Bioorganic & Medicinal Chemistry 19 (2011) 6505–6517

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and evaluation of novel macrocyclic antifungal peptides

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ARTICLE INFO

Article history:
Received 10 March 2011
Revised 10 August 2011
Accepted 14 August 2011
Available online 22 August 2011

Keywords:
Echinocandins
Macrocyclic peptides
Antifungal
β-(1,3)-D-Glucan synthase inhibitors
On-resin RCM

ABSTRACT

Echinocandins are a novel class of macrocyclic antifungal peptides that act by inhibiting the β-(1,3)-D-glucan synthase complex, which is not present in mammalian cells. Due to the large number of hydroxyl groups present in these complex macrocyclic lipopeptides, most structure–activity relationship studies have relied upon semisynthetic derivatives. In order to probe the influence of the cyclic peptide backbone on the antifungal activity we developed a successful strategy for the synthesis of novel echinocandins analogues by on-resin ring closing metathesis or disulfide formation. The specific minimum inhibitory activity of each mimic was determined against *Candida albicans*. Our results indicate that ring size is an important factor for antifungal activity.

1. Introduction

Invasive fungal infections represent a growing threat and in recent years the incidence and diversity of fungal infections has increased enormously.[1,2] Despite advances in antifungal therapies, systemic fungal infections continue to cause significant morbidity and mortality in immunocompromised patients. Resistance against and toxicity of the current antifungal agents underscores the urgent need for development of new antifungal compounds that act on novel targets.[3]

The echinocandins represent the most recent class of antifungal drugs that reached the market in the last decade.[4] They are large macrocyclic lipopeptide molecules that block fungal cell wall biosynthesis by inhibiting the β-(1,3)-D-glucan synthase complex. This complex is responsible for the production of the major fungal cell wall biopolymer β-(1,3)-D-glucan. Since this glucan is not utilized in human cells, inhibition of its biosynthesis provides for a selective approach to generate antifungal agents. By blocking the synthesis of β-(1,3)-D-glucan, the cell wall is weakened, which leads to lysis of the fungal cells. Different fungi have varying amounts of chitin, glucans, mannoproteins, and other cell wall constituents. Therefore, some species are more susceptible to the echinocandins than others. Since β-(1,3)-D-glucan is a major cell wall component of *Candida* and *Aspergillus* species, these species are more sensitive to echinocandins.[5]

To date, three echinocandins have been approved by the Food and Drug Administration (FDA). The first licensed echinocandin was caspofungin (**1**) in 2001, followed by micafungin (**2**) in 2005, and anidulafungin (**3**) in 2006 (Fig. 1).[6] These echinocandins are all semisynthetic derivatives of the natural fermentation products.[7–9]

Due to the large number of hydroxyl groups in these lipopeptides, total synthesis of the echinocandins is extremely challenging. The total synthesis of the least complex member of the family, echinocandin D (**4**), which lacks an ornithine hemiaminal function (Fig. 1), has been described in the mid 1980s.[10,11] However, all reported attempts at the synthesis of echinocandin C (**5**) have failed due to difficulties in formation of the hemiaminal moiety.[10,12] These synthetic limitations led to isolation and modification of semi-synthetic derivatives, which were used for most of the structure–activity relationship (SAR) studies.[13] In 1992 the first total synthesis of simplified echinocandin analogues was described by Zambias et al.[12] These structure–activity relationship data showed that several of the functional groups, primarily the hydroxyl groups on positions of R^1, R^2 and R^4 (Fig. 1), were not necessary for antifungal activity.[14] Partly inspired by this work, Klein et al. reported a similar study in 2000.[15] Both papers described derivatives that were obtained by peptide head-to-tail cyclization. Encouraged by these reports, we became interested in pursuing additional echinocandin analogues by employing alternate peptide cyclization approaches. We here report the synthesis and activity of novel echinocandin analogues (Fig. 2) obtained by ring closing metathesis (**6**) and disulfide formation (**7**). Using such approaches, the impact of the size and character of the macrocyclic peptide backbone on

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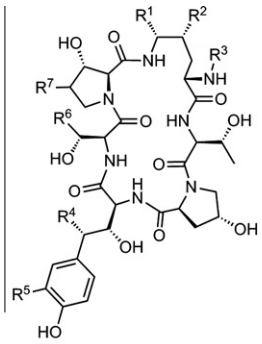
doi:10.1016/j.bmc.2011.08.034



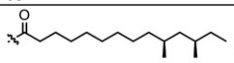
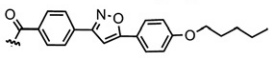
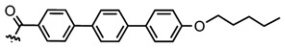
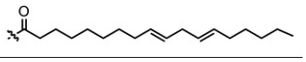
	R^1	R^2	R^4	R^5	R^6	R^7	R^3
Caspofungin (**1**)	$NHCH_2CH_2NH_2$	OH	OH	H	$CH_2CH_2NH_2$	H	
Micafungin (**2**)	OH	OH	OH	OSO_3H	CH_2CONH_2	Me	
Anidulafungin (**3**)	OH	OH	OH	H	Me	Me	
Echinocandin D (**4**)	H	H	H	H	Me	Me	
Echinocandin C (**5**)	OH	OH	H	H	Me	Me	

Figure 1. Structures of the FDA approved semisynthetic echinocandins (**1**–**3**) and two natural echinocandins (**4**,**5**).

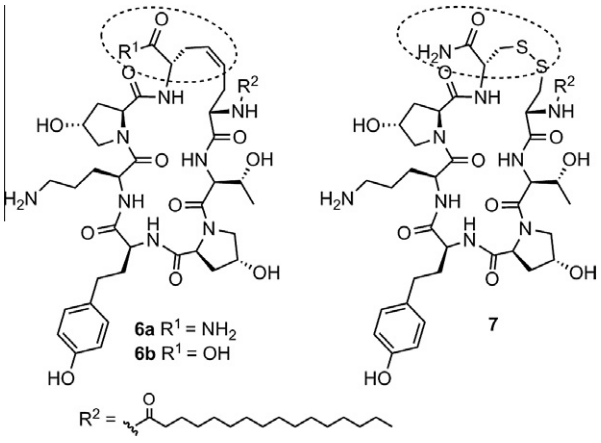


Figure 2. Structures of the designed synthetic echinocandin analogues.

antifungal activity can be investigated more extensively. In addition, these new mimics may be attractive alternatives since they also contain a position for further modification (i.e., the C-terminus; substituent R^1).

In comparison to caspofungin **1**, mimics **6** and **7** were designed to contain several alterations (Fig. 2). Consistent with SAR studies[14,15] a number of functional groups (primarily hydroxyl groups, i.e., R^1, R^2 and R^4) which are not necessary for antifungal activity, were omitted. Moreover, the lipophilic chain (R^3 in compounds **1**–**5)** was replaced by a palmitoyl group (R^2 in **6a** and **6b**), which closely mimics the fatty acid tail of caspofungin. Earlier SAR studies showed that a C12–C18 fatty acid chain gave an optimal activity.[16] In addition, the ornithine derived residue at the top of the caspofungin structure (**1**) was replaced by an allyl glycine residue together with introduction of an allyl glycine residue at the C-terminus leading to precursor **8**. Similarly, replacement by a cysteine residue at these positions gave precursor **11**. Cyclization of these precursors slightly enlarged the ring to a 23-membered ring as compared to the original 21-membered ring of caspofungin. According to the literature[17] there was room for alterations at this site of the molecule. Borromeo et al. reported an approach in which the ring of the hexapeptide was opened at the hemiaminal function and the ornithine residue was replaced to regenerate new cyclic hexapeptides.[17]

2. Results and discussion

Ring-closing metathesis (RCM) reactions have been previously carried out on peptides linked to a solid support.[18] However, these RCM reactions required long reaction times (18–48 h). Recently, Robinson et al. reported large acceleration of RCM on resin-bound peptides by microwave heating in the presence of chaotropic reagents.[19] This procedure was also applied to the preparation of our mimic **6**. As shown in Scheme 1, linear precursors were prepared by SPPS using either the Rink or Wang resin, ultimately providing the peptide amide (**6a**) or carboxylic acid (**6b**), respectively. Then, RCM reaction of resin-bound peptide **8** was performed using a 15 mol % solution of Grubbs II in DCM (containing 10 vol % LiCl/DMA 0.4 M) and microwave irradiation to provide the ring-closed product **9**. Removal of the Fmoc group, coupling of palmitic acid, and acidolytic cleavage from the resin with concomitant removal of the side chain protecting groups, gave the cyclic peptide **6** as a mixture of *cis*/*trans* isomers in an overall yield of 11% after purification by preparative HPLC. Attempted RCM reactions without microwave irradiation were less successful. For example, treatment of the linear resin-bound peptide with second generation Grubbs catalyst (20 mol %) in DCM containing 10 vol % LiCl/DMA (0.4 M) at 50 °C for 24 h gave only trace amounts of the cyclized product. After reduction of unsaturated peptides **6a** and **6b** macrocyclic peptides **10a** and **10b** were obtained.

Synthesis of the disulfide mimic **7** started with SPPS on the Rink resin as outlined in Scheme 2. Removal of the Fmoc group followed by introduction of the palmitoyl chain and cleavage from the resin yielded the deprotected peptide **11,** which was then cyclized via disulfide formation by oxidation with aq DMSO[20] yielding peptide **7** in an overall yield of 22% after purification by preparative HPLC.

The antifungal activity of each macrocyclic peptide analogue was evaluated by broth microdilution using Caspofungin[21] as a reference compound. Minimum inhibitory concentrations (MICs) were inspected visually and thereby quantified as the lowest concentration of compound resulting in inhibition of yeast growth

after overnight incubation at 30 °C (Table 1). Unfortunately, mimics **6, 7** and **10** did not show antifungal activity up to 100 μg/mL (corresponding to 96 μM). To more clearly determine the factors responsible for the observed lack of antifungal activity, further investigations were performed by varying the lipophilic 'tail' (substituent R^2, Fig. 3A), introduction of modifications at the C-terminus (Fig. 3B) and changing the peptide ring size (Fig. 3C).

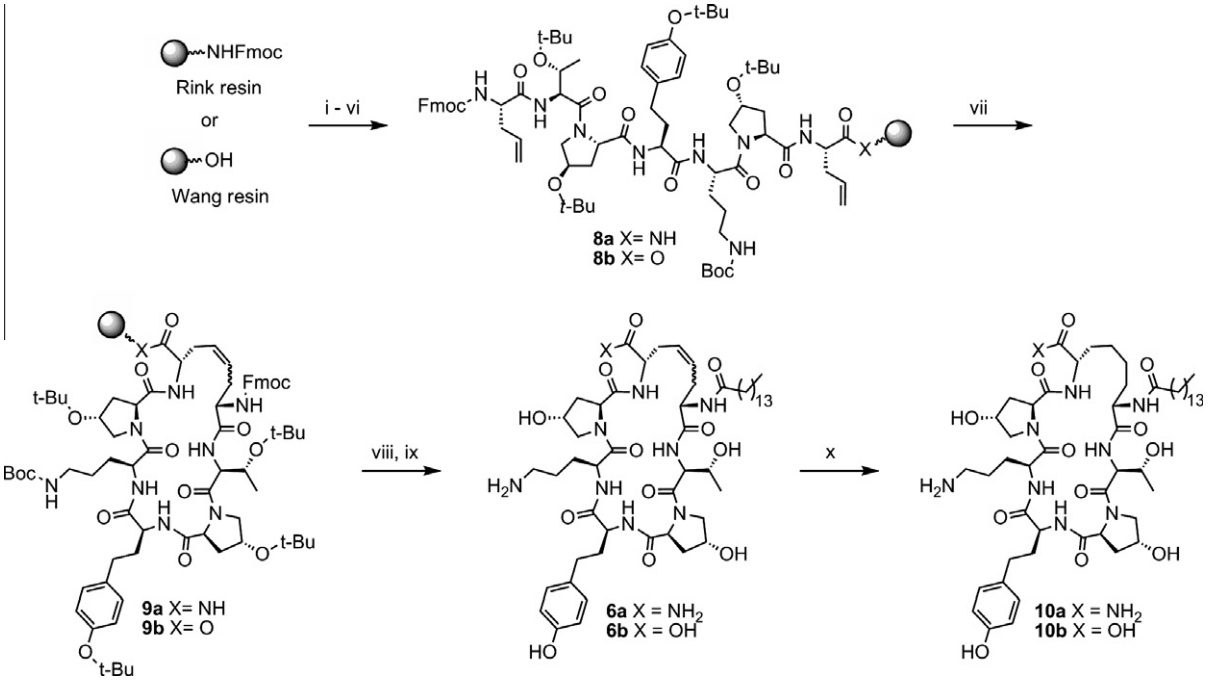
2.1. Influence of the lipophilic 'Tail'

The nature of the fatty acid chain and its influence on the activity of the echinocandins has been studied extensively. Several reports have described the preparation of semi-synthetic echinocandin analogues by enzymatic deacylation and chemical reacylation with alternative fatty acids.[16,22–26] It has been shown that neither the macrocyclic peptide itself, the fatty acid, nor a mixture of both show significant antifungal activity. These results clearly demonstrated that the intact echinocandin molecule is required for inhibition of antifungal growth.[23] Moreover, the nature of the side chain plays a very important role in the biological activity. In this regard, side chain length, overall lipophilicity, and other geometric factors such as rigidity, contribute to the SAR of these analogues.

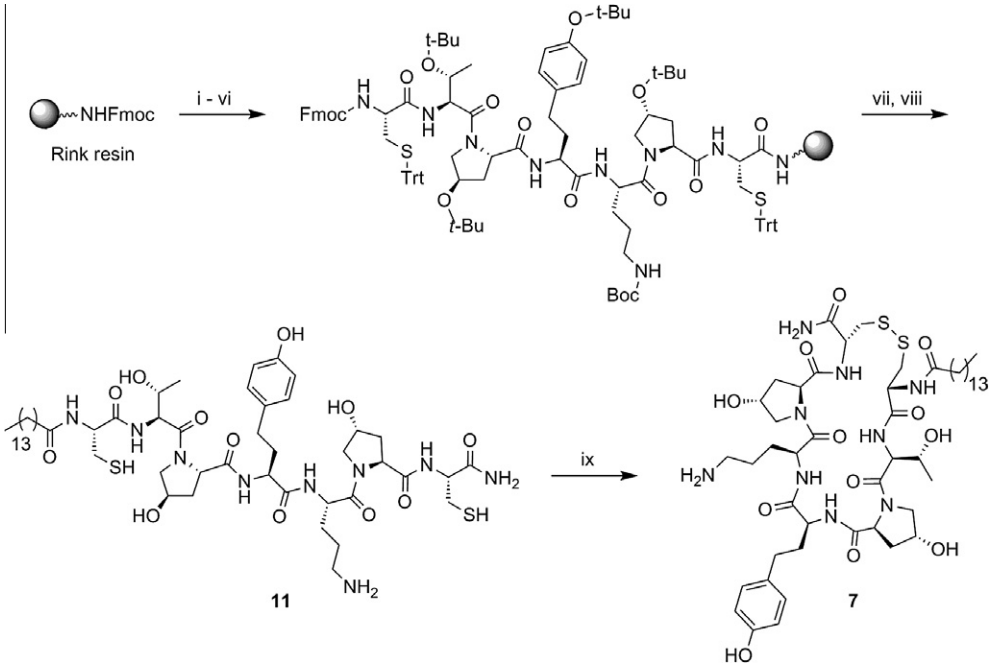
These previous studies have shown that the lipophilic chain in the echinocandins is a crucial determinant for antifungal potency. To determine if the choice of the lipophilic tail for mimics **6**, **7** and **10** might be partly responsible for the loss of antifungal activity, a series of fatty acid derivatives (**12a**,**b** and **13a**,**b**; Scheme 3), based on previous work by Klein et al., were prepared.[15] Their work established that head-to-tail backbone mimics bearing a terphenyl ('Ter') chain showed reasonable biological activity. Therefore, head-to-tail mimics were prepared with the palmitoyl (**12**) and the terphenyl[27] (**13**) fatty acid chain. The synthesis of these mimics proceeded readily and is outlined in Scheme 3 for mimics **12 and 13**. The linear precursor **14** was prepared by SPPS using the trityl resin. Removal of the ε-Fmoc group from ornithine, followed by mild acidolytic cleavage of the protected peptide from the resin and cyclization gave the protected macrocyclic peptide **15** in an overall yield of 90%. Removal of the IvDde-group[28] from ornithine with hydrazine and subsequent coupling of the fatty acid chain, followed by global deprotection and purification, gave cyclic peptides **12** and **13** in overall yields of 17–23%.

Minimum inhibitory concentrations (MICs) were evaluated and are given in Table 2. These results showed that mimic **13a** with a terphenyl lipophilic tail is 50-fold more active than **12a** with a palmitoyl chain. Moreover, mimics containing an ornithine (**12b**, **13b**) residue were fivefold more active than the mimics containing a threonine (**12a**, **13a**) residue at this position as is consistent with previously described results by Klein et al.[15]

Thus, compounds **12** and **13** having a terphenyl tail gave us a clue about the high importance of the lipophilic tail in general. This finding enticed us to synthesize from thereon all compounds a with a terphenyl chain. For example **6b** containing a terphenyl chain became **16**. For the synthesis of RCM mimic **16** with a terphenyl chain, the RCM conditions employed required optimization. The use of the Hoveyda–Grubbs II catalyst, longer reaction times, and adding the catalyst in portions did not improve the rate of conversion or yields of the ring-closed product. We did however find that changing the sequence of the reactions did allow for success at the RCM step. Thus, RCM was performed after coupling of the fatty acid chain instead of with the Fmoc precursor **8** as in Scheme 1 (step vii and viii were switched). Linear precursor **8** was prepared by SPPS on the Wang resin. Removal of the N-terminal Fmoc group, coupling of the terphenyl chain followed by RCM reaction of the resin-bound peptide using a 10 mol % solution of Grubbs II in DCM (containing 10 vol % 0.4 M LiCl in DMA) under microwave irradiation resulted in the ring-closed product. Cleavage from the resin with concomitant removal of side chain protecting groups gave cyclic peptide **16** as a mixture of *cis/trans* isomers in an overall yield of 15% after purification by preparative HPLC. After Pd/C assisted reduction of the double bond macrocyclic peptide **17** (Table 1) was obtained.



Scheme 1. Reagents and conditions: (i) Rink resin: (1) 20% piperidine in NMP; (2) Fmoc-Alg-OH, BOP, DIPEA, NMP; Wang resin: (1) Fmoc-Alg-OH, pyridine, DCBC, DMF; (2) Ac_2O/NMI/DiPEA/DMF (2/1/1/6, v/v/v); (ii–vi) (1) 20% piperidine in NMP; (2) Fmoc-Xxx-OH, BOP, DiPEA, NMP; (vii) Grubbs II (15 mol %), 10 vol % LiCl/DMA (0.4 M), MW, 75 min, 100 °C, DCM; (viii) (1) 20% piperidine in NMP; (2) palmitic acid, HATU, DiPEA, NMP; (ix) TFA/TIS/H_2O (95/2.5/2.5, v/v/v); (x) H_2, 10% Pd/C, EtOH, rt, 36 h.



Scheme 2. Reagents and conditions: (i) (1) 20% piperidine in NMP; (2) Fmoc-Cys(Trt)-OH, BOP, DiPEA, NMP; (ii–vi) (1) 20% piperidine in NMP; (2) Fmoc-Xxx-OH, BOP, DiPEA, NMP; (vii) (1) 20% piperidine in NMP; (2) palmitic acid, HATU, DiPEA, NMP; (viii) TFA/TIS/H_2O (95/2.5/2.5, v/v/v); (ix) H_2O, NH_4OAc, DMSO, pH 6.

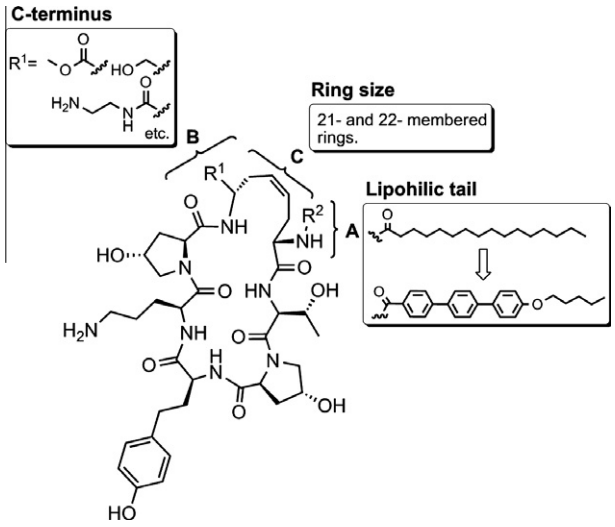


Figure 3. Factors studied for antifungal activity.

The minimum inhibitory concentrations (MICs) were evaluated and are shown in Table 1. Although these results showed that mimics **16** and **17** with a terphenyl chain did not reach their MIC-values at 100 μg/mL (corresponding to 87 μM), a difference in growth of *Candida albicans* cells was visible starting at 5 μg/mL (Fig. S1 in Supplementary data). Further investigation of this observation by light microscopy has led to a clearer view of the difference in growth that was observed. A clear-dose-dependent effect of our compounds in the growth pattern of *C. albicans* cells was visible. Higher concentrations of **16** resulted in enlarged rounded cells that agglutinated, showing large vacuoles and distorted cytoplasms. There was a clear reduction in the number of budding cells and when these appeared more buds were formed simultaneously. Although **16** was not fully active at concentrations up to 100 μg/mL a clear effect on yeast growth was observed (Fig. S2).

2.2. Influence of C-terminal modifications

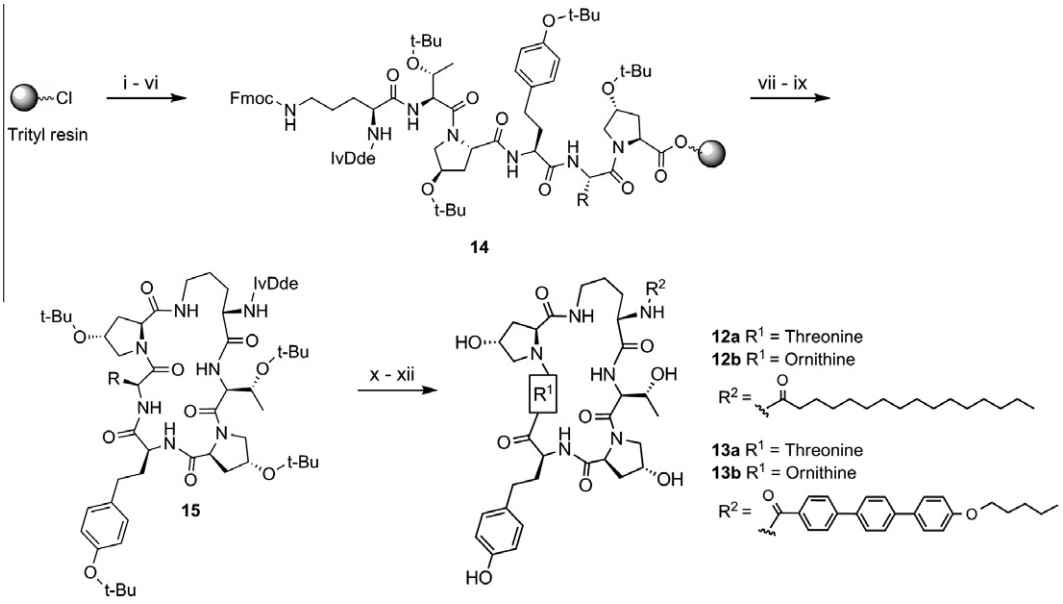
RCM mimic **16** contains a C-terminal moiety allowing for modification, which is convenient for the preparation of additional analogues. This moiety replaces the site of the hemiaminal group present in most echinocandins (Fig. 1, R^1 = OH), which is known to be unstable at pH >7.[28–30] This has led to several studies on derivatization of the hemiaminal moiety to improve stability.[31–37] In particular, these studies showed that aminoalkylethers (e.g., $R^1 = OCH_2CH_2NH_2$) bearing a basic amino group had enhanced antifungal properties.[31] Further optimization led to the closely related *N*-alkylamino aminal series[37] including clinically used caspofungin **1**.

Considering these factors, two C-terminally modified analogues incorporating basic groups were synthesized (Scheme 4). Synthesis of these mimics proceeded largely as described for mimic **16**, except in the last step. The resin-bound ring-closed product was cleaved from the resin with KCN in MeOH to give the fully *t*Bu/Boc protected methylester **18**. Saponification of **18** gave carboxylic acid **20**, which was coupled to Boc-protected ethylene diamine followed by acidolysis affording **21**. Reduction of methylester **18** followed by removal of the protecting groups gave macrocyclic peptide alcohol **22**. Reacting **18** with hydrazine afforded **23** (Scheme 4).

The minimum inhibitory concentrations (MICs) of mimics **19**, **21**, **22** and **23** were evaluated and are shown in Table 1. Although these results showed that these mimics did not reach their MIC-values at 100 μg/mL, the same dose-dependent change in the growth pattern of the *C. albicans* cells was observed for mimics **21**, **22** and **23** as for **16**.

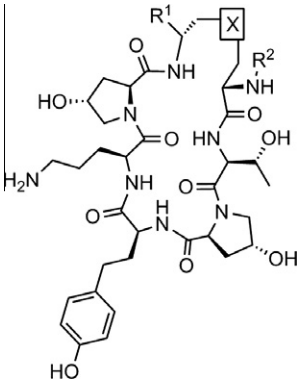
2.3. The ring size is crucial

The ring size of the RCM compounds (23-membered rings) is slightly larger than that of the natural echinocandins (21-membered rings). However, the resulting compounds were completely devoid of biological activity. This somewhat unexpected finding enticed us to investigate the influence of ring size on activity,



Scheme 3. Reagents and conditions: (i) (1) Fmoc-Hyp(*t*Bu)-OH, DIPEA, DCM; (2) DCM/MeOH/DiPEA (17/2/1, v/v/v); (ii–vi) (1) 20% piperidine in NMP; (2) Fmoc-Xxx-OH, BOP, DiPEA, NMP; (vii) 20% piperidine in NMP; (viii) HFIP/DCM (1/1, v/v); (ix) BOP, DiPEA, DMF; (x) NH_2NH_2/DMF (5 vol %); (xi) for **12**: palmitic acid, DCC, HOBt, DCM; for **13** Ter chain, DCC, HOBt, DCM; (xii) TFA/TIS/H_2O (95/2.5/2.5, v/v/v).

Table 1
Antifungal activity of analogues



	R^1	X	R^2	In vitro activity MIC (μg/mL) *Candida albicans* CBS 9975
Caspofungin (**1**)				0.025
6a	$C(O)NH_2$	CH=CH	Palm	>100
6b	C(O)OH	CH=CH	Palm	>100
7	$C(O)NH_2$	S–S	Palm	>100
10a	$C(O)NH_2$	CH_2CH_2	Palm	>100
10b	C(O)OH	CH_2CH_2	Palm	>100
16	C(O)OH	CH=CH	Ter	>100
17	C(O)OH	CH_2CH_2	Ter	>100
19	$C(O)OCH_3$	CH=CH	Ter	>100
21	$C(O)NHCH_2CH_2NH_2$	CH=CH	Ter	>100
22	CH_2OH	CH=CH	Ter	>100
23	$C(O)NHNH_2$	CH=CH	Ter	>100

and a 22-membered ring containing analogue **26** of echinocandin was synthesized by head-to-tail cyclization as is outlined in Scheme 5. Linear precursor **24** was prepared by SPPS on the trityl resin. Removal of the Fmoc group on lysine, coupling of the Ter chain, removal of the Psoc-protecting[38] group and mild acid cleavage from the resin gave protected linear peptide **25**. Solution-phase cyclization followed by protecting group removal afforded the macrocyclic peptide **26** in an overall yield of 21%. The minimum inhibitory concentration (MIC) of this echinocandin homolog was evaluated and shown in Table 2.

Table 2
Antifungal activity of head-to-tail analogues

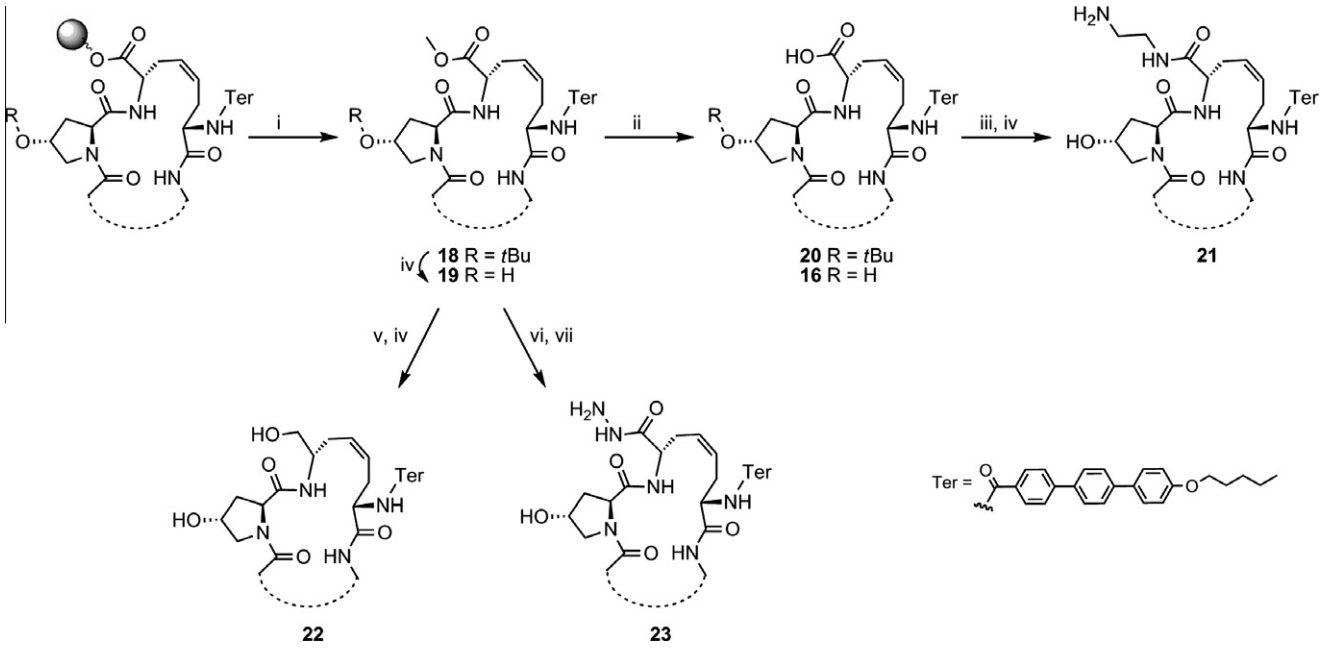
	R^1	R^2	*n*	In vitro activity MIC (μg/mL) *Candida albicans* CBS 9975
Caspofungin (**1**)	—	—	—	0.025
12a	Threonine	Palm	1	25
12b	Ornithine	Palm	1	4.38
13a	Threonine	Ter	1	0.47
13b	Ornithine	Ter	1	0.14
26	Ornithine	Ter	2	>100

Even expansion of the peptide macrocycle by just one carbon atom completely abolished antifungal activity which we had not expected in view of the limited added flexibility to the peptide macrocycle.

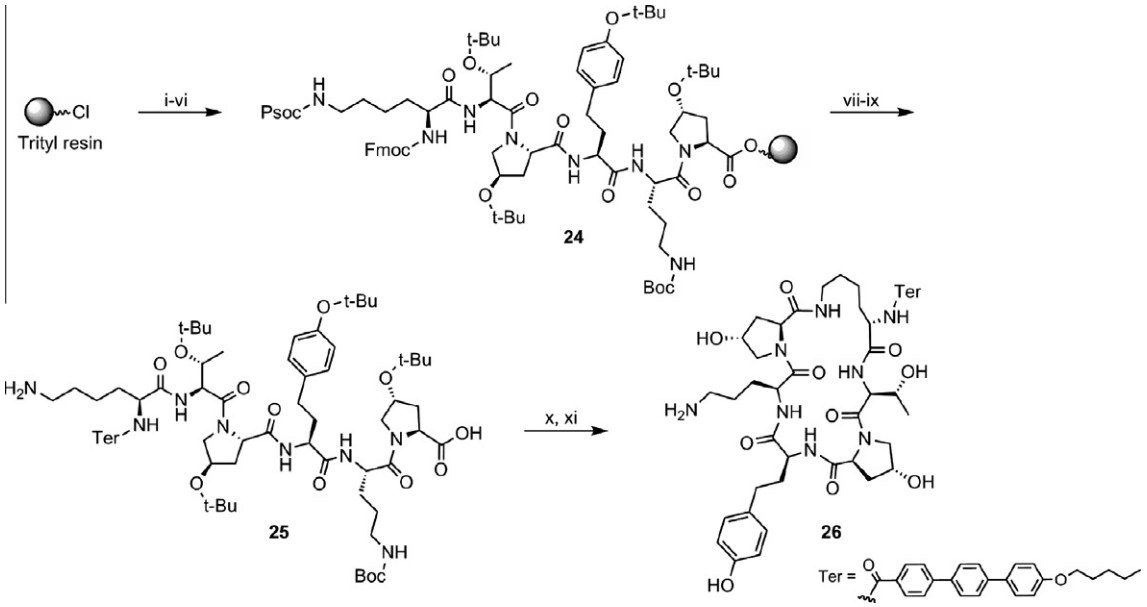
To assure that the low activities of our compounds are not strain dependent and hence the conclusions regarding the importance of the ring size are more general, we have tested a representative selection of the compounds against a panel of *Candida* strains. This panel included *Candida dubliensis*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei* and two strains of *C. albicans*. The results were in the same order of magnitude.

3. Conclusions

A series of new echinocandin analogues were synthesized by RCM and disulfide bond formation to explore the influence of the macrocyclic peptide ring structure of the echinocandins on antifungal activity. Evaluation of the antifungal properties of the resulting compounds showed no measurable activity for mimics **6**, **7** and



Scheme 4. Reagents and conditions: (i) KCN, MeOH; (ii) LiOH, THF; (iii) *N*-Boc-ethylene-diamine, HATU, DiPEA, NMP; (iv) TFA/TIS/H_2O (95/2.5/2.5, v/v/v); (v) $LiBH_4$, THF; (vi) $N_2H_4{\cdot}H_2O$, DMF; (vii) HCl/Et_2O.



Scheme 5. Reagents and conditions: (i) Fmoc-Hyp(*t*Bu)-OH, DIPEA, DCM; (ii–vi) (1) 20% piperidine in NMP; (2) Fmoc-Xxx-OH, BOP, DiPEA, NMP; (vii) (1) 20% piperidine in NMP; (2) ter tail, HATU, DiPEA, NMP; (viii) TBAF·H_2O, DCM; (ix) HFIP/DCM (1/1; v/v); (x) BOP, DiPEA, DMF; (xi) TFA/TIS/H_2O (95/2.5/2.5, v/v/v).

10. It is also known that the fatty acid chain plays a crucial role in relation to antifungal potency, therefore analogues **16** and **17**, bearing a terphenyl lipophilic chain, were synthesized. These analogues showed a dose-dependent effect on antifungal growth at concentrations starting at 5 μg/mL. The C-terminus is another modifiable site in the echinocandins, as such analogues (**21**–**23**) with a modified C-terminus were prepared and were shown to have the same dose-dependent effect on fungal growth. Remarkably, a slight enlargement of the macrocyclic peptide ring from a 21- (**13b**) to a 22-membered system (**26**) completely abolishes antifungal activity. Therefore, the preparation of other larger derivatives by either RCM or disulfide formation seems an unattractive avenue.

The mimics described here comprise a novel set of echinocandin-based analogues. The influence of ring size on the conformation(s) of the macrocyclic lipopeptides and the resulting impact on antifungal activity will be examined in future investigations. In addition, the double bond resulting from the RCM approach used here presents an additional site for derivatization. These modifications and their effects on antifungal activity are presently under investigation.

4. Experimental section

4.1. Chemistry

4.1.1. General

Unless stated otherwise, all chemicals were obtained from commercial sources and used without further purification. Piperidine, *N*,*N*-diisopropylethylamine (DiPEA), *N*-methylimidazole (NMI) peptide grade dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), 1-Methyl-2-pyrrolidinone (NMP), *tert*-butyl methylester (MTBE), trifluoroacetic acid (TFA) and HPLC grade solvents were purchased from Biosolve B.V. (Valkenswaard, The Netherlands) and used directly, with the exception of DMF, NMP and DCM, which were dried on molecular sieves (4 Å). The coupling reagents benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP), *N*-hydroxybenzotriazole (HOBt), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and N^a-9-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids were purchased from GL Biochem Ltd (Shanghai, China). The *tert*-butyl side chain protected homotyrosine (H-hTyr(*t*Bu)-OH) was purchased from Advanced Chemtech (Louisville, United States) and subsequently protected with the Fmoc group. Triisopropylsilane (TIS) was obtained from Merck (Darmstadt, Germany). 2,6-dichlorobenzoylchloride (DCBC), Second generation Grubbs and Hoveyda–Grubbs catalyst were obtained from Aldrich (Munich, Germany). The 2-chlorotritylchloride PS resin cross-linked with 1% DVB (200–400 mesh) was purchased from Hecheng Chemicals (Shanghai, China) (theoretical loading: 1.10 mmol/g). The Wang resin; Tentagel S PHB (theoretical loading: 0.26 mmol/g) and Rink resin; Tentagel S RAM (theoretical loading: 0.25 mmol/g) were purchased from RAPP Polymere (Tübingen, Germany).

All reactions were carried out at room temperature unless stated otherwise. Solid phase synthesis was performed in plastic syringes with a polyethylene frit. Microwave reactions were carried out on a Biotage Initiator system. Solution phase reactions were monitored by TLC on Merck pre-coated Silica 60 plates. Spots were visualized by UV light, ninhydrin and K_2CO_3/$KMnO_4$. Solid phase reactions were monitored with the chloranil test[39] in case of secondary amines or with the Kaiser test[40] in case of primary amines. Column chromatography was performed using Silicycle UltraPure silicagel (40–63 μm).

^{1}H NMR, TOCSY, ^{1}H–^{13}C HSQC and ROESY spectra were recorded using a Varian INOVA-500 spectrometer (500 MHz); chemical shifts (δ) were obtained in ppm relative to TMS. For measurements in DMSO, the residual solvent peak was used as a reference.

Analytical HPLC was performed on a Shimadzu automated HPLC system equipped with an evaporative light scattering detector (PL-ELS 1000) and a UV/Vis detector operated at 220/254 nm. Preparative HPLC runs were performed on an Applied Biosystems 400 solvent delivery system with an Applied Biosystems 757 UV/VIS absorbance detector. Two buffer systems were used for HPLC. The first will be referred to as TFA MeCN/H_2O buffer and consists out of buffer A: 0.1% TFA in MeCN/H_2O, 5/95, v/v and buffer B: 0.1% TFA in MeCN/H_2O, 95/5, v/v. For analytical HPLC a flow rate of 1.0 mL/min with a linear gradient of buffer B (100% in 20 min) from 100% buffer A was used. Preparative runs used a flow rate of 12 ml/min with a linear gradient of buffer B (100% in 40 min) from 100% buffer A. The second will be referred to as *i*-PrOH/MeOH/H_2O buffer and consists out of buffer A: 0.1% TFA in *i*-PrOH/MeOH/H_2O, 5/5/90, v/v/v and buffer B: 0.1% TFA in *i*-PrOH/MeOH/H_2O, 45/50/5, v/v/v. For analytical HPLC a flow rate of 0.5 mL/min with a linear gradient of buffer B (100% in 40 min) from 100% buffer A was used. Preparative runs used a flow rate of 6 ml/min with a linear gradient of buffer B (100% in 80 min) from 100% buffer A.

Peptides were characterized using ElectroSpray Ionization Mass Spectrometry (ESI-MS) on a QP8000 single quadrupole mass spectrometer in a positive ionization mode. High resolution mass spectrometry (HRMS) analyses were performed using MALDI TOF/TOF (Applied Biosystems).

4.2. General procedures

4.2.1. Solid phase peptide synthesis

Peptides were synthesized manually. Used abbreviations; Palm: palmitoyl, Ter: terphenyl, Alg: Allylglycine, Hyp: 4-hydroxyproline, hTyr: homotyrosine, IvDde: isovaleryl substituted dimedone, Psoc: (2-Phenyl-2-trimethylsilyl)ethoxycarbonyl. Each synthetic cycle consisted of the following steps.

4.2.1.1. Fmoc removal. The resin was treated with a 20% solution of piperidine in NMP (3×, each 10 min). The solution was removed by filtration and the resin was washed with NMP (3×, each 3 min) and DCM (3×, each 3 min).

4.2.1.2. Coupling step. A mixture of Fmoc-Xxx-OH (3 equiv), BOP (3 equiv) and DiPEA (6 equiv) in NMP (10 mL/mmol)) was added to the resin and N_2 was bubbled through the mixture for 2 h. The solution was removed by filtration and the resin washed with NMP (3×, each 3 min) and DCM (3×, each 3 min). Completion of the coupling was checked with Kaiser or chloranil test.

4.2.1.3. Capping of the remaining free amines. Capping solution [Ac_2O (50 mmol, 4.7 mL), HOBt (1.9 mmol, 220 mg), DiPEA (12.5 mmol, 2.2 mL) in 100 mL NMP] was added to the resin and N_2 was bubbled through the mixture for 20 min The solution was removed by filtration and the resin was washed with NMP (3×, each 3 min) and DCM (3×, each 3 min).

4.2.2. TFA cleavage

The resin was shaken in a mixture of TFA/TIS/H_2O (95/2.5/2.5, v/v/v) for 2 h. The peptide was precipitated in MTBE/hexane (1/1), the supernatant was removed and the crude peptide was washed twice with MTBE/hexane (1/1) and lyophilized from *tert*-BuOH/H_2O (1/1, v/v). The isolated peptide was analyzed by HPLC and characterized by MS.

4.2.3. Microwave-assisted RCM

A microwave vessel containing a magnetic stirrer bead was loaded under argon with resin peptide, catalyst and solvent. The vessel was capped and irradiated at 100 °C. At the end of the reaction period the resin-bound peptide was washed with DMF (3× 3 mL, each 3 min) DCM (3× 3 mL, each 3 min) and MeOH (3× 3 mL, each 3 min).

4.2.4. Hydrogenation

To a solution of the olefinic peptide in *t*BuOH/H_2O (1 mL, 3/1, v/v) 10% Pd/C was added. The reaction mixture was stirred under H_2 at atmospheric pressure overnight. The mixture was then filtered through a path of Celite and washed extensively with *t*BuOH. The mixture was concentrated and lyophilized from *tert*-BuOH/H_2O (1/1, v/v).

4.2.5. Head-to-tail cyclization

The peptide was dissolved in dry DMF (2 mL/μmol) and BOP (4 equiv) and DiPEA (8 equiv) were added. The mixture was stirred overnight followed by evaporation in vacuo. The product was redissolved in EtOAc and washed with 1 M $KHSO_4$ (2×), $NaHCO_3$ (2×) and H_2O (2×). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo.

4.2.6. Cleavage IvDde group

The peptide was dissolved in dry DMF (70 mL/mmol) and $N_2H_4{\cdot}H_2O$/DMF (5 v/v%) was added. The mixture was stirred for 2 h. Followed by the addition of brine and extraction with EtOAc (2×). The organic layer was washed with H_2O, dried over Na_2SO_4, filtered and concentrated in vacuo. The crude peptide was purified by column chromatography (DCM/MeOH 20/1, v/v/v).

4.3. *cyclo*[-(Palm)-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg]-NH_2 (6a)

The linear peptide Fmoc-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg-NH_2 (**8a**) was synthesized on Fmoc-Rink-Tentagel resin (1 g; 0.25 mmol) according to the general procedure for solid phase peptide synthesis. ESI-MS calcd for TFA deprotected **8a** $C_{54}H_{69}N_9O_{13}$: 1051.50, found: *m/z* 1052.20 $[M+H]^+$.

The resulting resin-bound peptide **8a** was subjected to the general microwave-assisted RCM procedure under the following conditions: Resin-bound peptide (421 mg; 105.2 μmol), DCM (3.8 mL), LiCl/DMA (degassed; 0.4 M; 0.38 mL), Grubbs II (15 mol %; 13.4 mg; 15.8 μmol), 100 °C for 75 min. ESI-MS calcd for TFA deprotected **9a** $C_{52}H_{65}N_9O_{13}$: 1023.47, found: *m/z* 1024.15 $[M+H]^+$.

The Fmoc group was cleaved and palmitic acid (108 mg; 421 μmol) was coupled overnight in the presence of HATU (160 mg; 421 μmol) and DIPEA (147 μL; 842 μmol) in NMP (3 mL). The resin was washed with NMP (3× 3 mL, each 3 min) and DCM (3× 3 mL, each 3 min) and subjected to the TFA-mediated cleavage procedure followed by lyophilization. The crude peptide was purified by column chromatography (*i*-PrOH/NH_4OH/H_2O, 15/1/1, v/v/v). After lyophilization peptide **6a** (12 mg; 11%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Adsorbosphere C8, 40 min run, TFA MeCN/H_2O buffers) and was found to be higher than 95% for a mixture of *E/Z* stereoisomers (t_R = 22.6 min and 22.8 min). ESI-MS calcd for $C_{53}H_{85}N_9O_{12}$: 1039.63, found: *m/z* 1040.61 $[M+H]^+$; HRMS calcd for $C_{53}H_{85}N_9O_{12}$ $[M+H]^+$ 1040.6396, found 1040.6403; ^{1}H NMR (DMSO, 500 MHz): δ Alg-1: 8.05 (NH), 4.16 (αCH), 2.36/2.16 (βCH), 5.74 (γCH); Thr-2: 7.354 (NH), 4.61 (αCH), 4.32 (βCH), 1.09 (γCH); Hyp-3: 4.43 (αCH), 2.13/1.79 (βCH), 4.30 (γCH), 3.64/3.56 (δCH); hTyr-4: 7.75 (NH), 4.00 (αCH), 2.04/1.90 (βCH), 2.46/2.36 (γCH); Orn-5: 7.23 (NH), 4.49 (αCH), 1.65/1.53 (βCH), 1.47 (γCH), 2.75 (δCH); Hyp-6: 4.46 (αCH), 2.02/1.87 (βCH), 4.37 (γCH), 3.78/3.66 (δCH); Alg-7: 7.93 (NH), 4.093 (αCH), 2.29 (βCH), 5.74 (γCH); Palm: 2.11 (CH_2), 1.45 (CH_2), 1.26 (CH_2), 1.23 (CH_2), 0.86 (CH_3) ppm. ^{13}C NMR (DMSO, 125 MHz): δ Alg-1: 52.2 (αCH), 34.5 (βCH), 126.2 (γCH); Thr-2: 56.6 (αCH), 66.0 (βCH), 19.4 (γCH); Hyp-3: 58.3 (αCH), 37.3 (βCH), 68.7 (γCH), 55.7 (δCH); hTyr-4: 52.4 (αCH), 32.6 (βCH), 31.1 (γCH); 115.5 (Ar-H), 129.8 (Ar-H); Orn-5: 52.8 (αCH), 28.0 (βCH), 24.0 (γCH), 39.0 (δCH); Hyp-6: 59.3 (αCH), 37.9 (βCH), 69.2 (γCH), 55.7 (δCH); Alg-7: 52.9 (αCH), 33.8 (βCH), 126.2 (γCH); Palm: 35.2 (CH_2), 28.9 (CH_2), 25.4 (CH_2), 22.2 (CH_2), 14.0 (CH_3).

4.4. *cyclo*[-(Palm)-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg]-OH (6b)

Peptide **6b** was obtained analogous to peptide **6a,** on Wang-Tentagel resin (1 g; 0.26 mmol), except for the loading step. For that Tentagel S PHB (1.0 g, 0.26 mmol) and Fmoc-Alg-OH (364 mg, 1.04 mmol) were dried in vacuo overnight over P_2O_5. DMF (5 mL) and pyridine (114 μL, 1.42 mmol) were added and the resin was shaken for 10 min until total dissolution. DCBC (149 μL, 1.04 mmol) was added and the resin was shaken over the weekend. The resin was drained and washed with DMF (5× 3 mL, each 2 min) and DCM (5× 3 mL, each 2 min). Subsequently, unreacted hydroxyl functions of the resin were acetylated by treatment with Ac_2O/NMI/DiPEA/DMF (5 mL; 2:1:1:6; v/v/v) for 30 min. The resin was drained and washed with DMF (3× 3 mL, each 2 min) and DCM (3× 3 mL, each 2 min). Hyp was coupled as the dipeptide Fmoc-Orn(Boc)-Hyp(*t*Bu)-OH.

ESI-MS calcd for TFA deprotected **8b** $C_{54}H_{68}N_8O_{14}$: 1052.49, found: *m/z* 1053.10 $[M+H]^+$.

ESI-MS calcd for TFA deprotected **9b** $C_{52}H_{64}N_8O_{14}$: 1024.45, found: *m/z* 1025.00 $[M+H]^+$.

The crude peptide was purified by column chromatography (*i*-PrOH/NH_4OH/H_2O, 10/1/1, v/v/v). After lyophilization peptide **6b** (12.2 mg; 11%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Adsorbosphere C8, 40 min run, TFA MeCN/H_2O buffers) and was found to be higher than 95% for a mixture of *E/Z* stereoisomers (t_R = 22.60 min and 22.81 min). ESI-MS calcd for $C_{53}H_{84}N_8O_{13}$: 1040.62, found: *m/z* 1041.15 $[M+H]^+$; HRMS calcd for $C_{53}H_{84}N_8O_{13}$ $[M+H]^+$ 1041.6236, found 1041.6243; ^{1}H NMR (CD_3OH, 500 MHz): δ Alg-1: 7.87 (NH), 4.17 (αCH), 2.60/2.13 (βCH), 5.48 (γCH); Thr-2: 7.95 (NH), 4.70 (αCH), 4.32 (βCH), 1.14 (γCH); Hyp-3: 4.37 (αCH), 2.18/1.73 (βCH), 4.40 (γCH), 3.68/3.61 (δCH); hTyr-4: 7.64 (NH), 4.02 (αCH), 2.14/1.93 (βCH), 2.48 (γCH); Orn-5: 7.64 (NH), 4.62 (αCH), 2.00/1.52 (βCH), 1.58 (γCH), 2.86 (δCH); Hyp-6: 4.51 (αCH), 2.20/1.99 (βCH), 4.34 (γCH), 3.68/3.42 (δCH); Alg-7: 8.23 (NH), 2.47/2.18 (βCH), 5.48 (γCH) ppm. ^{13}C NMR (CD_3OH, 125 MHz): δ Alg-1: 54.1 (αCH), 35.4 (βCH), 128.9 (γCH); Thr-2: 56.8 (αCH), 67.8 (βCH), 18.7 (γCH); Hyp-3: 61.1 (αCH), 37.2 (βCH), 70.2 (γCH), 55.7 (δCH); hTyr-4: 54.3 (αCH), 32.3 (βCH), 31.7 (γCH); 115.5 (Ar-H), 129.8 (Ar-H); Orn-5: 50.8 (αCH), 28.5 (βCH), 23.7 (γCH), 39.2 (δCH); Hyp-6: 59.3 (αCH), 37.9 (βCH), 69.8 (γCH), 55.5 (δCH); Alg-7: 54.7 (αCH), 34.3 (βCH), 128.9 (γCH).

4.5. *cyclo*[-(Palm)-Cys-Thr-Hyp-hTyr-Orn-Hyp-Cys]-NH_2 (7)

The linear peptide Fmoc-Cys-Thr-Hyp-hTyr-Orn-Hyp-Cys-NH_2 was synthesized on Fmoc-Rink-Tentagel resin (211 mg; 50.6 μmol) according to the general procedure for solid phase peptide synthesis. The Fmoc group was cleaved and palmitic acid (52 mg; 202.6 μmol) was coupled overnight in the presence of HATU (77 mg; 202.6 μmol) and DiPEA (71 μL; 405.2 μmol) in NMP (2 mL). The resin was washed with NMP (3× 2 mL, each 3 min) and DCM (3× 2 mL, each 3 min) and subjected to the TFA cleavage procedure and lyophilized. Mass spectral analysis (ESI-MS calcd for **11** $C_{51}H_{85}N_9O_{12}S_2$: 1079.58, found: *m/z* 1079.90 $[M+H]^+$) confirmed the formation of the linear peptide **11** and the peptide was cyclized by oxidation with DMSO. Peptide **11** (18 mg; 16.7 μmol) was dissolved in aq 2.5% AcOH (25 mL). The pH was adjusted to 6 with aq NH_4OH (25%). To this solution DMSO (5.5 mL) was added. After stirring at rt overnight, the solution was partially concentrated in vacuo and remaining DMSO was removed by a speedvac apparatus (42 °C, overnight). After preparative HPLC and lyophilization, peptide **7** (4 mg; 22%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 99% (t_R = 40.71 min). ESI-MS calcd for $C_{51}H_{83}N_9O_{12}S_2$: 1077.56, found: *m/z* 1078.35 $[M+H]^+$; HRMS calcd for $C_{51}H_{83}N_9O_{12}S_2$ $[M+H]^+$ 1078.5681, found 1078.5721; ^{1}H NMR (DMSO, 500 MHz): δ Cys-1: 8.18 (NH), 4.76 (αCH), 3.29/2.85 (βCH); Thr-2: 7.63 (NH), 4.70 (αCH), 4.29 (βCH), 1.05 (γCH); Hyp-3: 4.24 (αCH), 2.12/1.80 (βCH), 4.37 (γCH), 3.80/3.59 (δCH); hTyr-4: 7.34 (NH), 3.90 (αCH), 1.96/1.89 (βCH), 2.52/2.47 (γCH); Orn-5: 7.25 (NH), 7.24 (εNH), 4.45 (αCH), 1.80/1.61 (βCH), 1.51 (γCH), 2.80 (δCH); Hyp-6: 4.55 (αCH), 1.94 (βCH), 4.28 (γCH), 3.56/3.18 (δCH); Cys-7: 8.25 (NH), 4.30 (αCH), 2.85 (βCH); Palm: 2.11 (CH_2), 1.47 (CH_2), 1.26 (CH_2), 1.23 (CH_2), 0.86 (CH_3) ppm. ^{13}C NMR (DMSO, 125 MHz): δ Cys-1: 51.1 (αCH), 43.4 (βCH); Thr-2: 56.1 (αCH), 66.7 (βCH), 19.3 (γCH); Hyp-3: 61.0 (αCH), 37.1 (βCH), 69.3 (γCH), 55.9 (δCH); hTyr-4: 53.5 (αCH), 32.4 (βCH), 31.2 (γCH); 115.5 (Ar-H), 129.9 (Ar-H); Orn-5: 49.6

(αCH), 27.6 (βCH), 23.5 (γCH), 38.5 (δCH); Hyp-6: 59.0 (αCH), 37.8 (βCH), 68.5 (γCH), 53.5 (δCH); Cys-7: 51.5 (αCH), 43.4 (βCH); Palm: 35.3 (CH_2), 29.0 (CH_2), 25.4 (CH_2), 22.1 (CH_2), 14.0 (CH_3).

4.6. *cyclo*[-(Palm)-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg]-NH_2. (10a)

Hydrogenation of the cyclic unsaturated peptide **6a** (3.2 mg; 3.07 μmol) was carried out using the hydrogenation procedure in the presence of 3 mg 10% Pd/C. The saturated peptide **10a** (3.1 mg; quant) was obtained as a white solid. Purity was confirmed by analytical HPLC (Adsorbosphere C8, 40 min run, TFA MeCN/H_2O buffers) and was found to be higher than 99% (t_R = 22.62 min). ESI-MS calcd for $C_{53}H_{87}N_9O_{12}$: 1041.65, found: *m/z* 1042.59 $[M+H]^+$; HRMS calcd for $C_{53}H_{87}N_9O_{12}$ $[M+H]^+$ 1042.6552, found 1042.6530.

4.7. *cyclo*[-(Palm)-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg]-OH (10b)

Hydrogenation of the cyclic unsaturated peptide **6b** (4.7 mg; 4.52 μmol) was carried out using the hydrogenation procedure in the presence of 5 mg 10% Pd/C. The saturated peptide **10b** (4.6 mg; quant.) was obtained as a white solid. Purity was confirmed by analytical HPLC (Adsorbosphere C8, 40 min run, TFA MeCN/H_2O buffers) and was found to be higher than 95% (t_R = 22.57 min). ESI-MS calcd for $C_{53}H_{86}N_8O_{13}$: 1042.63, found: *m/z* 1043.59 $[M+H]^+$; HRMS calcd for $C_{53}H_{86}N_8O_{13}$ $[M+H]^+$ 1043.6393, found 1043.6416.

4.8. *cyclo*[-(Palm)-Orn-Thr-Hyp-hTyr-Thr-Hyp] (12a)

A polystyrene resin functionalized with a 2-chloro Trityl linker (600 mg; initial loading: 1.1 mmol/g) was loaded with Fmoc-Hyp(*t*Bu)-OH (810.8 mg; 1.98 mmol) in DCM (5 mL) in the presence of DiPEA (690 μL; 3.96 mmol) for 16 h. Subsequently, unreacted tritylchloride moieties were capped with methanol (DCM/MeOH/DiPEA; 3× 5 mL, each 2 min; 17/2/1; v/v/v). After drying in vacuo overnight, the amount of Fmoc-Hyp(*t*Bu)-OH coupled to the resin was determined by a Fmoc determination according to Meienhofer[41] and was found to be 0.536 mmol/g. The peptide sequence was synthesized according to the general procedure for solid phase peptide synthesis.

The Fmoc group was cleaved and the linear peptide was released from the resin by treatment of the resin with HFIP/DCM (6 mL, 1/1, v/v) for 2 h. This treatment was repeated once for 1 h. The mixture was concentrated to obtain the linear peptide. A portion of the linear peptide (187 mg; 0.15 mmol) was subjected to head-to-tail cyclization and the IvDde group was cleaved following the general procedures. The free amine of the linear peptide (54.2 mg; 54.2 μmol) was coupled with palmitic acid by dissolving the peptide in dry DCM (4 mL) and adding palmitic acid (15.3 mg; 59.6 μmol), DCC (12.3 mg; 59.6 μmol) and HOBt (8.0 mg; 59.6 μmol). The mixture was stirred overnight and the DCU filtered off over Celite. The crude peptide was purified by column chromatography (DCM/MeOH 30/1, v/v/v), Followed by treatment with a mixture of TFA/TIS/H_2O (3 mL, 95/2.5/2.5, v/v/v) for 2 h. The mixture was concentrated in vacuo, redissolved in a small amount of TFA and precipitated in MTBE/hexane (1/1). The supernatant was removed and the crude peptide washed twice with MTBE/hexane (1/1). After lyophilization, peptide **12a** (15.6 mg; 19%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 99% (t_R = 42.18 min). ESI-MS calcd for $C_{49}H_{79}N_7O_{12}$: 957.58, found: *m/z* 958.95 $[M+H]^+$; HRMS calcd for $C_{49}H_{79}N_7O_{12}$ $[M+H]^+$ 958.5865, found 958.5819; ^1H NMR (DMSO, 500 MHz): δ Orn-1: 7.80 (NH), 4.30 (αCH), 1.69/1.38 (βCH), 1.39 (γCH), 2.98 (δCH); Thr-2: 7.88 (NH), 4.23 (αCH), 4.01 (βCH), 1.21 (γCH); Hyp-3: 4.42 (αCH), 2.11/1.82 (βCH), 4.40 (γCH), 3.76 (δCH); hTyr-4: 8.46 (NH), 3.62 (αCH), 2.19/2.11 (βCH), 2.43/2.34 (γCH), 6.93/6.65 (Ar-H); Thr-5: 7.45 (NH), 4.12 (βCH), 1.02 (γCH); Hyp-6: 2.11/1.82 (βCH), 4.27 (γCH), 3.61 (δCH); Palm: 0.85 (CH_3), 1.26 (CH_2), 1.24 (CH_2), 1.44 (CH_2), 2.07 (CH_2) ppm. ^{13}C NMR (DMSO, 125 MHz): δ Orn-1: 50.9 (αCH), 28.9 (βCH), 24.0 (γCH), 37.9 (δCH); Thr-2: 58.5 (αCH), 66.5 (βCH), 19.1 (γCH); Hyp-3: 60.0 (αCH), 37.7 (βCH), 69.1 (γCH), 56.4 (δCH); hTyr-4: 53.7 (αCH), 31.0 (βCH), 31.2 (γCH), 129.8, 115.5 (Ar-H); Thr-5: 56.5 (αCH), 67.3 (βCH), 18.9 (γCH); Hyp-6: 59.3 (αCH), 37.7 (βCH), 69.0 (γCH), 56.2 (δCH); Palm: 13.9 (CH_3), 22.0 (CH_2), 25.3 (CH_2), 29.0 (CH_2), 31.4 (CH_2), 35.1 (CH_2).

4.9. *cyclo*[-(Palm)-Orn-Thr-Hyp-hTyr-Orn-Hyp] (12b)

Peptide **13a** was obtained analogously to peptide **12a** except for the amino acid sequence. The second amino acid coupled was Fmoc-Orn(Boc)-OH instead of Fmoc-Thr(*t*Bu)-OH. The loading of the resin was determined after first coupling: 0.543 mmol/g.

After lyophilization, peptide **12b** (18.5 mg; 22%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 40 min run, TFA MeCN/H_2O buffers) and was found to be higher than 99% (t_R = 23.18 min). ESI-MS calcd for $C_{50}H_{82}N_8O_{11}$: 970.61, found: *m/z* 971.20 $[M+H]^+$; HRMS calcd for $C_{50}H_{82}N_8O_{11}$ $[M+H]^+$ 971.6181, found 971.6187; ^1H NMR (DMSO, 500 MHz): δ Orn-1: 7.92 (NH), 7.67 (εNH), 4.14 (αCH), 1.64/1.56 (βCH), 1.54 (γCH), 2.79 (δCH); Thr-2: 8.27 (NH), 4.74 (αCH), 4.36 (βCH), 1.09 (γCH); Hyp-3: 4.39 (αCH); hTyr-4: 7.59 (NH), 4.03 (αCH), 1.86/1.75 (βCH), 2.43/2.37 (γCH); Orn-5: 7.70 (NH), 7.59 (εNH), 4.52 (αCH), 1.89/1.62 (βCH), 1.56 (γCH), 3.40/2.66 (δCH); Hyp-6: 4.40 (γCH), 3.76/3.51 (δCH); Palm: 2.02 (CH_2), 1.46 (CH_2), 1.32 (CH_2), 1.26 (CH_2), 0.86 (CH_3) ppm. ^{13}C NMR (DMSO, 125 MHz): δ Orn-1: 50.6 (αCH), 28.3 (βCH), 23.5 (γCH), 38.3 (δCH); Thr-2: 56.1 (αCH), 66.2 (βCH), 20.4 (γCH); Hyp-3: 60.8 (αCH); hTyr-4: 53.2 (αCH), 28.1 (βCH), 30.9 (γCH); 115.2 (Ar-H), 129.5 (Ar-H); Orn-5: 50.6 (αCH), 28.4 (βCH), 22.2 (γCH), 35.2 (δCH); Hyp-6: 69.3 (γCH), 55.4 (δCH); Palm: 35.2 (CH_2), 27.8 (CH_2), 25.9 (CH_2), 25.0 (CH_2), 13.6 (CH_3).

4.10. *cyclo*[-(Ter)-Orn-Thr-Hyp-hTyr-Thr-Hyp] (13a)

Peptide **13a** was obtained analogously to peptide **12a** until the coupling of the tail. Loading of the resin determined after first coupling: 0.536 mmol/g.

The free amine of the linear peptide (34.7 mg; 34.7 μmol) was coupled with the Ter chain by dissolving the peptide in DCM (4 mL) and adding 4-(4″-pentyloxy-1,1′:4′,1″-terphenyl)-carboxylic acid[27] (13.8 mg; 38.2 μmol), DCC (7.9 mg; 38.2 μmol) and HOBt (5.2 mg; 38.2 μmol). The mixture was stirred overnight and then the DCU was filtered off over Celite. The crude peptide was purified by column chromatography (DCM/MeOH 30/1, v/v/v), Followed by treatment with a mixture of TFA/TIS/H_2O (95/2.5/2.5; 3 mL) for 1 h. The mixture was concentrated in vacuo, redissolved in a small amount of TFA and precipitated in MTBE/hexane (1/1). The supernatant was removed and the crude peptide washed twice with MTBE/hexane (1/1). After lyophilization, peptide **13a** (13.1 mg; 23%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 99% (t_R = 39.72 min). ESI-MS calcd for $C_{57}H_{71}N_7O_{13}$: 1061.51, found: *m/z* 1062.65 $[M+H]^+$; HRMS calcd for $C_{57}H_{71}N_7O_{13}$ $[M+H]^+$ 1062.5188, found 1062.5227; ^1H NMR (DMSO, 500 MHz): δ Orn-1: 8.38 (NH), 7.46 (εNH), 4.53 (αCH), 1.91/1.54 (βCH), 1.53 (γCH), 3.09/3.02 (δCH); Thr-2: 7.95 (NH), 4.32 (αCH), 4.08 (βCH), 1.25 (γCH); Hyp-3: 4.43 (αCH), 2.14/1.84 (βCH), 4.40 (γCH), 3.77 (δCH); hTyr-4: 8.40 (NH), 3.68 (αCH), 2.18/2.13 (βCH), 2.44/2.36 (γCH), 6.95/6.66 (Ar-H); Thr-5:

7.48 (NH), 4.80 (αCH), 4.16 (βCH), 1.06 (γCH); Hyp-6: 4.38 (αCH), 2.11/1.84 (βCH), 4.29 (γCH), 3.64 (δCH); Ter: 0.91 (CH_3), 1.37 (CH_2), 1.42 (CH_2), 1.74 (CH_2), 4.01 (OCH_2) 7.66/7.04 (Ar-H Ph-3), 7.80/7.74 (Ar-H Ph-2), 7.99/7.81 (Ar-H Ph-1) ppm. ^{13}C NMR (DMSO, 125 MHz): δ Orn-1: 52.1 (αCH), 28.2 (βCH), 24.1 (γCH), 38.0 (δCH); Thr-2: 58.4 (αCH), 66.6 (βCH), 19.2 (γCH); Hyp-3: 60.1 (αCH), 37.6 (βCH), 69.2 (γCH), 56.4 (δCH); hTyr-4: 53.6 (αCH), 31.2 (βCH), 31.2 (γCH), 129.8/115.5 (Ar-H); Thr-5: 56.5 (αCH), 67.3 (βCH), 19.0 (γCH); Hyp-6: 59.3 (αCH), 37.6 (βCH), 69.1 (γCH), 56.2 (δCH); Ter: 13.8 (CH_3), 21.9 (CH_2), 27.7 (CH_2), 28.4 (CH_2), 67.7 (OCH_2), 128.2/115.4 (Ar-C Ph-3), 127.8/127.2 (Ar-C Ph-2), 128.8/126.6 (Ar-C Ph-1).

4.11. *cyclo*[-(Ter)-Orn-Thr-Hyp-hTyr-Orn-Hyp] (13b)

Peptide **13b** was obtained analogously to peptide **13a** except for the amino acid sequence. The second amino acid coupled was Fmoc-Orn(Boc)-OH instead of Fmoc-Thr(*t*Bu)-OH. Loading of the resin determined after first coupling: 0.543 mmol/g.

After lyophilization, peptide **13b** (9.4 mg; 17%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be 90% (t_R = 38.95 min). ESI-MS calcd for $C_{58}H_{74}N_8O_{12}$: 1074.54, found: *m/z* 1075.69 $[M+H]^+$; HRMS calcd for $C_{58}H_{74}N_8O_{12}$ $[M+H]^+$ 1075.5504, found 1075.5529; ^{1}H NMR (DMSO, 500 MHz): δ Orn-1: 8.62 (NH), 8.01 (εNH), 4.60 (αCH), 1.83/1.65 (βCH), 1.60 (γCH), 3.47/2.72 (δCH); Thr-2: 8.34 (NH), 4.78 (αCH), 4.39 (βCH), 1.13 (γCH); Hyp-3: 4.34 (αCH), 2.00/1.86 (βCH), 4.38 (γCH), 3.75/3.69 (δCH); hTyr-4: 7.60 (NH), 4.07 (αCH), 2.09/1.87 (βCH), 2.46/2.38 (γCH), 6.95/6.66 (Ar-H); Orn-5: 7.50 (NH), 4.69 (αCH), 1.83/1.65 (βCH), 1.60 (γCH), 2.90/2.84 (δCH); Hyp-6: 4.38 (αCH), 2.16/1.77 (βCH), 4.37 (γCH), 3.72/3.49 (δCH); Ter: 0.91 (CH_3), 1.37 (CH_2), 1.42 (CH_2), 1.75 (CH_2), 4.02 (OCH_2) 7.66/7.04 (Ar-H Ph-3), 7.81/7.75 (Ar-H Ph-2), 8.03/7.82 (Ar-H Ph-1) ppm. ^{13}C NMR (DMSO, 125 MHz): δ Orn-1: 51.8 (αCH), 29.0 (βCH), 22.9 (γCH), 35.7 (δCH); Thr-2: 56.5 (αCH), 66.5 (βCH), 19.5 (γCH); Hyp-3: 60.9 (αCH), 37.7 (βCH), 69.2 (γCH), 55.9 (δCH); hTyr-4: 52.2 (αCH), 33.4 (βCH), 31.1 (γCH), 129.9/115.5 (Ar-H); Orn-5: 49.7 (αCH), 29.0 (βCH), 22.9 (γCH), 38.9 (δCH); Hyp-6: 59.2 (αCH), 37.3 (βCH), 69.2 (γCH), 56.1 (δCH); Ter: 13.9 (CH_3), 21.9 (CH_2), 27.7 (CH_2), 28.4 (CH_2), 67.7 (OCH_2), 128.2/115.4 (Ar-C Ph-3), 127.9/127.2 (Ar-C Ph-2), 128.9/126.6 (Ar-C Ph-1).

4.12. *cyclo*[-(Ter)-Alg-Thr(tBu)-Hyp(tBu)-hTyr(tBu)-Orn(Boc)-Hyp(tBu)-Alg]-OMe (18)

Wang-Tentagel S PHB resin (4 g; 1.04 mmol) and Fmoc-Alg-OH (1.09 g, 4.16 mmol) were dried in vacuo overnight over P_2O_5. DMF (20 mL) and pyridine (555 μL, 6.86 mmol) were added and the resin was shaken for 10 min until total dissolution. DCBC (596 μL, 4.16 mmol) was added and the resin was shaken for two days. The resin was drained and washed with DMF (5× 30 mL, each 2 min) and DCM (5× 30 mL, each 2 min). Subsequently, unreacted hydroxyl functions of the resin were acetylated by treatment with Ac_2O/NMI/DiPEA/DMF (30 mL; 2/1/1/6; v/v/v) for 30 min. The resin was drained and washed with DMF (3× 30 mL, each 2 min) and DCM (3× 30 mL, each 2 min). The peptide sequence was synthesized according to the general procedure for solid phase peptide synthesis. Hyp was coupled as part of the dipeptide Fmoc-Orn(Boc)-Hyp(*t*Bu)-OH.

ESI-MS calcd for TFA deprotected **8b** $C_{54}H_{68}N_8O_{14}$: 1052.49, found: *m/z* 1053.52 $[M+H]^+$.

Removal of the Fmoc group was followed by coupling overnight of the ter tail (1.44 g; 4 mmol) in the presence of HATU (1.52 g; 4 mmol) and DIPEA (1.39 mL; 8 mmol) in NMP (30 mL). The resin was washed with NMP (3× 30 mL, each 3 min) and DCM (3× 30 mL, each 3 min). The resin-bound peptide was subjected to the microwave-assisted RCM procedure under the following conditions: Resin peptide (3.3 g; 0.86 mmol), DCM (27.5 mL), LiCl/DMA (degassed; 0.4 M; 2.7 mL), Grubbs II (9 mol %; 64 mg; 75.4 μmol), 100 °C for 60 min (performed in three batches due to the size (10 mL) of the microwave vessel).

The fully protected peptide **18** was obtained by cleavage from the resin by treatment with a catalytic amount of KCN in MeOH (20 mL) for 16 h. The resin was filtered and washed with MeOH (3× 15 mL) and the filtrate was concentrated in vacuo to yield the crude peptide. The peptide was purified by column chromatography (DCM/MeOH, 50/1, v/v). After lyophilization peptide **18** (191.5 mg; 15%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 99% (t_R = 50.05 min). ESI-MS calcd for $C_{83}H_{118}N_8O_{16}$: 1482.87, found: *m/z* 1483.79 $[M+H]^+$; HRMS calcd for $C_{83}H_{118}N_8O_{16}$ $[M+H]^+$ 1483.8744, found 1483.8715; ^{1}H NMR ($CDCl_3$, 500 MHz): δ *t*Bu: 1.53–1.37 (CH_3), 1.25–1.11 (CH_3); hTyr-4: 7.57/7.00 (Ar-H); Me ester: 3.71 (OCH_3); Ter: 0.95 (CH_3), 1.31 (CH_2), 1.41 (CH_2), 1.82 (CH_2), 4.01 (OCH_2) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ *t*Bu: 28.6 (CH_3), 28.2 (CH_3); hTyr-4: 128.7/115.5 (Ar-H); Me ester: 52.7 (OCH_3); Ter: 14.1 (CH_3), 22.6 (CH_2), 28.9 (CH_2), 29.2 (CH_2), 68.5 (OCH_2). Full NMR characterization of the protected peptide **18** was not possible due to the high intensity of the *t*Bu/Boc signals and the broadened amino acid signals. However, full NMR characterization of the deprotected peptide can be found at the experimental details of cyclic peptide **19**.

4.13. *cyclo*[-(Ter)-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg]-OH (16)

Peptide **16** was obtained analogous to peptide **18** except for the last step and obtained by using the TFA cleavage procedure for a small aliquot of resin. After preparative HPLC and lyophilization, peptide **16** was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 95% for a mixture of *E/Z* stereoisomers (t_R = 40.25 min and 40.43 min). ESI-MS calcd for $C_{61}H_{76}N_8O_{14}$: 1144.55, found: *m/z* 1145.67 $[M+H]^+$; HRMS calcd for $C_{61}H_{76}N_8O_{14}$ $[M+H]^+$ 1145.5559, found 1145.5547; ^{1}H NMR (DMSO, 500 MHz): δ Alg-1: 8.62 (NH), 4.73 (αCH), 2.88/2.55 (βCH), 5.46 (γCH); Thr-2: 7.66 (NH), 4.68 (αCH), 4.26 (βCH), 1.09 (γCH); Hyp-3: 4.23 (αCH), 1.79 (βCH), 4.36 (γCH), 3.78/3.58 (δCH); hTyr-4: 7.33 (NH), 3.87 (αCH), 1.95/1.88 (βCH), 2.52/2.47 (γCH), 6.95/6.65 (Ar-H); Orn-5: 7.62 (εNH), 7.21 (NH), 4.46 (αCH), 1.83/1.56 (βCH), 1.51 (γCH), 2.83 (δCH); Hyp-6: 4.28 (αCH), 1.91 (βCH), 4.53 (γCH), 3.58/3.16 (δCH); Alg-7: 8.15 (NH), 4.19 (αCH), 2.35 (βCH), 5.46 (γCH); Ter: 0.89 (CH_3), 1.21 (CH_2), 1.39 (CH_2), 1.73 (CH_2), 4.01 (OCH_2), 7.65/7.03 (Ar-H Ph-3), 7.79/7.73 (Ar-H Ph-2), 7.98/7.81 (Ar-H Ph-1) ppm.

4.14. *cyclo*[-(Ter)-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg]-OH (17)

Hydrogenation of the cyclic unsaturated peptide **16** (3.5 mg; 3.06 μmol) was carried out using the hydrogenation procedure in the presence of 3 mg of 10% Pd/C. The saturated peptide **17** (quant.) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 95% (t_R = 41.99 min). ESI-MS calcd for $C_{61}H_{78}N_8O_{14}$: 1146.56, found: *m/z* 1147.53 $[M+H]^+$; HRMS calcd for $C_{61}H_{78}N_8O_{14}$ $[M+H]^+$ 1147.5710, found 1147.5707; ^{1}H NMR (DMSO, 500 MHz): δ Alg-1: 8.60 (NH), 4.55 (αCH), 1.76 (βCH), 1.35 (γCH); Thr-2: 7.44 (NH), 4.64 (αCH), 4.30 (βCH), 1.14 (γCH); Hyp-3: 4.37 (αCH), 2.17/1.81 (βCH), 4.32 (γCH), 3.67/3.44 (δCH); hTyr-4: 7.68 (NH), 3.97 (αCH), 2.11/1.89 (βCH), 2.47/2.38 (γCH), 6.95/6.67 (Ar-H); Orn-5: 7.65 (εNH), 7.64 (NH), 4.64 (αCH), 1.89/

1.62 (βCH), 1.54 (γCH), 2.57/2.83 (δCH); Hyp-6: 4.47 (αCH), 2.46/1.85 (βCH), 4.38 (γCH), 3.73 (δCH); Alg-7: 8.33 (NH), 4.32 (αCH), 1.80/1.60 (βCH), 1.376 (γCH); Ter: 0.91 (CH_3), 1.36 (CH_2), 1.42 (CH_2), 1.75 (CH_2), 4.02 (OCH_2), 7.67/7.05 (Ar-H Ph-3), 7.81/7.76 (Ar-H Ph-2), 8.04/7.83 (Ar-H Ph-1) ppm. ^{13}C NMR (DMSO, 125 MHz): δ Alg-1: 54.0 (αCH), 31.4 (βCH), 24.0 (γCH); Thr-2: 56.1 (αCH), 66.3 (βCH), 19.1 (γCH); Hyp-3: 60.5 (αCH), 37.1 (βCH), 68.4 (γCH), 55.2 (δCH); hTyr-4: 52.1 (αCH), 32.2 (βCH), 30.7 (γCH), 129.6/115.2 (Ar-H); Orn-5: 49.3 (αCH), 28.6 (βCH), 23.2 (γCH), 38.4 (δCH); Hyp-6: 58.0 (αCH), 37.6 (βCH), 68.9 (γCH), 55.5 (δCH); Alg-7: 49.5 (αCH), 29.6 (βCH), 24.2 (γCH); Ter: 13.6 (CH_3), 21.6 (CH_2), 27.4 (CH_2), 28.1 (CH_2), 67.4 (OCH_2), 127.9/115.1 (Ar-H Ph-3), 127.6/126.9 (Ar-H Ph-2), 128.6/126.3 (Ar-H Ph-1) ppm.

4.15. *cyclo*[-(Ter)-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg]-OMe (19)

Fully protected peptide **18** (19.9 mg; 13.41 μmol) was treated with a mixture of TFA/TIS/H_2O (2 mL, 95/2.5/2.5, v/v/v) for 2 h. The mixture was concentrated in vacuo, re-dissolved in a small amount of TFA and precipitated in MTBE/hexane (1/1). The supernatant was removed and the crude peptide washed twice with MTBE/hexane (1/1). After preparative HPLC (Altima C8 semiprep, 120 min run, *i*-PrOH/MeOH/H_2O buffers) and lyophilization, peptide **19** (9.3 mg; 60%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 99% (t_R = 42.62 min). ESI-MS calcd for $C_{62}H_{78}N_8O_{14}$: 1158.56, found: *m/z* 1159.52 $[M+H]^+$; HRMS calcd for $C_{62}H_{78}N_8O_{14}$ $[M+H]^+$ 1159.5716, found 1159.5750; ^{1}H NMR (DMSO, 500 MHz): δ Alg-1: 8.62 (NH), 4.73 (αCH), 2.88/2.55 (βCH), 5.46 (γCH); Thr-2: 7.65 (NH), 4.68 (αCH), 4.26 (βCH), 1.09 (γCH); Hyp-3: 4.22 (αCH), 2.02/1.83 (βCH), 4.36 (γCH), 3.79/3.59 (δCH); hTyr-4: 7.33 (NH), 3.88 (αCH), 1.94/1.88 (βCH), 2.52/2.46 (γCH), 6.94/6.64 (Ar-H); Orn-5: 7.63 (εNH), 7.21 (NH), 4.46 (αCH), 1.82/1.57 (βCH), 1.51 (γCH), 2.82 (δCH); Hyp-6: 4.53 (αCH), 1.93/1.88 (βCH), 4.28 (γCH), 3.58/3.17 (δCH); Alg-7: 8.27 (NH), 4.29 (αCH), 2.35 (βCH), 5.46 (γCH); Me ester: 3.61 (CH_3); Ter: 0.89 (CH_3), 1.22 (CH_2), 1.38 (CH_2), 1.72 (CH_2), 4.00 (OCH_2), 7.64/7.03 (Ar-H Ph-3), 7.79/7.73 (Ar-H Ph-2), 7.98/7.81 (Ar-H Ph-1) ppm.

4.16. *cyclo*[-(Ter)-Alg-Thr(tBu)-Hyp(tBu)-hTyr(tBu)-Orn(Boc)-Hyp(tBu)-Alg]-OH (20)

A solution of 0.2 N LiOH (0.6 mL) was added drop-wise to a cooled (0 °C) solution of peptide **18** (61.8 mg; 41.7 μmol) in THF (1.5 mL). The reaction mixture was stirred at room temperature for 16 h followed by evaporation in vacuo. The product was re-dissolved in H_2O and neutralized with 1 N HCl, followed by lyophilization yielding peptide **20** (67 mg; quant.) as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 95% (t_R = 49.63 min). ESI-MS calcd for $C_{82}H_{116}N_8O_{16}$: 1468.85, found: *m/z* 1469.75 $[M+H]^+$; HRMS calcd for $C_{82}H_{116}N_8O_{16}$ $[M+H]^+$ 1469.8588, found 1469.8579; ^{1}H NMR ($CDCl_3$, 500 MHz): δ *t*Bu: 1.48–1.33 (CH_3), 1.22–1.08 (CH_3); hTyr-4: 7.53/6.96 (Ar-H); Ter: 0.92 (CH_3), 1.38 (CH_2), 1.28 (CH_2), 1.79 (CH_2), 3.98 (OCH_2) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ *t*Bu: 28.1 (CH_3), 28.0 (CH_3); hTyr-4: 128.3/115.1 (Ar-H); Ter: 13.7 (CH_3), 22.2 (CH_2), 28.5 (CH_2), 28.7 (CH_2), 68.0 (OCH_2). Full NMR characterization of the protected peptide **20** was not possible due to the high intensity of the *t*Bu/Boc signals and the broadened amino acid signals. However, full NMR characterization of the deprotected peptide can be found at the experimental details of cyclic peptide **16**.

4.17. *cyclo*[-(Ter)-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg]-$NHCH_2CH_2NH_2$. (21)

To a solution of peptide **20** (12.3 mg; 8.37 μmol), *N*-boc-ethylene-diamine (2.68 mg; 16.74 μmol) and HATU (3.18 mg; 8.37 μmol) in DCM (1 mL) DIPEA (2.92 μL; 16.74 μmol) was added and the reaction mixture was stirred for 48 h. As the reaction was not finished according to TLC (DCM/MeOH/AcOH, 19/1/0.1) another portion of *N*-boc-ethylene-diamine (2.7 mg; 17 μmol), HATU (3.2 mg; 8.4 μmol) and DIPEA (2.92 μL; 16.74 μmol) was added and the mixture was stirred for an additional 24 h. The mixture was concentrated in vacuo, the residue re-dissolved in EtOAc (5 mL) and washed with 1 N $KHSO_4$ (3× 5 mL), 5% $NaHCO_3$ (3× 5 mL) and brine (5 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The crude peptide was purified by column chromatography (DCM/MeOH/AcOH, 30/1/0.1, v/v/v) yielding the protected peptide (8.0 mg; 59%) as a white solid. ESI-MS calcd for $C_{89}H_{130}N_{10}O_{17}$: 1610.96, found: *m/z* 1611.53 $[M+H]^+$.

The protected peptide (12.7 mg; 7.8 μmol) was treated with a mixture of TFA/TIS/H_2O (1 mL, 95/2.5/2.5, v/v/v) for 2 h. The mixture was concentrated in vacuo, the residue re-dissolved in a small amount of TFA and precipitated in MTBE/hexane (1/1). The supernatant was removed and the crude peptide washed twice with MTBE/hexane (1/1). After preparative HPLC (Altima C8 semiprep, 120 min run, *i*-PrOH/MeOH/H_2O buffers) and lyophilization, peptide **21** (7.2 mg; 78%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 99% for a mixture of *E/Z* stereoisomers (t_R = 39.20 min and 39.48 min). ESI-MS calcd for $C_{63}H_{82}N_{10}O_{13}$: 1186.61, found: *m/z* 1187.65 $[M+H]^+$; HRMS calcd for $C_{61}H_{78}N_{10}O_{13}$ $[M+H]^+$ 1187.6141, found 1187.6152; ^{1}H NMR (DMSO, 500 MHz): δ Alg-1: 8.15 (NH), 4.25 (αCH), 2.40/2.19 (βCH), 5.61 (γCH); Thr-2: 7.67 (NH), 4.65 (αCH), 4.31 (βCH), 1.14 (γCH); Hyp-3: 4.35 (αCH), 2.16/1.83 (βCH), 4.41 (γCH), 3.77/3.72 (δCH); hTyr-4: 7.74 (NH), 3.96 (αCH), 2.11/1.95 (βCH), 2.48/2.37 (γCH) 6.96/6.67 (Ar-H); Orn-5: 7.71 (εNH), 7.46 (NH), 4.66 (αCH), 1.84/1.68 (βCH), 1.54 (γCH), 2.87 (δCH); Hyp-6: 4.48 (αCH), 2.06/1.89 (βCH), 4.32 (γCH), 3.67/3.44 (δCH); Alg-7: 7.99 (NH), 4.23 (αCH), 2.34 (βCH), 5.47 (γCH); $NHCH_2CH_2NH_2$ amide: 3.32/3.23 (CH_2), 2.86/2.82 (CH_2); Ter: 0.92 (CH_3), 1.38 (CH_2), 1.43 (CH_2), 1.75 (CH_2), 4.03 (OCH_2), 7.67/7.06 (Ar-H Ph-3), 7.82/7.76 (Ar-H Ph-2), 8.01/7.84 (Ar-H Ph-1) ppm.

4.18. *cyclo*[-(Ter)-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg]-[CH_2OH] (22)

$LiBH_4$ (11.1 μL; 22.17 μmol) was added to a solution of peptide **18** (18.8 mg; 12.67 μmol) in dry THF (2 mL) and the reaction mixture was stirred for 5 h. The reaction mixture was quenched by addition of satd aq $NaHCO_3$ and stirred for an additional 10 min. The aqueous layer was extracted with Et_2O (10 mL) and EtOAc (10 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated in vacuo. The crude peptide was purified by column chromatography (DCM/MeOH/AcOH, 20/1/0.1, v/v/v) yielding the protected peptide alcohol (10.2 mg; 55%) as a white solid. ESI-MS calcd for $C_{82}H_{118}N_8NaO_{15}$: 1477.86, found: *m/z* 1477.82 $[M+Na]^+$.

The peptide (10 mg; 6.9 μmol) was treated with a mixture of TFA/TIS/H_2O (2 mL; 95/2.5/2.5; v/v/v) for 2 h. The mixture was concentrated in vacuo, re-dissolved in a small amount of TFA and precipitated in MTBE/hexane (1/1). The supernatant was removed and the crude peptide washed twice with MTBE/hexane (1/1). After preparative HPLC (Altima C8 semiprep, 120 min run, *i*-PrOH/MeOH/H_2O buffers) and lyophilization, peptide **20** (3.7 mg; 26%) was obtained as a white solid. Purity was confirmed by analytical

HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 99% (t_R = 41.83 min). ESI-MS calcd for $C_{61}H_{78}N_8O_{13}$: 1130.57, found: *m/z* 1131.57 $[M+H]^+$; HRMS calcd for $C_{61}H_{78}N_8O_{13}$ $[M+H]^+$ 1131.5767, found 1131.5795; ^1H NMR (DMSO, 500 MHz): δ Alg-1: 8.67 (NH), 4.63 (αCH), 2.55/2.43 (βCH), 5.58 (γCH); Thr-2: 7.70 (NH), 4.64 (αCH), 4.28 (βCH), 1.13 (γCH); Hyp-3: 4.32 (αCH), 2.13/1.81 (βCH), 4.31 (γCH), 3.66/3.41 (δCH); hTyr-4: 7.75 (NH), 3.91 (αCH), 2.08/1.93 (βCH), 2.46/2.38 (γCH), 6.95/6.66 (Ar-H); Orn-5: 7.64 (εNH), 7.45 (NH), 4.63 (αCH), 1.91/1.61 (βCH), 1.55 (γCH), 2.94/2.83 (δCH); Hyp-6: 4.39 (αCH), 2.00/1.82 (βCH), 4.39 (γCH), 3.74/3.71 (δCH); Alg-7: 7.69 (NH), 3.71 (αCH), 2.29/1.94 (βCH), 5.54 (γCH); alcohol: 3.28/3.13 (CH_2OH); Ter: 0.91 (CH_3), 1.37 (CH_2), 1.42 (CH_2), 1.75 (CH_2), 4.02 (OCH_2), 7.67/7.05 (Ar-H Ph-3), 7.82/7.75 (Ar-H Ph-2), 8.01/7.83 (Ar-H Ph-1) ppm. ^{13}C NMR (DMSO, 125 MHz): δ Alg-1: 54.0 (αCH), 34.0 (βCH), 128.9 (γCH); Thr-2: 56.2 (αCH), 66.3 (βCH), 19.1 (γCH); Hyp-3: 60.5 (αCH), 36.9 (βCH), 68.4 (γCH), 55.1 (δCH); hTyr-4: 52.4 (αCH), 32.0 (βCH), 30.7 (γCH), 129.6/115.2 (Ar-H); Orn-5: 49.5 (αCH), 28.5 (βCH), 23.3 (γCH), 38.4 (δCH); Hyp-6: 58.2 (αCH), 38.0 (βCH), 69.0 (γCH), 55.5 (δCH); Alg-7: 50.0 (αCH), 33.0 (βCH), 127.7 (γCH); alcohol: 63.8 (CH_2OH); Ter: 13.6 (CH_3), 21.6 (CH_2), 27.4 (CH_2), 28.1 (CH_2), 67.4 (OCH_2), 127.9/115.1 (Ar-H Ph-3), 127.5/126.9 (Ar-H Ph-2), 128.5/126.3 (Ar-H Ph-1) ppm.

4.19. *cyclo*[-(Ter)-Alg-Thr-Hyp-hTyr-Orn-Hyp-Alg]-$NHNH_2$ (23)

To a solution of peptide **18** (9.0 mg; 6.06 μmol) in dry DMF (1 mL) $N_2H_4{\cdot}H_2O$ (0.2 mL) was added and the reaction mixture was stirred for 24 h. The volatiles were removed by evaporation in vacuo and coevaporation with MeOH (3×). Lyophilization yielded the protected peptide hydrazide (8.0 mg; 89%) as a white solid. ESI-MS calcd for $C_{82}H_{118}N_{10}NaO_{15}$: 1505.87, found: *m/z* 1505.80 [M+Na].

The protected peptide (8.0 mg; 5.4 μmol) was treated with HCl/Et_2O[42] for 2 h. The mixture was concentrated in vacuo, re-dissolved in a small amount of MeOH and precipitated in MTBE/hexane (1/1). The supernatant was removed and the crude peptide washed twice with MTBE/hexane (1/1). After preparative HPLC (Altima C8 semi-prep, 120 min run, *i*-PrOH/MeOH/H_2O buffers) and lyophilization, peptide according to the general procedure **23** (3 mg; 48%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 95% (t_R = 39.50 min). ESI-MS calcd for $C_{61}H_{78}N_{10}O_{13}$: 1158.57, found: *m/z* 1159.80 $[M+H]^+$; HRMS calcd for $C_{61}H_{78}N_{10}O_{13}$ $[M+H]^+$ 1159.5828, found 1159.5845; ^1H NMR (DMSO, 500 MHz): δ Alg-1: 8.72 (NH), 4.62 (αCH), 2.52/2.46 (βCH), 5.62 (γCH); Thr-2: 7.57 (NH), 4.65 (αCH), 4.30 (βCH), 1.13 (γCH); Hyp-3: 4.46 (αCH), 2.21/2.32 (βCH), 4.40 (γCH), 3.66/3.42 (δCH); hTyr-4: 7.71 (NH), 3.95 (αCH), 2.10/1.92 (βCH), 2.47/2.37 (γCH), 6.95/6.66 (Ar-H); Orn-5: 7.67 (εNH), 7.45 (NH), 4.65 (αCH), 1.85/1.65 (βCH), 1.52 (γCH), 2.92/2.83 (δCH); Hyp-6: 4.34 (αCH), 2.03/1.83 (βCH), 4.30 (γCH), 3.74 (δCH); Alg-7: 8.30 (NH), 4.31 (αCH), 2.15 (βCH), 5.63 (γCH); Ter: 0.91 (CH_3), 1.37 (CH_2), 1.42 (CH_2), 1.74 (CH_2), 4.02 (OCH_2), 7.76/7.05 (Ar-H Ph-3), 7.84/7.67 (Ar-H Ph-2), 8.02/7.82 (Ar-H Ph-1) ppm. ^{13}C NMR (DMSO, 125 MHz): δ Alg-1: 54.0 (αCH), 33.9 (βCH), 127.3 (γCH); Thr-2: 56.2 (αCH), 66.2 (βCH), 19.1 (γCH); Hyp-3: 58.0 (αCH), 34.1 (βCH), 69.0 (γCH), 55.1 (δCH); hTyr-4: 52.2 (αCH), 32.2 (βCH), 30.7 (γCH), 129.6/115.2 (Ar-H); Orn-5: 49.3 (αCH), 28.6 (βCH), 23.2 (γCH), 38.4 (δCH); Hyp-6: 60.6 (αCH), 37.6 (βCH), 68.4 (γCH), 55.5 (δCH); Alg-7: 50.5 (αCH), 37.0 (βCH), 129.3 (γCH); Ter: 13.5 (CH_3), 21.6 (CH_2), 27.4 (CH_2), 28.0 (CH_2), 67.4 (OCH_2), 126.9/115.1 (Ar-H Ph-3), 126.4/127.9 (Ar-H Ph-2), 128.5/127.5 (Ar-H Ph-1) ppm.

4.20. *cyclo*[-(Ter)-Lys-Thr-Hyp-hTyr-Orn-Hyp] (26)

Linear peptide Fmoc-Lys(Psoc)-Thr(*t*Bu)-Hyp(*t*Bu)-hTyr(*t*Bu)-Orn(Boc)-Hyp(*t*Bu)-OH (**24**) was synthesized on trityl resin (200 mg; 93.8 μmol) according to the general procedure for solid phase peptide synthesis. The Fmoc group was cleaved and the ter tail (101.4 mg; 281.4 μmol) was coupled overnight in the presence of HATU (107 mg; 281.4 μmol) and DiPEA (98 μL; 562.8 μmol) in NMP (2 mL). The resin was washed with NMP (3× 3 mL, each 3 min) and DCM (3× 3 mL, each 3 min). The Psoc group was cleaved by treatment with TBAF·$3H_2O$ (88.78 mg; 281.4 μmol) for 15 min in DCM (2 mL). Peptide **25** was obtained by treatment of the resin with HFIP/DCM (3 mL, 1/1, v/v) for 3 h. This was repeated once for 1 h. The mixture was concentrated to obtain peptide **25** (121.4 mg; 90%) as a foam. Characterization was carried out by ESI-MS (calcd for $C_{80}H_{118}N_8O_{15}$: 1430.87, found: *m/z* 1431.89 $[M+H]^+$).

Then, the crude linear peptide Ter-Lys-Thr(*t*Bu)-Hyp(*t*Bu)-hTyr(*t*Bu)-Orn(Boc)-Hyp(*t*Bu)-OH (**25**) was subjected to the head-to-tail cyclization according to the general procedure, followed by treatment with a mixture of TFA/TIS/H_2O (10 mL, 95/2.5/2.5, v/v/v) for 1 h. The mixture was concentrated in vacuo, re-dissolved in a small amount of TFA and precipitated in MTBE/hexane (1/1). The supernatant was removed and the crude peptide washed twice with MTBE/hexane (1/1). After preparative HPLC (Altima C8 semi-prep, 120 min run, *i*-PrOH/MeOH/H_2O buffers) and lyophilization, peptide **26** (20.4 mg; 23%) was obtained as a white solid. Purity was confirmed by analytical HPLC (Altima C8, 65 min run, *i*-PrOH/MeOH/H_2O buffers) and was found to be higher than 99% (t_R = 41.05 min). ESI-MS calcd for $C_{59}H_{76}N_8O_{12}$: 1088.56, found: *m/z* 1089.82 $[M+H]^+$; HRMS calcd for $C_{59}H_{76}N_8O_{12}$ $[M+H]^+$ 1089.5661, found 1089.5656; ^1H NMR (DMSO, 500 MHz): δ Lys-1: 8.53 (NH), 7.90 (εNH), 4.66 (αCH), 2.14/1.72 (βCH), 1.60/1.33 (γCH), 1.58/1.32 (δCH), 3.56/2.71 (εCH); Thr-2: 7.79 (NH), 4.71 (αCH), 1.09 (βCH), 1.09 (γCH); Hyp-3: 4.23 (αCH), 2.12/1.78 (βCH), 4.37 (γCH), 3.80/3.59 (δCH); hTyr-4: 7.43 (NH), 3.98 (αCH), 1.99/1.87 (βCH), 2.52/2.46 (γCH), 6.96/6.66 (Ar-H); Orn-5: 7.69 (εNH), 7.33 (NH), 4.51 (αCH), 1.87/1.63 (βCH), 1.56 (γCH), 2.89 (δCH); Hyp-6: 4.40 (αCH), 1.98/1.77 (βCH), 4.30 (γCH), 3.66/3.31 (δCH); Ter: 0.91 (CH_3), 1.36 (CH_2), 1.41 (CH_2), 1.74 (CH_2), 4.01 (OCH_2) 7.65/7.03 (Ar-H Ph-3), 7.80/7.74 (Ar-H Ph-2), 8.03/7.81 (Ar-H Ph-1) ppm. ^{13}C NMR (DMSO, 125 MHz): δ Lys-1: 53.4 (αCH), 31.0 (βCH), 23.3 (γCH), 26.9 (δCH), 38.4 (εCH); Thr-2: 55.7 (αCH), 66.4 (βCH), 19.2 (γCH); Hyp-3: 60.7 (αCH), 36.7 (βCH), 68.9 (γCH), 55.2 (δCH); hTyr-4: 53.0 (αCH), 32.3 (βCH), 30.7 (γCH), 129.1/114.6 (Ar-H); Orn-5: 49.4 (αCH), 27.7 (βCH), 23.3 (γCH), 38.3 (δCH); Hyp-6: 59.2 (αCH), 37.6 (βCH), 68.4 (γCH), 54.5 (δCH); Ter: 13.7 (CH_3), 21.6 (CH_2), 27.5 (CH_2), 28.1 (CH_2), 67.2 (OCH_2), 127.4/114.7 (Ar-C Ph-3), 127.1/126.4 (Ar-C Ph-2), 128.1/125.8 (Ar-C Ph-1).

4.21. Biological activity

Antifungal activity was evaluated by broth microdilution. The media used in this assay was Yeast Extract Peptone Dextrose (YPD) containing 1% yeast extract, 2% peptone, 1% dextrose in distilled water. Test compounds were dissolved in distilled water or 10% DMSO, depending on the solubility characteristics of the compounds, to a concentration of 1 mg/mL. After solubilization each compound was diluted five times in YPD medium rendering a stock solution of 200 μg/mL. Caspofungin,[20] purchased from the fabricant (Merck Sharp & Dohme B.V., Haarlem, Netherlands), was included as a control. Serial twofold dilutions of the test compounds in YPD medium were prepared as followed. To each well of a sterile Greiner bio-one Cellstar 96 well, U bottomed microtiter plate 100 μl of YPD was dispensed. Manually, 100 μl of

stock compounds was delivered to each well in colomn 1. Then using a 8-channel pipet, compounds in column 1 were serially diluted twofold.

The plates containing the diluted compounds were inoculated with 100 μl of the appropriate microorganism. The yeast strains used were isolates obtained from the CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands). The collection included *C. dubliensis*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and two strains of *C. albicans*. Stock cultures of the yeast strains in liquid media (YPD + 15% glycerol) were maintained at −80 °C. For use in this assay, yeast cultures were streaked on YPD agar plates and incubated for 24 h at 30 °C. Then, using a sterile disposable loop, cells from a colony were suspended in 5 mL of YPD media and aerated for 24 h at 30 °C on a shaker set at 300 rpm. The broth cultures were diluted 10 times with media and the optical density of this suspension was measured at a wavelength of 600 nm. The suspension was further diluted to an OD_{600} of 0.01 and this suspension was further diluted 1:100 in YPD media. This final dilution was used for inoculating the plates. Thus, the final number of cells per well is approximately 1.5×10^3 cells/ml.[43] The final volume/well, including organism and compound was 200 μl. The last row of the plate contained drug-free wells dedicated for growth and sterility controls for each organism tested.

Tests were incubated overnight at 30 °C prior to recording MICs. The in vitro activity was determined visually at 24 h of incubation as the lowest concentration of compound resulting in full inhibition of yeast growth.

Acknowledgments

We wish to thank Mr. H. W. Hilbers for recording ESI-MS mass spectra and Mr. C. Versluis and Mevr. J. M. A. Damen for recording HRMS spectra. From the CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands) we would like to thank Dr. T. Boekhout for providing us with the yeast strains and Dr. J. Dijksterhuis for his help with the visualization of yeast by light microscopy. And finally we thank Dr. D. T. S. Rijkers and Dr. N. I. Martin for critically reading the manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.08.034.

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