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Synthesis and Biological Activity of Novel Macrocyclic Antifungals: Modification of the Tyrosine Moiety of the Lipopeptidolactone FR901469

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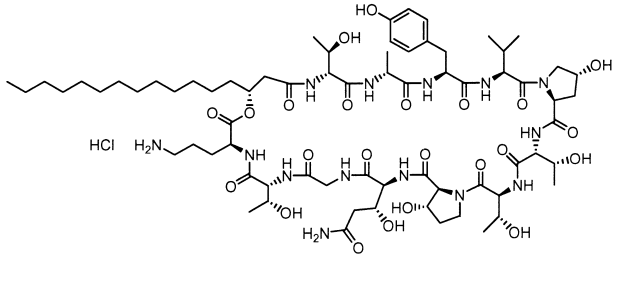
Abstract—A series of tyrosine-modified derivatives of the macrocyclic lipopeptidolactone FR901469 have been prepared and evaluated for in vitro and in vivo antifungal activity and for hemolytic activity towards red blood cells. Compound **14** displayed significantly reduced hemolytic potential at 1 mg/mL and a comparable protective effect to FR901469 in a mouse candidiasis model.

Introduction

As a part of our efforts to discover novel water-soluble antifungal agents suitable for parenteral administration, we recently described the isolation and antifungal activity of the novel macrocyclic lipopeptidolactone FR901469 (**1**) (Fig. 1).[1,2] This compound belongs to a new family of natural product fungal 1,3-β-glucan synthase inhibitors,[3] traditionally represented by the echinocandins and papulacandins, but more recently expanded by the discovery of arbocandins[4] and enfumafungin-type steroid derivatives.[5] Scientists at Nippon Roche recently described the discovery of aerothricins, a series of lipopeptides closely related to FR901469.[6,7] The validity of 1,3-β-glucan synthase as a clinically significant antifungal target has recently been clearly demonstrated and three compounds (caspofungin, micafungin, anidulafungin) are in various late-stage clinical trials.[3] Indeed, caspofungin very recently received FDA approval as a therapy for refractory invasive aspergillosis.

As described earlier,[1,2] FR901469 possesses potent antifungal activity, but also displays a tendency to cause lysis of red blood cells (100% at a concentration of 1 mg/mL). Reduction of this hemolysis was considered critical in order to improve the overall profile of this natural product, since work on echinocandin B suggested a relationship between hemolysis and toxicity.[8,9] We have already reported some of our efforts on the site-specific chemical modification of FR901469. In particular, we have described selective direct modification of the ornithine residue,[10] replacement of the lactone by an amide group,[11] and complete replacement of ornithine by a novel substituted glutamic acid residue.[12] In the course of these studies, it became apparent that the hemolysis associated with the natural product could be reduced significantly by chemical modification whilst maintaining the potent in vitro and, especially, in vivo antifungal activity. Examination of the structure of **1** reveals a number of amino acid residues that are



FR901469 (1)

Figure 1.

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potentially amenable to site-specific chemical modification. A particularly attractive candidate for selective and specific chemical modification is the tyrosine moiety, owing to the presence of the phenol moiety, leading to activation towards electrophilic aromatic substitution reactions. Furthermore, the relatively high acidity associated with the tyrosine hydroxyl group renders it amenable to selective alkylation reactions with a variety of alkylating reagents.

Earlier work in the pneumocandin series of compounds revealed the critical importance of the homotyrosine phenolic hydroxyl group for 1,3-β-glucan synthase inhibitory activity.[13,14] In particular, removal of this hydroxyl group by reduction or blocking as a phosphate ester completely abolished enzyme activity. In the work described in this paper, we aimed to examine whether the presence of the tyrosine phenol group in FR901469 is required for antifungal activity and to investigate whether tyrosine-specific modification can lead to novel compounds with an improved hemolytic profile compared to **1**. We herein report the synthesis and evaluation of a series of tyrosine-modified derivatives of FR901469.

Chemistry

The novel tyrosine-modified derivatives prepared in this work were obtained by the methods summarized in Schemes 1 and 2. Chlorination at the *ortho*-position adjacent to the tyrosine hydroxyl group was achieved with sodium hypochlorite in aqueous acetic acid (46%). Nitro derivative **3** was obtained by direct nitration with sodium nitrite in aqueous acetic acid (75%).[14] Reduction of **3** with sodium borohydride in the presence of 10% palladium on carbon in MeOH–AcOH–H_2O afforded amine **4** (39%). Conversion of acetate **5** to the β-alanine derivative **7** was achieved by nitration, reduction under hydrogenolysis conditions, acylation with β-alanine and deprotection of the *tert*-BOC group. Related analogues **9** and **15**–**20** were prepared via the common intermediate **8**, readily prepared from **1** via *tert*-butoxycarbonyl-protected derivative **6**. Nitration (78%) and hydrogenation (77%) of **6** afforded **8** in good yield. Acylation of **8** with various acids and acid derivatives afforded the respective amide derivatives in moderate yield (Table 1). These acylation reactions proceeded in modest yields due to formation of varying amounts of the corresponding bis-acylated species resulting from acylation of the phenol hydroxyl group in addition to the amino group. The desired mono-acylated intermediates could be readily obtained by reverse phase ODS column chromatography. Deprotection of protecting groups by stirring in neat TFA at room temperature afforded the final antifungal agents as amorphous solids.

The tyrosine hydroxyl-modified analogues **10**–**13** were obtained as shown in Scheme 2. Compound **10** was prepared in two steps from **6** by *O*-alkylation with *tert*-butyl bromoacetate in the presence of K_2CO_3 (84%), followed by deprotection with TFA (100%). Reprotection of the ornithine amino group as BOC, coupling of the carboxylic acid moiety with 1-*tert*-butoxycarbonylethylenediamine and removal of the BOC group afforded **12**. Compounds **11** and **13** were prepared from *Z*-protected FR901469 (**21**) by alkylation, *tert*-butyl ester deprotection, amidation and finally by hydrogenation to remove the carbobenzyloxy protecting group, as outlined in Scheme 2. Compound **14** was prepared by alkylation of nitrophenol **22** (an intermediate in the preparation of **8**), reduction, acylation and finally by single step removal of all protecting groups.

All reactions and purifications were monitored by reverse phase HPLC. Purifications were performed by reverse phase ODS column chromatography and products obtained by pooling of the appropriate fractions, adjusting to pH 3 with 1 N HCl, passage through a short column of Amberlyst A-26 (chloride form) and freeze-drying. All compounds were characterized by ^{1}H NMR, FAB-MS, IR and elemental analysis. Purity was assessed by HPLC.

Biological Methods

In vitro antifungal activity

The minimum inhibitory concentration (MIC) values shown in Table 2 were determined by the agar dilution method using Sabouraud Dextrose agar, as described in our earlier paper.[10] MIC was read as the lowest concentration required to inhibit visible growth of the organism. MICs in Table 3 were determined by the broth microdilution method, according to NCCLS M27-A guidelines.[15]

In vivo antifungal activity

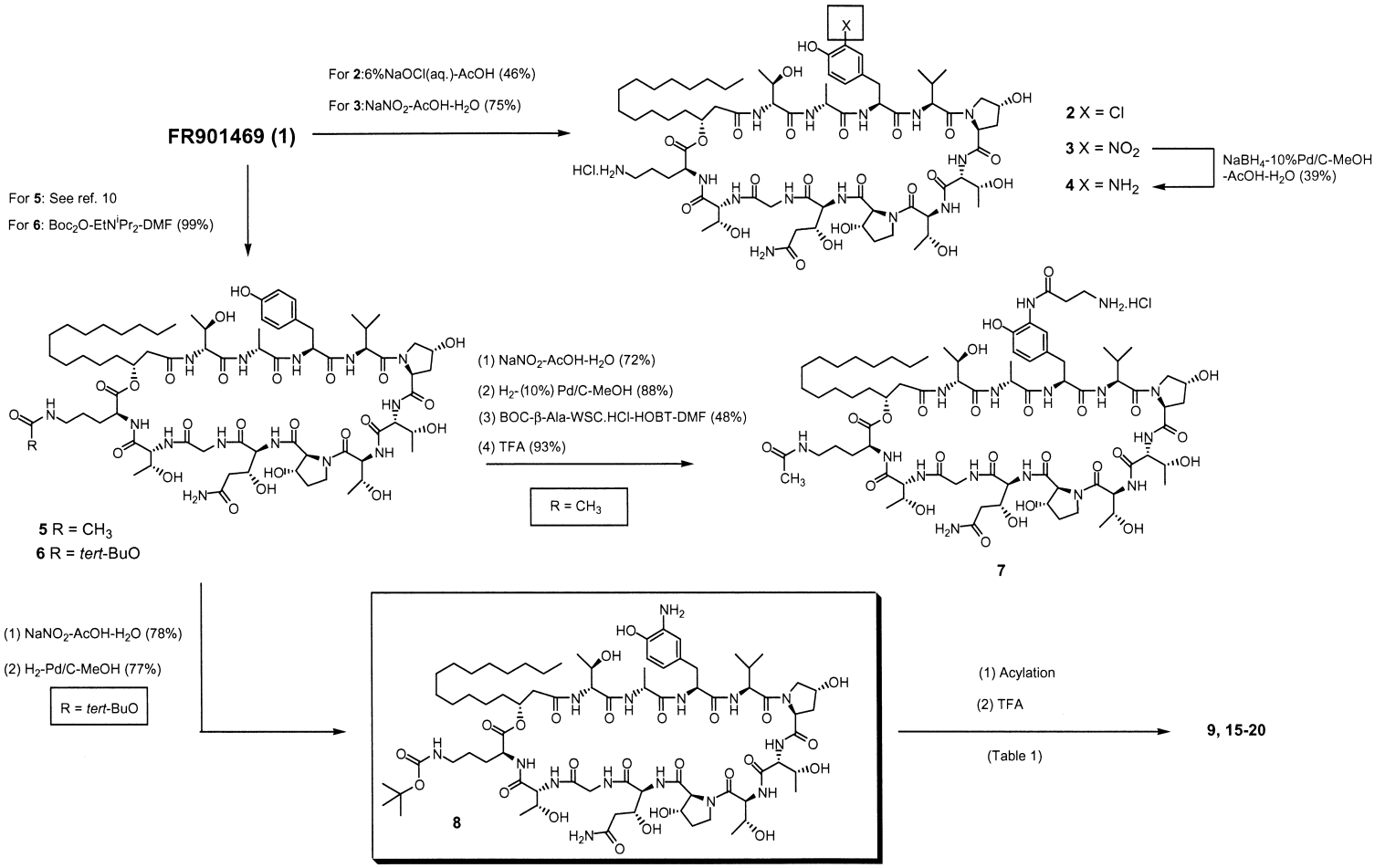
Disseminated candidiasis was induced in ICR mice by intravenous inoculation of 0.2 mL of a *Candida albicans* FP633 cell suspension via the lateral tail vein. A single dose of each compound was administered subcutaneously 60 min after challenge. ED_{50} was estimated on the basis of survival at day 14 after challenge.

Hemolytic activity

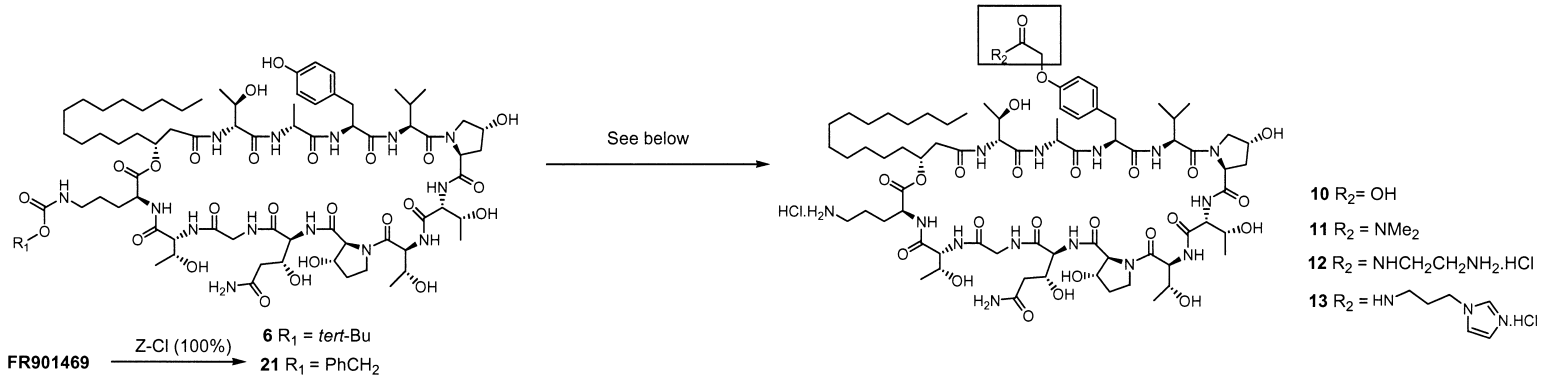
A microtiter red blood cell (RBC) hemolysis assay was used to determine the potential of compound to hemolyze ICR mouse RBCs at 1 mg/mL, as described earlier.[10]

Results and Discussion

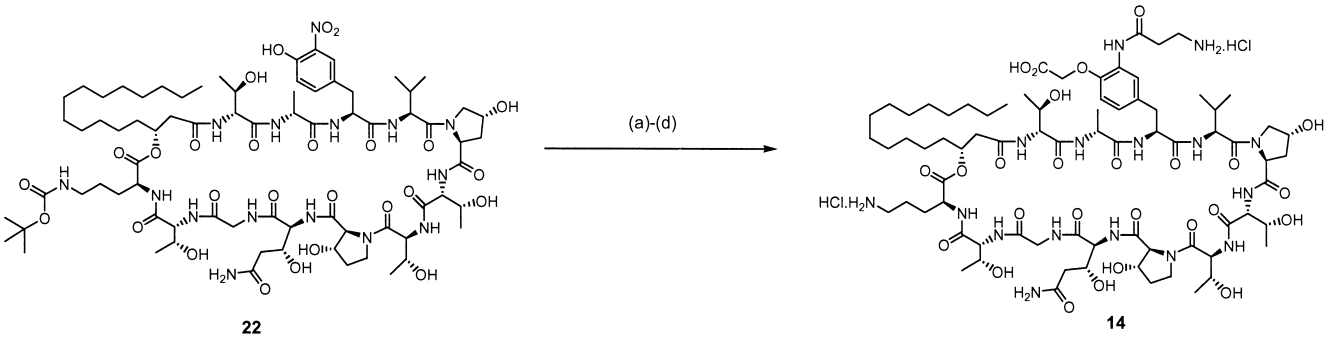
Introduction of a chloro or nitro group adjacent to the tyrosine phenol group of **1** resulted in no significant change in antifungal activity (**2** and **3**) (Tables 2 and 4). In particular, nitro derivative **3** displayed comparable in vivo efficacy in the candidiasis model. This result suggests that the tyrosine phenol in **1** and the homotyrosine phenol in the echinocandins may not have the same role in the inhibition mechanism of glucan synthase. This follows from results reported by Merck researchers, who established that introduction of nitro to pneumocandins



Scheme 1. Synthesis of tyrosine-modified FR901469 derivatives (1).



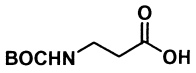
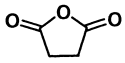
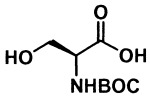
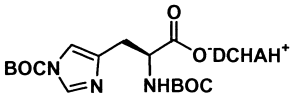
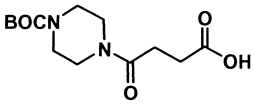
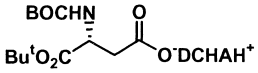
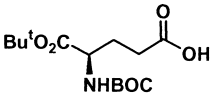
Reagents and conditions: Synthesis of **10** (from **6**): (a) $BrCH_2CO_2{}^tBu$-K_2CO_3-DMF-rt(84%), (b) TFA-rt(100%). Synthesis of **12** (from **10**): (a) Boc_2O-EtN^iPr_2-DMF-rt(61%), (b) $BOCNH(CH_2)_2NH_2$-HOBT-WSCD.HCl -DMF-rt(67%), (c) TFA-rt(100%). Synthesis of **11** and **13** (from **21**): (a) $BrCH_2CO_2{}^tBu$-K_2CO_3-DMF-rt(84%), (b) TFA-rt(56%). (c) Me_2NH.HCl-EtN^iPr_2-HOBT-WSCD.HCl-DMF-rt(74%) (for **11**); (c) 1-(3-aminopropyl)imidazole-HOBT-WSCD.HCl-DMF-rt(82%) (for **13**) then in both cases (d) H_2-10%Pd/C-MeOH(91%, **11**; 30% **13**).



Reagents and conditions: (a) $BrCH_2CO_2{}^tBu$-K_2CO_3-DMF-rt(87%), (b) H_2-10%Pd/C-MeOH(85%), (c) BOC-β-Ala-HOBT-WSCD.HCl-DMF-rt(36%), (d) TFA-rt (57%).

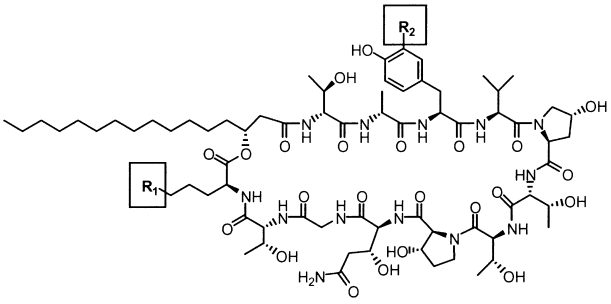
Scheme 2. Synthesis of tyrosine-modified FR901469 derivatives (2).

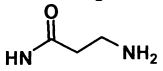
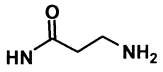
Table 1. Acylation and deprotection reactions of amine **8**

Final compound	Reagent	Method[a]	Acylation yield (%)	Deprotection yield (%)
9		A	31	81
15		B	—	20[b]
16		A	34	66
17		C	36	97
18		A	37	100
19		C	29	33
20		A	60	55

[a]A: Acid–HOBT–WSCD.HCl–DMF–rt; B: succinic anhydride–DMAP(cat)–DMF–rt; C: DCHA salt–EtN^iPr_2–HOBT–WSCD.HCl–DMF–rt (DCHA, dicyclohexylamine).
[b]Overall yield from **8**.

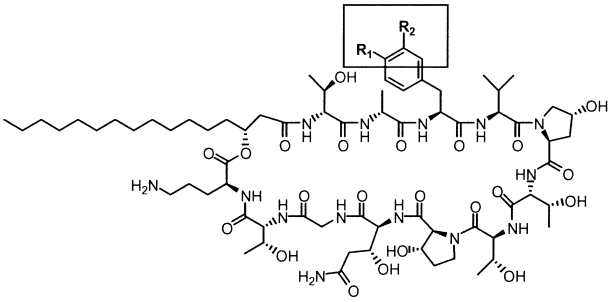
Table 2. In vitro antifungal and hemolytic activity of tyrosine-modified FR901469 derivatives (1)

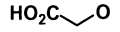
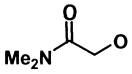
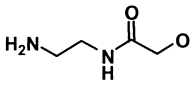
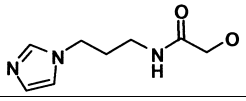
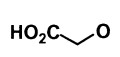
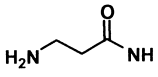
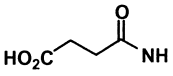
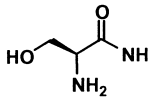
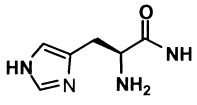
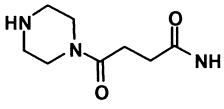
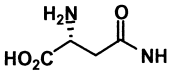
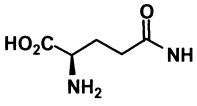


Compound[a]	R_1	R_2	MIC (μg/mL)						Hemolytic activity (%)
			C. a.[b]		*C. g.*	*C. t.*		*C. p.*	
			FP633	FP579	FP587	8001	8002	15001	
FR901469	NH_2	H	0.39	0.39	0.39	0.39	0.39	0.39	100
2	NH_2	Cl	0.39	0.78	0.78	0.78	0.78	0.78	97
3	NH_2	NO_2	0.39	0.78	0.78	0.78	0.78	0.78	100
4	NH_2	NH_2	0.78	1.56	0.78	1.56	0.78	1.56	100
9	NH_2		0.78	1.56	0.78	1.56	1.56	0.78	49
7	$NHCOCH_3$		1.56	1.56	1.56	1.56	1.56	1.56	23
5[10]	$NHCOCH_3$	H	0.39	0.78	0.78	1.56	1.56	1.56	5

[a]All compounds are hydrochloride salts, except **5**.
[b]*C. a.*, *Candida albicans*; *C. g.*, *Candida guilliermondi*; *C. t.*, *Candida tropicalis*; *C. p.*, *Candida parapsilosis*.

Table 3. In vitro antifungal and hemolytic activity of tyrosine-modified FR901469 derivatives (2)



Compound[a]	R_1	R_2	MIC (μg/mL)				Hemolytic activity (%)
			C. a.[b]		*A. f.*		
			FP633	13004	FP1305	8004	
FR901469	OH	H	0.25	0.25	0.25	0.5	100
10		H	0.5	0.5	0.125	1	76
11		H	0.5	1	0.25	1	85
12		H	1	1	0.5	1	45
13		H	1	2	1	2	53
14			0.5	0.5	0.125	0.5	23
15	OH		0.5	1	0.25	0.5	79
16	OH		1	2	0.5	2	84
17	OH		2	2	2	2	65
18	OH		1	1	0.5	1	46
19	OH		0.5	1	0.25	0.5	75
20	OH		0.5	0.5	0.5	1	60

[a]All compounds are hydrochloride salts.
[b]*C. a.*, *Candida albicans*; *A. f.*, *Aspergillus fumigatus*.

Table 4. In vivo antifungal activity: efficacy in disseminated murine candidiasis

Compound	ED_{50} (mg/kg)
FR901469	0.44–0.88[a]
3	0.45 (0.5)[b]
14	0.72 (1.5)
18	0.97 (2)
19	0.8 (1.6)
20	1.02 (2.1)
Amphotericin B	0.132
Fluconazole	>20

[a]Range for FR901469 over a number of experiments.
[b]Ratio of ED_{50}(drug)/ED_{50}(FR901469) for the same experiment.

led to a significant reduction in glucan synthase inhibition and MFC.[14] Amino derivative **4** had slightly weaker in vitro antifungal activity. However, these modifications had no effect at all on hemolysis at 1 mg/mL, indicating the need for further modification. Increasing polarity by introduction of a β-alanine residue (**9**) reduced hemolysis significantly (49% vs 100% at 1 mg/mL). Interestingly, compound **7** with the ornithine group blocked as acetamide and a β-alanyl residue on the tyrosine, had even lower hemolysis (23%), although not as low as acetamide **5**.[10]

A number of other acylated derivatives, replacing the β-alanine by various polar residues, were prepared and are summarized in Table 3. It is clear from the data presented for these derivatives (**15**–**20**) that addition of amino groups and/or carboxylic acid groups results in a better hemolytic profile, but antifungal activity is also reduced. Furthermore, the in vivo efficacies of compounds **18**, **19**, and **20** were approximately half that of FR901469, indicating no advantage over **1**.

Introduction of substituents to the tyrosine phenol group by alkylation was readily achieved. The carboxylic acid derivative **10** displayed strong in vitro antifungal activity, but did not have a significant effect on hemolysis (76% at 1 mg/mL). Amino derivative **12** had the lowest hemolytic activity amongst these simple derivatives, however MIC was considered too weak to warrant further consideration. The optimum result was obtained with compound **14**. Introduction of a carboxymethyl group to the phenol OH and a β-alanylamide to the *ortho*-position resulted in a compound with the lowest hemolysis (23%) and good in vivo activity in the candidiasis model.

In summary, a series of tyrosine-modified analogues of the unique macrocyclic lactone FR901469 has been prepared. Several derivatives with good in vivo antifungal efficacy and reduced hemolytic potential were identified. Compound **14** in particular displayed good in vivo efficacy in a candidiasis model and significantly reduced hemolysis at 1 mg/mL. The results described herein support the notion that the tyrosine phenol OH in **1** and the homotyrosine phenol OH in the echinocandins may not have the same role in the inhibition mechanism of 1,3-β-glucan synthase, since removal or blocking of this OH group in the echinocandins removes enzyme activity. An alternative explanation for the potent activity of tyrosine-modified analogues might be that FR901469 can also express antifungal activity by another mechanism not involving glucan synthesis, for example, membrane destabilization. With regards this possibility, researchers at Nippon Roche have reported that the tyrosine *O*-methyl derivative of **1** possessed good glucan synthase inhibitory activity.[7] Further studies on the glucan synthase inhibitory potency and membrane effects of these derivatives should help to clarify this point in more detail.

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