and an untreated control group. The results are summarized in Table 2. The compound **9** with both Orn-5- and HTyr-4-hydroxyls removed shows one log reduction in CFU by intraperitoneal injection.

3. Conclusion

The hemiaminal function of mulundocandin has been stabilized by formation of C–C and C–H derivatives at Orn-5 position without significant loss of activity. We have developed synthetic route for introduction of aryl group at Orn-5 position of mulundocandin (MCN) and activity is retained, which can further be exploited for generating novel series of echinocandin analogues for SAR studies. The stability study using TLC and HPLC analysis has established that all the synthesized derivatives are more stable than muludocandin.

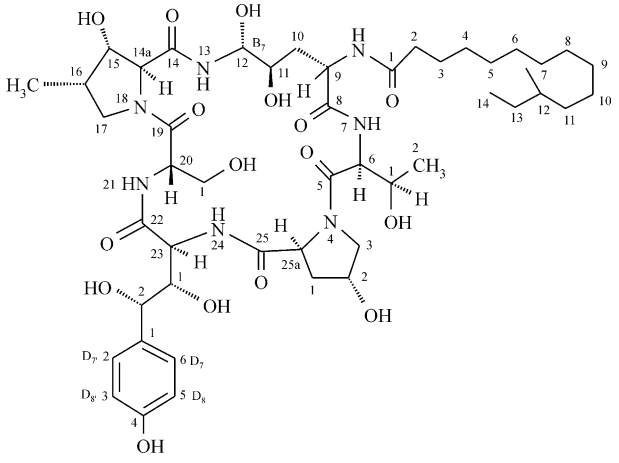


Figure 1. Mulundocandin (**1**).

3.1. Nomenclature of mulunddocandin

N1- [(6*S*, 9*S*, 14a*S*, 15*S*, 16*S*, 20*S*, 23*S*, 25a*S*, 2*R*, 11*R*, 12*R*)-23-((1*S*,2*S*)-1,2-dihydroxy-2-(4-hydroxyphenyl) ethyl)-2,11,12,15-tetrahydroxy-6-((1*R*)-1-hydroxyethyl)-20-hydroxymeth-yl-16-methyl-5,8,14,19,22,25-hexaoxoperhydrodiazolo [2,1-*c*:2,1-/][1,4,7,10,13,16] hexaazacyclohenicosin-9-yl]-12-methyltetradecanamide (Fig. 1).

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17. *12-(3,4-Dimethoxyphenyl)-mulundocandin* (**6**). To the three-neck 100 mL RB flask fitted with a nitrogen inlet, calcium chloride guard tube and rubber septum was added Orn-5-methoxymulundocandin **5** (0.11 g, 0.108 mmol) in 1,2-dimethoxyethane (3 mL) and mixture was stirred at −70 °C under nitrogen atmosphere. Titanium tetrachloride (25 μL) was added to the above solution and stirred for 5 min. 3,4-Dimethoxybenzene (0.04 mL, 3.13 mmol) was added to the above solution and stirred at −50 to −70 °C for 1.5 h. TLC analysis showed no starting compound but mixture of nonpolar compounds was formed. Reaction mixture was quenched with aq $NaHCO_3$, evaporated to smaller volume, poured into water (20 mL) and extracted with *n*-BuOH. The organic extract was evaporated to dryness and the crude residue was purified through HPLC. (Semipreparative RP-18 column, 10 μ, 16× 250 mm, λ = 220 and 270 nm, flow rate = 8 mL/min., isocratic system, 70% CH_3CN/H_2O). Lyophilization of the appropriate fractions gave **6** (0.01 g, 8.2%). Partial ^1H NMR: δ 7.38 (d, 1H, J = 1.47 Hz, ArH), 7.18 (d, 2H, J = 8.50 Hz, D_7-H & $D_{7'}$-H), 7.10 (dd, 1H, J = 8.55 Hz and 1.47 Hz, ArH), 6.9 (d, 1H, J = 8.55 Hz, ArH), 6.78 (d, 1H, J = 8.50, D_8-H & $D_{8'}$-H), 5.1 (s, 1H, B_7), 3.4–3.6 (2×s, 6H, 2×$ArOCH_3$). IR (KBr): ν_{max} 3300–3400, 2915, 1650, 1615, 1520, 1445, 1240, 1080 cm^{-1}. ESI MS (ES+): for $C_{56}H_{85}N_7O_{17}$; Calculated: 1128.317; Found: $(M+Na)^+ = 1150.6$. UV (MeOH) λ_{max} (ε M^{-1} cm^{-1}): 206 nm (22924).