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Synthesis and Antifungal Activities of Novel 1,3-β-D-Glucan Synthase Inhibitors. Part 1

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Abstract—Highly potent 1,3-β-D-glucan synthase inhibitors **10**, **11** and **13** have been identified by the chemical modification of the fungicidal macrocyclic lipopeptidolactone, RO-09-3655 (**1**), isolated from the cultured broth of *Deuteromycotinia* spp. D-Ornithine derivative (**10**) showed improved antifungal activity in the systemic candidiasis model in mice and reduced hepatotoxicity in vitro, as compared with **1**.

Introduction

There is an intensified medical need for new fungicidal agents with novel modes of action for the treatment of systemic fungal infections due to the rapid growth of the immunocompromised patient population and the development of resistance to the present azole therapies. 1,3-β-D-Glucan is one of the essential components in the cell wall of pathogenic fungi such as *Candida albicans*. Several 1,3-β-D-glucan synthase inhibitors that belong to the echinocandin family, such as MK991,[1] FK463[2] and LY303366,[3] are currently under clinical trials. We isolated novel fungicidal macrocyclic lipopeptidolactones **2** and **3** (Fig. 1) from the cultured broth of *Deuteromycotinia* spp. as potent 1,3-β-D-glucan synthase inhibitors[4] together with a known inhibitor FR901469[5] (**1** = RO-09-3655).

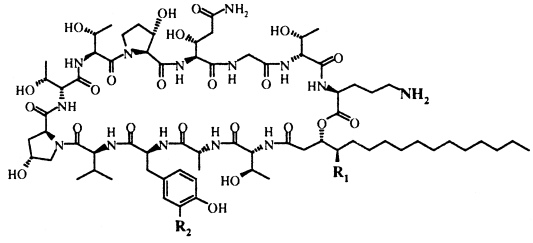
A chemical modification study to improve the hemolytic activity of **1** has been reported by Fujisawa researchers.[6]

In the biological evaluation of **1**, **2** and **3**, we independently found that they exhibited potent antifungal activities in the murine systemic candidiasis model, but **1** also showed hepatotoxicity after multiple dosing (30 mg/kg, iv, q.d.×14) in mice.[7]

Therefore, we conducted chemical modification studies[8] of **1** to improve the in vivo antifungal activity and reduce the hepatotoxicity, thereby identifying a β-1,3-D-glucan synthase inhibitor with a better therapeutic window. In this paper, we report our chemical modification of **1**, the preliminary SAR of the new derivatives, and the identification of **10** which has a better therapeutic window than **1**.

Chemistry

The preliminary SAR was examined by the chemo-selective modification of tyrosine, ornithine and β-hydroxyglutamine residues of **1**. The preparation of the



FR90146 (1 = RO-09-3655) : $R_1 = R_2 = H$

RO-09-3656 (2) : $R_1 = CH_3$, $R_2 = H$

RO-09-4279 (3) : $R_1 = H$, $R_2 = OH$

Figure 1. Structures of maclocyclic lipopeptidolactones.

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derivatives of **1** is summarized in Scheme 1. Reductive dimethylation of the ornithine moiety of **1** with formaldehyde afforded **5**. The hydroxy derivative (**6**) was prepared by treatment of **1** with 4-chlorobenzenediazonium hexafluorophosphate[9] in the presence of sodium carbonate in DMF followed by treatment with water. The *N*-acetyl analogue (**7**) was prepared by acetylation of **1** with acetic anhydride and pyridine in MeOH.

Acylation of **1** with *N*-Boc-amino acids was carried out by BOP and HOBt, followed by deprotection to give the corresponding amino acid conjugates (**8**, **9** and **10**). Reductive *N*-alkylation of the ornithine moiety of **1** with *N*-Boc-aminoacetaldehyde followed by removal of the Boc group with TFA gave **11**. Compound **12** was prepared from **4** by selective dehydration with Burgess reagent,[10] followed by deprotection. Compound **13** was prepared by the reduction of the primary amide of **4** with $BH_3{\cdot}Me_2S$ in THF followed by deprotection. The ethers **14** and **15** were prepared by *O*-alkylation of the phenolic hydroxyl group of **4** with an alkyl halide in the presence of potassium carbonate, followed by deprotection.

Biology

The 1,3-β-D-glucan synthase inhibitory activity of the new derivatives of **1** was measured with a purified enzyme[11] from *C. albicans* IFO1060. In vitro antifungal assay against *C. albicans* was performed by the NCCLS M27-A microdilution method[12] using modified media: Yeast Nitrogen Base (YNB) supplemented with 1% dextrose and 0.25% K_2HPO_4.

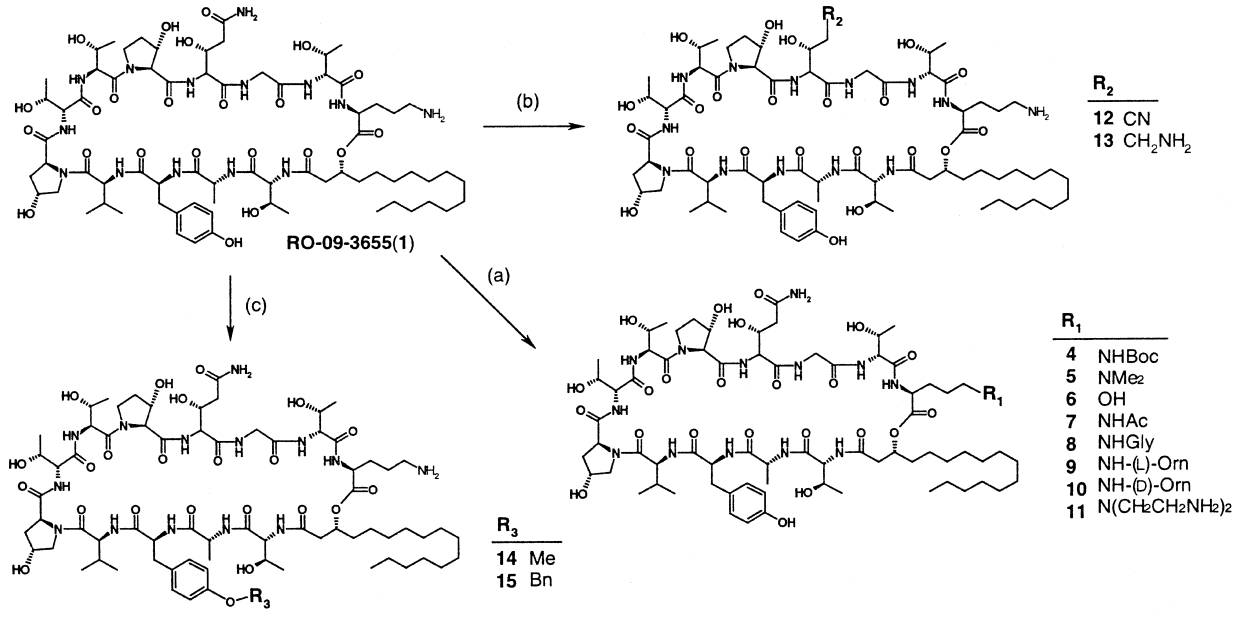
IC_{50} values were calculated as the concentration of drug yielding 50% OD (=50% inhibition) of the control growth. Antifungal activity with serum was determined in the presence of 80% calf serum, 2% dextrose, 10 μM $FeCl_3$, and 10 μM deferoxamin at 35 °C under 10% CO_2 atmosphere.

The in vivo anti-*Candida* activity was determined in a mouse model of systemic candidiasis. ICR mice ($n=5$) were infected intravenously with a lethal dose of *C. albicans* (CY1002) and treated intravenously with a single dose of the test compound. Efficacies of the compounds were calculated as the dose (mg/kg) effective for 50% survival (ED_{50}) on day 7.

To assess the hepatotoxicity, the in vitro inclusion–body formation assay[13] was performed using isolated mouse hepatocytes. ICR mouse hepatocytes were isolated by perfusion with a collagenase solution and incubated (monolayer culture) with the test compounds at 37 °C for 24 h. The cultured hepatocytes were fixed and stained with acid haematain. The inclusion-body formations were analyzed by an image analyzer and the lowest concentrations of the drugs that caused inclusion-body formations were evaluated.

Results and Discussion

The inhibitory activity (IC_{50}) of 1,3-β-D-glucan synthase (GS) of the derivatives of **1** is summarized in Table 1. Replacement of the primary amino group of **1** with a hydroxy group (**6**) or *N*-acetylation (**7**) resulted in a

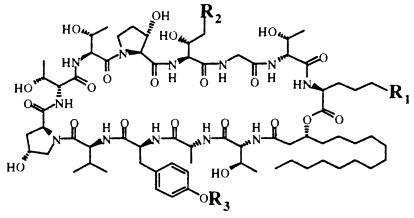


Scheme 1. Reagents: (a) synthesis of **4**: Boc_2O, Et_3N, MeOH, rt (93%); synthesis of **5**: HCHO, $NaBH_3CN$, AcOH, MeOH, rt (73%); synthesis of **6**: (i) $4\text{-}ClC_6H_4N_2{\cdot}PF_6$, Na_2CO_3, DMF, rt; (ii) H_2O, rt (22%, 2 steps); synthesis of **7**: Ac_2O, pyridine, MeOH, rt (53%); synthesis of **8**: (i) Boc-Gly-OH, BOP, HOBT, DIPEA, DMF, rt; (ii) TFA, 0 °C (46%, 2 steps); synthesis of **9**: (i) Boc-L-Orn(Boc)-OH, BOP, HOBT, DIPEA, DMF, rt; (ii) TFA, 0 °C (43%, 2 steps); synthesis of **10**: (i) Boc-D-Orn(Boc)-OH, BOP, HOBT, DIPEA, DMF, rt; (ii) TFA, 0 °C (54%, 2 steps); synthesis of **11**: (i) *N*-$BocNHCH_2CHO$, $NaBH_3CN$, AcOH, MeOH, rt; (ii) TFA, 0 °C (62%, 2 steps); (b) synthesis of **12** (starting from **4**): (i) Burgess reagent, CH_3CN, rt; (ii) TFA, 0 °C (14%, 2 steps); synthesis of **13** (starting from **4**); (i) $BH_3{\cdot}Me_2S$, THF, −10 °C; (ii) TFA, 0 °C (15%, 2 steps); (c) synthesis of **14** (starting from **4**): (i) MeI, K_2CO_3, DMF; (ii) TFA, 0 °C (69%, 2 steps); synthesis of **15** (starting from **4**): (i) BnBr, K_2CO_3, DMF; (ii) TFA, 0 °C (81%, 2 steps).

significant loss of activity, suggesting the importance of the presence of a basic amino group for this enzyme inhibitory activity.[14] Interestingly, when a glycine moiety (**8**) was introduced at the amino group of the ornithine residue of **1**, the enzyme inhibitory activity recovered, compared with the acetyl derivative (**7**), suggesting that the position of the basic amino group in the molecule is not so critical.

Thus, we introduced various basic amino acids (e.g., L- or D-ornithine, L- or D-lysine, etc.) and aminoalkyl group(s) to the ornithine residue of **1**. As a consequence, the enzyme inhibitory activities of D-ornithine derivative (**10**) and *N,N*-bis-(2-aminoethyl) derivative (**11**) were improved 2.4 and 6 times , respectively, as compared with that of **1**. Compound **13**, in which the carbamoyl group (R_2) of **1** was reduced to a primary amino group, also showed improved enzyme inhibitory activity.

Table 1. 1,3-β-D-Glucan synthase inhibitory activity of RO-09-3655 (**1**) derivatives



Compound	R_1	R_2	R_3	Enzyme inhibition GS IC_{50} (nM)
5	NMe_2	$CONH_2$	H	45
6	OH	$CONH_2$	H	>520
7	NHAc	$CONH_2$	H	230
8	NHGly	$CONH_2$	H	20
9	NH-(L)-Orn	$CONH_2$	H	8
10	NH-(D)-Orn	$CONH_2$	H	5
11	$N(CH_2CH_2NH_2)_2$	$CONH_2$	H	2
12	NH_2	CN	H	19
13	NH_2	CH_2NH_2	H	6
14	NH_2	$CONH_2$	Me	24
15	NH_2	$CONH_2$	Bn	>130
1	NH_2	$CONH_2$	H	12
LY303366	—	—	—	26

O-Methylation (**14**) of the phenolic hydroxy group of **1** still retained enzyme inhibitory activity, suggesting that this phenolic hydroxy group is not essential. However, the introduction of the more bulky benzyl group (**15**) resulted in significant loss of activity. Thus, the *N,N*-bis-(2-aminoethyl) derivative (**11**) was the most active among the derivatives we synthesized ($IC_{50}=2$ nM), and it was 13 times more active than the echinocandin analogue LY303366.

The in vitro and in vivo antifungal activities of **6**, **9**, **10**, **11** and **13** against *Candida albicans* CY 1002 are shown in Table 2. The in vitro antifungal activities were measured in the presence or absence of 80% calf serum. The hydroxy derivative (**6**) lacking a basic amino group showed a strong serum effect; i.e., the antifungal activity in the presence of serum was significantly reduced. As a consequence, its in vivo efficacy was very weak. A strong serum effect was also observed in LY303366, and its in vivo efficacy was not as strong as that expected from the in vitro antifungal activity without serum. In contrast, the serum effects of the derivatives having two or three basic amino groups (**9**, **10**, **11** and **13**) were weaker than that of **1**. Especially, **10**, **11** and **13** exhibited highly potent in vivo antifungal activity against the systemic candidiasis model after a single iv administration. The ED_{50} values of **11** and **13** were about 5 times lower than those of both LY303366 and **1**. These data suggest that the in vitro antifungal activity measured without serum does not directly translate into in vivo efficacy; but rather, the in vitro activity in the presence of serum appears to be more predictive for in vivo antifungal activity. We examined the hepatotoxicity of compound (**1**) derivatives, by measuring the minimal concentration of the compounds that formed inclusion-bodies in hepatocytes, since the preliminary toxicokinetic study of **1** suggested a correlation between the in vivo hepatotoxicity and the in vitro inclusion-body formation in the hepatocytes. The inclusion-body forming activities of **9**, **10**, **11** and **13** are shown in Table 2. In this in vitro assay, inclusion-body formation was observed at 1 μg/mL in LY303366, and at 10 μg/mL in the lead compound **1**. Among the derivatives tested, D-ornithine derivative (**10**) showed 3 times weaker hepatotoxicity, whereas the hepatotoxicity of L-ornithine derivative (**9**) remained as strong as that of **1**, for

Table 2. In vitro and in vivo anti-fungal activity against *Candida albicans* CY1002 and hepatotoxicity of RO-09-3655 (**1**) derivatives

Compound	In vitro anti-*Candida* activity (IC_{50}: μg/mL)[a]		In vivo efficacy[b] (ED_{50}: mg/kg, day 7)	Hepatotoxicity
	C. albicans CY10021	*C. albicans* CY1002 (with 80% serum)	Systemic candidiasis	Inclusion-body formation observed at (μg/mL)
6	0.04	16	>0.55	—
9	0.08	0.47	0.2	10
10	0.12	0.33	0.1	30
11	0.1	0.42	0.05	10
13	0.01	0.18	0.04	10
1	0.02	0.57	0.22	10
LY303366	0.004	0.58	0.22	1

[a]NCCLS M27-A microdilution method using modified media: Yeast Nitrogen Base (YNB) supplemented with 1% dextrose and 0.25% K_2HPO_4.
[b]Single dose iv treatment.

unknown reasons. These data suggest that more detailed modification studies of the ornithine moieties of **1**, **10** and **11**, with respect to the presence of the number of basic amino functional groups and the stereochemistry of a newly introduced amino acid, would lead to the identification of a compound with even better in vivo antifungal activity and to the further reduction of hepatotoxicity, namely a compound with the sufficient therapeutic index for a clinical candidate. Modification studies of this nature are currently in progress.

In conclusion, we identified highly potent 1,3-β-D-glucan synthase inhibitors (**10**, **11** and **13**) by the chemical modification of the fungicidal macrocyclic lipopeptidolactone, RO-09-3655 (**1**), isolated from the cultured broth of *Deuteromycotinia* spp. These compounds showed stronger in vivo antifungal activity than **1** in the systemic candidiasis model in mice. Among them, **10** additionally showed a 3-fold reduction of hepatotoxicity.

References and Notes

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12. Antifungal susceptibility assays were performed by the broth dilution method according to M27-A guidelines recommended by the National Committee for Clinical Laboratory Standards (NCCLS).
13. Details will be reported elsewhere.
14. Compound **6** showed in vitro antifungal activity despite lacking 1,3-β-D-glucan synthase inhibition, suggesting that it may have an alternative mechanism for antifungal activity.