was calculated as [(systolic - diastolic/3) + diastolic]. Zero points were determined prior to administration of peptides by injection of saline vehicle.

Histamine Assay. Histamine was assayed using a sensitive [^{125}I]histamine radio immunoassay (AMAC Inc., Westbrook, ME). The histamine determination makes use of the binding competition between the acylated test sample or histamine standard (0–5 ng/mL) and ^{125}I-acylated histamine to a mouse antihistamine mAb-coated test tube. An aliquot of 100 μL of standard (0.5 ng/mL histamine) or sample was acylated by adding the histamine standard or sample to a test tube containing 1 mg of a lyophilized acylating reagent and 50 μL of acylating buffer (borate buffer, pH 8.2). The acylating reagent was completely solubilized by vortexing each tube for 15–20 s. One mL of ^{125}I-acylated histamine (0.073 μCi/mL) was added to each 150 μL of acylated histamine standard or test sample and vortexed. Each histamine standard or sample was assayed in duplicate by adding 500-μL aliquots from the reaction mixture to the antihistamine mouse mAb-coated tubes and incubated at 4 °C for 18 h. After the 18-h incubation, all of the fluid was aspirated from each tube, and the empty tubes were counted in a gamma counter (Searle, Model 1185). Results are expressed as ng/mL histamine and extrapolated from the histamine standard curve. The assay has a sensitivity of 0.1 nM histamine, and intraassay coefficient of variation of 8.0 ± 0.4% and an interassay coefficient of variation of 9.9 ± 0.9%. The assay is also specific, as cross reactions with histidine or *N*-methylhistamine are very low ($<10^{-4}$).

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Preparation and Structure–Activity Relationships of Simplified Analogues of the Antifungal Agent Cilofungin: A Total Synthesis Approach

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The echinocandins are a well-known class of lipopeptides characterized by their potent antifungal activity against *Candida* species. The mechanism of action of the echinocandins is generally thought to be the inhibition of β-1,3-glucan synthesis, an important structural component in the cell wall of *Candida* species. Extensive structure–activity studies on the fatty acid side chain of echinocandin B (1) led to the preparation of the clinical candidate cilofungin (4). However, little is known about the cyclic peptide. We now report the preparation, by solid-phase synthesis, of a series of simplified analogs of cilofungin in which the unusual amino acids found in the echinocandins were replaced with more readily accessible natural amino acids. The solid-phase approach to the total synthesis of these analogs allowed us to conveniently explore structural modifications that could not be accomplished by chemical modification of the natural product. The simplest analog 5 showed no biological activity. Structural complexity was then returned to the system in a systematic fashion so as to reapproach the original cilofungin structure. Antifungal activity and the inhibition of β-1,3-glucan synthesis were monitored at each step of the process, thereby revealing the basic structure–activity relationships of the amino acids and the minimal structural requirements for biological activity in the echinocandin ring system. The results suggests that the 3-hydroxy-4-methylproline residue enhances activity but the L-homotyrosine residue is crucial for both antifungal activity and the inhibition of β-1,3-glucan synthesis.

Introduction

The echinocandins are a well known class of cyclic lipopeptides characterized by their potent antifungal activity against *Candida* species.[1] In addition, they are known inhibitors of β-1,3-glucan synthesis,[2] a common structural unit found in the cell walls of these fungi.[3] Extensive structure–activity studies of the fatty acid side chain of the echinocandin B ring system by other workers showed that the fatty side chain was essential for antifungal activity and ultimately led to the preparation of the clinical candidate cilofungin (4).[4] However, little is known about

(1) (a) Benz, F.; Knusel, F.; Nuesch, J.; Treichler, H.; Voser, W.; Nyfeler; Keller-Schierlein. Metabolic Products of Microorganisms. Echinocandin B, a Novel Polypeptide Antibiotic from *Aspergillus nidulans* var. *echinulatus*: Isolation and Structure. *Helv. Chim. Acta* **1974**, *57*, 2459–2477. (b) Keller-Juslen, C.; Kuhn, M.; Loosli, H-R.; Petcher, T. J.; Weber, H. P.; von Wartburg. Structure of the Cyclopeptide-Antibiotics SL 7810 (Echinocandin B). *Tetrahedron Lett.* **1976**, *46*, 4147–4150. (c) Iwata, K.; Yamamoto, Y.; Yamaguchi, H.; Hiratani, T. In Vitro studies of Aculeacin A, A New Antifungal Antibiotic. *J. Antibiot.* **1982**, *35*, 203–209. (d) Roy, K.; Mukhopadhyay, T.; Reddy, G. C. S.; Desikan, K. R.; Ganguli, B. N. Mulundocandin, a New Lipopeptide Antibiotic. I Taxonomy, Fermentation, Isolation and Characterization. *J. Antibiot.* **1987**, *40*, 275–280. (e) Tkacz, J. S. In *Emerging Targets In Antibacterial and Antifungal Therapy*; Sutcliffe, J., Georgopapadakou, N. H., Eds.; Chapman and Hall: New York, 1992; pp 495–523.

(2) (a) Sawistowska-Schroder, E. T.; Kerridge, D.; Perry, H. Echinocandin Inhibition of 1,3-β-D-Glucan synthase from *Candida albicans*. *FEBS Lett.* **1984**, *173*, 134–138. (b) Mason, R. E. The Mode of Action of Echinoncandin. *Bull. Br. Mycol. Soc.* **1977**, *11*, 144–152. (c) Miyata, M.; Kanabe, T.; Tanaka, K. Lysis of Growing Fission (Sic)-Yeast Cells Induced by Aculeacin A. A new Antifungal Antibiotic. *Arch. Microbiol.* **1980**, *127*, 11–16. (d) Gordee, R. S.; Zeckner, D. J.; Ellis, L. F.; Thakkar, A. L.; Howard, L. C. *In Vitro* and *In Vivo* Anti-Candida Activity and Toxicology of LY121019. *J. Antibiot.* **1984**, *37*, 1054–1065.

(3) Cassone, A. Cell Wall of Pathogenic Yeasts and Implications for Antimycotic Therapy. *Drugs Exp. Clin. Res.* **1986**, *12*, 635–643.

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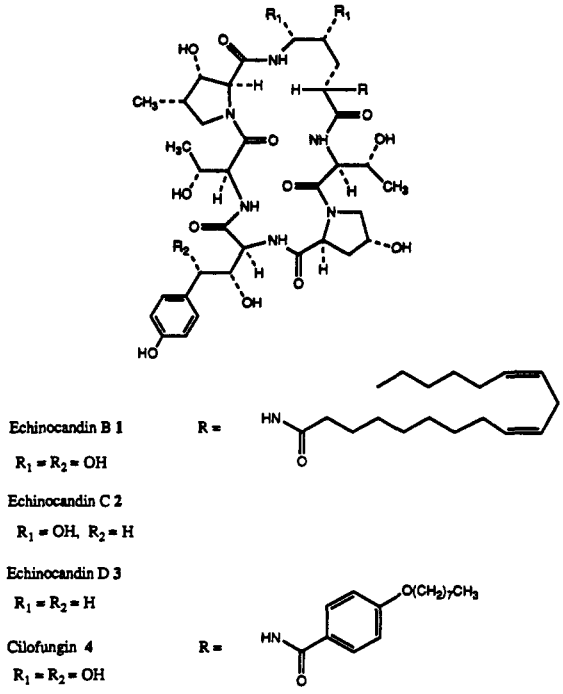


Figure 1. Structure of echinocandins B, C, D and cilofungin.

the structure–activity relationships of the peptide portion of these systems. Although the structures of echinocandins B (1), C (2), and D (3) are progressively less hydroxylated, they were reported to possess similar anti-*Candida* activity.[5] This finding, along with the recent reports of the total synthesis of echinocandin D,[6] suggested that simplified structures that retained biological activity might conveniently be prepared by solid-phase synthesis and that it might be possible to use these materials to investigate the structure–activity of the echinocandin peptide chain (Figure 1). We envisioned the solid-phase approach to the total synthesis of these materials as a method that could allow us to rapidly explore structural modifications that could not be accomplished by direct chemical modification of the natural product.

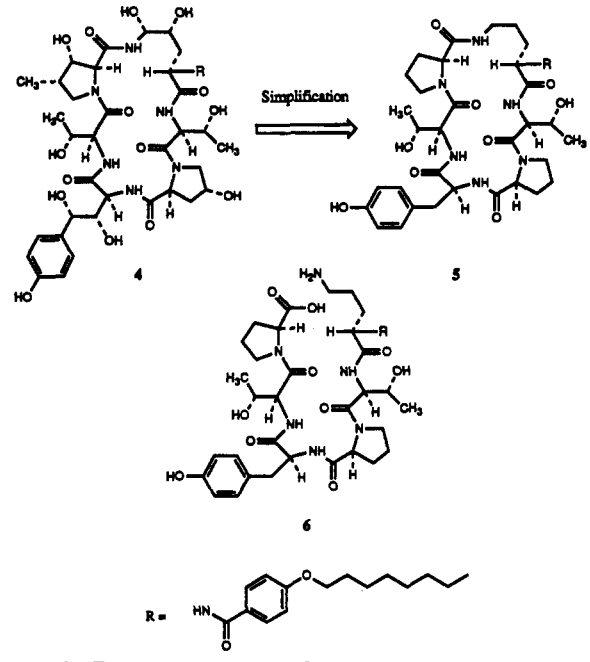


Figure 2. Base ring system and precursor.

Since the unusual amino acids found in 1–4 seemed likely to be structural features important to the biological activity of these compounds, our investigation was focused on them. We started replacing these amino acids with their more common counterparts while keeping the (octyloxy)benzoyl side chain of 4 intact. Thus, L-proline was substituted for (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline, L-tyrosine for 3,4-dihydroxy-L-homotyrosine, and L-ornithine for 3,4-dihydroxy-L-ornithine. In addition, L-proline was substituted for 4-hydroxy-L-proline. These changes led to the simplified hexapeptide 5. This structure was proposed as the initial target of our study, the base ring system (Figure 2).

The cyclization reaction reported in the total synthesis of echinocandin D also suggested that a linear hexapeptide containing an L-proline at the C-terminus and an α-*N*-[(octyloxy)benzoyl]-L-ornithine at the N terminus could serve as an excellent precursor of the simplified ring system we sought to investigate. We therefore proceeded with the preparation of 6. The synthesis was performed on a solid support using an automated peptide synthesizer.[7] After cleavage from the solid support, solution-phase cyclization of the carboxyl of the L-proline to the δ-amino of the L-ornithine gave the desired product 5. The compound was evaluated for antifungal activity against a panel of *Candida* species and for β-1,3-glucan synthesis inhibition and was found to be inactive. A combination of solid- and solution-phase syntheses was then used to prepare similar linear hexapeptides that reincorporated the unusual amino acids of the echinocandins back into their natural positions. This was followed by cyclization to give the corresponding ring systems. In this manner, structural detail was returned to the system in a controlled fashion so as to approach the structure of cilofungin. By monitoring antifungal activity against *Candida* and β-1,3-glucan synthesis inhibition at each step of the process, we hoped to pinpoint structural features important to the biology of these systems. The present work describes the synthesis of a series of these simplified analogs of cilofungin and their asso-

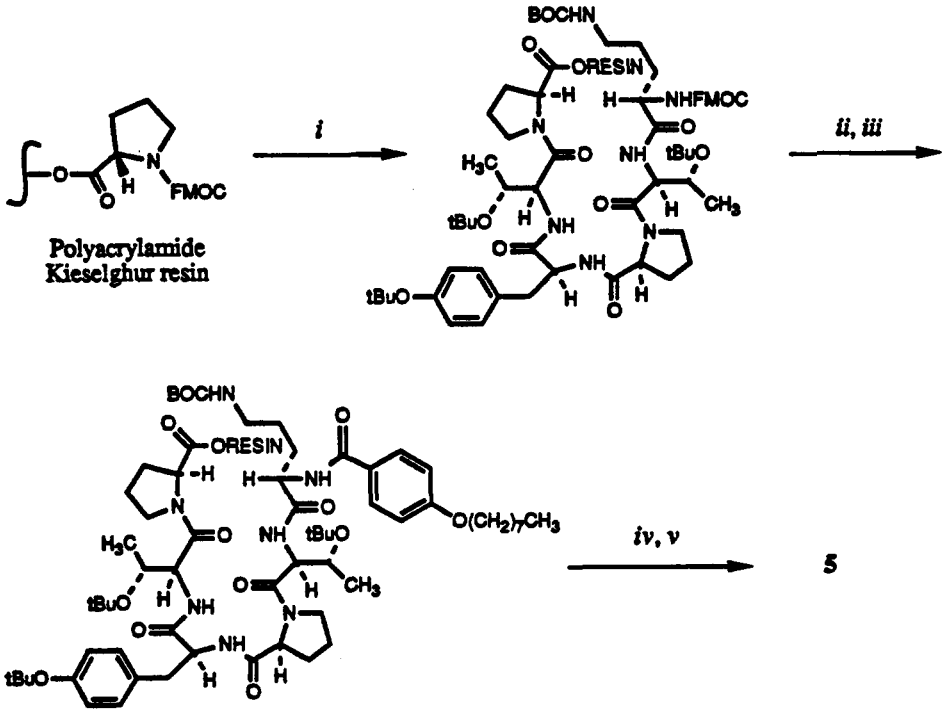
(4) (a) Debono, M.; Abbott, B. J.; Turner, J. R.; Howard, L. C.; Gordee, R. S.; Hunt, A. S.; Barnhart, M.; Molloy, R. M.; Willard, K. E.; Fukuda, D.; Butler, T. F.; Zeckner, D. J. Synthesis and Evaluation of LY121019, a Member of a Series of Semisynthetic Analogs of the Antifungal Lipopeptide Echinocandin B. *Ann. N.Y. Acad. Sci.* **1988**, *544*, 152–167. (b) Taft, C. S.; Selitrennikoff, C. P. Cilofungin Inhibition of (1-3)-β-Glucan Synthase: The Lipophilic Side Chain Is Essential for Inhibition of Enzyme Activity. *J. Antibiot.* **1990**, *43*, 433–437.

(5) (a) Traber, R.; Keller-Juslen, C.; Loosli, H.-R.; Kuhn, M.; von Wartburg, A. Cyclopeptid-Antibiotika aus *Aspergillus*-Arten. Struktur der Echinocandine C and D. *Helv. Chim. Acta* **1979**, *62*, 1252–1267. (b) Mizuno, K.; Yagi, A.; Satoi, S.; Takada, M.; Hayashi, M.; Asano, K.; Matsuda, T. Studies on Aculeacin. I. Isolation and Characterization of Aculeacin A. *J. Antibiot.* **1977**, *30*, 297–302. (c) Satoi, S.; Yagi, A.; Asano, K.; Mizuno, K.; Watanabe, T. Studies on Aculeacin. II. Isolation and Characterization of Aculeacins B, D, E, F and G. *J. Antibiot.* **1977**, *30*, 303–307.

(6) (a) Ohfune, Y.; Kurokawa, N. Total Synthesis of Echinocandins. 1. Stereocontrolled Synthesis of the constituent Amino Acids. *J. Am. Chem. Soc.* **1986**, *108*, 6041–6043. (b) Ohfune, Y.; Kurokawa, N. Total Synthesis of Echinocandins. 2. Total Synthesis of Echinocandin D via Efficient Peptide Coupling Reactions. *J. Am. Chem. Soc.* **1986**, *108*, 6043–6045. (c) Evans, D. A.; Weber, A. E. Synthesis of the Cyclic Hexapeptide Echinocandin D. New Approaches to the Asymmetric Synthesis of β-Hydroxy α-Amino Acids. *J. Am. Chem. Soc.* **1987**, *109*, 7151–7157.

(7) For a recent review of solid-phase synthesis, see: Barany, G.; Kneib-Cordonier, N.; Mullen, D. G. Solid Phase Synthesis: A Silver Anniversary Report. *Int. J. Peptide Prot. Res.* **1987**, *30*, 705–739 and references cited therein.

Scheme I[a]



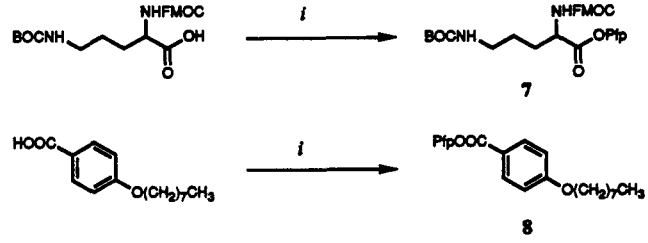
[a] (i) FMOC solid phase synthesis on a Milligen 9050 automated peptide synthesizer, (ii) 20% piperidine in methylene chloride on line in the synthesizer; (iii) *p*-(octyloxy)benzoic acid pentafluorophenyl ester coupling on line in the synthesizer; (iv) TFA, ethanedithiol, thioanisole; (v) DPPA, $NaHCO_3$, DMF.

ciated structure–activity data.

Chemistry

The linear hexapeptide precursor **6** was synthesized via α-*N*-fluorenylmethoxycarbonyl (Fmoc) protected amino acids activated as their pentafluorophenyl (–OPfp) or dihydrooxobenzotriazine (–ODhbt) esters. The standard protocols of the Milligen 9050 automated peptide synthesizer were employed on a commercially available polyamide–kieselguhr solid phase support with a C-terminal α-*N*-Fmoc-L-proline bonded to the resin (Scheme I). The C-terminal amino acid attached to the solid-phase support ultimately becomes the residue corresponding to the (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline in **4**. Commercially available α-*N*-Fmoc-L-proline-OPfp, α-*N*-Fmoc-L-threonine(Bu^t)-ODhbt and α-*N*-Fmoc-L-tyrosine (Bu^t)-OPfp were used as supplied. The OPfp activated ester of α-*N*-Fmoc-δ-*N*-Boc-L-ornithine (**7**) was prepared by DCC coupling of pentafluorophenol to α-*N*-Fmoc-δ-*N*-Boc-L-ornithine (Scheme II). The last amino acid unit in this and every other sequence in this study is the L-ornithine residue. This amino acid was orthogonally protected at the δ-amino group with the base-stable *tert*-butyloxycarbonyl (Boc) group. This allowed us to remove the α-*N*-Fmoc protecting group with 20% piperidine in methylene chloride under the standard synthesizer conditions while keeping the δ-amino group protected. The selective acylation of the α-amino group was performed directly in the synthesizer with pentafluorophenyl *p*-(octyloxy)-benzoate (**8**), prepared from *p*-(octyloxy)benzoic acid[8] by DCC coupling of pentafluorophenol (Scheme II). The

Scheme II[a]



[a] (i) DCC, pentafluorophenol, ethyl acetate.

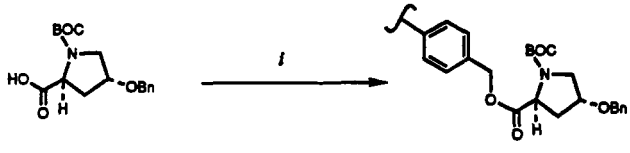
peptide chain was released from the resin with a TFA, ethanedithiol, thioanisole cleavage mixture. This also removed all remaining protecting groups, leaving the δ-amino group of the L-ornithine free for cyclization to the carboxyl of the C-terminal L-proline. The crude peptide was purified by reverse-phase HPLC. Final cyclization was accomplished with diphenyl phosphorazidate (DPPA) and solid sodium bicarbonate in DMF.[9] These cyclization conditions were found to be very general and were used throughout this work.

The remainder of the analogs may be separated into two categories: those that contain the (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline residue and those that do not. Accordingly, the syntheses can also be classified in a similar fashion. The preparation of the linear precursors **9a–g** of analogs that do not contain a (2*S*,3*S*,4*S*)-3-hydroxy-4-

(8) Debono, M.; Abbott, B. J.; Fukuda, D. S.; Barnhart, M.; Willard, K. E.; Molloy, R. M.; Michel, K. H.; Turner, J. R.; Butler, T. F.; Hunt, A. H. Synthesis of New Analogs of Echinocandin B by Enzymatic Deacylation and Chemical Reacylation of the Echinocandin Peptide: Synthesis of the Antifungal Agent Cilofungin (LY121019). *J. Antibiot.* **1989**, *42*, 389–397.

(9) For examples of the use of DPPA in the cyclization of peptides, see: (a) Brady, S. F.; Varga, S. L.; Freidinger, R. M.; Schwenk, D. A.; Mendlowski, M.; Holly, F. W.; Veber, D. F. Practical Synthesis of Cyclic Peptides, with an Example of Dependence of Cyclization Yield Upon Linear Sequence. *J. Org. Chem.* **1979**, *44*, 3101–3105. (b) Hamada, Y.; Shibata, M.; Shioiri, T. New Methods and Reagents in Organic Synthesis. 55. Total Synthesis of Patellamides B and C, Cytotoxic Cyclic Peptides From a Tunicate. 1. Their Proposed Structures Should Be Corrected. *Tetrahedron Lett.* **1985**, *26*, 5155–5158.

Scheme III[a]



11

[a] (i) Cs_2CO_3/DMF, chloromethyl Merrifield resin.

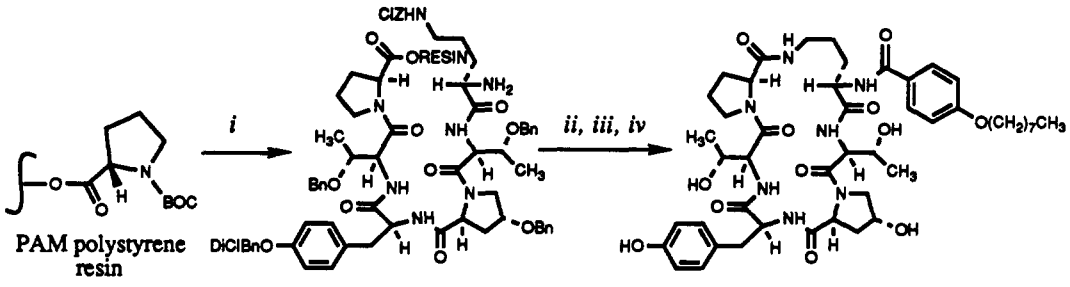
methylproline was performed by solid-phase methodology using Boc-protected amino acids and carbodiimide-based coupling chemistry. The commercial availability of α-*N*-Boc-*O*-benzyl-4-hydroxy-L-proline attracted us to the use of Boc-based chemistry as the analogous protected amino acid required for the Fmoc-based synthesis, α-*N*-Fmoc-L-4-hydroxy-L-proline(Bu^t)-OPfp, was not available. These syntheses start with commercially available phenylacetamidomethyl (Pam) or Merrifield polystyrene resins for solid-phase synthesis with the α-*N*-Boc-protected C-terminal L-amino acid already attached to the support. In the case of analogs containing a C-terminal 4-hydroxy-L-proline, the required resin **11** was prepared from commercially available chloromethyl Merrifield polystyrene resin and the cesium salt of *O*-Bn-α-*N*-Boc-4-hydroxy-L-proline, by a procedure similar to that reported by Gisin[10] (Scheme III). The desired peptide chain was then prepared by solid-phase synthesis using *t*-Boc chemistry on an ABI or Biosearch automated peptide synthesizer as described below. Deprotection of the Boc group was accomplished with TFA and coupling was performed with DCC/1-hydroxybenzotriazole (HOBt) or diisopropylcarbodiimide/HOBt activation. α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl (Cl-Z))-L-Orn was used as the protected form of ornithine to allow for the selective acylation of the α-amino group with the *p*-(octyloxy)benzoyl group. The δ-Cl-Z group is stable to TFA and is removed when the fully assembled linear lipohexapeptide is cleaved from the solid support by treatment with anhydrous HF at 0 °C in anisole.[11] This process removes all other protecting groups not removed by the TFA during the regular deprotection steps, leaving the δ-amino group of the L-ornithine free for cyclization to the carboxyl of the C-terminal L-amino acid. The coupling chemistry in this scheme also allowed us to use *p*-(octyloxy)benzoic acid and α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn without requiring Pfp esters as in the Fmoc example above.

An ABI model 430A automated peptide synthesizer was used to prepare **9a**. The standard chemical protocols of the synthesizer were used (Scheme IV). The resin was deprotected with TFA in dichloromethane (DCM) followed by neutralization with *N*,*N*-diisopropylethylamine (DIEA). The α-amino deprotected resin was then washed with DMF in preparation for the peptide bond formation with the incoming active ester. HOBt activated esters were prepared online in a separate reaction vessel by treatment of the protected amino acids with DCC and HOBt in methylene chloride and used to perform the coupling steps of the chemistry. After another washing step with DCM, the cycle was repeated. When the synthesis was completed, the desired peptide was cleaved from the resin with HF as described above. All other linear peptides in this series, **9b–g**, were prepared on a Biosearch SAM 2 automated peptide synthesizer. The standard program of the synthesizer was used to perform all the chemistry. All of these analogs contained a proline or a substituted proline in the C-terminal position with the exception of **9g** which contains a C-terminal threonine (Scheme V). Deprotection of the intermediate Boc-protected peptide chain was accomplished with 50% TFA in DCM with 2% anisole as scavenger. After deprotection the free amino group was released from its TFA salt by a base-wash cycle with 20% diisopropylethylamine in DCM followed by a neutral wash to remove any residual base. The coupling reagent employed was diisopropylcarbodiimide and HOBt in *N*-methylpyrrolidone (NMP). Final cleavage was performed with HF as described above. In all cases, cyclization was accomplished with DPPA as in the previous example to afford cyclic peptides **10b–g**. The synthesis of analogs containing a homotyrosine residue required the preparation of α-*N*-Boc-*O*-(2,6-dichlorobenzyl)-L-homotyrosine (**12d**), which was synthesized from (*S*)-*N*-(methoxycarbonyl)-*O*-methyl-L-homotyrosine (**12a**)[12] by a procedure similar to that reported by Yamashiro[13] (Scheme VI).

The peptides containing the (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline residue were prepared in a different fashion. Linear pentapeptides, **13a** and **15a**, with C-terminal L-threonine residues and containing a homotyrosine or tyrosine respectively, were prepared and acylated with the *p*-(octyloxy)benzoic acid side chain on a Biosearch automated peptide synthesizer via the method described above. Protection of **13a** with CBZ chloride and NaOH in 20% aqueous *tert*-butyl alcohol afforded a clean crude product that was a 3:1 mixture of mono **13b**:bis **13c** CBZ adducts (Scheme VII). This mixture was directly coupled to (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline methyl ester[6c] with [(diethylamino)propyl]ethylcarbodiimide and HOBt in DMF to afford a mixture of the coupled mono- (**13d**) and bis- (**13e**) CBZ protected peptides which was directly saponified to afford the desired linear hexapeptide **13f**. Catalytic hydrogenation of the crude product over 10% palladium on carbon gave the fully deprotected linear hexapeptide **13g** ready for cyclization. Final cyclization with DPPA and purification by HPLC were performed in the usual manner to give the desired **14**. The tyrosine-containing pentapeptide **15a** was protected with CBZ chloride as above, but the mono- (**15b**) and bis- (**15c**) CBZ adducts were isolated by HPLC, and only **15b** was carried through the sequence. Coupling to (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline methyl ester with [(diethylamino)propyl]ethylcarbodiimide and HOBt in DMF afforded the mono-CBZ methyl ester (**15d**). Saponification to the acid **15e** and hydrogenolysis of the CBZ group gave the desired linear lipohexapeptide **15f** ready for cyclization without any intermediate purification steps. Cyclization

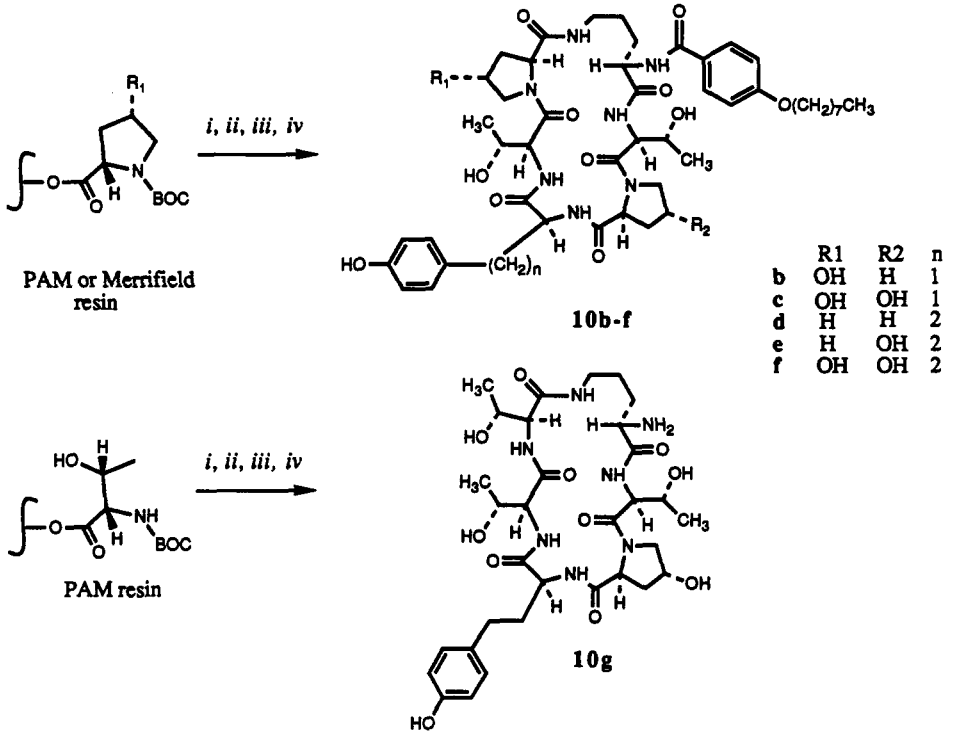
(10) Gisin, B. F. The Preparation of Merrifield-Resins Through Total Esterification With Cesium Salts. *Helv. Chim. Acta* **1973**, *56*, 1476–1482.
(11) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Co.: Rockford, IL, 1984; pp 85–90.
(12) Melillo, D. G.; Larsen, R. D.; Mathre, D. J.; Shukis, W. F.; Wood, W. W.; Colleluori, J. R. Practical Enantioselective Synthesis of a Homotyrosine Derivative and (R,R)-4-Propyl-9-hydroxynaphthoxazine, a Potent Dopamine Agonist. *J. Org. Chem.* **1987**, *52*, 5143–5150.
(13) Yamashiro, D.; Li, C. H. Adrenocorticotropins. 44. Total Synthesis of the Human Hormone by the Solid-Phase Method. *J. Am. Chem. Soc.* **1973**, *95*, 1310–1315.
(14) Schmatz, D. M.; Romancheck, M. A.; Pittarelli, L. A.; Schwartz, R. E.; Fromtling, R. A.; Nollstadt, K. H.; Vanmiddlesworth, F. L.; Wilson, K. E.; Turner, M. J. Treatment of *Pneumocystis carinii* pneumonia with 1,3-β-glucan synthesis inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5950–5954.
(15) Taft, C. S.; Stark, T.; Selitrenniaoff, C. T. Cilofungin (LY121019) Inhibits *Candida Albicans* β-1,3-Glucan Synthase. Antimicrobial Agents and Chemotherapy. **1988**, *32*, 1901–1903.

Scheme IV[a]



[a] (i) BOC solid phase synthesis on an ABI 430A automated peptide synthesizer; (ii) *p*-(octyloxy)benzoic acid, DCC/HOBT on line in the synthesizer, (iii) HF, anisole, 0 °C; (iv) DPPA, $NaHCO_3$, DMF.

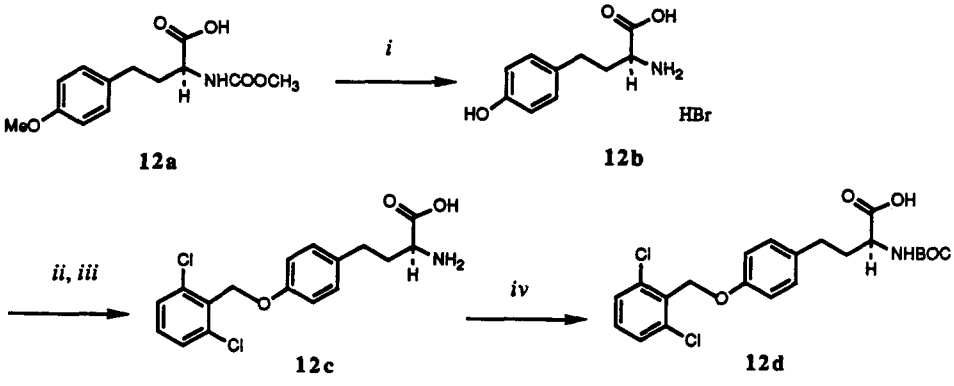
Scheme V[a]



	R1	R2	n
b	OH	H	1
c	OH	OH	1
d	H	H	2
e	H	OH	2
f	OH	OH	2

[a] (i) BOC solid phase synthesis on an Biosearch SAM II automated peptide synthesizer; (ii) *p*-(octyloxy)benzoic acid, diisopropylcarbodiimide/HOBT/NMP on line in the synthesizer, (iii) HF, anisole, 0 °C; (iv) DPPA, $NaHCO_3$, DMF.

Scheme VI[a]



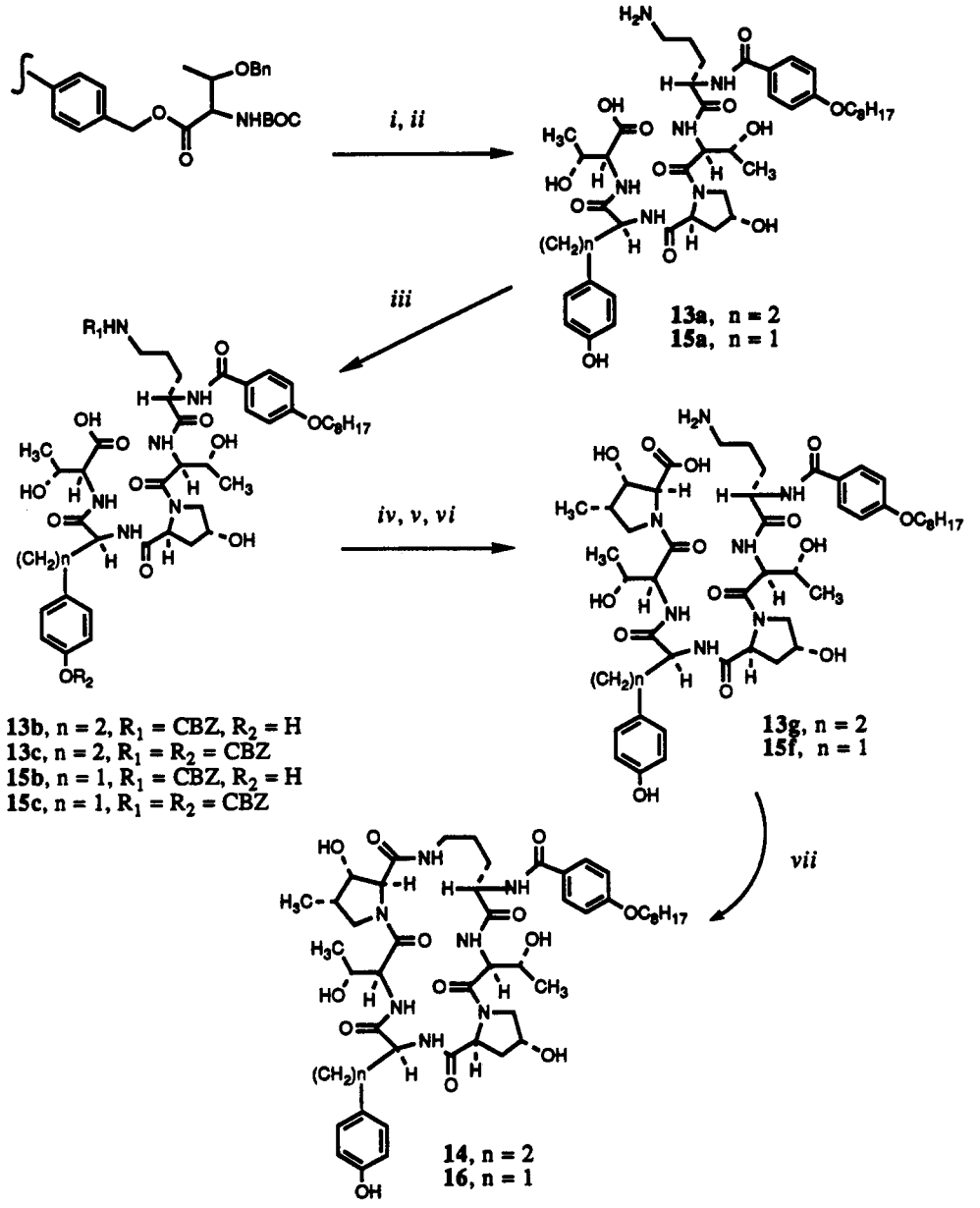
[a] (i) HBr/HOAc; (ii) NaOH, dioxane, $Cu_2SO_4 \cdot 5H_2O$, 2,6-dichlorobenzyl bromide; (iii) EDTA disodium salt; (iv) NaOH, di-*tert*-butyl pyrocarbonate, HCl.

and purification to give **16** were performed in the usual manner.

Results and Discussion

The simplified analog **5** showed no *Candida* fungicidal activity (Table I) or β-1,3-glucan synthesis inhibition (Table II). Incorporation of a 4-hydroxy-L-proline (i.e. **10a**) at the same position as found in cilofungin restored the first structural feature of the echinocandin B ring system. In addition, the analogs with 4-hydroxy-L-proline and L-proline in reversed positions, **10b**, and 4-hydroxy-L-proline substituted in both positions, **10c**, were also

Scheme VII[a]



[a] (i) BOC chemistry on an automated synthesizer; (ii) HF, anisole, 0 °C; (iii) CBZCl, NaOH, aqueous *tert*-butyl alcohol; (iv) (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline methyl ester hydrochloride, [(dimethylamino)propyl]ethylcarbodiimide hydrochloride, HOBT, TEA, DMF; (v) NaOH, MeOH; (vi) H_2, Pd/C; (vii) DPPA, $NaHCO_3$, DMF.

Table I. Minimum Fungicidal Concentrations (MFC)

	MFC (μg/mL)				
	C. albicans			*C. tropicalis*	*C. parapsilosis*
compd	MY1055	MY1208	MY1028	MY1012	MY1010
5	>64	>64	>64	>64	NT
10a	NT	>128	>128	>128	>128
10b	>128	>128	>128	>128	>128
10c	>128	>128	>128	>128	>128
16	>128	>128	>128	>128	>128
10d	4	4	NT	4	>128
10e	8	4	4	0.25	8
10f	8	8	8	8	>128
10g	8	8	8	4	>128
14	1	2	2	1	8
cilofungin	0.5	2	0.5	0.5	8

synthesized. None of these analogs showed antifungal or β-1,3-glucan synthesis inhibition activity. Recalling that the hydroxyl substituents of the ornithine residue of cilofungin are not necessary for antifungal activity as evidenced by the good antifungal activity reported for echinocandin D (Figure 1) suggested that the unusual amino acids (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline and 3,4-dihydroxy-L-homotyrosine were in fact independently or

Table II. Inhibition of *Candida* β-1,3-Glucan Synthesis

compd	*Candida* glucan synthesis IC_{50} (μM)	compd	*Candida* glucan synthesis IC_{50} (μM)
5	>10[a]	**10e**	10
10a	>10[a]	**10f**	10
10b	>10[a]	**10g**	>10[a]
10c	>10[a]	**14**	3
16	NT	cilofungin	1
10d	>10[a]		

[a] IC_{50} < 5% inhibition at 10 μM.

collectively critical to the biological activity of echinocandin-type structures.

In order to assess the contribution of the 3,4-dihydroxy-L-homotyrosine, L-homotyrosine was incorporated as a replacement for L-tyrosine in **5**, **10a**, and **10c** to give homologs **10d**, **10e**, and **10f**, respectively. All of the analogs in this series showed substantial antifungal activity against Candida while the two analogs containing a 4-hydroxyproline **10e** and **10f** also inhibited β-1,3-glucan synthesis. This experiment showed L-homotyrosine to be an essential structural determinant of biological activity.

We also sought to assess the relative importance of the (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline. Therefore our next step was the preparation of (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline and its incorporation into both the L-tyrosine and L-homotyrosine based ring systems. Both of these analogs contain 4-hydroxy-L-proline where it is found in naturally occurring echinocandins. The (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline-L-homotyrosine combination **14**, a tetrareduced version of **4**, showed better antifungal and β-1,3-glucan synthesis inhibition than any of the previous analogs tested, displaying much of the in vitro activity of **4**. Remarkably, the (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline-L-tyrosine combination **16** was completely inactive. Thus although the (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline enhanced the potency of the system and perhaps improved its spectrum with respect to *Candida parapsilosis*, it is not as essential as the L-homotyrosine residue. Indeed, it was also found that L-threonine could also be used to replace the (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline in the L-homotyrosine based ring system to produce an analog, **10g**, that still retained antifungal activity.

Conclusion

We have reported here on the solid-phase synthesis and biological activity of a series of simplified analogs of cilofungin designed to study the structure–activity relationships of the peptide portion of the system. The results of this investigation suggest that the L-homotyrosine residue found in echinocandin-type ring systems is a crucial structural element for good *Candida* antifungal activity and β-1,3-glucan synthesis inhibition. The presence of the (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline enhances the potency and improves spectrum with respect to *Candida parapsilosis*, but it is not as critical a structural determinant as the L-homotyrosine. Inspection of molecular models shows that the phenol ring of the homotyrosine and tyrosine analogs occupy completely different locations. This finding combined with the earlier work on the fatty acid side chain[17] suggests that the position of homotyrosine ring with respect to the fatty acid side chain may be the determining factor for antifungal activity.

Experimental Section

General. All reagents and solvents were analytical reagent grade and were used without further purification unless otherwise noted. The protected amino acids α-*N*-Boc-*O*-benzyl-L-Thr, α-*N*-Boc-4-benzyloxy-L-Pro, α-*N*-Boc-*O*-(2,6-dichlorobenzyl)-L-Tyr, α-*N*-Boc-L-Pro, α-*N*-Fmoc-δ-*N*-Boc-L-Orn, and substituted resins α-*N*-Boc-L-Pro Pam, α-*N*-Boc-*O*-benzyl-L-Thr Pam, and α-*N*-Boc-*O*-benzyl-L-Thr Merrifield resins were purchased from Bachem and used as supplied unless otherwise noted. α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn was obtained from Penninsula. All α-*N*-Fmoc-OPfp activated amino acids were obtained from Milligen in preweighed vials for use in the 9050 synthesizer unless otherwise indicated. ^1H nuclear magnetic resonance (NMR) spectra were obtained on a Varian XL 300 instrument as solutions in deuteriomethanol unless otherwise noted. Melting points were obtained on a Thomas-Hoover melting point apparatus and are uncorrected. Flash chromatography was performed essentially as described in the literature[16] using Kieselgel 60 (EM Science, 230–400 mesh) as stationary phase. Preparative HPLC was performed on a Waters Delta-Prep 3000 system with a Waters Model 490 detector and a Rheodyne 7125 injector with a 10.0-mL injection loop. Analytical HPLC was performed on a Waters Model 600E HPLC outfitted with a Rheodyne 7125 injector and Waters Model 990 photodiode array detector. All analytical HPLC work was performed on a 4.9 mm × 25 cm Zorbax C8 (7 μm) reverse-phase column under isocratic conditions at ambient temperature and monitored at a wavelength of 210 nm unless otherwise noted. Analytical thin-layer chromatography was performed using silica gel GHLF plates of 0.25-mm thickness obtained from Analtech.

Biology. *Candida* MFC Assay. (a) Media. The media used in this assay consists of Yeast Nitrogen base (Difco) with 1% dextrose (YNBD), YM broth (Difco), and Sabouraud dextrose agar (Difco).

(b) Antibiotic Preparation. Test compounds and controls were prepared as stock solutions of 512 μg/mL in either sterile distilled water or 10% DMSO, depending on the solubility characteristics of the compounds. A minimum of 4 mg/compound is required for the assay. After solubilization each compound was diluted 2-fold in YNBD as described below. The control drug used was cilofungin. Using the Cetus Pro/Pette or Labsystems Multidrop, 75 μL of YNBD was dispensed into each well of a sterile Costar 96-well, U-bottomed microtiter plate. Manually, 75 μL of stock compounds was delivered to each well in column 1. Using the Cetus Pro/Pette, compounds in column 1 were serially diluted 2-fold from 256 μg/mL to 0.125 μg/mL. Any alterations in the concentration range were noted.

(c) Microorganisms. Stock cultures of *Candida albicans* (MY 1028, 1055 and 1750), *Candida tropicalis* (MY 1012), and *Candida parapsilosis* MY 1010 were maintained at room temperature in distilled water. For use in this assay, yeast cultures were streaked on Sabouraud dextrose agar (SDA) and incubated for 24–48 h at 35–37 °C for verification of purity and viability. From each culture, three to five characteristic colonies were transferred to a fresh plate and incubated as described above for use in preparation of the inoculum.

(d) Preparation of Inocula. Using a sterile disposable loop, three to five colonies of each yeast culture were suspended in 10 mL of YM broth and incubated for 4 h at 35–37 °C on a shaker set at 225 rpm. The 4-h broth cultures were adjusted optically, using a Coleman Junior Spectrophotometer set at 530 nm, to that of a 0.5 McFarland barium sulfate standard at 86% transmission, resulting in a concentration of (1–5) × 10^6 cfu/mL. This suspension was further diluted 1:100 in YNBD to yield (1–5) × 10^4 cfu/mL.

(e) Procedure. Plates containing diluted compounds were inoculated with 75 μL/well of the appropriate microorganism using a Titertek 12-channel pipet. The final volume/well, including organism and compound, was 150 μL, the final inoculum was approximately 5 × 10^3 cfu/well, and the final antibiotic concentration range was 128 μg/mL to 0.06 μg/mL. At least two rows of drug-free wells were dedicated for growth and sterility controls for each organism tested. In addition, one set of microtiter plates were dedicated for sterility check of the compounds tested. Plates

(16) Cabib, E.; Kang, M. S. Fungal 1,3-β-Glucan Synthase. *Methods Enzymol.* **1987**, *138*, 637–642.

(17) Taft, C. S.; Selitrennikoff, Claude P. Cilofungin inhibition of (1,3)-beta-glucan synthase: the lipophilic side chain is essential for inhibition of enzyme activity. *J. Antibiot.* **1990**, *43*, 433–437.

were incubated for 48 h at 35–37 °C.

(f) Interpretation. (1) MIC. The MIC endpoint is defined as the lowest concentration of compound that completely inhibits growth (absence of detectable turbidity). Generally, the MIC is not an absolute value, but rather a concentration range that falls within a ±2-fold dilution limit. MICs are recorded at 24 h and, again, after 48 h of incubation. **(2) MFC.** After recording the MICs at 24 h, the microtiter plates are shaken gently on a Sarstedt TPM shaker to resuspend the cells. An MIC-2000 inoculator is used to transfer a 1.5-μL sample from each well of the 96-well microtiter plate to a MIC-2000 single reservoir inoculum plate containing SDA. The inoculated SDA and corresponding microtiter plates are incubated for 24 h at 35 °C. The MFC is defined as the lowest concentration of compound at which no growth or growth of ≤4 colonies occurs.

Glucan Synthase Inhibition Assay. (a) Reagents and Chemicals. Buffer A: 10% glycerol (1.2 M) in PBS containing PMSF (0.5 mM) and DTT (1 mM). Buffer B: same as buffer A, but without PMSF and DTT. Buffer C: Dulbeccoc's PBS, pH 7.0 (Gibco, Grand Island, NY) containing EDTA (5 mM) and DTT (50 mM). α-Amylase stock solution: The enzyme was obtained from Sigma Chemical Co., St. Louis, MO, supplied as a suspension in ammonium sulfate. This suspension (10.0 μL, 0.24 mg of protein, equivalent to 264 units) was diluted with 115 μL of buffer B. UDP-[6-^{3}H]glucose was obtained from Amersham Radiochemicals, Arlington Heights, IL, supplied at 250 μCi/250 μL (14.7 Ci/mmol); 5 μL of a 1:5 dilution (500 000 dpm) is equivalent to 77 pmol. GTP-γ-S was obtained from Sigma. Lipopeptide antifungal agent L-671,329[14] was obtained from the Sample Collection of Merck & Co., Rahway, NJ. All other chemicals were of reagent grade quality.

(b) *Candida albicans* Cell Culture (MY-1208). Cells were grown in Sabouraud dextrose broth (Difco) in 2-L baffled shake flasks each containing 500 mL, at 28 °C to early log phase, 6–8 h (150 rpm).

(c) Preparation of the Glucan Synthase System. The membrane associated synthase system was prepared from protoplasts essentially by the method of Taft et al.[15]

(d) Assay of Labled Glucose Incorporation with a Membrane System ex *C. albicans*. The assay of [^{3}H]glucan was conducted as a modification of the method previously described by Cabib and Kang for *S. cerevisiae*.[16] The total volume in wells of a microtiter plate designed for automated collection of the products of synthesis was 80 μL. The incubation system contained 6.6% DMSO, 125 mM TRIS-HCl (pH 7.5), 0.25 mM DTT, 0.15 mM PMSF, 0.4 M glycerol, 0.75 mM EDTA, 0.25% BSA, 40 mM GTP-γ-S, 2.5 mM laminaribiose, 30 units/system of α-amylase, synthase (500 μg of protein/mL assay system) contained in 15 μL and 25 μM UDPG, the required concentration added in 10 μL including UDP-[6-^{3}H]glucose (500 000 dpm). To determine the inhibitory effect of analogs, samples were added at 1.5 mM and serially diluted to a level of 0.04 μM. The required amount of inhibitor was contained in 4 μL of DMSO/H_2O (50%). The microtiter plates were covered with Parafilm and incubated for 60 min at 24 °C with agitation on a Minimix stand (Fischer Scientific). The reaction was terminated with the addition of TCA (10%, 100 μL/well), and the resulting insoluble materials were collected on a glass filter mat in a Wallac (LKB) cell harvester, followed by automated wash cycles (water and 95% ethanol). The incorporated radiolabel per well was determined in a Model 1205 Betaplate liquid scintillation counter (LKB, Wallac).

Chemistry. α-*N*-Fmoc-δ-*N*-Boc-L-Orn Pentafluorophenyl Ester (7). A solution of α-*N*-Fmoc-δ-*N*-Boc-L-Orn (1.5 g, 3.30 mmol) was prepared by warming in 12 mL of ethyl acetate and then allowing to cool to room temperature. To this stirring solution was added a solution of pentafluorophenol (0.61 g, 3.30 mmol) in ethyl acetate (3 mL). The reaction mixture was then cooled to 0 °C and stirred for 5 min when a solution of DCC (0.68 g, 3.30 mmol) in ethyl acetate (3 mL) was added in. After 15 min at 0 °C the reaction mixture was allowed to warm to room temperature for 1 h. The DCU was filtered off and the filtrate concentrated in vacuo. The resulting solids were triturated with ethyl acetate, filtered, and dried to give a white solid (1.39 g, 68%). This material was used as is. 7: mp 128–131 °C; MS (FAB) 565 (M + H − tBu), 521 (M + H − CO_2tBu); ^{1}H NMR ($CDCl_3$) δ 7.75 (d, 2 H, *J* = 6 Hz), 7.60 (d, 2 H, *J* = 6 Hz), 7.44 (m, 4 H), 5.62 (br s, 1 H), 4.74 (br s, 1 H), 4.60 (br s, 1 H), 4.50 (d, 2 H, *J* = 5 Hz), 4.26 (t, 1 H, *J* = 5 Hz), 3.24 (m, 2 H), 2.00 (br m, 2 H), 1.68 (m, 2 H), 1.48 (s, 9 H).

***p*-(Octyloxy)benzoic Acid Pentafluorophenyl Ester (8).** To a suspension of *p*-(octyloxy)benzoic acid (7.50 g, 30.0 mmol) in ethyl acetate (50 mL) was added pentafluorophenol (6.07 g, 33.0 mmol). The resulting mixture was cooled with an ice bath and dicyclohexylcarbodiimide (6.46 g, 33.0 mmol) was added. The mixture was allowed to stir at room temperature for 4 h and the dicyclohexylurea which had precipitated was collected by filtration and the filter cake washed with ethyl acetate. The filtrate was partitioned with water and the organic layer dried (Na_2SO_4) and concentrated to afford *p*-(octyloxy)benzoic acid pentafluorophenyl ester as a white crystalline solid (11.80 g, 94%): mp 46–47 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.12 (d, 2 H, *J* = 7.0 Hz), 6.97 (d, 2 H, *J* = 7.0 Hz), 4.03 (t, 2 H, 6.6), 2.0–1.2 (m), 0.87 (t, 3 H, *J* = 7.0 Hz).

Pro-Thr-Tyr-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (6). The continuous flow column of the Milligen 9050 peptide synthesizer was charged with Pepsyn KA resin (Milligen) containing 0.09 mmol of α-*N*-Fmoc-Pro/g of resin (2.0 g, 0.18 mmol α-*N*-Fmoc-Pro). The reagent rack was then loaded with α-*N*-Fmoc-L-proline-OPfp (0.8 mmol, 4.44 equiv), α-*N*-Fmoc-L-threonine(Bu^t)-ODhbt (1.6 mmol, 8.88 equiv) α-*N*-Fmoc-L-tyrosine (Bu^t)-OPfp (0.8 mmol, 4.44 equiv), along with α-*N*-Fmoc-δ-*N*-Boc-L-ornithine-OPfp (497 mg, 0.8 mmol, 4.44 equiv) and *p*-(octyloxy)benzoic acid-OPfp (333 mg, 0.8 mmol, 4.44 equiv). The synthesis was carried out using the standard protocols of the Milligen synthesizer. The resin was washed with DMF and deprotected with a 15-min cycle of 20% piperidine in DMF. The deprotection in this and all other cycles was monitored by UV at 365 nm. The deprotection was followed by a 15-min DMF wash. The acylations were performed by recycling a 0.33 M solution of the activated ester of the desired residue and HOBt for 30 min. This is followed by a DMF wash and the cycle is repeated. After the final cycle, the resin was washed with methylene chloride, transferred to a coarse filter frit, and washed with 2 × 10 mL ether. The resin was pulled dry and transferred to a polypropylene vial. The peptide was cleaved from the resin by slowly rocking with 15 mL of cleavage cocktail (95% TFA, 4% ethanedithiol, 1% thioanisole) for 3 h. The slurry was filtered and the filtrate collected. The resin was reexposed to fresh cleavage cocktail for another 0.5 h and refiltered. Finally the resin was washed with 2 × 15 mL acetic acid. All the filtrates were combined and concentrated down in vacuo. The residue was triturated with ether and filtered to give 126 mg off white solids. This material was redissolved in 20% aqueous acetic acid and extracted 2 × 8 mL ether. The aqueous phase was then concentrated by lyophilization to give the crude product (92 mg, 54%) >90% pure by HPLC analysis (45:55 H_2O/CH_3CN (0.1% TFA in both), 1.0 mL/min). This material was taken on to the cyclization step without further purification. HPLC retention time = 8.77 min; MS (FAB, Li spike) 930 (M + Li).

***cyclo*-Pro-Thr-Tyr-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (5).** To a solution of the crude 6 (80 mg, 0.087 mmol) in 12 mL of sieve-dried (3 A, 13 X), degassed DMF at 0 °C under N_2 was added diphenyl phosphorazidate (DPPA) (Aldrich) (21.4 μL, 0.096 mmol, 1.1 equiv) dropwise via syringe over the course of 2 min followed immediately by the addition of sodium bicarbonate (36.4 mg, 0.432 mmol, 5.0 equiv) in one portion. The reaction mixture was stirred for 48 h under nitrogen at 0 °C. The reaction mixture was then concentrated down in vacuo and the residue applied to a silica gel flash column. Elution with 90:10 $CHCl_3$/MeOH afforded the pure product (28 mg, 35%) as a white amorphous solid: HPLC retention time = 6.12 min (75:25 CH_3CN/H_2O, 1.0 mL/min); MS (FAB) calculated for $C_{47}H_{68}N_7O_{11}$ (M + H) 906.4976, found 906.5014.

Pro-Thr-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (9a). This synthesis was performed by using *t*-Boc solid-phase methodology on an ABI Model 430A automated peptide synthesizer. The reactor module of the synthesizer was loaded with 0.5 mmol of α-*N*-Boc-L-Pro Pam resin (ABI). All coupling, deprotection, and wash reagents were obtained in ready to use form from the manufacturer and were loaded onto the synthesizer as supplied. The amino acids used were, α-*N*-Boc-*O*-benzyl-L-Thr (1.24 g, 4.0 mmol), α-*N*-Boc-4-(benzyloxy)-L-Pro (343 mg, 2.0

mmol), α-*N*-Boc-*O*-(bromobenzyloxycarbonyl)-L-Tyr (989 mg, 2.0 mmol) (all from ABI) and α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn (429 mg, 2.0 mmol). The final acylation was performed with 4-(octyloxy)benzoic acid (500 mg, 2.0 mmol). All steps of the synthesis were performed using the standard chemical protocols of the synthesizer. The resin was deprotected with TFA in dichloromethane (DCM) followed by neutralization with *N*,*N*-diisopropylethylamine (DIEA). The α-amino deprotected resin was then washed with DMF in preparation for the peptide bond formation with the incoming active ester. HOBt activated esters were prepared online in a separate reaction vessel by treatment of the protected amino acids with DCC and HOBt in DCM and used to perform the coupling steps of the chemistry. After another washing step with DCM, the cycle is repeated. When the synthesis was complete, the resin was dried in vacuo. The peptide was cleaved from the resin with anhydrous HF. The resin was transferred to a KEL-F reactor, 3.0 mL of anisole was added, and the resin was allowed to swell. Additional 0.5-mL aliquots of anisole were added in until the resin became stirrable with a magnetic stir bar. The reactor was then secured on the HF line and cooled with liquid nitrogen and evacuated. Anhydrous HF (20.0 mL) was then distilled into the reactor from cobalt(III) fluoride. The reaction mixture was then warmed to 0 °C and stirred for 30 min. The HF was then removed by distillation at 0 °C into a liquid nitrogen cold trap. The final traces of HF were removed with a high vac line. The resin was washed with diethyl ether to remove the anisole and the crude product was then extracted from the resin with 3 × 20 mL aliquots of 50% aqueous acetic acid. Lyophilization afforded 585 mg of crude product containing minor impurities. Final purification was accomplished by reverse-phase chromatography. The product was dissolved in 2.0 mL of 20% acetic acid and applied to a Zorbax 25 mm × 25 cm C18 column in two batches. Gradient elution from 95:5 H_2O (0.1% TFA)/CH_3CN (0.1% TFA) to 10:90 at 6.0 mL/min over the course of 30 min afforded the pure (>93% by HPLC, Zorbax 4.9 mm × 25 cm C18 column, gradient elution from 95:5 H_2O (0.1% TFA)/CH_3CN (0.1% TFA) to 10:90, 1.0 mL/min, over 30 min) product (365 mg, 78%). HPLC retention time = 21.2 min; MS (FAB, Li spike) 946 (M + Li).

***cyclo*-Pro-Thr-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (10a).** To a solution of **9a** (100 mg, 0.107 mmol) in sieve-dried (13 A, 13 X), degassed DMF (15 mL) at −20 °C under nitrogen was added DPPA (25.5 μL, 32.5 mg, 0.118 mmol, 1.1 equiv) dropwise via syringe over the course of 2 min followed immediately by solid sodium bicarbonate (45 mg, 0.535 mmol, 5.0 equiv) all in one portion. The reaction mixture was stirred at −20 °C for 6 h and then warmed to 0 °C for 48 h. The DMF was concentrated off in vacuo. The residue was applied directly to a flash column and eluted with 12% MeOH in $CHCl_3$ to afford the pure product (60 mg, 59%). HPLC analysis >98% pure (50:50 H_2O/CH_3CN, 1.0 mL/min). HPLC retention time = 12.82 min; MS (FAB, Li spike) 928 (M + Li).

α-*N*-Boc-4-(benzyloxy)-L-proline Merrifield Resin (11). To a solution of α-*N*-Boc-4-(benzyloxy)-L-Pro (3.45 g, 10.72 mmol) in absolute ethanol (25 mL) was added 5.0 mL of H_2O. The pH was then adjusted to 7.7 with 2.0 M Cs_2CO_3 (2.7 mL, 5.4 mmol). The solution was then diluted with ethanol (100 mL) and concentrated down to dryness in vacuo. The residue was azeotroped with toluene (3 × 50 mL) and dried in a vacuum desiccator over P_2O_5. The resulting cesium salt was then taken up in dry DMF (65 mL) and stirred with 8.0 g of chloromethyl Merrifield resin (1.34 mmol of Cl/g, 10.72 mmol, 200–400 mesh from Bio-Rad) for 4 h. The resin was then washed sequentially with DMF, DMF/H_2O (1:1), DMF, methyl alcohol, and finally methylene chloride. After drying in vacuo, the final weight of the resin was 10.9 g, calculating out to a loading of 1.27 mmol of α-*N*-Boc-4-(benzyloxy)-L-proline/g of resin.

The following is representative of the Boc solid-phase synthesis of all other linear lipohexapeptides described in this work.

4-OH-Pro-Thr-Tyr-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (9b). The synthesis was carried out on a Biosearch SAM 2 peptide synthesizer. α-*N*-Boc-4-(benzyloxy)-L-Pro Merrifield resin with a nominal loading of 1.27 mmol/g (1.0 g, 1.27 mmol of 4-hydroxyproline), was loaded into the reactor module of the synthesizer. The amino acids used were α-*N*-Boc-*O*-benzyl-L-Thr (3.14 g, 10.16 mmol), α-*N*-Boc-4-(benzyloxy)-L-Pro (1.63 g, 5.08 mmol), α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn (2.18 g, 5.08 mmol), and α-*N*-Boc-*O*-(2,6-dichlorobenzyl)-L-Tyr (2.24 g, 5.08 mmol). The final acylation was performed with 4-(octyloxy)-benzoic acid (1.27 g, 5.08 mmol). A 4-fold molar excess of each residue was weighed out into a reservoir and dissolved in *N*-methylpyrrolidone (NMP) to a concentration 0.6 M. One equivalent of HOBt/millimole of amino acid was then added to each of the reservoirs and dissolved by sonication. The synthesizer was then charged with the requisite amino acids and octyloxybenzoic acid. The deblocking reagent was 45% TFA, 2.5% anisole, and 52.5% methylene chloride. The base wash solution was 20% diisopropylethylamine in methylene chloride. The coupling reagent used was 0.4 M diisopropylcarbodiimide. The capping cycle of the program was not used. The standard 1-h single couple cycle of the Biosearch tBoc program was used. When the synthesis was complete, the resin was collected and dried in vacuo overnight to give 1.94 g of substituted resin (95% of theory). Cleavage of the peptide from the resin was accomplished by the use of anhydrous HF. The resin was transferred to a KEL-F reactor, 3.0 mL of anisole was added, and the resin was allowed to swell. Additional 0.5-mL aliquots of anisole were added in until the resin became stirrable with a magnetic stir bar. The reactor was then secured on the HF line cooled with liquid nitrogen and evacuated. Anhydrous HF (10.0 mL/g of resin) was then distilled into the reactor from cobalt(III) fluoride. The reaction mixture was then warmed to 0 °C and stirred for 30 min. The HF was then removed by distillation at 0 °C into a liquid nitrogen cold trap. The final traces of HF were removed with a high vac line. The resin was washed with diethyl ether to remove the anisole, and the crude product was then extracted from the resin with 3 × 20 mL aliquots of 50% aqueous acetic acid. The extracts were diluted to 10% acetic acid with water and lyophilized to give 1.8 g of crude product. HPLC analysis of the crude product (40:60 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 2.0 mL/min) showed the crude linear lipohexapeptide to contain some rear running impurities. The final purification was accomplished by reverse-phase chromatography. The crude product was dissolved in 9.0 mL of mobile phase and purified in three batches on a Zorbax 25 mm × 25 cm C18 column with gradient elution from 100% H_2O (0.1% TFA) to 10:90 H_2O (0.1% TFA)/CH_3CN (0.1% TFA) at 8.0 mL/min over 1 h. The pure fractions, as determined by HPLC, were pooled and lyophilized to the product (580 mg, 58%) as an amorphous white solid. HPLC analysis for purity gave >87% and the product was carried on as is. HPLC retention time = 4.24 min; MS (FAB, Li spike) 946 (M + Li).

***cyclo*-4-OH-Pro-Thr-Tyr-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (10b).** To a solution of **9b** (300 mg, 0.32 mmol) in sieve-dried (13 A, 13 X), degassed DMF (45 mL) at −20 °C under N_2 was added DPPA (76.0 μL, 97.0 mg, 0.35 mmol, 1.1 equiv) dropwise via syringe over the course of 2 min followed immediately by solid sodium bicarbonate (135 mg, 1.6 mmol, 5.0 equiv) all in one portion. The reaction mixture was stirred at −20 °C for 6 h and then warmed to 0 °C for 40 h. The DMF was concentrated off in vacuo and the residue applied directly to a flash column and eluted with 15% MeOH in $CHCl_3$ to afford the pure product (200 mg, 68%) >95% pure by HPLC analysis (60:40 H_2O/CH_3CN (both 0.1% TFA), 1.0 mL/min). HPLC retention time = 8.72 min; MS (FAB) calculated for $C_{47}H_{68}N_7O_{12}$ (M + H) 922.4926, found 922.4901.

4-OH-Pro-Thr-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (9c). The synthesis was carried out on a Biosearch SAM 2 peptide synthesizer. α-*N*-Boc-4-(benzyloxy)-L-Pro Merrifield resin with a loading of 1.27 mmol/g (1.0 g, 1.27 mmol of 4-OH-Pro) was loaded into the reactor module of the synthesizer. The amino acids used were α-*N*-Boc-*O*-benzyl-L-Thr (3.14 g, 10.16 mmol), α-*N*-Boc-4-(benzyloxy)-L-Pro (1.63 g, 5.08 mmol), α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn (2.18 g, 5.08 mmol), and α-*N*-Boc-*O*-(2,6-dichlorobenzyl)-L-Tyr (2.24 g, 5.08 mmol). The final acylation was performed with 4-(octyloxy)benzoic acid (1.27 g, 5.08 mmol). After the standard synthetic procedure the resin was collected and dried in vacuo to give 2.14 g. The standard HF cleavage process afforded 800 mg of crude product which contained some rear running impurities by HPLC analysis (40:60 H_2O/CH_3CN (both 0.1% TFA), 2.0 mL/min). Final purification was accomplished by preparative reverse-phase HPLC. The crude product was dissolved in 8.0 mL of mobile phase and injected in

two batches onto a 25 mm × 25 cm Zorbax C8 column. Gradient elution from 100% H_2O (0.1% TFA) to 90:10 H_2O (0.1% TFA)/CH_3CN (0.1% TFA) at 8.0 mL/min over 1 h afforded 540 mg (47%) after lyophilization. Purity as determined by HPLC analysis was >84% and the product was carried on to the cyclization step as is. HPLC retention time = 3.56 min; MS (FAB, Li spike) 962 (M + Li).

***cyclo*-4-OH-Pro-Thr-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (10c).** To a solution of **9c** (300 mg, 0.315 mmol) in sieve-dried (13 A, 13 X), degassed DMF (45 mL) at −20 °C under N_2 was added DPPA (75.0 mL, 96.0 mg, 0.347 mmol, 1.1 equiv) dropwise via syringe over the course of 2 min followed immediately by solid sodium bicarbonate (132 mg, 1.6 mmol, 5.0 equiv) all in one portion. The reaction mixture was stirred at −20 °C for 6 h and then warmed to 0 °C for 40 h. The DMF was concentrated off in vacuo. The residue was applied directly to a flash column and eluted with 18% MeOH in $CHCl_3$ to afford 200 mg of off-white solids after trituration with ether. This material was taken up in 50 mL of 5% 2-propanol in $CHCl_3$ and extracted with saturated sodium bicarbonate (10 mL), 5% sodium bisulfite (10 mL), and saturated NaCl (10 mL). The organic layer was dried over sodium sulfate, filtered, and concentrated in vacuo to afford 130 mg of off-white solids: single spot to TLC (18% MeOH in $CHCl_3$), >94% pure by HPLC analysis (50:50 H_2O/CH_3CN (both 0.1% TFA), 2.0 mL/min). The product was rechromatographed on silica gel with 15% MeOH in $CHCl_3$ to remove the off color cast. Pure fractions were pooled and concentrated in vacuo to afford product (95 mg, 32%) as a white solid of very high purity (>98.5% by HPLC analysis). HPLC retention time = 6.63 min; MS (FAB) calculated for $C_{47}H_{68}N_7O_{13}$ (M + H) 938.4875, found 938.4875.

L-Homotyrosine Hydrobromide (12a). A solution of (*S*)-*N*-(methylcarbamoyl)homotyrosine methyl ether (5.00 g, 18.72 mmol) in 30% HBr in acetic acid (25 mL) was heated to 60 °C for 76 h. A solid was deposited. The reaction mixture was diluted with ether (50 mL) and the product isolated by filtration and washed with ether. The pale yellow solid was taken up in 25 mL of 2-propanol with warming and diluted with ether (50 mL). The solution was filtered from a small amount of greenish oil and then concentrated to a yellow oil which was triturated with ether to give the hydrobromide of homotyrosine as a white solid (4.34 g, 84%): mp 234–236 °C; ^1H NMR δ 7.06 (d, 2 H, *J* = 8.4 Hz), 6.73 (d, 2 H, *J* = 8.4 Hz), 3.96 (t, 1 H, *J* = 6.6 Hz), 2.69 (m, 2 H), 2.15 (m, 2 H).

***O*-(2,6-Dichlorobenzyl)-L-homotyrosine (12b).** To a solution of NaOH (1.76 g, 44.2 mmol) in water (14 mL) was added **12a** (4.00 g, 14.5 mmol) and to the resulting solution was added a solution of copper(II) sulfate pentahydrate (1.81 g, 7.25 mmol) in water (7 mL). The mixture was heated to 55 °C and then cooled to room temperature and diluted with methanol (60 mL). To the resulting mixture was added 2,6-dichlorobenzyl bromide (4.63 g, 19.29 mmol) and the reaction allowed to stir at room temperature for 22 h. The blue solid which had precipitated was collected by filtration and washed sequentially with 25% water in methanol, methanol, and acetone (50-mL aliquots each). After air-drying the resulting blue solid was added to a boiling solution of 250 mL of water and 250 mL of ethanol containing EDTA (5.0 g as the disodium salt). After stirring for a few minutes at the boiling point, the solution (which had a small amount of fine white solid crystallizing) was decanted away from a small residual amount of heavy blue solid. The product was allowed to crystallize overnight in the refrigerator. The product was collected by filtration and washed sequentially with water and ethanol and then dried in a desiccator under vacuum. Product was a white solid and was taken on without further purification (2.85 g, 55%). **12b**: mp 238–243 °C; ^1H NMR δ 7.45 (d, 2 H, *J* = 9 Hz), 7.36 (dd, 1 H, *J* = 9 Hz), 7.19 (d, 2 H, *J* = 8.4 Hz), 6.96 (d, 2 H, *J* = 8.4 Hz), 2.70 (m, 2 H), 2.12 (m, 2 H).

***O*-(2,6-Dichlorobenzyl)-α-*N*-*t*-Boc-L-homotyrosine (12c).** To a solution of aqueous sodium hydroxide (0.225 g, 5.65 mmol) in water (6 mL) and dioxane (6 mL) was added *O*-(2,6-dichlorobenzyl)homotyrosine (2.00 g, 5.65 mmol), and to the resulting solution was added di-*tert*-butyl pyrocarbonate (1.08 g, 6.21 mmol, Aldrich). The mixture was allowed to stir at room temperature for 2.5 h. To the resulting aqueous mixture was added ethyl acetate (25 mL) and the reaction mixture acidified with 2 N HCl (6 mL). Additional ethyl acetate and water were added and the layers separated. As a rather intractable emulsion formed, 20% NaCl was added as necessary. The organic layers from three extractions with ethyl acetate were combined, dried, and concentrated. This material was purified by flash chromatography using 3% MeOH in methylene chloride containing 1% acetic acid as eluant to afford (*S*)-*O*-(2,6-dichlorobenzyl)-α-*N*-Boc-homotyrosine as a white crystalline solid (1.38 g, 54%) after two concentrations from toluene to remove residual acetic acid: mp 111–113 °C; ^1H NMR δ 7.45 (d, 1 H, *J* = 8.7 Hz), 7.45 (d, 1 H, *J* = 7.2 Hz), 7.35 (dd, 1 H, *J* = 7.2, 8.7 Hz), 7.15 (d, 2 H, *J* = 8.7 Hz), 6.95 (d, 2 H, *J* = 8.7 Hz), 5.26 (s, 2 H), 4.05 (m, 1 H), 2.66 (m, 2 H), 2.07 (m, 1 H), 1.92 (m, 1 H), 1.46 (s, 9 H).

Pro-Thr-Homotyr-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (9d). The synthesis was carried out on a Biosearch SAM 2 peptide synthesizer. α-*N*-Boc-L-Pro Pam resin with a nominal loading of 0.72 mmol/g (712 mg, 0.47 mmol of proline) was loaded into the reactor module of the synthesizer. The amino acids used were α-*N*-Boc-*O*-benzyl-L-Thr (1.16 g, 3.76 mmol), α-*N*-Boc-L-Pro (405 mg, 1.88 mmol), α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn (807 mg, 1.88 mmol), and **12c** (853 mg, 1.88 mmol). The final acylation was performed with 4-(octyloxy)benzoic acid (470 mg, 1.88 mmol). After the standard synthetic procedure, the dried resin was collected (1.12 g). The standard HF cleavage conditions afforded 290 mg of white amorphous solids. HPLC analysis of the crude product (55:45 H_2O/CH_3CN (both 0.1% TFA), 1.0 mL/min) showed the presence of some front and rear running impurities. Final purification was accomplished by reverse-phase HPLC. The product was dissolved in mobile phase (8.0 mL) and injected in four batches onto a 25 mm × 25 cm Zorbax C8 column. Isocratic elution with 55:45 H_2O/CH_3CN both 0.1% TFA at 7.0 mL/min and lyophilization of the pure fractions afforded the pure (>95% by HPLC analysis) product (160 mg, 36%). HPLC retention time = 7.16 min; MS (FAB) 938 (M + H).

***cyclo*-Pro-Thr-Homotyr-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (10d).** To a solution of **9d** (143 mg, 0.153 mmol) in sieve dried (3 A, 13 X), degassed DMF (22 mL) at −20 °C under N_2 was added DPPA (36 μL, 0.17 mmol, 1.1 equiv) via syringe over the course of 2 min followed immediately by the addition of solid sodium bicarbonate (65 mg, 0.765 mmol, 5.0 equiv) all in one portion. The reaction mixture was stirred at −20 °C, warmed to −5 °C, and stirred until the reaction was deemed to be complete by HPLC analysis (35:65 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 1.0 mL/min). The reaction was complete after 56 h. The DMF was concentrated off in vacuo and the residue taken up in mobile phase (40:60 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 10 mL) and injected in two portions onto a 25 mm × 25 cm Zorbax C8 column and eluted isocratically at 7 mL/min. The pure fractions were determined by HPLC, pooled, and lyophilized to afford the pure (>95% by HPLC analysis) product (75 mg, 54% yield). HPLC retention time = 9.85 min; MS (FAB) 920 (M + H).

Pro-Thr-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (9e). The synthesis was carried out on a Biosearch SAM 2 peptide synthesizer. α-*N*-Boc-L-Pro Pam resin with a nominal loading of 0.72 mmol/g (625 mg, 0.45 mmol of proline) was loaded into the reactor module of the synthesizer. The amino acids used were α-*N*-Boc-*O*-benzyl-L-Thr (1.11 g, 3.6 mmol), α-*N*-Boc-4-(benzyloxy)-L-Pro (388 mg, 1.88 mmol), α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn (806 mg, 1.88 mmol), and **12c** (854 mg, 1.88 mmol). The final acylation was performed with 4-(octyloxy)benzoic acid (470 mg, 1.88 mmol). After the standard synthetic procedure the resin was collected and dried in vacuo. The standard HF cleavage conditions afforded a white amorphous solid (250 mg, 58%). Crude product was carried on to the next step as is (purity >80% as determined by HPLC analysis (40:60 H_2O/CH_3CN), both 0.1% TFA, 1.0 mL/min). HPLC retention time = 7.68 min; MS (FAB) 954 (M + H).

***cyclo*-Pro-Thr-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (10e).** To a solution of **9e** (234 mg, 0.246 mmol) in sieve dried (3 A, 13 X), degassed DMF (22 mL) at −20 °C under N_2 was added DPPA (58 μL, 0.27 mmol, 1.1 equiv) via syringe over the course of 2 min followed immediately by the addition of solid sodium bicarbonate (103 mg, 1.23 mmol, 5.0 equiv) all in one portion. The reaction mixture was stirred at −20 °C for 6 h and warmed to −5 °C for 24 h and finally 12 h at 0 °C. The

DMF was concentrated off in vacuo. The residue was taken up in mobile phase (60:40 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 10 mL), filtered, and injected in two batches onto a 25 mm × 25 cm Zorbax C8 column and eluted isocratically at 10 mL/min. The pure fractions as determined by HPLC (40:60 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 1.0 mL/min) were pooled and lyophilized to afford the pure (>95% by HPLC analysis) product (90 mg, 40% yield). HPLC retention time = 9.85 min; MS (FAB) calculated for $C_{48}H_{70}N_7O_{12}$ (M + H) 936.5082, found 936.5118.

4-OH-Pro-Thr-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (9f). The synthesis was carried out on a Biosearch SAM 2 peptide synthesizer. α-*N*-Boc-4-(benzyloxy)-L-Pro Merrifield resin with a nominal loading of 1.27 mmol/g (250 mg, 0.32 mmol of 4-hydroxy-Pro) was loaded into the reactor module of the synthesizer. The amino acids used were α-*N*-Boc-*O*-benzyl-L-Thr (792 mg, 2.56 mmol), α-*N*-Boc-L-4-(benzyloxy)-Pro (411 mg, 1.28 mmol), α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn (549 mg, 1.28 mmol), and **12c** (582 mg, 1.28 mmol). The final acylation was performed with 4-(octyloxy)benzoic acid (313 mg, 1.28 mmol). After the standard synthetic procedure the resin was collected and dried in vacuo to give 440 mg. The standard HF cleavage process afforded 290 mg of crude product which contained some front running impurities by HPLC analysis (50:50 H_2O/CH_3CN (both 0.1% TFA), 1.0 mL/min). Final purification was accomplished by preparative reverse-phase HPLC. The crude product was dissolved in 10.0 mL of mobile phase and injected in four batches onto a 25 mm × 25 cm Zorbax C8 column and eluted with 45:55 H_2O/CH_3CN (both 0.1% TFA) at 7.0 mL/min. Pure fractions as determined by HPLC analysis were combined and lyophilized to afford the pure (94.5% by HPLC) product (85 mg, 29%). HPLC retention time = 9.79 min; MS (FAB) 970 (M + H).

***cyclo*-4-OH-Pro-Thr-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (10f).** To a solution of **9f** (126 mg, 0.130 mmol) in sieve dried (3 A, 13 X), degassed DMF (35 mL) at −20 °C under N_2 was added DPPA (31 μL, 0.14 mmol, 1.1 equiv) via syringe over the course of 2 min followed immediately by the addition of solid sodium bicarbonate (55 mg, 5.0 equiv) all in one portion. The reaction mixture was stirred at −20 °C for 6 h, warmed to −5 °C, and stirred until the reaction was complete (40 h) by HPLC analysis (45:55 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 1.0 mL/min). The DMF was concentrated off in vacuo. The purification of the residue was attempted by flash chromatography on silica gel with 13% MeOH in $CHCl_3$ to elute. This process afforded 94 mg of insufficiently pure material. Another pass through at flash chromatography yielded 55 mg of material that still required further purification. Final purification was accomplished by reverse-phase HPLC. The product was taken up in mobile phase (45:55 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 10 mL) and injected onto a 25 mm × 25 cm Zorbax C8 column in series and eluted at 7.0 mL/min. The pure fractions were determined by HPLC, pooled, and lyophilized to afford the pure (>95% by HPLC analysis) product (28 mg, 22% yield). HPLC retention time = 10.00 min; MS (FAB) calculated for $C_{48}H_{70}N_7O_{13}$ (M + H) 952.5031, found 952.5028.

Thr-Thr-Homotyr-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (9g). The synthesis was carried out on a Biosearch SAM 2 peptide synthesizer. α-*N*-Boc-*O*-benzyl-L-Thr Pam resin with a nominal loading of 0.72 mmol/g (625 mg, 0.45 mmol of Thr) was loaded into the reactor module of the synthesizer. The amino acids used were α-*N*-Boc-*O*-benzyl-L-Thr (1.11 g, 3.6 mmol), α-*N*-Boc-4-(benzyloxy)-L-Pro, α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn (772 mg, 1.8 mmol), and **12c** (818 mg, 1.8 mmol). The final acylation was performed with 4-(octyloxy)benzoic acid (450 mg, 1.8 mmol). After the standard synthesis procedure the resin was collected and dried in vacuo to give 1.1 g. The standard HF cleavage process afforded 327 mg of crude product (76%). HPLC analysis (45:55 H_2O/CH_3CN), both 0.1% TFA, 1.0 mL/min) showed the product to be pure enough (>90%) to carry on to the next step as is. HPLC retention time = 8.37 min; MS (FAB) 958 (M + H).

***cyclo*-Thr-Thr-Homotyr-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (10g).** To a solution of **9g** (305 mg, 0.321 mmol) in sieve dried (13 A, 13 X), degassed DMF (45 mL) at −20 °C under N_2 was added DPPA (75.0 mL, 96.0 mg, 0.347 mmol, 1.1 equiv) dropwise via syringe over the course of 2 min followed immediately by solid sodium bicarbonate (132 mg, 1.6 mmol, 5.0 equiv) all in one portion. The reaction mixture was stirred at −20 °C for 6 h and then warmed to 0 °C for 144 h and finally 10 °C for 4 h. HPLC analysis (50:50 H_2O (0.1% TFA/CH_3CN (0.1% TFA), 1.0 mL/min) showed the reaction mixture to be a 3:1 ratio of product to starting material. The DMF was concentrated off in vacuo. The residue was taken up in mobile phase and injected in two batches onto a 25 mm × 25 cm Zorbax C8 column and eluted with 50:50 H_2O (0.1% TFA)/CH_3CN (0.1% TFA) at 10.0 mL/min. Pure fractions were pooled and lyophilized to afford the pure (>95% by HPLC analysis) product (60 mg, 20%). HPLC retention time = 14.78 min; MS (FAB) calculated for $C_{47}H_{70}N_7O_{13}$ (M + H) 952.5032, found 952.5023.

Thr-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (13a). The synthesis was carried out on a Biosearch SAM 2 peptide synthesizer. One gram of α-*N*-Boc-*O*-benzyl-L-Thr Merrifield resin with a nominal loading of 0.57 mmol/g (0.57 mmol of Thr) was loaded into the reactor module of the synthesizer. The amino acids used were α-*N*-Boc-*O*-benzyl-L-Thr (705 mg, 2.28 mmol), α-*N*-Boc-4-(benzyloxy)-L-Pro (733 mg, 2.28 mmol), α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn (978 mg, 2.28 mmol), and **12c** (1.04 g, 2.28 mmol). The final acylation was performed with 4-(octyloxy)benzoic acid (570 mg, 2.28 mmol). After the standard synthetic procedure the resin was collected and dried in vacuo to give 1.55 g. The standard HF cleavage process afforded 355 mg of crude product (72%) which contained some rear running impurities by HPLC analysis (45:55 H_2O/CH_3CN), both 0.1% TFA, 1.0 mL/min). Final purification was accomplished by preparative reverse-phase HPLC. The crude product was dissolved in 8.0 mL of mobile phase and injected in four batches onto two 25 mm × 25 cm Zorbax C8 columns in series and eluted with 45:55 H_2O (0.1% TFA)/CH_3CN (0.1% TFA) at 10.0 mL/min. Pure fractions as determined by HPLC analysis were combined and lyophilized to afford the pure (94.5% by HPLC) product (148 mg, 30%). HPLC retention time = 9.01 min; MS (FAB) 957 (M + H).

Thr-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-δ-CBZ-Orn (13b) and Thr-*O*-CBZ-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-δ-CBZ-Orn (13c) Linear Pentapeptides. To a 0 °C solution of **13a** (138 mg, 0.161 mmol) in 10 mL of 20% aqueous *tert*-butyl alcohol was added 80.5 μL of 2 N NaOH (0.161 mmol, 1.0 equiv). The solution became cloudy and the pH was measured at 7.0. Another 46.3 μL of 2 N NaOH (0.0925 mmol, 0.57 equiv) was added. The reaction mixture became a clear solution. This was quickly followed by the addition of 12.6 μL (0.55 equiv) of CBZ chloride. An additional 3.0 μL of 2 N NaOH was added to keep the reaction mixture pH between 7 and 8. After 10 min of stirring at 0 °C, the reaction mixture had become cloudy again. Another aliquot of 46.3 μL (0.57 equiv) of 2 N NaOH was added and the reaction mixture became a solution again. This was followed immediately by the addition of 12.6 μL (0.55 equiv) of CBZ chloride. The pH was again kept between 7 and 8 by the addition of 8.0 μL of 2 N NaOH. The reaction mixture was allowed to stir for 10 min at 0 °C, and an aliquot was removed for HPLC analysis (70:30 H_2O/(9:1 CH_3CN/H_2O), both 0.1% TFA, 1.0 mL/min). The analysis showed a significant amount of starting material in the reaction mixture. An 8.0-μL aliquot of 2 N NaOH was then added followed by 2.5 μL of CBZ chloride and the reaction allowed to stir for 1.5 h at 0 °C. HPLC analysis showed a small amount of starting peptide remaining. A final addition of 8.0 μL of 2 N NaOH followed by 1.0 μL of CBZ chloride was added and the reaction mixture stirred at 0 °C for another 0.5 h. HPLC analysis showed the reaction to be complete. The mono-CBZ- and bis-CBZ-protected peptides had formed in a ratio of approximately 3:1, respectively. The reaction mixture was frozen and put on the lyophilizer overnight to give 183 mg of crude mixture of products which was taken on to the next step as is. Mono-CBZ (**13b**) HPLC retention time = 6.76 min; bis-CBZ (**13c**) HPLC retention time = 18.20 min.

(2*S*,3*S*,4*S*)-3-Hydroxy-4-methyl-ProOMe-Thr-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-δ-CBZ-Orn (13d) and (2*S*,3*S*,4*S*)-3-Hydroxy-4-methyl-ProOMe-Thr-O-CBZ-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-δ-CBZ-Orn (13e) Linear Hexapeptides. To a 0 °C solution of the starting mixture of CBZ peptides **13b** and **13c** (124 mg, 0.125 mmol (based

on mono-CBZ only) and (2S,3S,4S)-3-hydroxy-4-methylproline methyl ester hydrochloride (27 mg, 0.138 mmol, 1.1 equiv) in 3.0 mL of dry DMF under nitrogen was added triethylamine (19.3 μL, 14 mg, 0.138 mmol, 1.1 equiv) followed by hydroxybenzotriazole (18 mg, 0.131 mmol, 1.05 equiv) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (25 mg, 0.131 mmol, 1.05 equiv). The reaction mixture was stirred for 2 h at 0 °C and then overnight at room temperature. HPLC analysis (30:70 H_2O/(9:1 CH_3CN/H_2O), 1.0 mL/min) showed the reaction complete. HPLC analysis gave a 2.3:1 ratio of mono-:bis-CBZ products. The reaction mixture was filtered through a plug of glass wool and concentrated down to dryness in vacuo. The residue was partitioned between 3.0 mL of H_2O and 14.0 mL of ethyl acetate. The aqueous layer was removed and back extracted with 1.0 mL of ethyl acetate. The combined organic extracts were washed with 3.0 mL of 1 N sodium hydrogen sulfate, 3.0 mL of saturated sodium bicarbonate, and 3.0 mL of saturated sodium chloride. The organic layer was dried over sodium sulfate, filtered, and concentrated to give 113 mg (80%) of amorphous solids. Taken on to the saponification as is. A pure sample of each of these materials was isolated by semi-prep HPLC for identification purposes: mono-CBZ (**13d**) HPLC retention time = 8.28 min; MS (FAB) 1132 (M + 1). Bis-CBZ (**13e**) HPLC retention time = 22.64 min; MS (FAB) 1266 (M + 1).

(2S,3S,4S)-3-Hydroxy-4-methyl-Pro-Thr-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-δ-CBZ-Orn (13f) Linear Hexapeptide. To a solution of the crude mixture (2.3:1 mono-CBZ:bis-CBZ) of peptides (79 mg of mono-CBZ (**13d**), 70.7 μmol; 34 mg bis-CBZ (**13e**), 27.2 μmol; 113 mg, 97.9 μmol total based on calculation from HPLC ratio) in 1.6 mL of methyl alcohol under nitrogen was added 215.4 μL of 1 N NaOH (2.2 equiv). After 4 h HPLC analysis (30:70 H_2O/(9:1 CH_3CN/H_2O), 1.0 mL/min) showed the reaction mixture to be 2:1 mixture of starting mono-CBZ ester to desired product. An additional aliquot of 1 N NaOH was added in (79 μL, 0.8 equiv) and the reaction allowed to proceed for another 8 h. HPLC analysis showed the reaction to be complete. The reaction mixture was partitioned between 25 mL of 1 N sodium hydrogen sulfate and 75 mL of ethyl acetate. The organic layer was collected and the aqueous layer was reextracted with 1 × 75 mL ethyl acetate and finally 1 × 77 mL chloroform. The combined organic extracts were dried over sodium sulfate and concentrated down to dryness. The residue was taken up in 50 mL of 50:50 H_2O/CH_3CN (both 0.1% TFA), lyophilized to give the crude product (78 mg, 70%), and taken on to the next step as is. HPLC retention time = 6.1 min; MS (FAB) 1118 (M + 1).

(2S,3S,4S)-3-Hydroxy-4-methyl-Pro-Thr-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (13g) Linear Hexapeptide. To a degassed solution of the crude **13f** (75 mg) in methyl alcohol under nitrogen was added 30 mg of 10% Pd/C. The flask was flushed with hydrogen and hydrogenation was conducted under balloon hydrogen pressure at room temperature overnight. HPLC analysis (30:70 H_2O/(9:1 CH_3CN/H_2O), 1.0 mL/min) showed the reaction to be complete. The catalyst was filtered off and the filtrate concentrated down to dryness in vacuo. The residue was taken up in 50 mL of 70:30 H_2O/CH_3CN and lyophilized to give the product (63 mg, 98%) as an amorphous white solid. The crude product was taken on to the cyclization as is. MS (FAB) 984 (M + 1).

***cyclo*-(2S,3S,4S)-3-Hydroxy-4-methyl-Pro-Thr-Homotyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (14).** To a solution of the crude linear hexapeptide (60 mg, 0.061 mmol) in sieve dried (3 A, 13 X), degassed DMF (35 mL) at −20 °C under N_2 was added DPPA (31 μL, 0.143 mmol, 1.1 equiv) via syringe over the course of 2 min followed immediately by the addition of solid sodium bicarbonate (55 mg, 5.0 equiv) all in one portion. The reaction mixture was stirred at −20 °C for 6 h. The reaction was then warmed to 0 °C and allowed to proceed until judged complete by HPLC analysis (30 h) (40:60 H_2O/(90:10 CH_3CN/H_2O) (both 0.1% TFA), 1.0 mL/min). The DMF was concentrated off in vacuo. The purification of the residue was attempted by flash chromatography with 13% MeOH in $CHCl_3$ to elute. This purification yielded 94 mg of insufficiently pure material. Reverse-phase chromatography was tried instead. The material was taken up in mobile phase (40:60 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA) (5.0 mL) and injected onto a 25 mm × 25 cm Zorbax C8 column and eluted at 7.0 mL/min to give the pure (>99% by HPLC) product (9.5 mg, 16% yield). HPLC retention time 9.90 min; MS (FAB) 966 (M + H).

Thr-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (15a). The synthesis was carried out on a Biosearch SAM 2 peptide synthesizer. α-*N*-Boc-*O*-benzyl-L-Thr Merrifield resin with a nominal loading of 0.57 mmol/g (1.5 g, 0.86 mmol of Thr) was loaded into the reactor module of the synthesizer. The amino acids used were α-*N*-Boc-*O*-benzyl-L-Thr (1.06 g, 3.44 mmol), α-*N*-Boc-4-(benzyloxy)-L-Pro (1.11 g, 3.44 mmol), α-*N*-Boc-δ-(2-chlorobenzyloxycarbonyl)-L-Orn (1.48 g, 3.44 mmol), α-*N*-Boc-*O*-(2,6-dichlorobenzyl)-L-Tyr (1.52 g, 3.44 mmol). The final acylation was performed with 4-(octyloxy)benzoic acid (861 mg, 3.44 mmol). After the standard synthetic procedure the resin was collected and dried in vacuo to give 2.6 g. The standard HF cleavage process afforded 725 mg (100%) of crude product which contained some rear running impurities by HPLC analysis (30:70 H_2O/CH_3CN (both 0.1% TFA), 1.0 mL/min). Final purification was accomplished by preparative reverse-phase HPLC. The crude product was dissolved in 12.0 mL of mobile phase and injected in three batches onto a 25 mm × 25 cm Zorbax C8 column and eluted with 30:70 H_2O/CH_3CN (both 0.1% TFA) at 10 mL/min. Pure fractions as determined by HPLC analysis were combined and lyophilized to afford the pure (96.5% by HPLC) product (525 mg, 73%). HPLC retention time = 4.38 min; MS (FAB) 943 (M + H).

Thr-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-δ-CBZ-Orn (15b) and Thr-*O*-CBZ-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-δ-CBZ-Orn (15c) Linear Pentapeptides. Linear pentapeptide Thr-Tyr-4-hydroxy-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (TFA salt) (250 mg, 0.297 mmol) was dissolved in 4.0 mL of 50% aqueous *tert*-butyl alcohol, diluted with 12 mL water, and cooled to 0 °C. The solution was neutralized by the addition of 149 μL of 2 N NaOH and then rendered alkaline by the addition of 85 μL of 2 N NaOH (0.57 equiv). This was immediately followed by the addition of 24.3 μL of CBZ chloride (0.55 equiv) and allowed to stir for 20 min at 0 °C. The pH is kept between 7 and 8 by the addition of 2 N NaOH as required. At first the reaction mixture is a solution but gradually gets cloudly with time. At the end of this period another 85 μL (0.57 equiv) of 2 N NaOH was added followed by 24.3 μL of CBZ chloride and the stirring at 0 °C continued for another 20 min. This process was repeated again and the reaction mixture analyzed by HPLC (30:70 H_2O/(90:10 CH_3CN/H_2O) (both 0.1% TFA), 1.0 mL/min). A small amount of the starting peptide remained so a final aliquot (43 μL, 0.29 equiv) of 2 N NaOH was added followed by the CBZ chloride (12 μL, 0.27 equiv) and the stirring continued for another 20 min. The reaction mixture was diluted with 100 mL of mobile phase and lyophilized to give 320 mg of white amorphous solids. The same process was repeated on another 250 mg, and the combined solids were dissolved in 16 mL of mobile phase and injected in 4 × 4.0 mL batches onto two Zorbax 25 mm × 25 cm C8 columns in series (30:70 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 10.0 mL/min). Fractions were analyzed by HPLC. Purified fractions were pooled and lyophilized. Yields pure materials: 40 mg starting material, mono-CBZ (**15b**); 80 mg (>95% by HPLC), HPLC retention time = 7.50 min; MS (FAB) 999 (M + Na); bis-CBZ (**15c**): 28 mg (>92% pure by HPLC), HPLC retention time = 20.20 min; MS (FAB, Li spike) 1123 (M + Li).

(2S,3S,4S)-3-Hydroxy-4-methyl-Pro-OMe-Thr-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-δ-CBZ-Orn (15d). To a 0 °C solution of **15b** (75 mg, 76.8 μmol) and (2S,3S,4S)-3-hydroxy-4-methylproline methyl ester hydrochloride (17 mg, 84.5 μmol, 1.1 equiv) in 2.0 mL of dry DMF was added triethylamine (12.4 μL, 9.0 mg, 84.5 μmol, 1.1 equiv) followed by HOBt monohydrate (11.0 mg, 80.6 μmol, 1.05 equiv) and finally 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (16 mg, 80.6 μmol, Aldrich). The reaction mixture was stirred overnight at 0 °C. HPLC analysis (30:70 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 1.0 mL/min) showed the reaction to be complete. The reaction mixture was partitioned between 3 mL of water and 12 mL of ethyl acetate. The aqueous layer was back extracted with an additional 2 × 2 mL ethyl acetate, and the combined organics were washed with 1 × 2 mL of 1 N sodium hydrogen sulfate, 1 × 2 mL of saturated sodium bicarbonate, and 1 × 2 mL of saturated sodium chloride. The organics were dried over sodium

sulfate and concentrated in vacuo to give very clean (>90% by HPLC) crude product (77 mg, 89.5%). This material was taken on to the next step as is. HPLC retention time = 9.25 min; MS (FAB) 1118 (M + H), 1140 (M + Na).

(2*S*,3*S*,4*S*)-3-Hydroxy-4-methyl-Pro-Thr-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-δ-CBZ-Orn (15e). To a solution of **15d** (75 mg, 0.067 mmol) in 1.5 mL of methyl alcohol was added 1 N NaOH (147 μL, 0.147 mmol, 2.2 equiv). The reaction mixture was stirred under nitrogen for 6 h. HPLC analysis (30:70 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 1.0 mL/min) showed the reaction to be incomplete. An additional 1.1 equiv of 1 N NaOH was added and the reaction allowed to stir overnight at 0 °C. The following morning the reaction was still incomplete. Another 1.1 equiv of 1 N NaOH was added and the stirring continued for another 6 h. HPLC analysis still showed some starting peptide. Another 0.55 equiv of 1 N NaOH was added and the reaction allowed to warm to room temperature for 6 h. HPLC showed the reaction to be complete. The reaction mixture was partitioned between ethyl acetate (60 mL) and 1 N sodium hydrogen sulfate (20 mL). The organic layer was collected and the aqueous phase back extracted with an additional 2 × 60 mL ethyl acetate and 1 × 60 mL methylene chloride. The combined organics were dried over sodium sulfate and concentrated in vacuo. The gummy residue was redissolved in 20% aqueous acetonitrile and lyophilized to yield a fluffy white solid (67 mg, 90.5%). This material was taken on to the next step as is. HPLC retention time = 6.80 min; MS (FAB) 1126 (M + Na).

(2*S*,3*S*,4*S*)-3-Hydroxy-4-methyl-Pro-Thr-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (15f). A solution of **15e** (60 mg, 0.0544 mmol) in 15 mL of methyl alcohol was degassed and purged with nitrogen. Palladium on carbon (10%, 30 mg) was added and the system flushed with hydrogen. The hydrogenation was carried out overnight at balloon pressure. HPLC analysis (30:70 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 1.0 mL/min) showed the reaction to be complete. The catalyst was removed by filtration and the filtrate concentrated in vacuo. The residue was redissolved in 20% aqueous acetonitrile and lyophilized to yield a fluffy white solid (45 mg, 83%). This material carried on to the cyclization step as is. HPLC retention time = 5.0 min; MS (FAB) 970 (M + H).

***cyclo*-(2*S*,3*S*,4*S*)-3-Hydroxy-4-methyl-Pro-Thr-Tyr-4-OH-Pro-Thr-α-*N*-(octyloxybenzoyl)-Orn (16).** To a −20 °C of **15f** (41 mg, 0.042 mmol) in sieve (3 A, 13 X) dried, degassed DMF under nitrogen was added the DPPA (12.7 mg, 10.0 μL, 0.046 mmol, 1.1 equiv) followed immediately by solid sodium bicarbonate (17.6 mg, 0.21 mmol, 5.0 equiv). The reaction mixture was stirred at −20 °C for 9 h and then warmed to 0 °C for another 9 h. HPLC analysis (45:55 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 1.0 mL/min) showed the reaction to be complete. The reaction mixture was filtered and the DMF removed in vacuo. Purification was accomplished by HPLC (10 mm × 25 cm Zorbax C8 semi prep column, 45:55 H_2O/(90:10 CH_3CN/H_2O), both 0.1% TFA, 4.0 mL/min flow). Fractions were analyzed by HPLC. Pure cuts were pooled together and lyophilized to give the pure (>95% by HPLC) product as a white fluffy solid (16 mg, 38%). HPLC retention time = 8.50 min; MS (FAB) 952 (M + H).

Registry No. **5**, 141806-00-0; **6**, 141806-01-1; **7**, 123180-69-8; **8**, 106159-24-4; **9a**, 141806-02-2; **9b**, 141806-03-3; **9c**, 141806-04-4; **9d**, 141806-05-5; **9e**, 141806-06-6; **9f**, 141806-07-7; **9g**, 141806-08-8; **10a**, 141806-09-9; **10b**, 141806-10-2; **10c**, 141806-11-3; **10d**, 141806-12-4; **10e**, 141806-13-5; **10f**, 141806-14-6; **10g**, 141806-15-7; **12a**, 110936-12-4; **12b**, 141899-12-9; **12c**, 141806-16-8; **12d**, 141806-17-9; **13a**, 141806-18-0; **13b**, 141806-19-1; **13c**, 141806-20-4; **13d**, 141806-21-5; **13e**, 141806-22-6; **13f**, 141806-23-7; **13g**, 141806-24-8; **14**, 141806-25-9; **15a**, 141806-26-0; **15b**, 141806-27-1; **15c**, 141806-28-2; **15d**, 141806-29-3; **15e**, 141806-30-6; **15f**, 141806-31-7; **16**, 141806-32-8; Cbz-Cl, 501-53-1; Fmoc-Pro-OPfp, 86060-90-4; Fmoc-Thr(Bu-*t*)-ODhbt, 119767-84-9; Fmoc-Tyr(Bu-*t*)-OPfp, 86060-93-7; Boc-Thr(Bn)-OH, 15260-10-3; Boc-Hyp(Bn)-OH, 54631-81-1; Boc-Tyr(BrZ)-OH, 47689-67-8; Boc-Orn(ClZ)-OH, 118554-00-0; Boc-Pro-OH, 15761-39-4; Fmoc-Orn(Boc)-OH, 109425-55-0; 2,6-$Cl_2C_6H_3CH_2Br$, 20443-98-5; 4-[$Me(CH_2)_7O$]C_6H_4COOH, 2493-84-7; (2*S*,3*S*,4*S*)-3-hydroxy-4-methylproline methyl ester hydrochloride, 111002-66-5.

Supplementary Material Available: 300-MHz ^1H NMR spectra in CD_3OD of **5**, **10a–g**, **14**, and **16** (10 pages). Ordering information is given on any current masthead page.

2-Substituted 1-Azabicycloalkanes, a New Class of Non-Opiate Antinociceptive Agents

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2-Substituted 1-azabicycloalkanes (3- and 5-aryloctahydroindolizines **2** and **11**, 3-cyclohexyloctahydroindolizine **12**, 4-aryloctahydroquinolizines **13**, and 3-arylhexahydropyrrolizines **14**) constitute a new class of non-opiate antinociceptive agents. These compounds demonstrated activity in the mouse abdominal constriction test and many were active in the mouse tail-flick test. *trans*-3-(2-Bromophenyl)octahydroindolizine (**2a**) did not bind to the opiate receptor nor did it affect arachidonate metabolism. 3-Aryloctahydroindolizines were prepared by catalytic hydrogenation of 1-aryl-3-(2-pyridinyl)-2-propen-1-ones. The X-ray crystal structure of (−)-**2a** was determined and absolute stereochemistry assigned as 3-*R*,8a-*R*.

Introduction

The mechanisms of pain and its remission (analgesia) have received intensive scientific study. However, despite an ever growing body of knowledge of endogenous nociceptive and antinociceptive systems, clinical treatment of pain today is dominated by two classes of analgesics: the cyclooxygenase inhibitors (aspirin and other NSAIDs) and the opiates (morphine and its synthetic derivatives). The aspirin-like compounds are generally thought of as peripherally acting analgesics with clinical indications for mild to moderate pain. The opiates, on the other hand, produce their action via an interaction with specific receptors in the central nervous system with clinical indications for moderate to severe pain.

The pharmacological profiles of the centrally acting analgesics typically are different from the profiles of peripherally acting analgesics. The mouse abdominal constriction test[1] can be used to detect both peripherally and

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(1) Collier, H. O. J.; Dinneen, L. C.; Johnson, C. A.; Schneider, C. Abdominal Constriction Response and Its Suppression by Analgesic Drugs in the Mouse. *Br. J. Pharmacol. Chemother.* **1968**, *32*, 295–310.

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