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# Total Synthesis and Antifungal Evaluation of Cyclic Aminohexapeptides

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**Abstract**—The need for new therapies to treat systemic fungal infections continues to rise. Naturally occurring hexapeptide echinocandin B (**1**) has shown potent antifungal activity via its inhibition of the synthesis of β-1,3 glucan, a key fungal cell wall component. Although this series of agents has been limited thus far based on their physicochemical characteristics, we have found that the synthesis of analogues bearing an aminoproline residue in the 'northwest' position imparts greatly improved water solubility (> 5 mg/mL). The synthesis and structure–activity relationships (SAR) based on whole cell and upon in vivo activity of the series of compounds are reported. 

## Introduction

The recent demand for more effective treatment of systemic fungal infections has been due, in part, to the increase in the immunosuppressed patient population. The vulnerability of these patients toward commensal infections caused by organisms such as *Candida albicans* and other previously innocuous and rare fungal organisms such as *Fusarium* has also increased this need.[1] The toxicity of older agents such as amphotericin B[2] and the potential for resistance to the well-known azole agents, such as fluconazole,[3] have encouraged further study for the identification of even more potent analogues or new agents having unrelated and novel modes of action. Recently, research efforts have been focused on the inhibition of those particular targets which exhibit fungicidal rather than fungistatic properties, since the penultimate clearance of these slowly invasive infections is paramount for successful therapy in patients whose immune system is challenged.

β-1,3-Glucan is present as the major structural carbohydrate component in the cell wall of many pathogenic fungi.[4] The producing enzyme, β-1,3-glucan synthetase, is a membrane-bound protein utilizing UDP-glucose as the substrate for polymer synthesis. Along with other components, including a GDP-binding protein,[5a] this complex extrudes glucan microfibrils into the interstitial space of the cell where other glycosyltransferases complete the crosslinking steps required to construct the growing cell wall. It has been shown that interference of this process leads to a weakened cell wall and subsequent cell content leakage due to the internal osmotic pressure ultimately results in cell death.[6]

Over the last 10 years the search for novel inhibitors of glucan synthesis has primarily identified two naturally occurring structural classes which have entered clinical study or have shown encouraging preclinical data: (1) the lipopeptide series, typified by echinocandin B (**1**)[7]

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(Fig. 1) and (2) the liposaccharide series, typified by papulacandin.[8] Both series of compounds exhibit potent inhibition of glucan synthesis and display whole cell antifungal activity; however, only the lipopeptide series has exhibited good in vivo activity in rodent models which was similar to that of the industry standard, amphotericin B. A lead series of modified echinocandin B analogues was prepared by the Lilly group leading to LY-303366 (**2**)[9] as a potential drug candidate. The Merck group isolated pneumocandin $B_0$ (**3a**) and, following several functional group modifications, produced L-743,872 (**3b**) as a clinical candidate.[10]

In 1992 the total synthesis of simplified echinocandin analogues using solid-phase techniques was reported by Merck.[11] Structure–activity relationship data from this work showed that several of the functional groups, primarily the hydroxyl groups, were not necessary for antifungal activity, leading to simplified analogues which retained reasonable antifungal activity such as **4** (Fig. 2; compare to structure **1**). This result encouraged us to investigate extending this approach in a more comprehensive manner toward further simplification of this hexapeptide agent. Furthermore, in order to increase the solubility of these defunctionalized analogues, we replaced the 4-methyl-3-hydroxyproline residue of the echinocandins with *cis*-4-amino-(L)-proline. We have found that this aminoproline (Amp) moiety improves both the water solubility and antifungal activity relative to those of the proline or hydroxyproline fragments. Preliminary data toward this end have been presented from a parallel synthesis study.[12] We report herein the effects on antifungal activity of further modifications to several of the amino acid residues in **5(*A, B, C*)** and the lipophilic side-chain in **5(*G*)**.

## Results and Discussion

### Chemistry

The initial stages of this work utilized solid phase synthesis for preparation of the acyclic amino acids. The homotyrosine (hTyr) derivative **6b** was protected as the trimethylsilylethyl (TSE) ester **7** (Scheme 1). This residue was attached to *p*-carboxymethoxybenzyl linker via the phenolic hydroxyl group to give **8b** and loaded onto the BHA resin (see Experimental). Removal of the fluorenylmethoxycarbonyl (Fmoc) group produced **9b** which has the resin-linked hTyr available for chain elongation. In order to procure larger quantities of products for biological testing, preparation of analogues was also carried out via a solution phase approach starting with the di-*t*-butyl protected hTyr derivative **9a**.

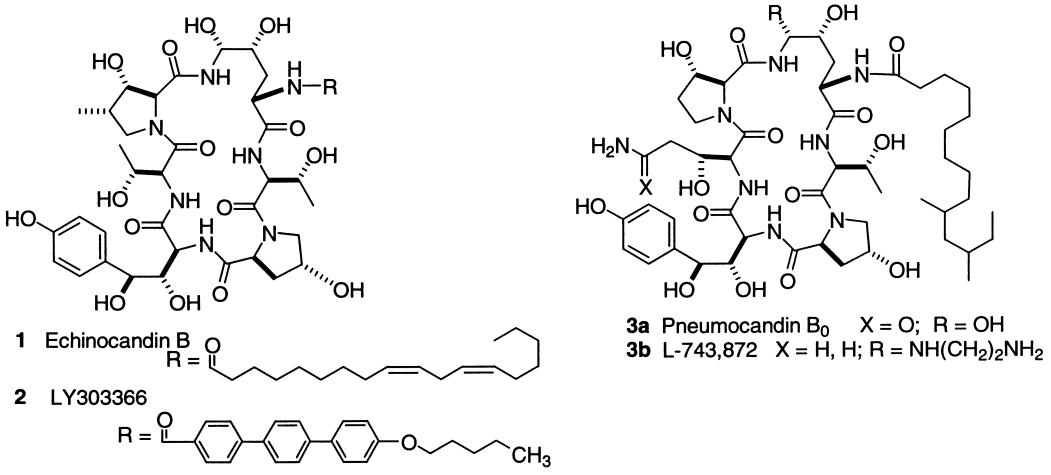



**Figure 1.**

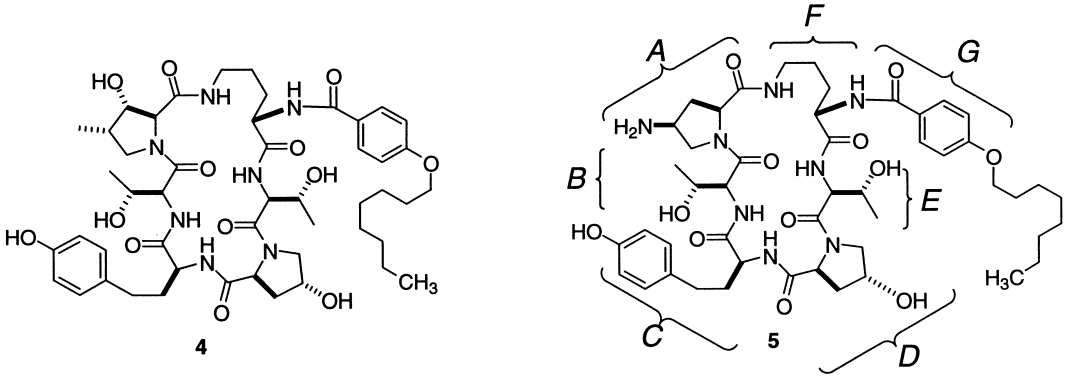
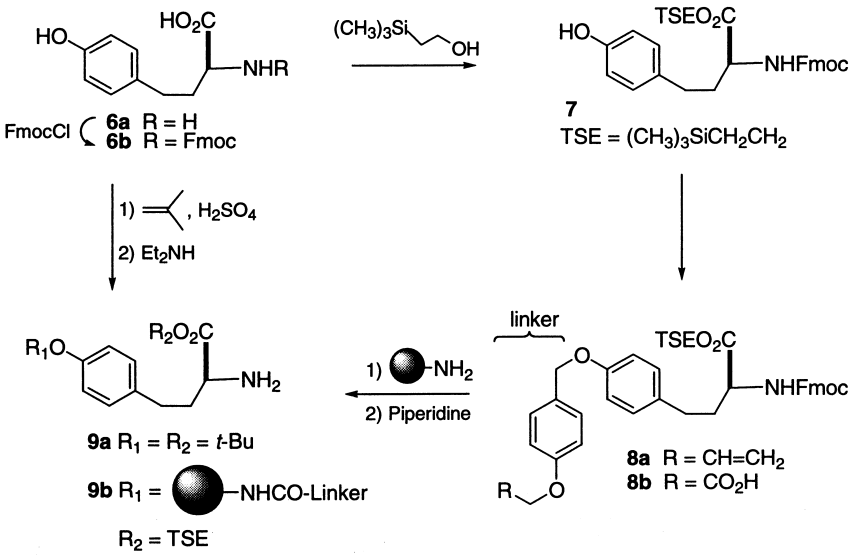


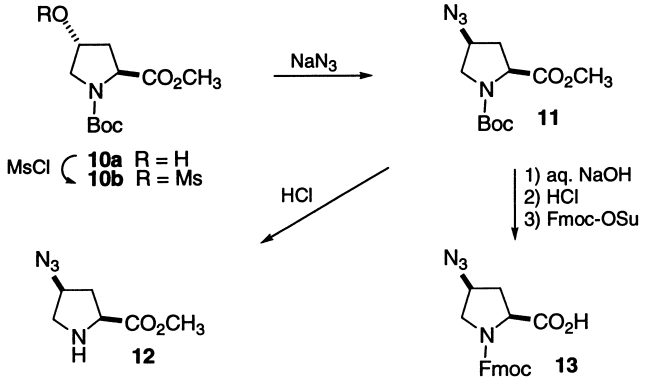

**Figure 2.**



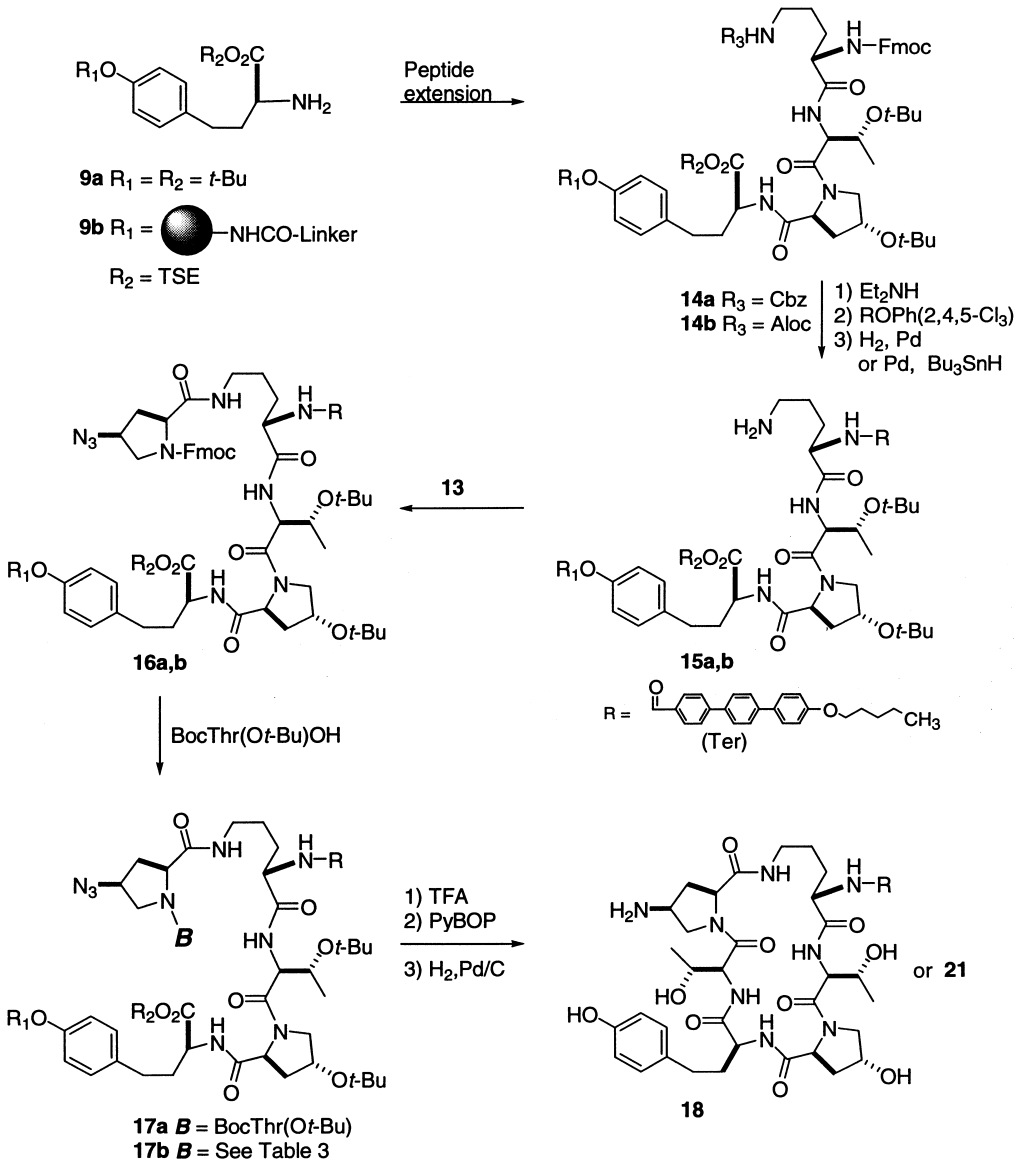

**Scheme 1.**

Free hydroxyl groups and the C-terminal acids of other residues were generally protected as the corresponding *t*-butyl ethers and esters as needed with the N-terminus protected as its Fmoc derivative. Preparation of the azidoproline building blocks used in all approaches commenced from the commercially available BocHyp OMe (**10a**) (Scheme 2) via the *trans*-4-mesylate followed by azide displacement. The product azide was either unblocked to amine **12**, or hydrolyzed and reprotected as Fmoc-acid **13**. A specific example of the hexapeptide preparation is shown in Schemes 3–5. The majority of changes to the proline and threonine positions (residues ***A*** and ***B*** in **5**) were carried out via the intermediate tetrapeptides **14a** and **14b** (Scheme 3), which were, in turn, prepared via a linear coupling/deprotection sequence starting from the deprotected or resin bound hTyr **9a** or **9b**, respectively.[13] The aromatic side-chains were coupled by initial removal of the Fmoc group of **14** and subsequent acylation of this amine via the active side-chain esters as shown for **15**, typically as the corresponding 2,4,5-trichlorophenyl esters.[9] Hydrogenolytic cleavage of the carbobenzyloxycarbonyl (Cbz) group gave the free amine aminopeptide **15**. Standard solution phase coupling conditions were used to extend the chain to hexapeptide **17** in a linear sequence using **13** followed by BocThr(O*t*-Bu)OH. Precyclitive deprotection of the *t*-butyl ethers, carbamates, and esters was carried out using 90% trifluoroacetic acid (TFA) at ambient temperature to give the unprotected hexapeptide **17**. Direct ring closure of this hexapeptide was carried out using either diphenylphosphoryl azide (DPPA) or benzotriazole-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate (PyBOP) and usually afforded 30–60% yield of the cyclized product. Reduction of the azido group with triphenylphosphine (TPP) or catalytic hydrogenation with simultaneous removal of Cbz groups was performed to produce the final aminopeptide analogues shown in Tables 1 and 3.

Scheme 4 illustrates the modified approach for studying variations in the hTyr structure. The precursor pentapeptide, **22**, was constructed from the proline residue at its C-terminus and was coupled with the modified final amino acid **26** via standard conditions. Furthermore, the final deprotection, cyclization, and reduction of the azide in **27** proceeded as for **17** (Scheme 3) to give the hTyr analogues **28** (Table 4).




**Scheme 2.**




**Scheme 3.**

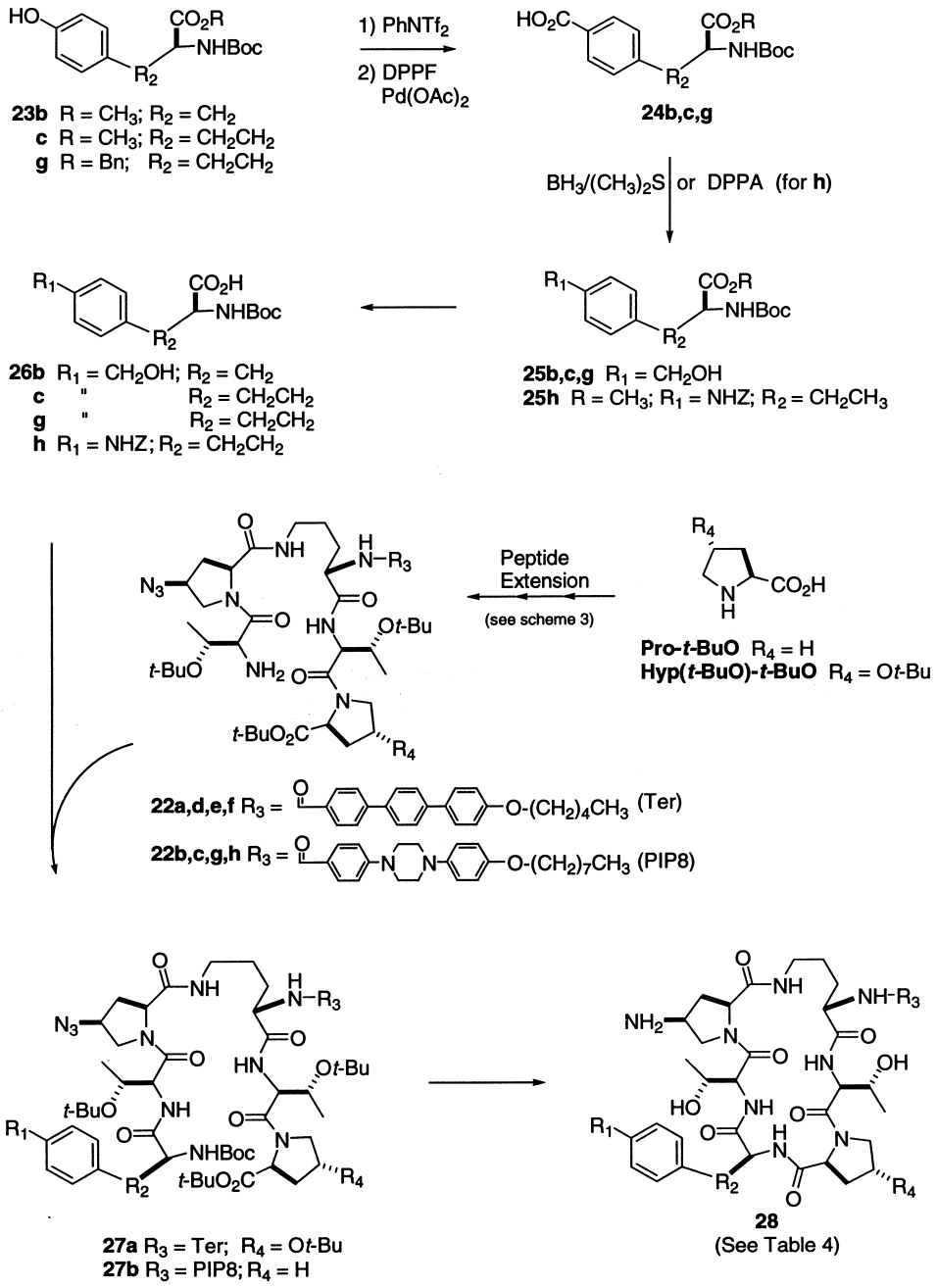
Alternatively, the cyclic hexapeptide was constructed via a '3 + 3' format as in Scheme 5 where the two tripeptides, Azp-AA-Hty **29** and Orn-Thr-AA **30**, are first coupled at the *C-D* position to form linear hexapeptide **31**. Deprotection and cyclization then produces intermediate **32** which has two, or in some cases, three orthogonally protected amino groups available for further modification. Deprotection of the BocOrn group allows for attachment of the side-chain (Table 5). Reduction of the azide in **33** with TPP then allows for alkylation or acylation of the proline amine (Table 2). Finally, in compounds with the Orn(Z) *B* residue, hydrogenolysis is carried out and leads to the final compounds in Table 6.

Characterization of the final compounds is shown in Table 7. In addition to mass spectral analysis, high resolution mass spectral data were obtained for all but two of the analogues which proved difficult to analyze. These two analogues did show >92% purity from HPLC analysis and the desired mass spectral data.

## Structure–Activity Relationships

### Assay descriptions

The mode of action of these compounds has been determined to be inhibition of β-1,3-glucan synthesis as previously described.[5b] The in vitro antifungal activity was evaluated by broth microdilution using amphotericin B as a control. The in vitro activity was quantitated visually as the lowest concentration of compound resulting in inhibition of yeast growth.[14] Those analogues which showed promising in vitro activity or bore novel structural elements were tested in our in vivo mouse model. This acute model has been described previously[15] and afforded protection results after 10

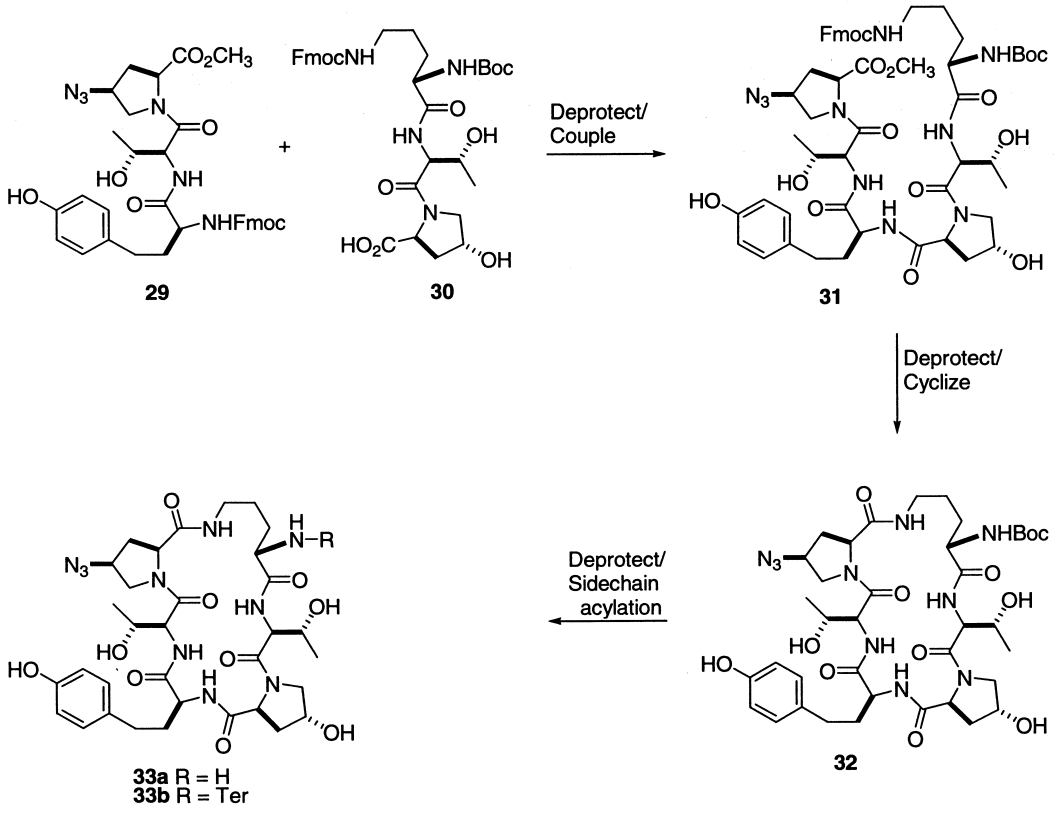



**Scheme 4.**

days. The in vivo data in Tables 1–6 are reported as a ratio to standard amphotericin B to normalize run-to-run variation.

### A. Aminoproline position

Initial structure–activity studies showed that the 4β-aminoproline analogue, **18**, was most active among the stereo- and regioisomeric proline amines listed in Table 1. Furthermore, **18** also showed improved activity as compared to the corresponding 4β-hydroxyl analogue **19b**. The aminomethylproline[16] analogue, **19f**, also exhibited moderate activity. While the unsubstituted proline analogue **19d** exhibited good in vitro activity, this compound was quite insoluble and difficult to formulate for in vivo testing. Introduction of an amino group on the proline ring not only increased the water solubility of these compounds, but also enhanced the in vitro and in vivo antifungal activities of these analogues. Structure–activity studies showed that the 4β-aminoproline analogue **18** was the most active among the stero- and regioisomeric proline amines listed in Table 1. In addition, ease of synthesis proved to be an advantage for **18** as compared to the 3-aminoproline analogue **19a**. While hydroxyprolines **19b** and **19c** exhibited reasonable in vitro activity, no enhancement of the in vivo activity was observed. Similarly, aminoproline analogue **19f** exhibited a moderate boost only in the in vitro




**Scheme 5.**

activity. The aminoproline position proved to be quite sensitive to gross structural modification of this residue. For example, increasing the size of the ring as in **19e** led to a relatively inactive compound. Furthermore, we know from previous work that analogues bearing the opposite (D)-proline stereochemistry exhibited greatly decreased activity.[17] These results suggest that the proline ring may play an important role in the conformational character of the macro-ring structure.

The effect of aminoproline *N*-alkyl and *N*-acyl substituents on the antifungal activity was also studied (Table 2). It was found that as the length of the alkyl residue was extended from methyl **20b** to benzyl **20a** and then to octyl **20c**, the whole cell antifungal activity decreased. This same trend was seen in the case of the acylated amines **20d–g**. Other carboxyl derivatives such as carbamates and ureas, **20k** and **20j**, were also studied. In general the bulk of the corresponding alkyl residues in these series seemed to have a greater effect on diminishing activity than the functionality of these groups. One of the most active substituents on the proline was found to be the guanidino group **20h** which, in addition to retaining good activity, also exhibited excellent solubility characteristics.

## B. Thr position

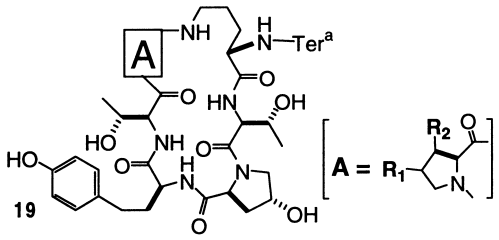
Prior to our work, some data were available for modifications at the ***B*** threonine position such as replacement by serine and glycine as described by Merck[11] and Lilly[18] respectively, in their particular systems. The naturally occurring pneumocandin compounds contain a hydroxyglutamine at this position which had been reduced to an ornithine residue.[19] In our system, minor changes to the threonine group had little effect on the activity (Table 3). Substitution of this residue with serine **21a**, ethylglycine **21b**, and even glycine **21c** did not greatly diminish this activity; however, inversion of the stereochemistry **21d** or disubstitution at the α-carbon **21e** led to analogues with greatly diminished activity. It is not clear whether these effects are due to a conformational change in the ring caused by the α-carbon configuration or result from the positioning of the amino acid side-chain itself. Because of this effect, the (L)-stereochemistry was retained in all other analogues.

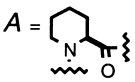
A wide variety of functional groups at this position were found to be compatible with retention of good whole cell antifungal activity. Amine-containing residues (Orn or Dap) had a distinct advantage over carboxylic acid groups (Glu) in the in vivo assay. Carboxylic acid groups arising from glutamic acid **21g**, phenols such as tyrosine **21f**, and amines such as ornithine **21h** or arginine **21j**, all were quite active. In summary, ornithine was considered as the optimal group for this position based upon antifungal activity, water solubility, and cost.

## C. hTyr

Previous work by Merck[11] established that homotyrosine (hTyr) showed activity similar to that of the naturally occurring dihydroxyhomotyrosine whereas the

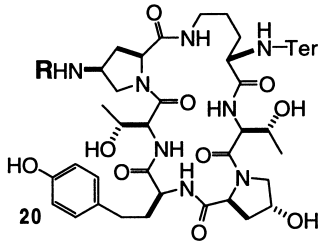
**Table 1.** Antifungal activity of residue *A* analogues




| | | | In vitro activity (μg/mL) | | | In vivo activity |
|---|---|---|---|---|---|---|
| | $R_1$ | $R_2$ | *Candida albicans* ATCC 62376 | *Candida albicans* ATCC 38247 | *Candida glabrata* ATCC 15545 | $ED_{50}/ED_{50}$AmpB |
| **18** |  | H | 0.2 | 0.39 | 0.2 | 10.6 |
| **19a** | H |  | 0.2 | 0.39 | 0.78 | — |
| **19b** |  | H | 0.39 | 1.56 | 3.12 | — |
| **19c** | H |  | 0.2 | 0.78 | 0.39 | > 20 |
| **19d** | H | H | 3.12 | 6.25 | 12.5 | > 20 |
| **19e** |  | | 6.25 | > 100 | 12.5 | — |
| **19f** |  | H | 0.78 | 0.39 | 0.78 | > 20 |

[a]Ter = -(O)C-Ph-Ph-Ph-O-$(CH_2)_4CH_3$.

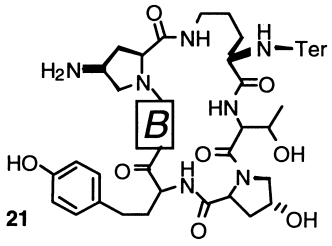
**Table 2.** Antifungal activity of substituted aminoproline analogues




| | | In vitro activity (μg/mL) | | | In vivo activity |
|---|---|---|---|---|---|
| **20** | R | *Candida albicans* ATCC 62376 | *Candida albicans* ATCC 38247 | *Candida glabrata* ATCC 15545 | $ED_{50}/ED_{50}$AmpB |
| **a** | Bn | 0.39 | 6.25 | 1.25 | — |
| **b** | $CH_3$ | 0.2 | 0.39 | 0.39 | 8.4 |
| **c** | Octyl | 6.25 | 50 | 6.25 | — |
| **d** | Ac | 0.2 | 0.39 | 0.39 | > 20 |
| **e** | Octanoyl | 50 | > 100 | > 100 | — |
| **f** | Benzoyl | 6.25 | 25 | 6.25 | — |
| **g** | Succinoyl | 0.78 | 3.12 | 6.25 | > 20 |
| **h** | Guanidinyl | 0.39 | 0.39 | 0.39 | 26 |
| **i** | Boc | 12.5 | > 100 | 100 | — |
| **j** | $CH_3OC(O)$ | 0.39 | 1.25 | 3.12 | — |
| **k** | $CH_3NHC(O)$ | 0.2 | 0.78 | 0.39 | — |

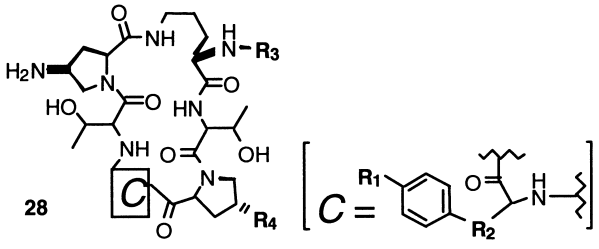
corresponding tyrosine and phenylalanine analogues were much less active. In our system, the results from the tyrosine (Tyr) **28a** and homophenylalanine (hPhe) analogues **28d** (Table 4) were consistent with those previously described, i.e., both were less active than the parent hTyr, with Tyr less active of the two. The three atom oxygen-bridged analogue **28e** prepared from benzylserine shows similar activity to its two-carbon parent; however, the corresponding inverted stereoisomer **28f** was also prepared and, as in the previous cases, exhibited

**Table 3.** Antifungal activity of residue *B* analogues




| 21 | *B* | In vitro activity (μg/mL) | | | In vivo activity |
|---|---|---|---|---|---|
| | | *Candida albicans* ATCC 62376 | *Candida albicans* ATCC 38247 | *Candida glabrata* ATCC 15545 | $ED_{50}/ED_{50}$AmpB |
| **a** | (L)-Serine | 0.1 | 0.2 | 0.1 | 4.98 |
| **b** | (L)-Ethylglycine | 0.1 | 0.39 | 0.1 | 6.49 |
| **c** | (L)-Glycine | 0.2 | 1.56 | 1.56 | — |
| **d** | (D)-Threonine | 12.5 | 6.25 | 3.12 | — |
| **e** | α,α-Dimethylglycine | 50 | 25 | 6.25 | — |
| **f** | (L)-Tyrosine | 1.56 | 1.56 | 0.78 | — |
| **g** | (L)-Glutamic acid | 0.39 | 1.56 | 0.78 | >20 |
| **h** | (L)-Ornithine | 0.2 | 0.39 | 0.2 | 3.01 |
| **i** | (L)-Diaminopropionic acid (Dap) | 1.56 | 0.78 | 0.39 | 21.8 |
| **j** | (L)-Arginine | 0.39 | 0.78 | 0.2 | 11.2 |

**Table 4.** Antifungal activity of residue *C* analogues




| 28 | $R_1$ | $R_2$ | $R_3$ | $R_4$ | In vitro activity (μg/mL) | | | In vivo activity |
|---|---|---|---|---|---|---|---|---|
| | | | | | *Candida albicans* ATCC 62376 | *Candida albicans* ATCC 38247 | *Candida glabrata* ATCC 15545 | $ED_{50}/ED_{50}$AmpB |
| **a** | HO | $CH_2$ | Ter[a] | OH | 50 | 25 | 6.25 | — |
| **b** | $HOCH_2$ | $CH_2$ | PIP8[b] | H | 12.5 | 12.5 | >100 | — |
| **c** | $HOCH_2$ | $CH_2CH_2$ | PIP8 | H | 1.56 | 3.12 | 1.56 | >20 |
| **d** | H | $CH_2CH_2$ | Ter | OH | 1.56 | 3.12 | 3.12 | >20 |
| **e** | H | $CH_2$-O-$CH_2$ | Ter | OH | 1.56 | 3.12 | 3.12 | — |
| **f** | H (*D*) | $CH_2$-O-$CH_2$ | Ter | OH | >100 | 25 | 25 | — |
| **g** | $CH_3$ | $CH_2CH_2$ | PIP8 | H | 3.12 | 6.25 | 3.12 | — |
| **h** | $NH_2$ | $CH_2CH_2$ | PIP8 | H | 0.2 | 0.78 | 0.78 | >20 |

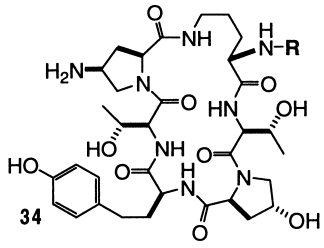
[a]Ter = -(O)C-Ph-Ph-Ph-O-$(CH_2)_4CH_3$.
[b]PIP8 = (O)C-Ph-piperazine-Ph-O-$(CH_2)_6CH_3$.

much lower activity. The importance of the phenolic hydroxyl group was studied by testing the hydroxymethyl **28c**, methyl **28g**, and amino **28h** group replacements, though only the latter derivative had near optimal antifungal activity. All one-carbon linked analogues **28a,b** exhibited poor activity regardless of retention of the phenolic or hydroxymethyl group. These SAR results suggest that these chain shortened analogues may suffer a conformational change due to closer interactions of the aryl group with the macrocycle ring.

## D. Side-chain

Problems related to hemolysis led the Lilly group to modify the side-chain of echinocandin B.[9] Their SAR work led initially to a 4-(*n*-octyloxy)benzoyl(*p*-OB) chain (Cilofungin, **2**, R = *p*-OB), which later evolved to the 4-(*n*-pentyloxy)terphenoyl group as being the optimal side-chain for their system. Aspects of lipophilicity, length, and shape of this side-chain were also considered as variables in our work, though not as comprehensively

**Table 5.** Antifungal activity of side-chain analogues



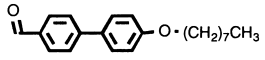
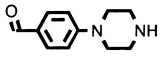
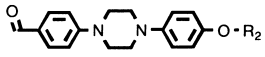
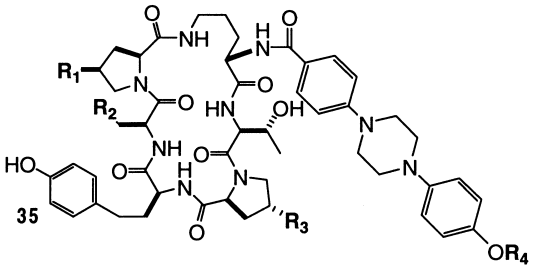
| 34 | R | In vitro activity (μg/mL) | | | In vivo activity |
|---|---|---|---|---|---|
| | | *Candida albicans* ATCC 62376 | *Candida albicans* ATCC 38247 | *Candida glabrata* ATCC 15545 | $ED_{50}/ED_{50}$AmpB |
| **a** | Ac | > 100 | > 100 | > 100 | — |
| **b** | $C(O)(CH_2)_{13}CH_3$ | 0.78 | 0.78 | 1.25 | > 20 |
| **c** | Bz | > 100 | > 100 | > 100 | — |
| **d** |  | 0.2 | 0.39 | 12.5 | 4 |
| **e** |  | > 100 | > 100 | > 100 | — |
| **f** |  $R_2$=H | 25 | 50 | 50 | — |
| **g** | $CH_3$ | 12.5 | 3.12 | 12.5 | — |
| **h** | $C_5H_{11}$ | 0.2 | 0.2 | 0.69 | > 20 |
| **i** | $C_8H_{17}$ | 0.2 | 0.2 | 0.39 | 7.63 |
| **j** | $C_{12}H_{25}$ | 0.78 | 1.56 | 0.39 | > 20 |
| **k** | $CH_2$-(*c*-$C_6H_{11}$) | 0.2 | 0.2 | 0.2 | 10 |

**Table 6.** Antifungal activity of combination analogues



| 35 | $R_1$ | $R_2$ | $R_3$ | $R_4$ | In vitro activity (μg/mL) | | | In vivo activity |
|---|---|---|---|---|---|---|---|---|
| | | | | | *Candida albicans* ATCC 62376 | *Candida albicans* ATCC 38247 | *Candida glabrata* ATCC 15545 | $ED_{50}/ED_{50}$AmpB |
| **a** | $NH_2$ | $NH_2$ | OH | *n*-$C_5H_{11}$ | 0.78 | 0.78 | 0.78 | 13.6 |
| **b** | Guanidino | $NH_2$ | OH | *n*-$C_5H_{11}$ | 0.39 | 1.56 | 1.56 | 13.5 |
| **c** | $NH_2$ | $NH_2$ | OH | *n*-$C_8H_{17}$ | 0.78 | 0.78 | 0.78 | 3.8 |
| **d** | Guanidino | $NH_2$ | OH | *n*-$C_8H_{17}$ | 0.39 | 0.78 | 0.39 | 2.16 |
| **e** | $NH_2$ | $H_2NCH_2CH_2$ | OH | *n*-$C_8H_{17}$ | 0.2 | 0.39 | 0.2 | 1.4 |
| **f** | Guanidino | $H_2NCH_2CH_2$ | OH | *n*-$C_8H_{17}$ | 0.78 | 0.78 | 0.39 | 1.5 |
| **g** | $NH_2$ | $H_2NCH_2CH_2$ | H | *n*-$C_8H_{17}$ | 0.2 | 0.39 | 0.2 | 0.38 |
| **h** | Guanidino | $H_2NCH_2CH_2$ | H | *n*-$C_8H_{17}$ | 0.2 | 0.2 | 0.2 | 0.2 |

as in the previous study. Whereas the acetyl and benzoyl analogues (**34a** and **34c**) are inactive on our hexapeptide (Table 5), extending or appending a hydrophobic chain onto either recovers the activity. Side-chain length and rigidity components were further studied by addition of ring units, e.g., from 4-(*n*-octyloxy)benzoyl, to 4′-(octyloxyphenyl)benzoyl and 4″-(*n*-octyloxy)terphenoyl, which showed increasing activity.

The Fujisawa group[20] patented a pneumocandin-like series bearing a 1,4-diphenylpiperazine based side-chain for improved potency which also increased water solubility. We prepared several analogues using this side-chain and also several truncated versions which showed that an unsubstituted piperazine **34e** or phenolic group **34f** led to a loss of activity. As the length of the alkyl residue ($R_2$ in Table 5) is increased to an eight carbon

**Table 7.** Characterization of target compounds

| Compound | MS (FAB) *m/z* | HRMS Calculated for | HRMS Measured | HPLC[a] %/$t_R$ | Compound | MS (FAB) *m/z* | HRMS Calculated for | HRMS Measured | HPLC[a] %/$t_R$ |
|---|---|---|---|---|---|---|---|---|---|
| Table 7a | | | | | Table 7d | | | | |
| **18** | 1061 [M+H$^+$] | $C_{57}H_{73}N_8O_{12}$ 1061.5348 | 1061.5363 | 100/13.9 | **28a** | 1047 [M+H$^+$] | $C_{56}H_{70}N_8O_{12}$ 1047.5191 | 1047.516 | 83/14.2[e] |
| **19a** | 1099 [M+K$^+$] | $C_{57}H_{73}N_8O_{12}$ 1061.5348 | 1061.5327 | 100/13.4 | **28b** | 1095 [M+H$^+$] | $C_{58}H_{83}N_{10}O_{11}$ 1095.6243 | 1095.6238 | 98/10.7 |
| **19b** | 1062 [M+H$^+$] | $C_{57}H_{71}N_7O_{13}$ 1062.5188 | 1062.5165 | 81/7.4 | **28c** | 1109 [M+H$^+$] | $C_{59}H_{85}N_{10}O_{11}$ | f | 93/11.3 |
| **19c** | 1062 [M+H$^+$] | $C_{57}H_{71}N_7O_{13}$ 1062.5188 | 1062.5182 | 100/14.7 | **28d** | 1045 [M+H$^+$] | $C_{57}H_{72}N_8O_{11}$ 1045.5399 | 1045.540 | 96/12.3[d] |
| **19d** | 1046 [M+H$^+$] | $C_{57}H_{71}N_7O_{12}$ 1046.5239 | 1046.5263 | 95/16.2 | **28e** | 1061 [M+H$^+$] | $C_{57}H_{72}N_8O_{12}$ 1061.5348 | 1061.537 | 98/12.4[d] |
| **19e** | 1060 [M+H$^+$] | $C_{58}H_{74}N_7O_{12}$ 1060.5395 | 1060.5408 | 87/16.3 | **28f** | 1095 [M+H$^+$] | $C_{58}H_{83}N_{10}O_{11}$ 1095.6243 | 1095.6245 | 90/12.5 |
| **19f** | 1075 [M+H$^+$] | $C_{58}H_{75}N_8O_{12}$ 1075.5504 | 1075.5485 | 100/13.3 | **28g** | 1094 [M+H$^+$] | $C_{59}H_{85}N_{10}O_{10}$ 1094.6450 | 1094.6450 | 98/13.9 |
| Table 7b | | | | | **28h** | 1094 [M+H$^+$] | $C_{58}H_{84}N_{11}O_{10}$ | f | 97/10.6 |
| **20a** | 1151 [M+H$^+$] | $C_{64}N_{79}N_8O_{12}$ 1151.5817 | 1151.5844 | 83/17.65 | Table 7e | | | | |
| **20b** | 1075 [M+H$^+$] | $C_{58}H_{75}N_8O_{12}$ 1075.5504 | 1075.5514 | 100/14.4 | **34a** | 761 [M+H$^+$] | $C_{35}H_{53}N_8O_{11}$ 761.3834 | 761.3853 | 89.9/21.4 |
| **20c** | 1173 [M+H$^+$] | $C_{65}H_{89}N_8O_{12}$ 1173.6600 | 1173.6625 | 84/22 | **34b** | 943 [M+H$^+$] | $C_{48}H_{78}N_8O_{11}$ 943.5868 | 943.5860 | 92.8/13.6 |
| **20d** | 1103 [M+H$^+$] | $C_{59}H_{75}N_8O_{13}$ 1103.5454 | 1103.5469 | 100/14.7 | **34c** | 823 [M+H$^+$] | $C_{40}H_{54}N_8O_{11}$ 823.3990 | 823.3981 | 97.1/3.8 |
| **20e** | 1187 [M+H$^+$] | $C_{65}H_{87}N_8O_{13}$ 1187.6393 | 1187.6409 | 79/12.0 | **34d** | 1027 [M+H$^+$] | $C_{54}H_{74}N_8O_{12}$ 1027.5504 | 1027.5507 | 90/14.6 |
| **20f** | 1203 [M+K$^+$] | $C_{64}H_{77}N_8O_{13}$ 1165.5610 | 1165.5626 | 100/18.6 | **34e** | 907 [M+H$^+$] | $C_{44}H_{62}N_{10}O_{11}$ 907.4678 | 907.4694 | 94.3/8.1[b] |
| **20g** | 1161 [M+H$^+$] | $C_{61}H_{77}N_8O_{15}$ 1161.5508 | 1161.5491 | 100/13.6 | **34f** | 999 [M+H$^+$] | $C_{50}H_{67}N_{10}O_{12}$ 999.4940 | 999.4959 | 89/3.8 |
| **20h** | 1103 [M+H$^+$] | $C_{58}H_{75}N_{10}O_{12}$ 1103.5566 | 1103.5544 | 100/14.2 | **34g** | 1013 [M+H$^+$] | $C_{51}H_{68}N_{10}O_{12}$ 1013.5096 | 1013.5095 | 88.2/3.8 |
| **20i** | 1161 [M+H$^+$] | $C_{62}H_{81}N_8O_{14}$ 1161.5872 | 1161.5875 | 90/19.2 | **34h** | 1069 [M+H$^+$] | $C_{55}H_{76}N_{10}O_{12}$ 1069.5722 | 1069.5726 | 93.1/5.5 |
| **20j** | 1119 [M+H$^+$] | $C_{59}H_{75}N_8O_{14}$ 1119.5403 | 1119.541 | 100/15.8 | **34i** | 1111 [M+H$^+$] | $C_{58}H_{83}N_{10}O_{12}$ 1111.6192 | 1111.6188 | 92.8/10 |
| **20k** | 1156 [M+K$^+$] | $C_{59}H_{76}N_9O_{13}$ 1118.5563 | 1118.5575 | 100/14.6 | **34j** | 1167 [M+H$^+$] | $C_{62}H_{91}N_{10}O_{12}$ 1167.6818 | 1167.6830 | 83/18 |
| Table 7c | | | | | **34k** | 1095 [M+H$^+$] | $C_{57}H_{79}N_{10}O_{12}$ 1095.5879 | 1095.5903 | 93/7 |
| **21a** | 1085 [M+K$^+$] | $C_{56}H_{70}N_8O_{12}K$ 1085.4750 | 1085.4745 | 98/13.2 | Table 7f | | | | |
| **21b** | 1083 [M+K$^+$] | $C_{57}H_{72}N_8O_{11}K$ 1083.4958 | 1083.4937 | 99/13.8 | **35a** | 1054 [M+H$^+$] | $C_{54}H_{76}N_{11}O_{11}$ 1054.5726 | 1054.5740 | 70/5.4 |
| **21c** | 1055 [M+K$^+$] | $C_{55}H_{68}N_8O_{11}K$ 1055.4645 | 1055.4653 | 97/13.2 | **35b** | 1096 [M+H$^+$] | $C_{55}H_{78}N_{13}O_{11}$ 1096.5944 | 1096.5917 | 87/5.1 |
| **21d** | 1085 [M+K$^+$] | $C_{56}H_{70}N_8O_{12}K$ 1085.4750 | 1085.4745 | 91/13.2 | **35c** | 1096 [M+H$^+$] | $C_{57}H_{82}N_{11}O_{10}$ 1095.615 | 1095.6201 | 90/9.8 |
| **21e** | 1045 [M+H$^+$] | $C_{57}H_{73}N_8O_{11}$ 1045.5399 | 1045.5399 | 98/14.1 | **35d** | 1138 [M+H$^+$] | $C_{58}H_{84}N_{13}O_{11}$ 1138.6413 | 1138.6416 | 90/9.8 |
| **21f** | 1123 [M+H$^+$] | $C_{62}H_{75}N_8O_{12}$ 1123.5504 | 1123.547 | 99/14.1[c] | **35e** | 1124 [M+H$^+$] | $C_{59}H_{86}N_{11}O_{11}$ 1124.6508 | 1124.6493 | 95/8.9 |
| **21g** | 1089 [M+H$^+$] | $C_{58}H_{73}N_8O_{13}$ 1089.5297 | 1089.5293 | 100/13.2 | **35f** | 1166 [M+H$^+$] | $C_{60}H_{88}N_{13}O_{11}$ 1166.6726 | 1166.6686 | 90/9.3 |
| **21h** | 1112 [M+K$^+$] | $C_{58}H_{75}N_9O_{11}K$ 1112.5223 | 1112.5223 | 100/11.7 | **35g** | 1108 [M+H$^+$] | $C_{59}H_{86}N_{11}O_{10}$ 1108.6559 | 1108.6534 | 98/11.8[e] |
| **21i** | 1084 [M+K$^+$] | $C_{56}H_{72}N_9O_{11}$ 1046.5351 | 1046.5347 | 98/11.0[c] | **35h** | 1150 [M+H$^+$] | $C_{60}H_{87}N_{13}O_{10}$ 1150.6777 | 1150.677 | 98/11.9[e] |
| **21j** | 1116 [M+H$^+$] | $C_{59}H_{78}N_{11}O_{11}$ 1116.5882 | 1116.588 | 95/12.1 | | | | | |

[a]Purity determined by reversed phase HPLC analysis. Unless otherwise stated, reversed phase HPLC analysis was carried out using the following method: column: dynamax C18 (25 cm L, 4.6 mm ID, 5 m, 300 A); flow rate: 1 mL/min; mobile phase: B acetonitrile, A 0.1% TFA in water; gradient: linear from 45 to 75% B in 14.56 min, from 75 to 95% B in 0.44 min; detection: UV, 254 nm.
[b]Same method as (a) except: gradient: linear from 10 to 75% B in 14.56 min.
[c]Same method as (a) except: flow rate: 2 mL/min; column: Waters Delta-Pak C18 (20 cm L, 8 mm ID, 15 m, 300 A).
[d]Same method as (c) except: gradient: linear from 45 to 95% B in 15 min.
[e]Same method as (c) except: gradient: linear from 25 to 95% B in 15 min.
[f]We were unable to obtain HRMS data of these samples prior to exhaustion of sample although the mass spectral and HPLC data clearly showed the expected molecular weight and purity.

chain as for **34i**, antifungal activity increases, whereas longer residues show poorer results. In terms of the whole cell and in vivo antifungal activity, and water solubility, we found the octyloxyphenyl-piperazinylbenzoyl **34i** and cyclohexylmethyl **34k** side-chains to be optimal.

### E. Other residues

Regarding the remaining residues in the cyclic hexapeptide, it was shown that replacing the Hyp *D* (Fig. 2) ring with Amp, similar to the *A* 'corner' of the molecule, led to compounds showing poor activity; however, substitution of Hyp with Pro afforded compounds which retained activity but minimized cost and protecting group requirements.[21] Other modifications to the *D*, *E*, and *F* residues that have been carried out will be reported at a later date.[22]

### F. Combination analogues

The SAR information from the previous studies was used to design and prepare an optimized set of analogues relative to their potency, water solubility,[23] and efficacy. The optimum groups were chosen from the study of residue *A* (4β-amino and guanidino groups), from residue *B* (diaminopropionic (Dap) acid and ornithine (Orn) groups), and from residue *G* (*n*-octyl- and *n*-pentyloxyphenylpiperazinylbenzoyl side-chains) (Table 6). The guanidinoproline group showed equal or slightly better activity as compared to the Amp group with improved solubility characteristics, and thus was considered the best choice at residue *A*. At residue *B,* placement of Dap resulted in analogues **35c,d** with no clear advantage over the less costly Orn analogues **35e,f**. Optimum efficacy was obtained with the octyloxy moiety (**35c,d**) in the side-chain residue *G* over the pentyloxy analogues **35a,b**. While the last four entries in Table 6 were all quite potent as antifungal agents both in the whole cell and mouse model assays, entry **35h** was chosen as the best compound in our series based on the total biological and chemical profile.

## Conclusion

We have shown that incorporation of an aminoproline residue into the ring of these cyclic hexapeptide antifungal agents leads to improvements of potency, in vivo efficacy, along with obvious effects on physico-chemical characteristics such as water solubility. We have shown that by combining several optimally modified moieties in this series, the resultant analogue exhibits 5-fold greater in vivo activity in our mouse model as compared to amphotericin B.

## Experimental

All reactions were performed at room temperature (25 °C) unless otherwise stated. Standard work up refers to quenching coupling mixtures with methylene chloride (DCM) and 10% citric acid. The organic layer was washed consecutively with saturated $NaHCO_3$, $H_2O$, and brine, and then dried over $Na_2SO_4$, filtered and the solvent evaporated. All purifications were performed with silica gel (E. Merck silica gel 60, 230–400 mesh) flash chromatography unless otherwise stated. Methylene chloride (DCM) was distilled from calcium hydride, and tetrahydrofuran (THF) was distilled from sodium–benzophenone. The reagents used for coupling were *N*-hydroxybenzotriazole (HOBT), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDAC), diisopropylcarbodiimide (DIPC), dicyclohexylcarbodiimide (DCC), *N*-ethoxycarbonyl-2-ethoxy-1,2-dehydroquinoline (EEDQ, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC), and benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP). Mass spectral data were recorded with a Finnigan SSQ 7000 instrument, and high resolution MS were obtained on a Kratos MS 50 instrument.

### Biological evaluation

**Whole cell antifungal assay.** Antifungal activity was evaluated by broth microdilution. Compounds were dissolved in DMSO, then diluted in yeast nitrogen broth (Difco Laboratories, Detroit, MI) containing 5 g/L dextrose, pH 7.0, in serial 2-fold steps. Amphotericin B was included as a control. The tests were inoculated with the yeast strains at approximately $10^4$ colony-forming units per mL prepared from an overnight culture on Sabouraud dextrose agar. The cultures were diluted to a turbidity equivalent to 0.5 McFarland standard, then inoculated at a final dilution of 1:100. Tests were incubated for 18–20 h at 35 °C. The in vitro activity was determined visually as the lowest concentration of compound resulting in inhibition of yeast growth. The yeast strains were clinical isolates purchased from the American Type Culture Collection, Rockville, MD. *C. albicans* ATCC 62376 and *C. glabrata* ATCC 15545 were susceptible isolates and *C. albicans* ATCC 38247 is resistant to polyenes.

**Acute *Candida* mouse model.**[24] For efficacy evaluation of antifungal compounds, female CF-I outbred mice (Charles River Laboratories) of approximately 8 weeks of age and 25 g body mass were inoculated intravenously with undiluted overnight culture of *C. albicans* CAF2 grown in Sabauroud broth. The inocula were approximately $2\times10^6$ cfu/mouse, and generally resulted in mortality of untreated mice within 24 h post inoculation. Test compounds were formulated in 2% DMSO/PBS for injection and were administered by intraperitoneal injection at 1 h post inoculation. There were 10 mice per dosage group.

Mice were monitored daily for survival for 10 days. The percent survival rate indicates the percentage of mice per treatment group surviving to the termination of the trial at day 10 post inoculation. Curative therapy was determined by plating kidney homogenates of individual surviving mice on Sabouraud dextose agar. Kidney homogenates absent of *C. albicans* growth were indicative of curative efficacy. The percent cure rate indicates the percentage of mice per treatment group with sterilized

kidneys. The mean effective dose to protect 50% of the mice ($ED_{50}$ value) was calculated from cumulative mortality by logarithmic-probit analysis of a plotted curve of survival versus dosage. The mean $ED_{50}$ value for the control, amphotericin B, was 1.62 mg/kg with the standard deviation (S.D.) being 1.148.

**Fmoc-hTyrOH (6b).** To a 0 °C solution of homotyrosine hydrobromide **6a** (4 g, 15 mmol) in 30% aq dioxane (40 mL) was added 20% aq sodium carbonate (35 mL) followed by a solution of FmocCl (4 g, 16 mmol) in dioxane (20 mL). The mixture was allowed to warm to 25 °C for 2 h and neutralized with 2 N HCl. The mixture was extracted twice with ethyl acetate, the organic layer washed with brine, dried over sodium sulfate, and the solvents were evaporated. The crude residue was purified by chromatography using hexane:ethyl acetate (2:1) to give 4.67 g (77%) of **6b**.

**hTyr(O*t*-Bu)O*t*-Bu (9a).** A mixture of **6b** (20.8 g, 50 mmol) and isobutylene (400 mL) was combined in a metal bomb with sulfuric acid (4.2 mL) and DCM (400 mL) for 3 days. Saturated sodium bicarbonate was added (400 mL) and the aqueous layer extracted twice with DCM. The solvents were evaporated and the crude residue was purified using hexane:ethyl acetate (8:1–4:1) to give 16.7 g (63%) of the di-*t*-butyl Fmoc-hTyr. This material was dissolved in DMF (250 mL) and treated with diethylamine (30 mL, 10 equiv) for 4 h. The solvents were evaporated to give crude **9a** which was used directly for the synthesis of tetrapeptide **14a**.

***N*-Boc-4-(*R*)-mesyl-(L)-proline methyl ester (10b).** A solution of Boc-(*R*)-HypOMe (**10a**) (6.2 g, 25.3 mmol) in pyridine (50 mL) was treated with methanesulfonyl chloride (4 mL) at 0 °C, and was warmed to 25 °C overnight. The reaction mixture was diluted with ethyl acetate and washed with 0.5 N HCl and brine. The organic layer was separated and the solvent was evaporated. The residue was triturated with heptane and the solids were filtered to give 8 g (98%) of **10b**.

***N*-Boc-4-(*S*)-azido-(L)-proline methyl ester (11).** A solution of **10b** (7.5 g, 23 mmol) and sodium azide (7.5 g, 115 mmol) in dimethylformamide (30 mL) was heated at 60 °C overnight. After cooling to 25 °C the reaction mixture was diluted with ethyl acetate and washed with water. The aqueous layer was extracted with ethyl acetate, the organic layers combined, and washed with brine. The solvents were evaporated to give 6.74 g of BocAzpOMe (**11**) as an oil which was used directly in the next step.

**4-(*S*)-Azido-(L)-proline methyl ester hydrochloride (12).** A solution of **11** in 2.8 N HCl in ethyl acetate (260 mL) was stirred for 1 h. The solvent was evaporated and the residue treated with methyl *t*-butyl ether (MTBE) at 0 °C. The solids were filtered, washed with MTBE, and dried in vacuo for 2 h to give **12** which was used directly for the next step.

**Fmoc-4-(*S*)-AzpOH (13).** To a solution of **12** (27.3 g, 0.1 mol) in methanol (50 mL) and dioxane (50 mL) was added 2 N sodium hydroxide solution (100 mL) and the mixture was stirred for 1 h. After evaporation of the methanol, dioxane (275 mL) was added along with concd HCl to pH 2 followed by more HCl (19 mL). The mixture was warmed to 50 °C for 2 h and cooled to 0 °C. Sodium carbonate (32.3 g, 0.3 mol) was added to pH 9 followed by Fmoc-OSu (27 g, 80 mmol) in dioxane (75 mL). After 2 h at 0 °C, the mixture was warmed to 25 °C for 18 h. The mixture was filtered through Celite, diluted with ethyl acetate (1.2 L) and washed with 2 N HCl (250 mL), brine, dried, and the solvent was evaporated. The residue was purified using 1% methanol/DCM with 0.1% acetic acid to give 22.8 g (60%) of **13**.

**Tetrapeptide Fmoc(Z)Orn-Thr(O*t*-Bu)-Hyp(O*t*-Bu)-hTyr(O*t*-Bu)O*t*-Bu (14a).** (i) To a 0 °C solution of **9a** (8.6 g, 28 mmol) and FmocHyp(OtBu) (11.43 g, 28 mmol) were added CMC (17.74 g, 42 mmol) and HOBT (5.66 g, 42 mmol), and the mixture was stirred overnight and allowed to warm to 25 °C. After the standard work up, the residue was purified using 2–15% ethyl acetate/DCM as the eluent to give 16.7 g (86%) of FmocHyp-(O*t*-Bu)hTyr(O*t*-Bu)O*t*-Bu.

(ii) The product from above was treated with diethylamine (25 mL) in DMF (275 mL) for 2 h. The solvents were evaporated, and the crude residue was dissolved in DCM (200 mL) and cooled to 0 °C. To this solution were added FmocThr(O*t*-Bu) (10.3 g, 24 mmol), CMC (15.2 g, 36 mmol), and HOBT (4.8 g, 36 mmol) and the mixture was stirred overnight and allowed to warm to 25 °C. After the standard work up, the residue was purified with 2.5% ethyl acetate/DCM to give 17.3 g (84%) of FmocThr(O*t*-Bu)-Hyp(O*t*-Bu)-hTyr(O*t*-Bu)O*t*-Bu.

(iii) Preparation of FmocOrn(Z)OH: To a 0 °C suspension of Orn(Z)OH (19.9 g, 75 mmol) in dioxane (190 mL) and aqueous sodium carbonate (9 g, 84 mmol in 70 mL water) was added FmocOSu (27.8 g, 82 mmol). The mixture was stirred overnight and allowed to warm to 25 °C. The mixture was diluted with ethyl acetate and water, and the pH was adjusted to 1 before twice extracting with ethyl acetate, drying over $Na_2SO_4$, and evaporating the solvent to give FmocOrn(Z)OH which was used directly in the next step.

(iv) The product from (ii) above (15.2 g, 24 mmol) was treated with diethylamine (19 mL) in DMF (230 mL) for 2 h. The solvents were evaporated and the crude residue was dissolved in DCM (150 mL) and cooled to 0 °C. To this solution was added FmocOrn(Z)OH (8.75 g, 18 mmol) from (iii) above, CMC (11.3 g, 27 mmol) and HOBT (3.63 g, 27 mmol) and the mixture was stirred overnight and allowed to warm to 25 °C. After the standard work up the residue was purified using 25–50% ethyl acetate/DCM to give 16.8 g (67%) of Fmoc(Z)Orn-Thr(O*t*-Bu)-Hyp(O*t*-Bu)-Tyr(O*t*-Bu)O*t*-Bu **14a**.

**RCONHOrn-Thr(O*t*-Bu)-Hyp(O*t*-Bu)-hTyr(O*t*-Bu)O*t*-Bu (15a).** (i) Tetrapeptide **14a** (0.5 g, 0.52 mmol) was treated with diethylamine (0.5 mL) in DMF (25 mL) for 2 h. The solvents were evaporated and the residue was

treated with 2,4,5-trichlorophenyl 4-(4′-pentyloxybipheny1)benzoate[20] (0.31 g, 0.57 mmol) in DMF (60 mL) at 60 °C for 3 h. The solvents were evaporated, and the residue was purified using 50% ethyl acetate/hexane as the eluent to give 0.45 g (69%) of the acylated product.

(ii) The product from above (0.5 g, 0.4 mmol) was treated with $Pd(OH)_2$ on carbon (20% Pd by wt, 83 mg) in ethanol (10 mL) and acetic acid (0.065 mL). A hydrogen balloon was used for the reduction which took place over 4 h. The catalyst was filtered and the solvents were evaporated. The residue was dissolved in ethyl acetate and washed consecutively with saturated sodium bicarbonate, water, and brine and dried over sodium sulfate. The solvents were evaporated and the product amine **15a** (74 mg, 100%) was used directly for the next step.

**Azido pentapeptide (16a).** To a solution of **15a** (5 g, 4.6 mmol) in DCM (50 mL) at 0 °C was added **13** (2.3 g, 6 mmol), CMC (3 g, 7.1 mmol) and HOBT (1 g, 7.4 mmol) and the mixture was stirred overnight at 25 °C. After the standard work up, the residue was purified using ethyl acetate:hexane (7:3) to give 5.5 g (83%) of the protected pentapeptide **16a**.

**Azido hexapeptide (17a).** Pentapeptide **16a** (2.29 g, 1.5 mmol) was treated with diethylamine (1.7 mL) in DMF (20 mL) for 2 h. The solvents were evaporated and the residue was dissolved in DCM (25 mL) at 0 °C. To this solution was added the N-Boc amino acid (2.1 equiv) (for **18**: BocThr(*t*-BuO)OH, 0.85 g, 3.l mmol), CMC (1.99 g, 4.7 mmol) and HOBT (0.63 g, 4.7 mmol) and the mixture was warmed to 25 °C overnight. Standard work up afforded 1.99 g (85%) crude **17**.

**Cyclic hexapeptide (18).** Product **17** (1.08 mmol) was treated with 90% trifluoroacetic acid (10 mL) at 0–25 °C for 5 h. The solvent was evaporated and the crude residue was treated with PyBOP (1.68 g, 3.2 mmol), HOBT (0.44 g, 3.2 mmol) and diisopropylethylamine (DIPEA) (1.23 mL) in DMF (1000 mL) for 24 h. The solvent was evaporated and the residue was dissolved with minimal amounts of methanol followed by water to effect precipitation. The solid product was filtered and purified using 5–12.5% methanol in DCM to give 0.977 g (68%) of the cyclized azide. This azide was treated with 10% Pd/C (0.2 g) in methanol (60 mL) with a hydrogen balloon overnight. The reaction mixture was filtered and the solvent was evaporated. The crude product was purified using 15–20% methanol/10% ammonia/DCM to give 0.52 g (66%) of **18**.

## Section A. Preparation of residue *A* analogues (19,20)

**a. Preparation of aminoproline replacements (19a–f).** Residue *A* analogues appearing in Table 1 were prepared in a similar manner from **14a** as **18**. The aminomethyl proline leading to analogue **19f** was prepared as in ref 16.

**19a.** Intermediate **15a** was treated with the Fmoc-3β-azidoProOH as in preparation of **16a** above to give 82% pentapeptide **16**. The deprotection and coupling to BocThr(O-*t*-Bu)OH gave 69% **17**. Deprotection with TFA and cyclization afforded 24% azido intermediate which was reduced with 10% Pd/C in ethanol to give **19a** in 41% yield after HPLC purification.

**19b.** Coupling to resin **15b** (0.52 g, 0.5 mmol/g) with Fmoc-3β-HypOH was followed by Fmoc deprotection, coupling to BocThrOH, and cleavage to the acyclic hexapeptide in 28% total yield. Cyclization afforded 15% of **19b**.

**19c.** Coupling to **15a** in a similar manner with Fmoc-3α-HypOH, 87%; deprotection/coupling to BocThr(O-*t*-Bu)OH, 80%; TFA deprotection/cyclization to **19c**, 43%.

**19d.** Coupling to resin **15b** (1 g, 0.46 mmol/g) with FmocProOH (see section B.a below) was followed by Fmoc deprotection and coupling to BocThrOH, and cleavage to acyclic hexapeptide amino acid, 47%. Cyclization as above gave 45% of **19d**.

**19e.** Coupling to resin **15b** (0.5 g, 0.5 mmol/g) with Fmoc hProOH was followed by Fmoc deprotection, coupling to BocThrOH, and cleavage to acyclic hexapeptide, 58%. Cyclization as above gave 89% of **19e**.

**19f.** Coupling to **15a** with Fmoc-4-carbobenzyloxyaminomethyl-ProOH, 97%. In this case the Fmoc deprotection/coupling steps and the TFA deprotection/cyclization/hydrogenolytic deprotection were carried out directly without purification to give **19f** (31% total yield for five steps).

**b. Preparation of *N*-alkylated prolines (20a–c). 20a.** To a solution of **18** (0.2 g, 0.19 mmol) in dichloroethane (10 mL) and DMF (10 mL) was added benzaldehyde (0.022 mL, 0.21 mmol) followed by sodium cyanoborohydride (0.016 g) and acetic acid (0.011 mL) and the reaction mixture was stirred for 18 h. The solvents were evaporated and the residue was dissolved in methanol:DCM (8 mL, 3:1) and precipitated with water (35 mL). The crude solids were purified using 5% methanol/DCM with 0.5% ammonium hydroxide to give 0.13 g (66%) **20a**.

**20b.** To a solution of **20a** (0.13 g, 0.11 mmol) in DMF (4 mL) were added 37% formaldehyde (0.5 mL), sodium cyanoborohydride (0.014 g), and acetic acid (0.013 mL) to give after purification using 10% methanol/DCM/0.5% ammonium hydroxide 0.1 g (84%) of the *N*-benzyl-*N*-methyl intermediate.

This intermediate (0.07 g, 0.06 mmol) was treated with 5% Pd/C (0.07 g) in methanol:chloroform (10 mL, 4:1) and shaken under hydrogen balloon pressure for 18 h. The catalyst was filtered, the solvents evaporated, and the residue purified using 20% methanol/DCM/0.5% ammonium hydroxide to give 0.064 g (100%) of **20b**.

**20c.** In a similar manner to **20a**, octanal was used to alkylate **18** to give after purification using 15% methanol/DCM/0.5% ammonium hydroxide 0.03 g (36%) **20c**.

**c. Preparation of *N*-acyl proline analogues (20d–k). 20d.** Compound **18** (0.15 g) was treated with acetic anhydride (0.014 mL) in DMF (4 mL) for 3 h. The solvents were evaporated and the residue purified using 10% methanol/DCM/0.5% ammonium hydroxide to give 0.1 g (66%) **20d**.

**20e.** A solution of 2,4,5-trichlorophenyl octanoate was prepared by combining octanoic acid (9.4 mL, 0.062 mmol) 2,4,5-trichlorophenol (12.3 mg, 0.062 mmol), DCC (12.8 mg, 0.062 mmol) and catalytic DMAP in DCM (2 mL) at 0 °C for l h. This mixture was filtered and combined with **18** (30 mg, 0.028 mmol) and DIPEA (5 mL) in DMF (0.5 mL). This mixture was stirred overnight, the solvents were evaporated, and the crude residue was purified using 10% MeOH/DCM to give 16 mg (50%) **20e**.

**20f.** To a solution of **18** (0.05 g, 0.047 mmol) in DMF (0.5 mL) and triethylamine (6.6 mL, 0.047 mmol) was added benzoyl chloride (0.0107 g, 0.047 mmol). After 3 h the reaction was complete and the solvents were evaporated and the residue purified using 10% methanol/DCM/1% formic acid to give 29 mg (50%) **20f**.

**20g.** To a solution of **18** (0.025 g, 0.024 mmol) in DMF (0.5 mL) and triethylamine (4 mL, 0.028 mmol) were added dimethylaminopyridine (DMAP) (0.0033 g, 0.028 mmol) and succinic anhydride (0.0026 g, 0.026 mmol). After 1 h the reaction was complete and the solvents were evaporated and the residue was purified using 5% methanol/DCM/1% formic acid to give 15 mg (57%) **20g**.

**20h.** To a solution of **18** (0.1 g, 0.096 mmol) in DMF (1 mL) and triethylamine (40 mL) were added *N,N′*-bis-*t*-butoxycarbonylthiourea (0.029 g, 0.105 mmol) and mercuric chloride (29 mg, 0.105 mmol). After 2 h the solvents were evaporated, and the crude residue was purified using 15% methanol/DCM to give 116 mg bis-Boc intermediate which was directly treated with 90% trifluoroacetic acid (3 mL) for 3.5 h. The solvents were evaporated and the residue purified by HPLC to give 11 mg (65%) of **20h**.

**20i.** To a solution of **18** (0.04 g, 0.038 mmol) in DMF (0.5 mL) and l,2-dichloroethane (1 mL) were added TEA (0.011 mL) and $Boc_2O$ (12.3 mg, 0.057 mmol) and the mixture was stirred for 17 h. The solvents were evaporated and the residue purified using 10% methanol/DCM to give 31.4 mg (73%) **20i**.

**20j.** To a solution of **18** (0.03g. 0.028 mmol) in DMF (0.5 mL) was added a solution of 1 M methyl chloroformate in DCM (0.03 mL, 0.029 mmol) and the mixture was stirred for 2 h. The solvents were evaporated and the residue purified using 15% methanol/DCM to give 24 mg (90%) **20j**.

**20k.** To a solution of **18** (0.053g, 0.05 mmol) in DMF (0.5 mL) was added a solution of 1 M methyl isocyanine in DCM (0.05 mL) and the mixture was stirred overnight. The solvents were evaporated and the residue purified using 10% methanol/DCM to give 33 mg (59%) **20k**.

## Section B. Solid-phase synthesis of hexapeptides: preparation of residue *B* analogues

**Fmoc-Homotyroslne trimethylsilylethyl (TSE) ester (7).** To a cooled solution of EDAC (2.35 g, 12.3 mmol), trimethylsilylethanol (7.5 mL) and catalytic DMAP in THF (30 mL) was added **6b** (4.4 g, 11 mmol) in THF (30 mL). After 5 h the reaction was diluted with ethyl ether and washed with 2 N HCl. The organic layer was separated, washed with brine and the solvent was evaporated. The residue was purified using hexane:ethyl acetate (5:1) to give 4 g (73%) of the ester **7**.

**Fmoc-Homotyrosine 4′-allyloxyphenylmethyl ether TSE ester (8a).** To a cooled solution of **7** (4 g, 8 mmol) dissolved in THF (30 mL) with 4-allyloxybenzyl alcohol (1.52 g, 9.3 mmol) and triphenylphosphine (2.4 g, 9.3 mmol) was added diethylazodicarboxylate (1.6 g, 9.3 mmol). The mixture was warmed to 25 °C, the solvents evaporated, and the residue purified using hexane:ethyl acetate (5:1) to give 3.85 g (75%) of **8a**.

**Fmoc-Homotyrosine 4′-carboxymethylphenylmethyl ether TSE ester (8b).** A solution of **8a** (2.5 g, 3.8 mmol) in methanol:DCM (1:1) (20 mL) cooled to −78 °C was bubbled in ozone for 20 min. Dimethyl sulfide was added and the solvents were evaporated. This crude residue was dissolved in 50% aq THF (50 mL) and combined with 2-methylbut-2-ene (9.4 mL, 2 M in THF), a freshly prepared solution of sodium chlorite (80%) and potassium phosphate. After 3 h the mixture was diluted with ethyl acetate and washed with 10% sodium bisulfite, brine and the solvents were evaporated. The residue was purified using hexane:ethyl acetate (3:1–1:2 with 1% acetic acid) to give 2.36 g (92%) of **8b**.

**a. Preparation of resin 9b.** (i) Fmoc-**9b**: the hTyr-linker portion was loaded onto the resin in the following manner:[25] to a solution of **8b** in DCM:*N*-methylpyrrolidone (NMP) (40 mL, 3:1) was added HOBT (1.2 equiv) at 0 °C in a separatory funnel followed by DIPC (1.2 equiv). After 30 min this mixture was added to base-washed BHA resin (Novabiochem; 0.6–0.9 mmol/g) and rinsed with more DCM (10 mL). This mixture was shaken overnight and washed the following day with DCM (3×50 mL for 5 min) followed by MeOH (3×50 mL for 5 min). Endcapping was carried out by shaking the resin with acetic anhydride (4 equiv/mol of resin) overnight followed by washing with DCM (3×50 mL for 5 min), DMF (3×5 mL for 5 min), and MeOH (3×5 mL at m for 5 min) to give resin after drying by vacuum evaporation. The loading number was calculated to be 0.46 mmol/g of resin Fmoc-**9b**.

(ii) Standard Fmoc deprotection the resin Fmoc-**9b** from above was washed with DMF (2×75 mL at m for 5 min), treated with 20% piperidine in DMF (2×75 mL at m for 15 min) and washed with DMF (6×50 mL for 5 min) to give **9b** ready for coupling reactions.

**b. Preparation of pentapeptide 15b.** (i) To resin **9b** (10 g, 5 mmol) in NMP (40 mL) were added FmocHyp(*t*-BuO)OH (9.4 g, 20 mmol), HOBT (3 g, 22 mmol), and DIPC (3.46 mL, 22 mmol) and the mixture was shaken overnight. The resulting resin was filtered. Standard washing procedure: the resin was washed consecutively with DMF (3×50 mL for 5 min), DCM (3×50 mL for 5 min), and DMF:DCM (3×50 mL, 1:1, for 5 min).

(ii) Resin from (i) above was deprotected as in a(ii) for Fmoc-**9b** followed by combining FmocThr(O*t*-Bu)OH (8 g, 20 mmol) in NMP (50 mL), HOBT (3 g, 22 mmol), DIPC (3.46 mL, 22 mmol) and the mixture was shaken overnight. The resulting resin was washed as in b(i).

(iii) One half of the resin from above was deprotectcd as in a(ii) above followed by combining FmocOrn(Aloc) OH (2.2 g, 2.5 mmol) in NMP (50 mL), HOBT (0.76 g, 5.5 mmol), DIPC (0.87 mL, 5.5 mmol) and the mixture was shaken overnight. This resin was washed as in b(i) and dried to give the peptide resin **14b**.

(iv) Resin **14b** (10 g, 4.6 mmol) was deprotected as in a(ii) and reacted with 2,4,5-trichlorophenyl 4″-pentyloxyterphenoate (5 g, 9.2 mmol) and DIPEA (1.42 g, 5.5 mmol) in NMP (50 mL). This mixture was shaken at 60 °C overnight. The next day it was washed as in b(i) to give acylated resin. The acylated resin was treated with tri-*n*-butyltin hydride (4 g, 13.8 mmol), HOAc (1.5 mL), DCM (40 mL), and $Pd(Ph_3P)_4$ (0.22 g, 0.18 mmol). After shaking overnight this resin was washed with DCM (2×50 mL for 5 min), 10% TEA in DCM (2×50 mL for 5 min), DCM (2×50 mL for 5 min), MeOH (2×50 mL for 5 min) and DCM (2×50 mL for 5 min) to give dried resin **15b**.

**c. Preparation of residue *B* analogues (21a–j).** (i) Resin **15b** was treated with **13** (4.4 equiv), HOBT (4 equiv), DIPC (4 equiv) in NMP. After shaking overnight resin **16b** was washed as in b(i), deprotected as in a(ii), and treated with a Boc-protected threonine (Thr) replacement (see Table 3), HOBT (4 equiv), and DIPC (4 equiv) in NMP. After shaking overnight this resin was washed as in b(i) to give **17b**.

(ii) The peptide was cleaved from resin **17b** by shaking with 90% TFA for 2–3 h. The resin was filtered, rinsed with 90% TFA, and the TFA was evaporated. The residue was triturated with ether, and the solids were filtered and dried. The crude residue was dissolved in DMF (10 mL) and combined with HOBT (2.5 equiv), DIPEA (5 equiv), and PyBOP (2.5 equiv) and stirred overnight. The next day the solvents were evaporated, the residue was dissolved in MeOH and precipitated by adding water. The crude solids were filtered and dried to give the *N*-protected cyclic hexapeptide which was purified using 5–15% methanol in DCM.

(iii) This hexapeptide was shaken with 10% Pd/C for 2–3 h in ethanol with a hydrogen balloon, and the mixture was filtered, rinsed with more MeOH, and the washings were collected and evaporated. The residue was triturated with ether, the crude solids were filtered and dried before purification by HPLC or flash chromatography on silica gel to give the final cyclic hexapeptide, **21**.

The following Boc-protected amino acids were used as Thr replacements, and the total yield from **15b** for (i/ii) and (iii) were, respectively: (L)-BocSer(*t*-Bu)OH, **21a**, 8%, 68%; (L)-Boc-ethylGlyOH, **21b**, 8%, 26%; (L)-Boc-GlyOH, **21c**, 15%, 64%; (D)-BocThr(*t*-Bu)OH, **21d**, 9%, 28%; (L)-Boc-a,a-dimethylGlyOH, **21e**, 13%, 37%; (L)-BocTyr(*t*-Bu)OH, **21f**, 13%, 42%; (L)-BocGlu(*t*-Bu)OH, **21g**, 21%, 32%; (L)-BocOrn(Z)OH, **21h**, 8%, 27%; (L)-BocDap(Z)OH, **21i**,13%, 38%; (L)-BocArg(di-Z)OH, **21j**, 11%, 32%.

## Section C. Preparation of hTyr analogues (28a–h)

**a. Preparation of homotyrosine (hTyr) replacement acids (26a–h).** Homotyrosine (hTyr) replacement acids **26** for couplings were either commercially available (**a,d,e,f**) or prepared as follows:

**26b.** (i) To a solution of BocTyrOMe (**23b**) (2.5 g, 8.47 mmol) and $PhN(Tf)_2$ (3.18 g, 8.47 mmol) in DCM (12 mL) at 0 °C was added TEA (1.3 mL) and the mixture was warmed to 25 °C overnight. The mixture was washed with 1 M $NaHCO_3$, and the solvents were evaporated. The crude residue was purified using ethyl acetate:hexane (1:4) to give 3.2 g (88%) oil BocTyr (OTf)OMe. A solution of this triflate (2 g, 4.68 mmol), potassium acetate (2.3 g, 23.4 mmol), $Pd(OAc)_2$ (0.105 g, 0.47 mmol), DPPF (0.52 g, 0.94 mmol) in DMF (10 mL) was heated at 60 °C for 2 days. The mixture was combined with ethyl acetate and satd $NaHCO_3$ and filtered. The aqueous layer was separated, acidified with 10% citric acid, extracted with ethyl acetate and the organic layer was separated and the solvent was evaporated to give 0.62 g (41%) of the crude solid (**24b**).

(ii) To a solution of **24b** (0.62 g, 1.91 mmol) in THF (10 mL) at 0 °C was added 10 M borane:dimethyl sulfide complex (0.9 mL, 8.9 mmol) and the mixture was warmed to 25 °C for 2 h. The solvents were evaporated and the residue partitioned between ethyl acetate and satd $NaHCO_3$. The organic layer was separated and evaporated. The crude residue was purified using ethyl acetate:hexane (2:3) to give 0.45 g (76%) BocTyr($CH_2$ OH)OMe (**25b**).

(iii) To a solution of BocTyr($CH_2OH$)OMe (0.43 g, 1.39 mmol) in THF (2.5 mL) at 0 °C was added lithium hydroxide (0.07 g, 1.67 mmol) in water (1.5 mL) and stirred for 2 h. The mixture was diluted with ethyl acetate, the aqueous layer was separated, acidified with 10% citric acid, and extracted with EtOAc. Evaporation of the solvent gave 0.4 g (97%) of **26b**.

**26c.** (i) To a solution of hTyr (2.3 g, 8.3 mmol) in MeOH (20 mL) was added thionyl chloride (1.18 g, 10 mmol) and the mixture was refluxed for 6 h. The solvents were evaporated and the crude residue was purified using 15% MeOH/DCM to give 2 g (98%) of methyl ester. To a solution of ester (2 g, 8.1 mmol) in dioxane (80 mL) was added 1 M $NaHCO_3$ (26 mL)

followed by $Boc_2O$ (2.43 mL, 10.5 mmol) and the mixture was stirred overnight. The solvents were evaporated and the residue was partitioned between ethyl acetate and water, the organic layer was separated and washed with 10% citric acid, brine, and the solvent was evaporated. The residue was purified using 5% MeOH/DCM to give 1.7 g (67%) of Boc-hTyrOMe (**23c**).

(ii) To a solution of **23c** (1.7 g, 5.5 mmol) was added $PhN(Tf)_2$ (2.16 g, 5.8 mmol) and TEA (0.85 mL, 6.1 mmol) and the mixture was stirred overnight. The solvent was evaporated and partitioned between 1 M $NaHCO_3$ and ether, and the organic layer was separated and evaporated. The crude residue was purified using 15% MeOH/DCM to give 2 g (82%) of the triflate. A solution of triflate (2 g, 4.53 mmol), potassium acetate (2.2 g, 25 mmol), $Pd(OAc)_2$ (0.3 g, 1.36 mmol), DPPF (1 g, 1.8 mmol) in DMF (10 mL) was heated at 60 °C for 16 h. The mixture was combined with ethyl acetate and satd $NaHCO_3$ and filtered. The aqueous layer was separated, acidified with 10% citric acid, extracted with ethyl acetate and the organic layer was separated and the solvent was evaporated to give 1.25 g (82%) of the aryl acid **24c**.

(iii) To a solution of acid (0.7 g, 2 mmol) in THF (20 mL) was added 10 M borane:dimethyl sulfide complex (1 mL, 10 mmol) and the mixture was stirred for 12 h. The solvents were evaporated and the residue partitioned between ethyl acetate and satd $NaHCO_3$, the organic layer was separated and evaporated. The crude residue was purified using 35% ethyl acetate/hexane to give 0.51 g (76%) Boc-hTyr($CH_2OH$)OMe (**25c**).

(iii) To a solution of Boc-hTyr($CH_2OH$)OMe (0.51 g, 1.57 mmol) in THF (2.5 mL) at 0 °C was added lithium hydroxide (0.08 g, 1.9 mmol) in water (1.5 mL). The mixture was warmed to 25 °C over 1.5 h and the solvents were evaporated. The residue was partitioned between water and ethyl acetate, and the aqueous layer was separated, acidified with 10% citric acid, extracted with ethyl acetate, and the organic layer was separated and evaporated to give 0.47 g (96%) Boc-hTyr($CH_2$OH)OH (**26c**).

**26g.** (i) Boc-hTyr (2.1 g, 7 mmol) was dissolved in MeOH (40 mL) and water (4 mL) and 20% $CsCO_3$ was added to pH 7 and the solvents were evaporated. The residue was dissolved in DMF (20 mL), and benzyl bromide (0.88 mL) was added and the mixture was stirred for 16 h. After the standard work up, the crude residue was purified using 5% MeOH/DCM to give 0.95 g (35%) (**23g**).

(ii) To a solution of the benzyl ester (0.85 g, 2.2 mmol) in DCM (10 mL) at 0 °C were added $PbNTf_2$ (0.87 g, 2.37 mmol) and TEA (0.34 mL), and the mixture was stirred for 16 h. The solvents were evaporated and the crude residue was purified using 15% ethyl acetate/hexanes to give 1.13 g (99%) triflate. To a suspension of triflate from (ii) (1.13 g, 2.22 mmol), potassium acetate (1.09 g, 11.1 mmol), palladium acetate (0.05 g, 0.22 mmol), DPPF (0.25 g, 0.44 mmol) in DMF (10 mL) was bubbled in gaseous CO and the mixture was heated to 60 °C for 8 h. The solvents were evaporated and the crude residue was purified using 50% ethyl acetate/hexanes to give 0.8 g (87%) acid **24g**.

(iii) To a solution of acid from (ii) (0.8 g, 1.9 mmol) in THF (12 mL) was added 10 M $BH_3S(CH_3)_2$ (0.97 mL, 9.7 mmol) and the mixture was stirred for 4 h. The mixture was cooled to 0 °C and quenched with MeOH (10 mL). The solvents were evaporated and the crude residue was purified using 35% ethyl acetate/hexanes to give 0.33 g (43%) of the alcohol **25g**.

(iv) A solution of alcohol (0.33 g, 0.83 mmol) in MeOH (5 mL) and 10% Pd/C (0.05 g) was hydrogenated with a hydrogen balloon for 1.5 h. The mixture was filtered and the crude residue was purified using 20% MeOH/DCM to give 0.175 g (73%) of the methyl analogue **26g**.

**26h.** To a solution of **24c** (0.35 g, 1 mmol) in THF (20 mL) was added DPPA (0.246 mL, 1.1 mmol) followed by TEA (0.16 mL, 1.1 mmol) and the mixture was heated at 60 °C for 2 h. The solvents were evaporated, the residue was dissolved in toluene (5 mL) and benzyl alcohol (0.139 mL, 1.3 mmol) was added and the mixture was heated to 110 °C for 4 h. The solvents were evaporated and the crude residue was purified using 25% ethyl acetate/hexanes to give 0.28 g (61%) benzyl carbamate **25h**. To a solution of carbamate (0.28 g, 0.63 mmol) in THF (2 mL) at 0 °C was added LiOH (0.032 g, 0.76 mmol) in water (1 mL) and the mixture was stirred for 3 h. The solvents were evaporated and after the usual work up the crude residue was purified using 10% MeOH/DCM to give 0.27 g (99%) acid **26h**.

**b. Preparation of pentapeptide (22).** (i) To a solution of ProO*t*-Bu (5 g, 29.2 mmol) and FmocThr(*t*-BuO)OH (12.8 g, 32.2 mmol) in DCM (150 mL) at 0 °C were added EDAC (8.6 g, 45 mmol) and HOBT (5.8 g, 43 mmol). The mixture was warmed to 25 °C overnight. The solvent was evaporated and the residue was combined with ethyl acetate and 10% citric acid. The organic layer was washed with satd $Na_2CO_3$, and dried with $Na_2SO_4$. The solvent was evaporated to give crude solids which were used directly for the next step.

(ii) This dipeptide was dissolved in DMF (50 mL) and diethylamine (15 mL) for 4 h. The solvents were removed and the crude residue was combined with FmocOrn(Z)OH (15.7 g, 32 mmol), EDAC (6.8 g, 36 mmol), and HOBT (4.8 g, 36 mmol) and stirred overnight. The solvent was removed and the crude residue was combined with ethyl acetate and 10% citric acid. The organic layer was washed with satd $NaHCO_3$, and dried with $Na_2SO_4$. The solvent was evaporated and the crude residue was purified using 2.5% MeOH in DCM to give 11 g (47% from ProO*t*-Bu) of FmocOrn(Z)-Thr(*t*-OBu)-ProO*t*-Bu.

(iii) Tripeptide from (ii) (2.67 g, 3.3 mmol) was treated with diethylamine (4 mL) in DMF (20 mL) for 4 h. The solvents were evaporated and the crude residue was dissolved in DMF (40 mL) and treated with DIPEA

(1.74 mL) and 2,4,5-trichlorophenyl 4-(*N*-piperazinyl-*N*′-(4-*n*-octyloxyphenyl)benzoate (PIP8) (for **b,c,g,h**) or 2,4,5-trichlorophenyl 4-((4-*n*-pentyloxyphenyl)-4-phenyl)benzoate (Ter) (for **a,d,e,f**) (2.16 g, 3.7 mmol) and heated to 50 °C for 2 days. The solvents were evaporated, and the crude residue was purified using ethyl acetate:hexane (3:1) to give 1.39 g (43% from (ii)) of PIP8Orn(Z) Thr(*t*-OBu)ProO*t*-Bu.

(iv) A solution of tripeptide from (iii) above (2.6 g, 2.7 mmol) in ethanol (50 mL) and acetic acid (0.35 mL) was treated with $Pd(OH)_2$ (0.44 g) with a hydrogen balloon. After 5 h the catalyst was filtered and the solvent was evaporated. The residue was partitioned between ethyl acetate and satd $NaHCO_3$, the organic layer was separated, washed with brine and dried with $Na_2SO_4$. The solvent was evaporated to give 2 g crude amine. This crude amine was combined with FmocAzpOH (**13**) (1.11 g, 2.9 mmol), CMC (1.7 g, 4 mmol), and HOBT (0.54 g, 4 mmol) in DCM (30 mL). After the standard work up the residue was purified using 3% MeOH/DCM to give 1.87 g (60%) of the tetrapeptide.

(v) To a solution of the tetrapeptide (1.86 g, 1.56 mmol) in acetonitrile (12 mL) at 0 °C was added diethylamine (3 mL) and the mixture was warmed to 25 °C for 2 h. The solvents were evaporated, and the residue was combined with FmocThr(*t*-BuO)OH (0.7 g, 1.8 mmol), EDAC (0.313 g, 1.6 mmol), HOBT (0.25 g, 1.8 mmol) in NMP (25 mL) at 0 °C before adding NMM (0.2 mL). The mixture was stirred at 25 °C overnight, and the solvents were evaporated. The crude residue was purified using 8% MeOH/DCM to give 1.8 g (85%) of the pentapeptide **22**.

**c. General procedure for preparation of the hTyr analogues (28a–h).** (i) To a solution of the pentapeptide **22a,b** (1 g, 0.74 mmol) in acetonitrile (9 mL) at 0 °C was added diethylamine (2 mL) and the mixture was warmed to 25 °C for 2 h. The solvents were evaporated, and the residue was purified using 8% MeOH/DCM to give 0.77 g (92%) of amine. To a 0 °C solution of this amine (0.2 g, 0.17 mmol) in DCM (2 mL) were added CMC (3 equiv), HOBT (3 equiv) and the hTyr replacement (1.25–2 equiv), and this mixture was warmed to 25 °C overnight. The solvents were evaporated and after the standard work up the crude residue was purified using 5% MeOH/DCM to give the acyclic hexapeptide **27a,b**.

(ii) Hexapeptide **27a,b** was treated at 0 °C with 90% aq trifluoroacetic acid and then warmed to 25 °C for 3 h. The mixture was precipitated with diethyl ether and the solids filtered to give the crude amino acid. To a solution of this material dissolved in DMF at 0 °C was added DPPA (1.1 equiv). The mixture was warmed to 25 °C overnight. The solvents were evaporated and after the standard work up, the crude residue was purified using 5–10% MeOH/DCM to give the cyclized product.

(iii) To a solution of the cyclic hexapeptide (0.055 g, 0.048 mmol) in THF:water (4:1) was added triphenylphosphine (4 equiv) and the mixture was heated at 80 °C for 1 h. The solvents were evaporated and the crude residue was purified using 7.5–10% MeOH/DCM with 1% $NH_4OH$. The material was dissolved in MeOH and excess acetic acid, and the solvents were evaporated to give the acetate salt **28**.

**28a.** For example, the yields of coupling of **22a** and **26a**, TFA treatment and cyclization of **27a**, and TPP reduction of azide as described above giving **28a** were 98, 55 and 63%, respectively.

**28b.** The same reaction for **22b** and **26b**, **27b**, resulting in **28b**, gave 57, 30, 94%.

**28c.** The same reaction for **22b** and **26c**, **27c**, resulting in **28c**, gave 73, 19, 77%.

**28d.** The same reaction for **22a** and **26d**, **27d**, resulting in **28d**, gave 99, 71, 60%.

**28e.** The same reaction for **22a** and **26e**, **27e**, resulting in **28e**, gave 99, 37, 67%.

**28f.** The same reaction for **22a** and **26f**, **27f**, resulting in **28f**, gave 79, 47, 67%.

**28g.** The same reaction for **22b** and **26g**, **27g**, resulting in **28g**, gave 97, 26, 94%.

**28h.** The same reaction for **22b** and **26h**, **27h**, resulting in **28h**, gave 51, 33, 67%.

## Section D. Side-chain analogues

**a. Preparation of cyclic hexapeptides via the '3 + 3' approach. Fmoc-hTyr-Thr-AzpOMe (29).** (i) BocThrOH (11.32 g, 52 mmol), **12** (10.67 g, 52 mmol), and HOBT (8.72 g, 64 mmol) were dissolved in NMP (70 mL) and THF (170 mL) and cooled to 0 °C. NMM (6.95 mL) was added followed by addition of EDAC (10.84 g, 57 mmol), and the reaction mixture was allowed to warm to 25 °C overnight. After the standard work up, the residue was redissolved in ethyl acetate, precipitated in a mixture of 10% MTBE in heptane, stirred for 30 min at 0 °C, filtered and dried in vacuo to give 15.47 g (75%) BocThrAzpOMe.

(ii) Treatment of BocThrAzpOMe (12.35 g, 33 mmol) with HCl/ethyl acetate as for **12** gave 9.93 g (100%) ThrAzpOMe(HCl).

(iii) ThrAzpOMe(HCl) (9.9 g, 33 mmol), FmochTyrOH (13.52 g, 33 mmol), and HOBT (5.55 g, 41 mmol) were dissolved in NMP (150 mL) and THF (150 mL) and cooled to 0 °C. NMM (5.43 mL) was added followed by addition of EDAC (6.67 g, 35 mmol), and the reaction mixture was kept at 0 °C overnight. The mixture was warmed to 25 °C, concentrated, and after the standard work up, the organic layer was washed with 10% aqueous sodium bicarbonate, brine, and the solvent was evaporated. The residue was dissolved in ethyl acetate, precipitated in a mixture of 10% MTBE in heptane, stirred for 30 min at 0 °C, filtered and dried

in vacuo to give 21.04 g (97%) Fmoc-hTyrThr-Pro ($N_3$)OMe (**29**).

**BocOrn(Fmoc)ThrHypOH (30).** (i) CbzThrOH (22 g, 87 mmol), HypOBn(HCl) (22.39 g, 87 mmol), and HOBT (14.81 g, 109 mmol) were dissolved in NMP (240 mL) and THF (240 mL) and cooled to 0 °C. NMM (14.4 mL) was added followed by addition to EDAC (17.7 g, 93 mmol), and the reaction mixture was allowed to warm to 25 °C overnight. The mixture was concentrated, and after the standard work up, the residue was dissolved in ethyl acetate, precipitated in a mixture of 10% MTBE in heptane, stirred for 30 min at 0 °C, filtered and dried in vacuo to give 37.5 g (95%) CbzThrHypOBn.

(ii) To a solution of CbzThrHypOBn (22 g, 48 mmol) and *p*-toluenesulfonic acid (10.09 g, 53 mmol) in isopropanol (200 mL) was added 5% Pd/C and the mixture was hydrogenated via hydrogen balloon pressure for 16 h. The catalyst was filtered and washed with methanol and the solvents were evaporated to give a residue ThrHyp OH(TsOH) which was used directly for the next step.

(iii) To a 0 °C solution of BocOrn(Fmoc)OH (25 g, 55 mmol) and *N*-hydroxysuccinimide (HOSu) in THF (300 mL) was added a solution of DCC (12.46 g, 60 mmol) in THF and the reaction was stored at 0 °C overnight. The solids were filtered, the filtrate concentrated, and combined with heptane to precipitate a white solid which was filtered and rinsed with more heptane. The crude residue was dissolved in water (110 mL) with sodium bicarbonate (10 g) and cooled to 0 °C. A solution of BocOrn(Fmoc)OSu was added with stirring and the mixture was allowed to warm to 25 °C for 20 h. The THF was evaporated and the pH brought to 7. The mixture was diluted with water and extracted with ethyl acetate. The organic layer was discarded and the pH of the aqueous layer was adjusted to 4 with 20% citric acid and extracted with ethyl acetate. The solvents were evaporated and the crude residue was purified using ethyl acetate:methanol:acetic acid (100:5:1–100:10:3). The product was then precipitated from hexanes:diethyl ether (4:1) to give 28.57 g (88%) of the tripeptide BocOrn(Fmoc)ThrHypOH (**30**).

**Preparation of cyclic hexapeptide (32).** (i) A solution of FmocHtyThrAzpOMe (**29**) (6.73 g, 10 mmol) in acetonitrile (150 mL) was cooled to 0 °C and diethylamine (35 mL) was added and the mixture was stirred for 1 h. The solvent was evaporated and the crude residue (4.6 g) was used directly without further purification.

(ii) To a solution of BocOrn(Fmoc)ThrHypOH (**30**) (5.22 g, 8 mmol) and crude HtyThrAzpOMe from above in DCM (150 mL) was added a solution of EEDQ (2.73 g, 11 mmol) and the mixture stirred for 40 h. The reaction mixture was filtered through Celite and the solvents were evaporated. After the standard work up, the crude residue was purified using 4–10% methanol in ethyl acetate to give 9.2 g (70%) of the linear hexapeptide BocOrn(Fmoc)ThrHypHtyThrAzpOMe (**31**).

(iii) Hexapeptide **31** (9 g, 6.8 mmol) was dissolved in 90% aq ethanol (150 mL) and cooled to 0 °C. To this solution was added 1 N sodium hydroxide (35 mL) keeping the pH at 12 at 0–25 °C until the hydrolysis was complete. The pH was adjusted to 5, the ethanol evaporated, and the aqueous layer was extracted with ethyl acetate to remove impurities. The aqueous layer was evaporated and the residue was dissolved in methanol, salts filtered, and the solvent was evaporated and crude residue dried in vacuo. The crude residue was dissolved in dimethylformamide (1.3 L) and cooled to −20 °C. Diphenylphosphoryl azide (2.2 mL, 10.2 mmol) was added with stirring followed by sodium bicarbonate (4.1 g). The reaction mixture was kept at 25 °C until the coupling was complete. The solvent was evaporated and the residue was dissolved in 10% methanol/ethyl acetate. Precipitated solids were filtered and the filtrate was washed with 10% aq sodium bicarbonate and brine. The solvents were evaporated and the crude material was purified using 8% methanol/DCM to give 5 g (69%) of the cyclic hexapeptide **32**.

**b. Preparation of side-chain analogues (34a–k).** To a solution of **32** (4.1 g, 4.7 mmol) in chloroform (4 mL) at 0 °C was added 90% TFA (14 mL) and the mixture allowed to warm to 25 °C. After stirring for 2 h ether was added to precipitate the solids which were filtered, rinsed with ether and vacuum dried to give 4 g (99%) of the trifluoroacetic acid salt of **33a**.

**34a.** To a solution of **33a** (50 mg, 67 mmol) in THF (4 mL) and DIPEA (24 mL, 0.17 mmol) was added acetic anhydride (8 mg, 0.08 mmol). After 2 days the solvents were evaporated and the crude residue was purified using 20% methanol in DCM with 5% ammonium hydroxide to give 33 mg (62%) of the *N*-acetyl intermediate. This material was treated with triphenylphosphine (40 mg, 0.15 mmol) in 20% aq THF (6 mL) at reflux for 2 h. The solvents were evaporated and the crude residue was purified by using 30% methanol in DCM with 5% ammonium hydroxide to give 28.7 mg (99%) of **34a**.

**34b.** To a solution of **33a** (72 mg, 0.098 mmol) in DMF (4 mL) and DIPEA (31 mg, 0.24 mmol) was added 2,4,5-trichlorophenyl pentadecanoate (83 mg, 0.19 mmol). After 2 days the solvents were evaporated and the crude residue was precipitated with 20% methanol in DCM and ether to give 54 mg azide which was hydrogenated with 5% Pd/C (20 mg) in ethanol (5 mL) equipped with a hydrogen balloon. The catalyst was filtered, the solvents were evaporated and the crude residue was purified by using 20% methanol in DCM with 5% ammonium hydroxide to give 48 mg (53%) of **34b**.

**34c.** To a solution of **33a** (75 mg, 0.1 mmol) in THF (4 mL) and DIPEA (34 mL, 0.25 mmol) was added benzoic anhydride (27 mg, 0.12 mmol). After 1 h the solvents were evaporated and the crude residue was purified using 20% methanol in DCM with 5% ammonium hydroxide to give 70 mg (82%) of the *N*-benzoate. This material was treated with triphenylphosphine (86 mg,

0.33 mmol) in 20% aq THF (6 mL) at reflux for 4 h. The solvents were evaporated and the crude residue was purified by using 15% methanol in DCM with 5% ammonium hydroxide to give 28.7 mg (67%) of **34c**.

**34d,f–k.** Side-chains used to acylate **33a** to give **34d–k** were all reacted as the corresponding 2,4,5-trichlorophenyl esters (1.5 equiv) with DIPEA (2.5 equiv) in DMF at 50 °C until complete. The solvents were evaporated and the crude residues were purified using 10–20% methanol/DCM with 5% ammonium hydroxide to give the pure azidoproline precursors **34d–k**, all of which except **34e** (see below) were reduced by heating with TPP (4 equiv) in 20% aq THF until complete. The solvents were evaporated and the crude residues were purified with similar solvents to give the final **34d,f–k**.

**34e.** In the case of the azido-precursor to **34e**, the product from above was debenzylated and reduced by treating **33** (R = COPh-*p*-(*N*-piperazine)) (70 mg, 0.065 mmol) with 10% Pd/C (70 mg) in methanol (5 mL) equipped with a hydrogen balloon and stirred overnight. The catalyst was filtered, the solvents evaporated, and the residue purified using 10% methanol in DCM with 2% ammonium hydroxide to give 35 mg (51%) of **34e**.

Side-chains for **34f–k** were prepared as in ref 20 via alkylation of the phenolic group. In a similar manner, the biphenyl side-chain for **34d** was prepared by octylation of methyl 4′-hydroxy-4-biphenyl-carboxylate as in this reference.

### Section E. Preparation of combination analogues (35a–h)

**35a.** Compound **35a** was prepared in a similar manner to **18** by replacing the terphenyl side-chain with 4-(*N*-(4-(*n*-pentyloxy)phenyl)piperazinyl)benzoyl group and the residue *B* threonine with BocDap(Cbz)OH. For this compound, the cyclization was accomplished using DPPA in the presence of $NaHCO_3$ as described in **32** (iii). Sequential reduction of the azide with $Ph_3P$ in THF–$H_2O$ and deprotection of the Cbz group with hydrogen balloon, 10% Pd on carbon in THF:EtOH (1:1) and AcOH (a few drops) gave **35a**.

**35b.** Compound **35b** was prepared from the amino precursor of **35a**. After reduction of the azide with $Ph_3P$ in THF–$H_2O$, the free amine was guanidinylated as for **20h**. Deprotection of the Cbz group was carried out as described above to give **35b**.

**35c.** Compound **35c** was prepared in a similar manner to **18** by replacing the terphenyl side-chain with octyloxyphenylpiperazinylbenzoyl group and the residue *B* threonine with BocDap(Cbz)OH. Reduction of the azido and Cbz groups was carried out using hydrogen balloon, 10% Pd on carbon in THF:EtOH (1:1) and AcOH.

**35d.** Compound **35d** was prepared in a similar manner to **35b** by replacing the pentyloxy side-chain with octyloxyphenylpiperazinylbenzoyl group.

**35e.** Compound **35e** was prepared in a similar manner to **35c** by replacing the residue *B* BocDap(Cbz)OH with BocOrn(Cbz)OH. Reduction of the azido and Cbz groups was carried out with hydrogen balloon, 10% Pd on carbon in THF.

**35f.** Compound **35f** was prepared in a similar manner to **35b**. Deprotection of the Cbz group was carried out using hydrogen balloon, 20% $Pd(OH)_2$ on carbon in THF in the presence of AcOH.

**35g.** Compound **35g** was prepared in a similar manner to **34i** by replacing the residue *B* BocThrOH with BocOrn(Cbz)OH and replacing the residue *D* HypOBn (HCl) with ProOBn(HCl). Sequential reduction of the azide with $Ph_3P$ in THF–$H_2O$ and deprotection of the Cbz group with hydrogen balloon, 20% $Pd(OH)_2$ on carbon in EtOH in the presence of AcOH gave **35g**.

**35h.** Compound **35h** was prepared in a similar manner to **35b**. Deprotection of the Cbz group was carried out using hydrogen balloon, 20% $Pd(OH)_2$ on carbon in EtOH in the presence of AcOH.

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