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Mutual influence of backbone proline substitution and lipophilic tail character on the biological activity of simplified analogues of caspofungin†Monique P. C. Mulder,^{‡a} Peter Fodran,^{‡b} Johan Kemmink,^a Eefjan J. Breukink,^c John A. W. Kruijtzter,^a Adriaan J. Minnaard^{*b} and Rob M. J. Liskamp^{*a}

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The echinocandins represent the most recent class of antifungal drugs. Previous structure–activity relationship studies on these lipopeptides have relied mainly upon semisynthetic derivatives due to their complex chemical structures. A successful strategy for the rapid enantioselective synthesis of the branched fatty acid chain of caspofungin and analogues was developed to synthesize several simplified analogues of caspofungin. The specific minimum inhibitory activity of each mimic was determined against a panel of *Candida* strains. This approach gave access to new fully synthetic derived caspofungin mimics with high and selective antifungal activities against *Candida* strains. In addition, the data suggested an important role of the hydroxy proline residue in the bioactive conformation of the macrocyclic peptide ring structure.

Introduction

Over the past decades, the incidence and diversity of life-threatening fungal infections have increased dramatically. In part this is due to an increase of the immunosuppressed patient population.^{1,2} In addition, the toxicity and growing antifungal resistance of the current antifungal agents underscore the urgent need for development of new antifungal compounds that act on unrelated and novel targets.^{3,4}

Amphotericin B used for treatment of severe mycoses is hampered by its nephrotoxicity and many other side effects. Azoles are the most widely used antifungal agents because of their high therapeutic index. However, their widespread use has resulted in severe drug resistance.⁵ Development of echinocandin like cyclolipohexapeptides for the treatment of invasive fungal infections represented a breakthrough in antifungal chemotherapy.⁶ This family of antifungal drugs interferes with the synthesis of β -(1,3)-glucan, a major component of the framework of the

fungal cell wall, by inhibiting the β -(1,3)-glucan synthase enzyme.⁷ In view of their mode of action as well as their pharmacological and toxicological profiles, the echinocandin compounds exhibit the most promising target selectivity, as 1,3- β -glucan is only found in fungi and not in mammalian cells.⁷ This resulted in less toxic effects, as compared to the other classes of antifungal agents. Moreover, so far the clinically approved agents of this class of compound have rarely met fungal resistance.⁸

The echinocandins are a broad group of lipopeptide antifungal agents that typically consists of a cyclic hexapeptide acylated with a lipophilic tail on its N-terminus. The first licensed echinocandin was caspofungin (**1**, Fig. 1), a semi-synthetic compound based on pneumocandin B₀, in 2001. Since then, two other semi-synthetic echinocandins, micafungin (2005) and anidulafungin (2006), have been approved by the Food and Drug Administration (FDA).⁹ These clinically relevant echinocandins are all semisynthetic derivatives of the natural fermentation products.^{10–12}

The complex chemical structures of the echinocandins have led to extensive structure–activity relationship (SAR) studies by semisynthetic modification of the natural product.¹³ This approach relies on the availability of the lipopeptides in large quantities from fermentation and of course these semisynthetic modifications cannot access a complete SAR of the echinocandins. With respect to this, synthetic approaches offer an opportunity to obtain valuable additional SAR information.

In 1992 the first total synthesis of simplified echinocandin analogues was described by Zambias *et al.*¹⁴ Structure–activity relationship data of these analogues showed that several of the functional groups, primarily the hydroxyl groups, were not

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‡ These authors contributed equally to this work.

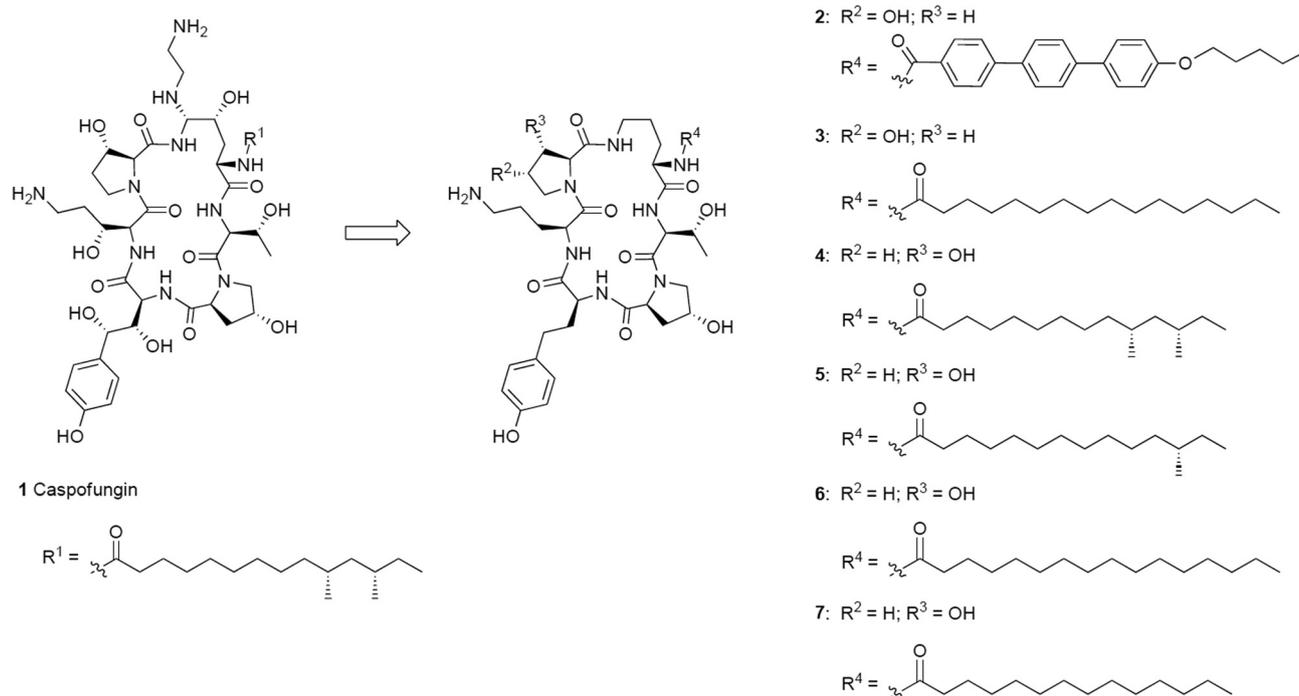


Fig. 1 Structures of caspofungin (**1**), previously described mimics (**2** and **3**)¹⁶ and the designed synthetic caspofungin analogues (**4**–**7**).

essential for antifungal activity.¹⁴ Partly inspired by this work, Klein *et al.* reported a similar study in 2000.¹⁵ The total synthesis approach has provided valuable echinocandin SAR data that would be difficult or impossible to obtain *via* modification of the natural products themselves.¹⁶

These reports and our own findings¹⁷ showed that the role of the fatty acid tail was very dominant. Our previous analogues (e.g. **3**, Fig. 1) were designed to contain a palmitoyl chain,¹⁷ instead of the dimethylmyristoyl chain, which is consistent with SAR studies showing that a regular C12–C18 fatty acid side chain gives optimal activity.¹⁹ Unfortunately, mimics equipped with this chain showed low to no activity. Therefore, based on work by Klein *et al.*,¹⁵ simplified head-to-tail analogues were synthesized equipped with a terphenyl fatty acid side chain (e.g. **2** Fig. 1). Terphenyl lipophilic tail containing analogue **2** was 30-fold more active than mimic **3** with a palmitoyl chain.¹⁷

Other reports in the literature discussed the importance of both the stereochemistry and methyl branching patterns of fatty acids in biological recognition.^{20,21} This raised the interest in an investigation of the influence of the presence and stereochemistry of methyl groups in the dimethylmyristoyl chain on the antifungal activity.

Although extensive structure–activity studies have been performed in the past on the nature of the fatty acid side chain and its influence on the activity of the echinocandins,^{18,22–26} the role of the stereochemistry and number of methyl groups in the natural dimethylmyristoyl chain of caspofungin (**1**) has not been investigated so far.

Here, we report the synthesis and antifungal activity of a simplified caspofungin analogue (**4**) bearing its natural (10*R*,12*S*)-dimethylmyristoyl chain.²⁷ In addition, the influence of the methyl groups in this chain on antifungal activity was determined by synthesis of several analogues and evaluation of their

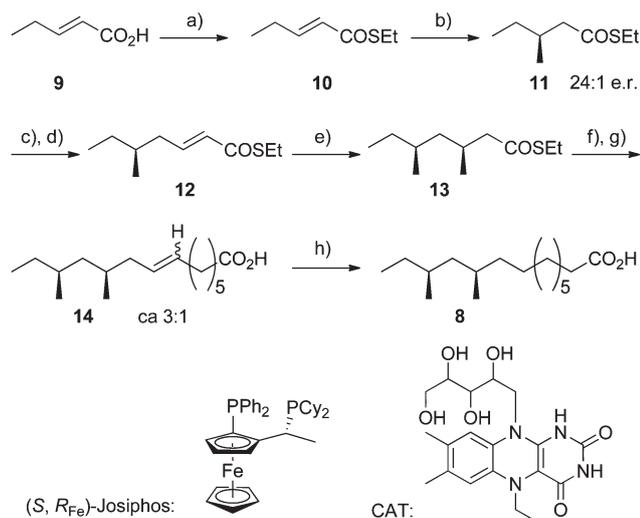
antifungal activity. Also analogues were included with either one (**5**) or no (**6**, **7**) methyl groups (Fig. 1).

Results and discussion

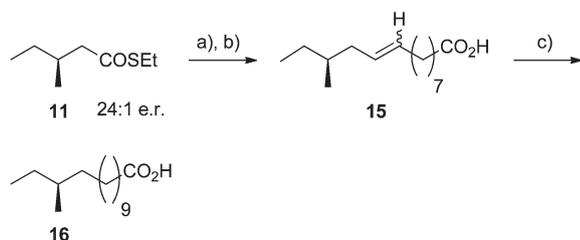
Chemistry

Both the relative and absolute configuration of the fatty acid side chain of caspofungin (**1**) have been elucidated by Leonard *et al.*²⁷ Their reported stereoselective synthesis of the (*R,S*)-enantiomer was conducted in 8 steps and provided (10*R*,12*S*)-dimethylmyristic acid **8** in a 90 : 10 (*syn/anti*) ratio of diastereomers. Based on this, we designed an efficient asymmetric synthesis using the copper/Josiphos^{28a,b} catalysed addition of methylmagnesium bromide for the construction of the stereogenic centers (Scheme 1).²⁷

Commercially available (*E*)-pent-2-enoic acid **9** was converted into thioester **10** with Steglich conditions.²⁹ This thioester underwent copper/Josiphos catalyzed enantioselective conjugate addition of MeMgBr smoothly, affording the desired compound **11** in 92% yield with a high *er* (24 : 1).^{28b} Subsequent DIBAL-H reduction followed by the Horner–Wadsworth–Emmons^{28b} reaction gave the elongated thioester **12** in good yield (80%) with an excellent *E* : *Z* ratio (20 : 1). After separation of the unwanted *Z*-stereoisomer with column chromatography, the α,β -unsaturated thioester **12** was subjected to a second conjugate addition. This reaction afforded a 20 : 1 mixture of C3 epimers which was separable again by column chromatography. The desired diastereomer **13** was isolated in 80% yield. After DIBAL-H reduction and Wittig reaction with a carboxylate functionalized ylide, a mixture of double bond isomers **14** in an approximate 3 : 1 ratio was obtained in 47% combined yield. In order to avoid any epimerization of the nearby stereocenter, the double bond of



Scheme 1 Synthesis of (10*R*,12*S*)-dimethylmyristic acid **8**. (a) EtSH, DCC, DMAP, DCM, 0–21 °C, 3 h, 95%; (b) MeMgBr, CuBr–(*S,R*_{Fe})-Josiphos, MTBE, –78 °C, 16 h, 92%; (c) DIBAL-H, DCM, –50 °C, 1 h; (d) (MeO)₂P(O)CH₂COSEt, *n*BuLi, THF, 0–21 °C, 16 h, 70%; (e) MeMgBr, CuBr–(*S,R*_{Fe})-Josiphos, MTBE, –78 °C, 16 h, 80%; (f) DIBAL-H, DCM, –50 °C, 1 h; (g) Br–Ph₃P⁺(CH₂)₆CO₂H, LiHMDS, THF, 21 °C, 20 min, 47%; (h) NH₂NH₂·H₂O, O₂, Cat, EtOH, 21 °C, 70%.



Scheme 2 Synthesis of (12*S*)-methylmyristic acid **16**. (a) DIBAL-H, DCM, –50 °C, 1 h; (b) Br[–]Ph₃P⁺(CH₂)₈CO₂H, LiHMDS, THF, 21 °C, 20 min, 60% over two steps; (c) NH₂NH₂·H₂O, O₂, Cat, EtOH, 21 °C, 80%.

14 was subsequently reduced by diimide, *in situ* formed by catalytic oxidation of hydrazine,³⁰ affording **8** in 70% yield.

Overall, the side chain **8** of caspofungin was synthesized in a linear 8 step synthesis with an overall yield of 16%. The catalytic enantioselective conjugate addition in combination with a Wittig reaction using a carboxylate functionalized ylide represents an efficient approach towards methyl-branched fatty acids. It is important to mention that several of the intermediates in the synthesis were rather volatile, which complicated isolation.

The synthesis of the mono methyl analogue **16** was performed in a similar manner (Scheme 2). Thioester (**11**) was reduced by DIBAL-H and after a Wittig reaction, a mixture of *E* and *Z* isomers (**15**) was isolated in 60% yield. Reduction of the resulting double bond was achieved by *in situ* generated diimide³⁰ and gave **16** in 80% yield.

Now that we had both the (10*R*,12*S*)-dimethylmyristic acid **8** and its mono methyl analogue (12*S*)-methylmyristic acid **16** in hand, attention was directed toward the synthesis of the head-to-tail mimics. The synthesis of these mimics proceeded readily

and is outlined in Scheme 3 for mimic **4**. Linear precursor **17** was prepared by SPPS using the trityl resin as follows. After removal of the ϵ -Fmoc group from ornithine dimethylmyristic acid **8** was coupled. Mild acidolytic cleavage of the Mtt group using trifluoroethanol and acetic acid also liberated the peptide chain from the resin and gave the linear fully protected peptide precursor **18**. Cyclization and subsequent deprotection afforded the macrocyclic peptide **4** in an overall yield of 51% after purification by preparative reverse phase HPLC. Synthesis of macrocyclic peptides **5–7** was performed analogously to the synthesis of mimic **4** in Scheme 3. For the preparation of peptides **5–7** other fatty acids, *i.e.* methylmyristic acid, palmitic acid and myristic acid, were included in step (h) instead of dimethylmyristic acid **8** (Scheme 3). Peptides **5–7** were obtained in overall yields of 48–88% after purification by preparative reverse phase HPLC.

Structure–activity relationships

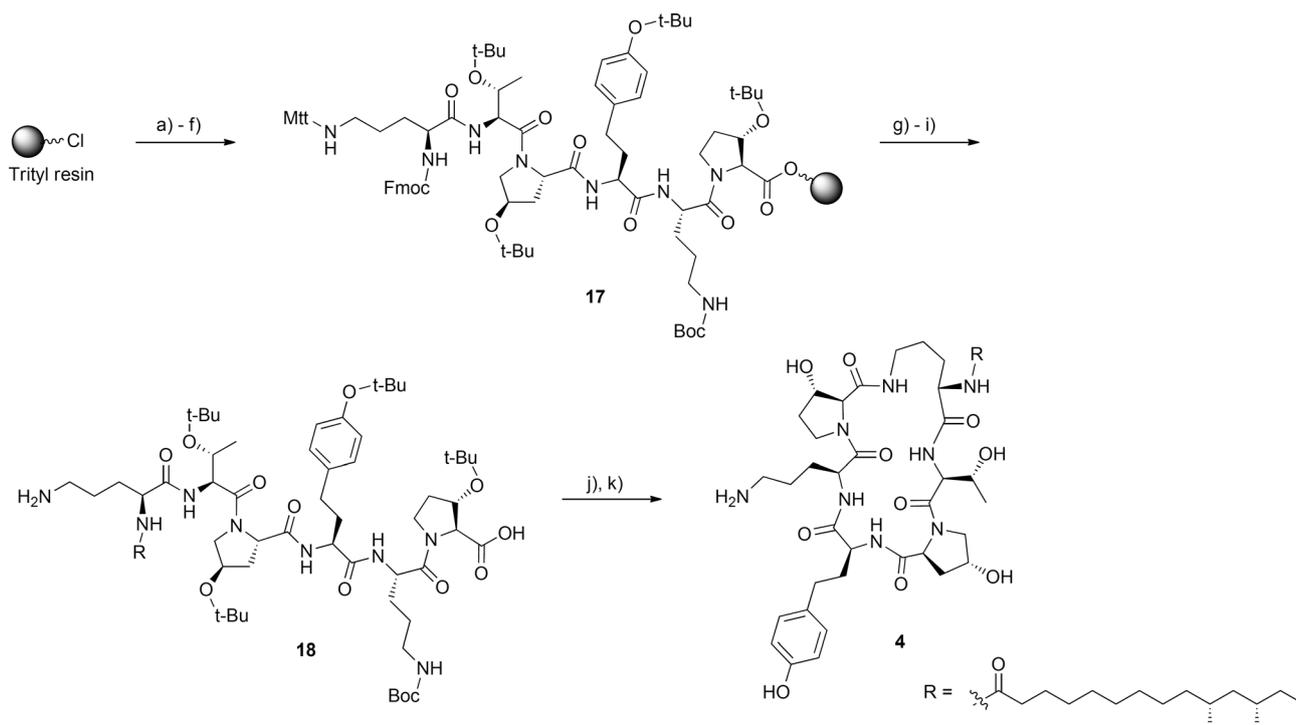
The antifungal activity of each analogue (**4–7**) was evaluated using a broth microdilution microtiter assay against a panel of common *Candida* species.³¹ This panel included *C. dubliniensis*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and two strains of *C. albicans*. Caspofungin **1**³² was used as a reference compound and our previously described compounds (**2** and **3**)¹⁷ were also included in the test for comparison. The results of these tests are expressed as the minimum inhibitory concentration (MIC) value, the minimum concentration of compound which completely inhibits visible fungal growth, and are shown in Table 1.

The simplified caspofungin analogue **4**, bearing the natural side chain, showed substantial antifungal activity against the *Candida* strains (0.07–0.19 $\mu\text{g mL}^{-1}$) with a somewhat higher MIC value for *C. krusei* (2.25 $\mu\text{g mL}^{-1}$) and proved to be inactive against *C. parapsilosis*. Only a 5-fold decrease in the inhibitory activity of **4**, compared with that of caspofungin (**1**), was observed against *C. albicans* and the closely related *C. dubliniensis*.³³ In addition, analogue **4** proved to have a more selective antifungal spectrum than caspofungin (**1**).

In order to assess the contribution of the methyl groups in the dimethylmyristoyl chain **8** of caspofungin (**1**), the mono methyl derivative methylmyristic acid **16**, as well as mono-methylated palmitic and myristic fatty acid chains were incorporated as a replacement for **8** in analogue **4** to give **5**, **6** and **7**, respectively.

Remarkably, all of the analogues in the series showed substantial antifungal activity against *Candida*. Similar to analogue **4**, these analogues were more selective than caspofungin, having higher MIC values for *C. krusei* and showing no activity against *C. parapsilosis*. These results also showed that there is no obvious relationship between the presence of the methyl groups in the dimethylmyristoyl chain **8** of caspofungin (**1**) and antifungal activity.

Most surprisingly analogue **6** with a palmitoyl chain, as is also present in analogue **3**, showed a 10–35-fold increase in antifungal activity against *Candida*. The only difference between these analogues is the position of the hydroxyl group in the left-upper hydroxyproline (residue Hyp-6) of the cyclic hexapeptide (R² and R³ in Fig. 1).



Scheme 3 Reagents and conditions: (a) (1) Fmoc-3-Hyp(*t*Bu)-OH, DIPEA, DCM; (2) DCM/MeOH/DIPEA (17/2/1, v/v/v); (b)–(f) (1) 20% piperidine in NMP; (2) Fmoc-Xxx-OH, BOP, DIPEA, NMP; (g) 20% piperidine in NMP; (h) dimethylmyristic acid **8**, HATU, DIPEA, NMP; (i) TFE/AcOH/DCM (2/1/7, v/v/v); (j) BOP, DIPEA, DMF; (k) TFA/TIS/H₂O (95/2.5/2.5, v/v/v).

Table 1 *In vitro* antifungal activity of caspofungin and analogues against common *Candida* species displayed as MIC values in $\mu\text{g mL}^{-1}$

Peptide	<i>Candida albicans</i> CBS9975	<i>Candida albicans</i> CBS8758	<i>Candida dubliniensis</i> CBS7987	<i>Candida glabrata</i> CBS138	<i>Candida krusei</i> CBS573	<i>Candida parapsilosis</i> CBS604	<i>Candida tropicalis</i> CBS94
Caspofungin 1	0.023	0.039	0.014	0.027	0.006	0.281	0.006
2	0.140	0.156	0.063	0.188	0.078	>4.25	0.063
3	>4.25	>4.25	1.75	3.8	>4.25	>4.25	3.8
4	0.117	0.188	0.070	0.469	2.25	>4.25	0.094
5	0.188	0.281	0.047	0.625	1.875	>4.25	0.188
6	0.313	0.406	0.102	0.234	0.375	3.5	0.109
7	0.203	0.25	0.047	0.438	0.813	>4.25	0.063

This somewhat surprising influence of the hydroxyl position in the proline residue warranted the synthesis of three additional analogues. The synthesis of these macrocyclic peptides **19–21** was performed analogously to the synthesis of mimic **4** in Scheme 3 and their antifungal activity was evaluated and expressed as MIC values as is shown in Table 2.

These analogues confirm the finding that the position of the hydroxyl group in the top-left proline residue (R^2 and R^3 in Hyp-6) is crucial for antifungal activity. Analogues **3**, **20** and **21** ($R^2 = \text{OH}$) bearing the same lipophilic tail as analogues **6**, **4** and **7** ($R^3 = \text{OH}$), respectively, were practically inactive or showed a tremendous decrease in antifungal activity against *Candida*. Clearly $R^2 = \text{OH}$ is only allowed in caspofungin derivative **2** bearing a terphenyl fatty acid side chain. Here analogue **19** with $R^3 = \text{OH}$ and analogue **2** with $R^2 = \text{OH}$ both showed substantial antifungal activity against *Candida*. Analogue **19** was even more active than analogue **2** and had activities comparable to those of

caspofungin (**1**). In addition the selectivity of inhibition of *Candida parapsilosis* by **19** was remarkable and was not shown by any of the other analogues (see Tables 1 and 2).

These results suggest that the position of the hydroxyl group in the top-left hydroxyproline of the cyclic hexapeptide backbone (R^2 and R^3 in Hyp-6, Fig. 1) in combination with the character of the lipophilic fatty acid chain was a crucial factor for antifungal activity.

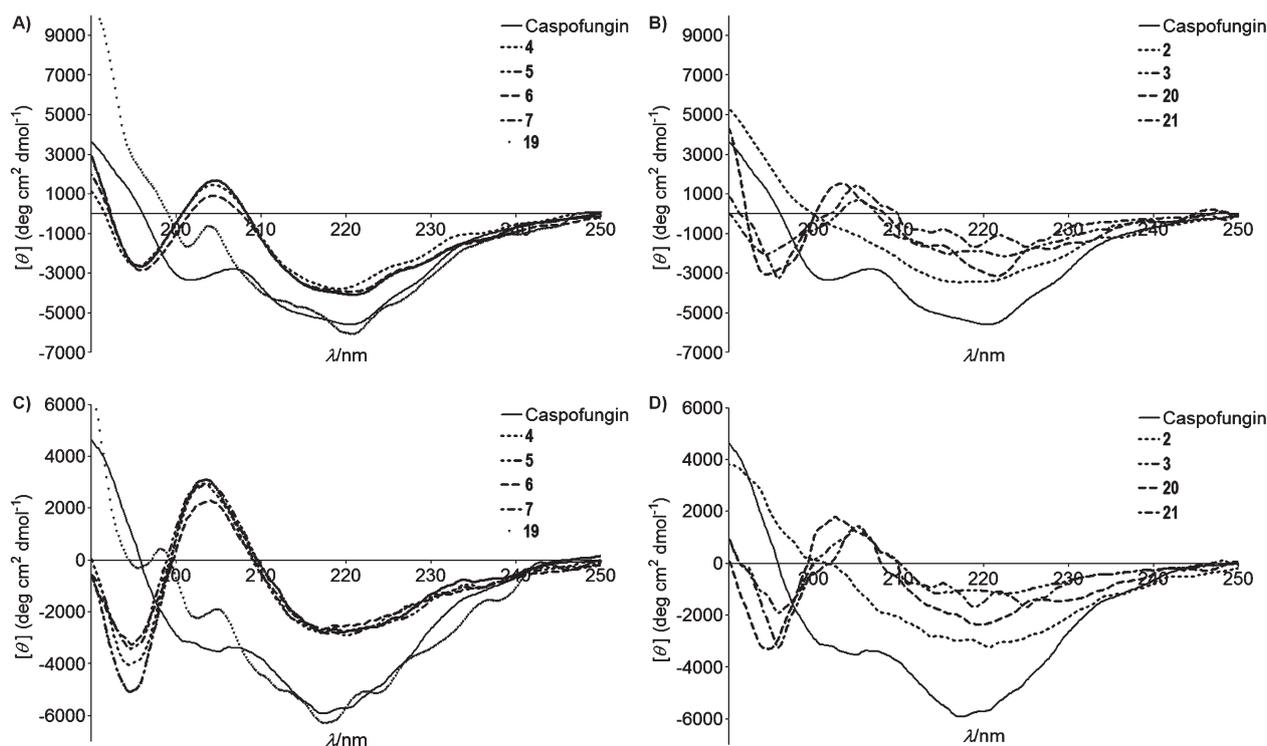
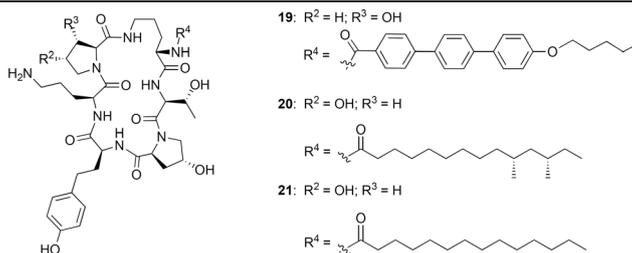
In attempts to interpret this finding we evaluated the analogues by circular dichroism (CD) in order to probe their conformational properties.

Circular dichroism spectroscopy

In addition to secondary structure prediction, circular dichroism spectroscopy can give valuable insights into peptide structure.³⁴

Table 2 *In vitro* antifungal activity of caspofungin analogues against common *Candida* species displayed as MIC values in $\mu\text{g mL}^{-1}$

Peptide	<i>Candida albicans</i> CBS9975	<i>Candida albicans</i> CBS8758	<i>Candida dubliniensis</i> CBS7987	<i>Candida glabrata</i> CBS138	<i>Candida krusei</i> CBS573	<i>Candida parapsilosis</i> CBS604	<i>Candida tropicalis</i> CBS94
19	0.051	0.039	0.031	0.027	0.027	0.75	0.141
20	1.63	3.0	1.5	2.5	>4.25	>4.25	2.5
21	>4.25	>4.25	3.0	>4.25	>4.25	>4.25	>4.25

**Fig. 2** CD spectra of caspofungin and analogues (2–7, 19–21). (A) + (B) Measured in MeCN/H₂O (1/1, v/v); (C) + (D) measured in TFE/H₂O (1/1, v/v). All peptides were measured at 0.1 mM concentration.

As was described by Ovchinnikov and Ivanov,³⁵ CD spectra can provide evidence for the perturbation or alteration of the conformational state in a series of related peptides.³⁴ Therefore, CD may provide an attractive technique to evaluate our analogues and determine whether the observed antifungal activity can be correlated with the conformational state.

Thus, the secondary structure of caspofungin (1) and analogues (2–7, 19–21) was investigated by circular dichroism as presented in Fig. 2. In addition to measurements in MeCN/H₂O, CD spectra were measured in TFE/H₂O, since TFE is known to enhance secondary structure formation of peptides.³⁶

As is shown in Fig. 2, spectra of the cyclic peptides 4–7 in both MeCN/H₂O and TFE/H₂O are similar. Peptides 4–7 are characterized by a negative band at *ca.* 220 nm and a positive band at 205 nm. In general, these peptides (4–7) showed CD spectra characteristic for a type II β -turn.^{37–40} However, their pattern below 215 nm is clearly different from caspofungin (1). This difference may be explained by the deletion of several hydroxyl groups in our analogues. Moreover, analogue 19 showed a distinctly different pattern at wavelengths below 210 nm and higher ellipticities of the band compared to peptides 4–7. This observation may be explained by a large contribution

of the aromatic residues present in the side chain of analogue **19** as is supported by CD spectra of other analogues (data in ESI†).

Remarkably, cyclic peptides **3**, **20** and **21** ($R^2 = OH$; Fig. 2B and D) exhibit different spectra than series **4–7** ($R^3 = OH$; Fig. 2A and C). Differences are apparent in the ellipticities of the bands, which could indicate that these peptides have a more disordered structure with less β -turn character. The only structural difference between these analogues is the position of the hydroxyl group in the left-upper hydroxyproline of the hexapeptide ring (R^2 and R^3 in Hyp-6, Fig. 1). Apparently, merely the position of the OH-function was responsible for the largely different CD-spectra and possibly conformational behaviour of the peptides.

For further insights into the influence of the OH-function on the conformation of our analogues, models were built and superimposed with the reported crystal structure of Echinocandin B (ECBN)¹² (data in ESI, Fig. S3†). Overall, the more active analogue **6** appeared to have a better superimposition with the crystal structure. Analogue **3** had a more puckered structure with the Hyp-6 residue situated more in the back of the molecule as compared to analogue **6**. In addition, the Orn-5 residue was pointing out more to the front of the macrocyclic peptide ring structure in **3**. These models support the finding that the position of the OH-function in Hyp-6 induces subtle changes in the conformation of the peptides. Apparently, these add up and were sufficient for a large effect on the biological activity.

Conclusion

A versatile approach for the rapid enantioselective synthesis of the dimethylmyristoyl chain of caspofungin and a one methyl group containing analogue is described. These fatty acids were coupled to simplified analogues of the caspofungin skeleton. In this way access was obtained to new fully synthetic derived caspofungin mimics with high and selective antifungal activities against *Candida*. Unexpectedly, no obvious relationship between the presence of the methyl groups in the dimethylmyristoyl tail **8** of caspofungin (**1**) and antifungal activity was observed. The dimethyl containing lipophilic tail derivative **4** did not lead to significant improvement of its bioactivity as compared to that of the non-methylated lipophilic chain. In fact the activities of non-methylated derivatives **6** and **7** and the methylated derivatives **4** and **5** were quite similar in most species. The position of the hydroxyl group in the top-left proline residue (R^2 and R^3 in Hyp-6) was apparently more crucial: when this group is moved from the beta (R^3) to the gamma (R^2) position, activity is lost when the fatty acid side chain is an acyl side chain. Clearly $R^2 = OH$ is only allowed in caspofungin derivative **2** bearing a terphenyl fatty acid side chain. Thus, the position of the hydroxy group in the top-left hydroxyproline of the cyclic hexapeptide backbone (R^2 and R^3 in Hyp-6, Fig. 1) in combination with the character of the lipophilic fatty acid chain was a crucial factor for antifungal activity. The position of the hydroxy group turned out to be very important for the general activity against *Candida* species as was shown by the higher activity of analogue **19** compared to analogue **2**, but also for selectivity of inhibition. Compound **19** was capable of a much more selective inhibition of *Candida parapsilosis* than analogue **2**.

Extensive NMR studies showed that in solution these peptides comprise many rapidly exchanging conformations, which result in averaged NMR spectra, and we have not been able to detect any conformational preferences of the peptides with either the hydroxyl group at the R^2 or R^3 position. So far, crystallization attempts of these peptides were also unsuccessful. We assume that the inherent flexibility of the cyclic peptides is the main reason for this. CD measurements showed subtle but distinctive changes indicative of structural changes in the conformation of these cyclic peptides. The results of this investigation suggest that the top-left proline residue somehow plays an important role in the bio-active conformation of the macrocyclic peptide ring structure.

Finally, we have observed that the choice of the fatty acid derivative (rigid as in **2** or flexible as in **3**) in combination with the conformational character of the ring may be a determining factor for antifungal activity.

Experimental section

Reagents, materials and analysis methods

Unless stated otherwise, all chemicals were obtained from commercial suppliers and used without further purification. Piperidine, *N,N*-diisopropylethylamine (DiPEA), peptide grade dichloromethane (DCM), *N,N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), *tert*-butyl methyl ether (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), *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) and *N* α -9-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids were purchased from GL Biochem Ltd (Shanghai, China). Protected homotyrosine (H-hTyr(*t*Bu)-OH) was purchased from Advanced Chemtech (Louisville, United States) and furnished with the Fmoc group. Fmoc-Orn(Mtt)-OH was obtained from Nova Biochem and Fmoc-3-Hyp(*t*Bu)-OH was synthesized according to a literature procedure.⁴¹ Triisopropylsilane (TIS) was obtained from Merck (Darmstadt, Germany). The 2-chlorotriylchloride PS resin cross-linked with 1% DVB (200–400 mesh) was purchased from Hecheng Chemicals (Shanghai, China) (theoretical loading: 1.10 mmol g⁻¹).

All reactions were carried out at room temperature unless stated otherwise. Solid phase synthesis was performed in plastic syringes with a polyethylene frit. Synthesis in solution was monitored by TLC on Merck pre-coated silica 60 plates. Spots were visualized by UV light, K₂CO₃/KMnO₄ or Seebach stain. Solid phase reactions were monitored with the chloranil test⁴² in the case of secondary amines or with the Kaiser test⁴³ in the case of primary amines. Column chromatography was performed using Silicycle UltraPure silica gel (40–63 μ m).

¹H NMR spectra were recorded on a Varian AMX 400 MHz spectrometer and chemical shifts are given in ppm relative to TMS (0.00 ppm). ¹³C NMR spectra were recorded using the attached proton test (APT) sequence on a Varian AMX 400 (101 MHz) spectrometer and chemical shifts are given in ppm

relative to CDCl_3 (77.0 ppm). For measurements in DMSO, the residual solvent peak was used as a reference. For the peptides, ^1H NMR, TOCSY, ^1H - ^{13}C HSQC and ROESY spectra were recorded using a Varian INOVA-500 spectrometer (500 MHz).

Purity of the peptides was confirmed by analytical HPLC using an Alltima C8 column (4.6×250 mm; $5 \mu\text{m}$) at a flow rate of 0.5 mL min^{-1} using a linear gradient of buffer B (100% in 40 min) from 100% buffer A. 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 using an Alltima C8 column (22×250 mm; $10 \mu\text{m}$) at a flow rate of 6 mL min^{-1} with a linear gradient of buffer B (100% in 80 min) from 100% buffer A on an Applied Biosystems 400 solvent delivery system with an Applied Biosystems 757 UV/VIS absorbance detector. The buffer system used consists of buffer A: 0.1% TFA in MeCN/ H_2O , 20/80, v/v and buffer B: 0.1% TFA in *i*-PrOH/MeCN/ H_2O , 45/50/5, v/v/v.

ESI-MS spectra were obtained in the positive ion mode on a Shimadzu QP8000 single quadrupole mass spectrometer. HRMS analyses were performed on a MALDI TOF/TOF (Applied Biosystems).

Chemistry

General procedures for solid phase synthesis. Peptides were synthesized manually. Used abbreviations: DMT-Myr: (10*R*,12*S*)-dimethylmyristoyl; Me-Myr: (12*S*)-methylmyristoyl; Myr: myristoyl; Palm: palmitoyl; Ter: terphenyl; 3-Hyp: 3-hydroxyproline; hTyr: homotyrosine; Mtt: 4-methyltrityl. Each synthetic cycle consisted of the following steps.

Fmoc-group removal: The resin was treated with a 20% solution of piperidine in NMP ($3 \times$, each 10 min). The solution was removed by filtration and the resin was washed with NMP ($3 \times$, each 3 min) and DCM ($3 \times$, each 3 min).

Coupling step: A mixture of Fmoc-Xxx-OH (3 equiv.), BOP (3 equiv.) and DiPEA (6 equiv.) in NMP (10 mL mmol^{-1}) 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 ($3 \times$, each 3 min) and DCM ($3 \times$, each 3 min). Completion of the coupling was checked with the Kaiser or chloranil test.

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] (10 mL mmol^{-1}) 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 ($3 \times$, each 3 min) and DCM ($3 \times$, each 3 min).

General procedure for the preparation of the caspofungin analogues (4–7). A polystyrene resin functionalized with a 2-chloro trityl chloride linker (550 mg; initial loading: 1.1 mmol g^{-1}) was loaded with Fmoc-3-Hyp(*t*Bu)-OH (999.4 mg; 2.44 mmol) in DCM (5 mL) in the presence of DiPEA (850 μL ; 4.88 mmol) for 16 h. The solution was removed by filtration and the resin washed with DCM (6×5 mL, each 3 min). After drying *in vacuo* overnight, the amount of Fmoc-3-Hyp(*t*Bu)-OH coupled to the resin was determined by an Fmoc determination according to Meienhofer *et al.*⁴⁴ and was found to be

$0.520 \text{ mmol g}^{-1}$. Subsequently, unreacted trityl chloride moieties were capped with methanol (DCM/MeOH/DiPEA; 3×5 mL, each 2 min; 17/2/1; v/v/v). The peptide sequence was synthesized according to the general procedure for solid phase peptide synthesis. The resin-bound peptide **17** was divided in portions for the synthesis of the analogues **4–7**.

The Fmoc group was cleaved and the fatty acid side chain was coupled by adding the tail (3 equiv.), HATU (3 equiv.) and DiPEA (6 equiv.) in NMP (3 mL) to the resin. The mixture was shaken overnight, subsequently the coupling solution was removed by filtration and the resin washed with NMP (3×4 mL, each 3 min) and DCM (3×4 mL, each 3 min). Completion of the coupling was checked with the Kaiser test. Then, mild acidolytic cleavage, by treatment with TFE/AcOH/DCM (2/1/7, v/v/v, 5 mL) for 2 h, of the Mtt group as well as cleavage from the resin gave protected peptide precursor **18**. The mixture was concentrated *in vacuo* and the peptide was precipitated in MTBE/hexanes ($3 \times$, 1/1, v/v).

The linear peptide was then subjected to head-to-tail cyclization by dissolving it in dry DMF ($2 \text{ mL } \mu\text{mol}^{-1}$) followed by the addition of BOP (4 equiv.) and DiPEA (8 equiv.). The mixture was stirred for 48 h and then evaporated *in vacuo*. The product was redissolved in EtOAc and washed with 1 M KHSO_4 ($2 \times$), NaHCO_3 ($2 \times$) and H_2O ($2 \times$). The organic layer was dried over Na_2SO_4 , filtered and concentrated *in vacuo*. Final protecting group removal by treatment with a mixture of TFA/TIS/ H_2O (2 mL, 95/2.5/2.5, v/v/v) for 2 h and precipitation in MTBE/hexanes ($3 \times$, 1/1, v/v) afforded the crude peptide. After lyophilization from *tert*-BuOH/ H_2O (1/1, v/v) the peptide was purified by preparative HPLC.

Cyclo[(DMT-Myr)-Orn-Thr-Hyp-hTyr-Orn-3-Hyp] (4). Peptide **4** was obtained according to the general procedure for the preparation of the caspofungin analogues using 149.4 μmol of resin bound peptide **17**. After lyophilization, peptide **4** (74.5 mg; 51%) was obtained as a colourless solid.

Purity was confirmed by analytical HPLC and was found to be higher than 99% ($R_t = 41.28$ min). ESI-MS calcd for $\text{C}_{50}\text{H}_{82}\text{N}_8\text{O}_{11}$: 970.61, found: m/z 971.90 $[\text{M} + \text{H}]^+$; HRMS calcd for $\text{C}_{50}\text{H}_{82}\text{N}_8\text{O}_{11}$ $[\text{M} + \text{H}]^+$ 971.6181, found 971.6124; ^1H -NMR (CD_3OH , 500 MHz): δ Orn-1: 8.34 (NH), 4.48 (αCH), 2.31/1.79 (βCH), 1.68 (γCH), 3.60/2.85 (δCH); Thr-2: 8.73 (NH), 4.90 (αCH), 4.53 (βCH), 1.22 (γCH); Hyp-3: 4.49 (αCH), 2.27/1.97 (βCH), 4.50 (γCH), 3.78/3.70 (δCH); hTyr-4: 7.62 (NH), 4.81 (αCH), 1.96/1.67 (βCH), 2.98/2.92 (γCH), 6.92/6.69 (Ar-H); Orn-5: 7.50 (NH), 4.30 (αCH), 2.21/1.92 (βCH), 2.17 (γCH), 2.57 (δCH); Hyp-6: 4.28 (αCH), 4.08 (βCH), 2.27/1.97 (γCH), 3.78/3.71 (δCH); Tail: 2.25 ($\text{C}(\text{O})\text{CH}_2$), 1.81/1.75 (CH_2), 1.67/1.61 (CH_2), 1.49, 1.42, 1.35, 1.33/1.27, 1.31/1.06, 1.28 (CH_2), 1.24/0.92 (CHCH_2CH), 1.09, 0.87 (CH_3), 0.85 (CH_3) ppm. ^{13}C -NMR (CD_3OH , 125 MHz): δ Orn-1: 53.1 (αCH), 38.1 (βCH), 24.0 (γCH), 37.6 (δCH); Thr-2: 57.9 (αCH), 68.9 (βCH), 19.7 (γCH); Hyp-3: 62.4 (αCH), 34.2 (βCH), 71.4 (γCH), 56.9 (δCH); hTyr-4: 50.8 (αCH), 30.0 (βCH), 40.6 (γCH), 130.7/116.5 (Ar-H); Orn-5: 54.7 (αCH), 34.4 (βCH), 27.0 (γCH), 32.6 (δCH); Hyp-6: 74.5 (αCH), 69.4 (βCH), 34.2 (γCH), 46.5 (δCH); Tail: 45.7 (CHCH_2CH), 37.8, 36.7 ($\text{C}(\text{O})\text{CH}_2$), 32.6, 30.9, 30.0, 27.7, 26.9 (CH_2), 23.9 (CH_2), 30.8 (CH_2), 30.1, 20.05 (CH_3), 11.2 (CH_3).

Cyclo[-(Me-Myr)-Orn-Thr-Hyp-hTyr-Orn-3-Hyp] (5). Peptide **5** was obtained according to the general procedure for the preparation of the caspofungin analogues using 39 μmol of resin bound peptide **17**. After lyophilization, peptide **5** (17.9 mg; 48%) was obtained as a colourless solid.

Purity was confirmed by analytical HPLC and was found to be higher than 95% ($R_t = 40.48$ min). ESI-MS calcd for $\text{C}_{49}\text{H}_{80}\text{N}_8\text{NaO}_{11}$: 979.58, found: m/z 979.85 $[\text{M} + \text{Na}]^+$; HRMS calcd for $\text{C}_{49}\text{H}_{80}\text{N}_8\text{O}_{11}$: $[\text{M} + \text{H}]^+$ 957.6025, found 957.6016; $^1\text{H-NMR}$ (CD_3OH , 500 MHz): δ Orn-1: 8.15 (NH), 8.33 (ϵNH), 4.48 (αCH), 2.30/1.79 (βCH), 1.67 (γCH), 3.59/2.84 (δCH); Thr-2: 8.72 (NH), 4.89 (αCH), 4.53 (βCH), 1.22 (γCH); Hyp-3: 4.48 (αCH), 2.27/1.96 (βCH), 4.49 (γCH), 3.77/3.70 (δCH); hTyr-4: 7.72 (NH), 4.80 (αCH), 1.95/1.66 (βCH), 2.98/2.91 (γCH), 6.99/6.68 (Ar-H); Orn-5: 7.52 (NH), 4.30 (αCH), 2.20/1.91 (βCH), 2.17 (γCH), 2.56 (δCH); Hyp-6: 4.28 (αCH), 4.07 (βCH), 2.27/1.96 (γCH), 3.77/3.70 (δCH); Tail: 2.24 ($\text{C}(\text{O})\text{CH}_2$), 1.80/1.75 (CH_2), 1.66/1.59 (CH_2), 1.32 (CH_2), 1.30, 1.30/1.27, 1.30/1.09, 1.29 (CH_2), 1.27 (CH_2), 1.13, 0.87 (CH_3), 0.85 (CH_3) ppm. $^{13}\text{C-NMR}$ (CD_3OH , 125 MHz): δ Orn-1: 53.1 (αCH), 38.1 (βCH), 24.0 (γCH), 37.6 (δCH); Thr-2: 57.9 (αCH), 68.9 (βCH), 19.8 (γCH); Hyp-3: 62.3 (αCH), 34.2 (βCH), 71.3 (γCH), 56.8 (δCH); hTyr-4: 50.8 (αCH), 30.0 (βCH), 40.6 (γCH), 130.7/116.5 (Ar-H); Orn-5: 54.6 (αCH), 34.4 (βCH), 26.9 (γCH) 32.6 (δCH); Hyp-6: 74.5 (αCH), 69.4 (βCH), 34.2 (γCH), 46.5 (δCH); Tail: 37.6, 36.6 ($\text{C}(\text{O})\text{CH}_2$), 35.4, 30.7 (CH_2), 30.3, 30.1 (CH_2), 27.9, 26.9 (CH_2), 23.9 (CH_2), 23.8 (CH_2), 19.3 (CH_3), 11.4 (CH_3).

Cyclo[-(Palm)-Orn-Thr-Hyp-hTyr-Orn-3-Hyp] (6). Peptide **6** was obtained according to the general procedure for the preparation of the caspofungin analogues using 39 μmol of resin bound peptide **17**. After lyophilization, peptide **6** (24.5 mg; 65%) was obtained as a colourless solid.

Purity was confirmed by analytical HPLC and was found to be higher than 99% ($R_t = 42.28$ min). ESI-MS calcd for $\text{C}_{50}\text{H}_{82}\text{N}_8\text{O}_{11}$: 971.23, found: m/z 972.45 $[\text{M} + \text{H}]^+$; HRMS calcd for $\text{C}_{50}\text{H}_{82}\text{N}_8\text{O}_{11}$: $[\text{M} + \text{H}]^+$ 971.6181, found 971.6197; $^1\text{H-NMR}$ (CD_3OH , 500 MHz): δ Orn-1: 8.16 (NH), 4.48 (αCH), 2.30/1.78 (βCH), 1.67 (γCH), 3.59/2.84 (δCH); Thr-2: 8.72 (NH), 4.88 (αCH), 4.52 (βCH), 1.21 (γCH); Hyp-3: 4.49 (αCH), 2.26/1.96 (βCH), 4.49 (γCH), 3.77/3.70 (δCH); hTyr-4: 7.72 (NH), 4.81 (αCH), 1.96/1.66 (βCH), 2.97/2.91 (γCH), 6.99/6.68 (Ar-H); Orn-5: 7.52 (NH), 4.30 (αCH), 2.20/1.91 (βCH), 2.17 (γCH), 2.57 (δCH); Hyp-6: 4.28 (αCH), 4.07 (βCH), 2.26/1.96 (γCH), 3.77/3.71 (δCH); Tail: 2.24 ($\text{C}(\text{O})\text{CH}_2$), 1.78/1.75 (CH_2), 1.66/1.60 (CH_2), 1.31 (CH_2), 1.30 (CH_2), 1.28 (CH_2), 1.27 (CH_2), 0.89 (CH_3) ppm. $^{13}\text{C-NMR}$ (CD_3OH , 125 MHz): δ Orn-1: 53.1 (αCH), 38.1 (βCH), 24.0 (γCH), 37.6 (δCH); Thr-2: 58.0 (αCH), 68.9 (βCH), 19.7 (γCH); Hyp-3: 62.3 (αCH), 34.2 (βCH), 71.3 (γCH), 56.9 (δCH); hTyr-4: 50.8 (αCH), 30.0 (βCH), 40.6 (γCH), 130.7/116.5 (Ar-H); Orn-5: 54.6 (αCH), 34.4 (βCH), 27.0 (γCH), 32.7 (δCH); Hyp-6: 74.5 (αCH), 69.4 (βCH), 34.2 (γCH), 46.5 (δCH); Tail: 36.7 ($\text{C}(\text{O})\text{CH}_2$), 32.8 (CH_2), 30.5 (CH_2), 30.0 (CH_2), 27.0 (CH_2), 23.9 (CH_2), 23.4 (CH_2), 14.1 (CH_3).

Cyclo[-(Myr)-Orn-Thr-Hyp-hTyr-Orn-3-Hyp] (7). Peptide **7** was obtained according to the general procedure for the

preparation of the caspofungin analogues using 39 μmol of resin bound peptide **17**. After lyophilization, peptide **7** (32.4 mg; 88%) was obtained as a colourless solid.

Purity was confirmed by analytical HPLC and was found to be higher than 99% ($R_t = 39.33$ min). ESI-MS calcd for $\text{C}_{48}\text{H}_{78}\text{N}_8\text{O}_{11}$: 942.58, found: m/z 944.00 $[\text{M} + \text{H}]^+$; HRMS calcd for $\text{C}_{48}\text{H}_{78}\text{N}_8\text{O}_{11}$: $[\text{M} + \text{H}]^+$ 943.5868, found 943.5867; $^1\text{H-NMR}$ (CD_3OH , 500 MHz): δ Orn-1: 8.16 (NH), 8.34 (ϵNH), 4.48 (αCH), 2.31/1.78 (βCH), 1.67 (γCH), 3.59/2.85 (δCH); Thr-2: 8.72 (NH), 4.89 (αCH), 4.53 (βCH), 1.22 (γCH); Hyp-3: 4.49 (αCH), 2.27/1.97 (βCH), 4.50 (γCH), 3.78/3.70 (δCH); hTyr-4: 7.62 (NH), 4.81 (αCH), 1.96/1.66 (βCH), 2.97/2.92 (γCH), 6.99/6.69 (Ar-H); Orn-5: 7.53 (NH), 4.30 (αCH), 2.21/1.92 (βCH), 2.17 (γCH), 2.57 (δCH); Hyp-6: 4.28 (αCH), 4.08 (βCH), 2.27/1.96 (γCH), 3.78/3.71 (δCH); Tail: 2.25 ($\text{C}(\text{O})\text{CH}_2$), 1.81/1.75 (CH_2), 1.66/1.60 (CH_2), 1.32 (CH_2), 1.31 (CH_2), 1.29 (CH_2), 1.27 (CH_2), 0.90 (CH_3) ppm. $^{13}\text{C-NMR}$ (CD_3OH , 125 MHz): δ Orn-1: 53.1 (αCH), 38.1 (βCH), 24.0 (γCH), 37.6 (δCH); Thr-2: 58.0 (αCH), 69.0 (βCH), 19.7 (γCH); Hyp-3: 62.3 (αCH), 34.2 (βCH), 71.3 (γCH), 56.9 (δCH); hTyr-4: 50.8 (αCH), 29.9 (βCH), 40.6 (γCH), 130.7/116.5 (Ar-H); Orn-5: 54.7 (αCH), 34.4 (βCH), 27.0 (γCH), 32.7 (δCH); Hyp-6: 74.5 (αCH), 69.4 (βCH), 34.2 (γCH), 46.5 (δCH); Tail: 36.7 ($\text{C}(\text{O})\text{CH}_2$), 32.9 (CH_2), 30.4 (CH_2), 30.0 (CH_2), 27.0 (CH_2), 23.9 (CH_2), 23.4 (CH_2), 14.2 (CH_3).

(E)-S-Ethyl pent-2-enethioate (10). To a cooled solution (0 $^\circ\text{C}$) of (*E*)-pentenoic acid **9** (5.01 g; 50 mmol), DCC (11.35 g; 55 mmol) and DMAP (611 mg; 5 mmol, 10 mol%) in pentane (500 mL), neat EtSH (7.22 mL; 100 mmol) was added dropwise. The mixture was allowed to slowly reach room temperature and stirred for 16 h. After filtration through a short silica pad, volatiles were evaporated. The crude residue was distilled (Kugelrohr) and afforded product **10** as a colorless liquid (6.85 g; 95%).

$^1\text{H NMR}$ (400 MHz, CDCl_3): δ 6.96–6.84 (m, 1H), 6.06 (dd, $J = 15.5, 1.5$ Hz, 1H), 2.95–2.84 (m, 2H), 2.18 (qd, $J = 7.2, 3.7$ Hz, 2H), 1.28–1.01 (m, 3H), 0.99 (s, 3H) ppm. $^{13}\text{C NMR}$ (101 MHz, CDCl_3): δ 190.03 (Cq), 146.46 (CH), 127.79 (CH), 25.18 (CH_2), 22.95 (CH_2), 14.78 (CH_3), 12.05 (CH_3). Spectral data correspond to literature.^{28a}

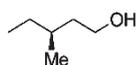
(S)-S-Ethyl 3-methylpentanethioate (11). (*S*, R_{Fe})-Josiphos-EtOH adduct (43.2 mg; 67 μmol) and $\text{CuBr}\cdot\text{Me}_2\text{S}$ (12.8 mg; 62 μmol) were stirred in freshly distilled MTBE (56 mL) until the mixture remained homogeneous (typically 10–30 min). Then the mixture was cooled to -78 $^\circ\text{C}$ and after 10 min a solution of MeMgBr in Et_2O (2.7 mL; 8.1 mmol) was added dropwise during 10 min. After 15 min of stirring a solution of thioester **10** (721 mg; 5 mmol) in MTBE (6.2 mL) was added over 3 h by a syringe pump. The reaction mixture was stirred for an additional 16 h at -78 $^\circ\text{C}$, quenched with EtOH (5 mL) and allowed to reach ambient temperature. Then a solution of NH_4Cl (1 M; 50 mL) was added. The organic layer was separated and the aqueous layer extracted with Et_2O (3×20 mL). The combined organic layers were dried over MgSO_4 and carefully evaporated (the product is volatile). The residual yellow liquid was purified by flash chromatography (Et_2O :pentane; 1:4) to afford

(*S,S*)-ethyl 3-methylpentanethioate **11** (800 mg; 80%) as a colourless liquid.

HRMS: calcd for $C_8H_{16}OS$ $[M + H]^+$ 161.0994, found 161.0996; 1H NMR (400 MHz, $CDCl_3$): δ 2.87 (q, $J = 7.4$ Hz, 2H), 2.53 (dd, $J = 14.4, 6.1$ Hz, 1H), 2.34 (dd, $J = 14.4, 8.1$ Hz, 1H), 2.17–1.72 (m, 1H), 1.45–1.30 (m, 1H), 1.24 (t, $J = 7.4$ Hz, 4H), 1.08–0.72 (m, 6H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 199.36, 51.01, 32.63, 29.23, 23.24, 19.02, 14.78, 11.21. NMR spectra contain traces of solvents ($\approx 5\%$). Spectral data correspond to literature.^{28a}

The enantiomeric ratio was determined on the corresponding methyl ester by chiral stationary phase gas chromatography on a Chiraldex G-TA column (30 m \times 0.25 mm), 60 °C, retention times: 5.94 (*R*)/6.05 (*S*) min: 85 : 1 (er), 97% ee (as reported in the literature^{28a}) $[\alpha]_D^{25} = +8.4$ ($c = 1.0$ in $CHCl_3$).

The absolute configuration was determined on the alcohol:^{28a}



$[\alpha]_D^{25} = +7.4$ ($c = 0.95$ in $CHCl_3$).

Literature⁴⁵ reports the opposite enantiomer with $\alpha_D = -8.5$ ($c = 1$ in $CHCl_3$).

(*S,E*)-S-Ethyl 5-methylhept-2-enoethioate (12). A solution of (*S,S*)-ethyl 3-methylpentanethioate **11** (801 mg; 5 mmol) in CH_2Cl_2 (50 mL) was cooled to -55 °C and then a solution of DIBAL (1 M in CH_2Cl_2 ; 6 mL; 6 mmol) was added. The mixture was stirred until complete conversion of the starting material (*ca.* 1.5 h). Subsequently, the mixture was poured into a saturated Rochelle's salt (potassium sodium tartrate) solution and stirred until the phases separated (mostly within 2 h). Layers were separated and the aqueous layer was extracted with CH_2Cl_2 (3 \times 15 mL). The combined layers were dried and carefully evaporated (the product is volatile) until the weight corresponded to a quantitative yield (501 mg).

To a cooled solution (0 °C) of HWE reagent (1.8 g; 7.5 mmol) in THF (25 mL) a solution of *n*-BuLi (1.6 M in hexanes; 3.44 mL; 5.55 mmol) was added dropwise. The reaction mixture was stirred for 20 min at 0 °C. Then (*S*)-3-methylpentanal (501 mg; 5 mmol) in a small amount of THF (0.3 mL) was added and the reaction mixture was stirred overnight (16 h). The reaction was quenched with water (10 mL). Layers were separated and the aqueous layer extracted with Et_2O (3 \times 15 mL). The combined organic layers were dried over $MgSO_4$ and carefully evaporated (the product is volatile). The residue was purified by flash chromatography on SiO_2 (MTBE : pentane; 250 : 1) and afforded (*S,E*)-S-ethyl 5-methylhept-2-enoethioate **12** (510 mg; 55%) as a colorless liquid.

HRMS calcd for $C_{10}H_{18}OS$ $[M + H]^+$ 187.1151, found 187.1152; $[\alpha]_D^{25} = +8.2$ ($c = 1.8$ in $CHCl_3$); 1H NMR (400 MHz, $CDCl_3$): δ 6.85 (dtd, $J = 8.6, 7.6, 1.2$ Hz, 1H), 6.08 (dd, $J = 15.5, 1.3$ Hz, 1H), 3.05–2.83 (m, 2H), 2.17 (ddd, $J = 7.2, 6.5, 3.5$ Hz, 1H), 2.09–1.91 (m, 1H), 1.53 (dq, $J = 13.3, 6.9$ Hz, 1H), 1.36 (dt, $J = 14.5, 6.8$ Hz, 1H), 1.26 (ddd, $J = 7.4, 4.9, 1.2$ Hz, 3H), 0.86 (td, $J = 7.0, 3.2$ Hz, 6H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ 189.95, 144.27, 129.69, 39.21, 34.19, 29.18, 22.99, 19.14, 14.79, 11.34 + 2 peaks at 65.81 and 15.24 as Et_2O residues.

(*3S,5S*)-S-Ethyl 3,5-dimethylheptanethioate (13). (*S,R_{Fc}*)-Josiphos-CuBr complex (29.1 mg; 39 μ mol) was dissolved in freshly distilled MTBE (23.7 mL) until the mixture remained homogeneous (typically 10–30 min). Then the mixture was cooled to -78 °C and after 10 min a solution of $MeMgBr$ in Et_2O (3 M in Et_2O ; 1.1 mL; 3.42 mmol) was added dropwise.⁴⁶ After 15 min of stirring, a solution of thioester **12** (490 mg; 2.63 mmol) in MTBE (2.6 mL) was added over 3 h by a syringe pump. The mixture was stirred for an additional 16 h at -78 °C. The reaction was quenched by addition of $EtOH$ (2 mL) and the mixture was allowed to reach ambient temperature. Then an aqueous solution of NH_4Cl (1 M, 30 mL) was added, the organic layer separated and the aqueous layer extracted with Et_2O (3 \times 20 mL). The combined organic layers were dried over $MgSO_4$ and carefully evaporated (the product is volatile). The residual yellow liquid was purified by flash chromatography (MTBE : pentane; 1 : 250) to afford (*3S,5S*)-S-ethyl 3,5-dimethylheptanethioate **13** (417.7 mg; 78%) as a colorless liquid.

HRMS: calcd for $C_{11}H_{22}OS$ $[M + H]^+$ 203.147, found 203.110; $[\alpha]_D^{25} = +4.2$ ($c = 1.4$ in $CHCl_3$); 1H NMR (400 MHz, $CDCl_3$): δ 2.86 (q, $J = 7.4$ Hz, 2H), 2.52 (dd, $J = 14.4, 5.4$ Hz, 1H), 2.28 (dd, $J = 14.4, 8.5$ Hz, 1H), 1.43–1.27 (m, 2H), 1.24 (t, $J = 7.4$ Hz, 4H), 1.14–0.94 (m, 3H), 0.92 (d, $J = 6.6$ Hz, 3H), 0.85 (d, $J = 6.5$ Hz, 6H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ 199.33, 51.29, 44.07, 31.53 (CH1), 29.00, 28.66 (CH1), 23.23, 20.15 (CH₃), 19.48 (CH₃), 14.79 (CH₃), 11.07 (CH₃).

(*10S,12S*)-Dimethyltetradec-7-enoic acid (14). A solution of (*S,S*)-ethyl 3-methylpentanethioate (417 mg; 2.06 mmol) in CH_2Cl_2 (20 mL) was cooled to -55 °C and a solution of DIBAL (1 M in CH_2Cl_2 ; 2.5 mL; 2.5 mmol) was added. The mixture was stirred until complete conversion of the starting material (*ca.* 1.5 h), then poured into saturated aqueous Rochelle's salt and stirred until phases separated (mostly within 2 h). Layers were separated and the aqueous layer was extracted with CH_2Cl_2 (3 \times 15 mL). The combined layers were dried and carefully evaporated (the product is volatile) to a weight corresponding to a quantitative yield (294 mg).

To a stirred suspension of 7-(bromotriphenylphosphoranyl)-heptanoic acid (1.65 g; 3.5 mmol) in THF (2 mL) at ambient temperature a solution of LiHMDS (1 M in THF; 6.2 mL; 6.18 mmol) was added dropwise. The mixture was stirred until the suspension turned into a deep red solution. Then a solution of (*3S,5S*)-3,5-dimethylheptanal (293 mg; 2 mmol) in a small amount of THF (300 μ L) was added and the reaction mixture was stirred until complete consumption of the starting material (2 h). The mixture was acidified to pH = 1 by dilute aq. HCl and extracted with Et_2O (3 \times 20 mL). The combined organic layers were dried and evaporated. The resulting thick liquid was purified by column chromatography (Et_2O : pentane; 1 : 4) and afforded **14** (331 mg; 47%) as a colorless liquid (a mixture of *E* and *Z* isomers).

HRMS: calcd for $C_{16}H_{30}O_2$ $[M + H]^+$ 255.232, found 255.235; $[\alpha]_D^{25} = +12.2$ ($c = 1.8$ in $CHCl_3$); 1H NMR (400 MHz, $CDCl_3$): δ 5.51–5.22 (m, 2H), 2.35 (t, $J = 7.5$ Hz, 2H), 2.13–0.55 (m, 25H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ 131.22, 130.17, 129.03, 128.67, 44.23, 44.00, 39.77, 34.26, 34.03, 32.35, 31.61, 31.55, 30.70, 30.42, 29.29, 29.16, 28.72,

28.49, 27.08, 24.57, 24.51, 20.16, 20.06, 19.68, 11.14 – additional peaks observed due to the inseparable *E/Z* mixture. Spectral data correspond to the literature.²⁷

(10*R*,12*S*)-Dimethyltetradecanoic acid (8). To a vigorously stirred solution of (10*S*,12*S*)-dimethyltetradec-7-enoic acid **14** (310.1 mg; 1.2 mmol) and flavine catalyst (49.6 mg; 0.12 mmol, 10 mol%) in EtOH (1 mL) under an oxygen atmosphere, hydrazine hydrate (1.6 mL; 31.7 mmol) was added in one portion. Vigorous stirring continued for 16 h. Then the reaction mixture was acidified to pH = 1 by dilute aq. HCl and extracted with Et₂O (3 × 20 mL). The combined organic layers were dried and evaporated. The residual red liquid was purified by column chromatography (Et₂O : pentane; 1 : 4) and afforded **8** (524 mg; 81%) as a colorless thick liquid.

HRMS: calcd for C₁₆H₃₂O₂ [M + H]⁺ 255.232, found 255.216; [α]_D = +14.1 (*c* = 1.3 in CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 11.20 (bs, 1H), 2.34 (t, *J* = 7.5 Hz, 2H), 1.95–1.51 (m, 1H), 1.57–0.32 (m, 31H) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 180.15 (Cq), 44.70, 36.85, 34.21, 31.56 (CH), 30.00 (CH), 29.93, 29.46, 29.24, 29.20, 29.07, 26.86, 24.73, 20.26 (CH₃), 19.72 (CH₃), 11.17(CH₃).

Spectral data correspond to the literature.²⁷

(*S*)-12-Methyltetradec-9-enoic acid (15). To a stirred solution of (*S*)-*S*-ethyl 3-methylpentanethioate **11** (80 mg; 0.5 mmol) in CH₂Cl₂ (0.75 mL) at –50 °C, DIBAL-H was added. After complete conversion of the thioester (2 h), the reaction mixture was poured into a saturated solution of Rochelle's salt. After clear layer separation, the organic layer was separated and the aqueous layer extracted with Et₂O (3 × 10 mL). The combined organic layers were dried and carefully evaporated until the weight corresponded to a quantitative yield (43 mg).

Then, to a stirred suspension of 9-(bromotriphenyl phosphoranyl)nonanoic acid (425 mg; 0.85 mmol) in THF (0.75 mL) a solution of LiHMDS (1 M in THF; 1.5 mL; 1.5 mmol) was added until the solution stayed deep red. To this solution, 3-methyl-pentanal in a small amount of THF (300 μL) was added. The reaction mixture was stirred until complete conversion of the starting material (2 h) and acidified to pH = 1 by dilute aq. HCl. The resulting solution was extracted with Et₂O (3 × 20 mL) and the combined organic layers were dried and evaporated. The resulting thick liquid was purified by column chromatography (Et₂O : pentane; 1 : 4) and afforded **15** (65.9 mg; 55%) as a colorless liquid (as a mixture of *E* and *Z* isomers).

HRMS: calcd for C₁₅H₂₉O₂ [M + H]⁺ 241.216, found 241.217; [α]_D = +0.3 (*c* = 0.5 in CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 5.37 (dt, *J* = 6.0, 4.6 Hz, 2H), 2.35 (t, *J* = 7.5 Hz, 2H), 2.01 (d, *J* = 5.4 Hz, 2H), 1.68–1.59 (m, 2H), 1.40–1.21 (m, 14H), 0.96–0.80 (m, 6H) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 177.89, 130.32, 128.66, 35.11, 34.13, 33.63, 29.69, 29.46, 29.19, 28.94, 28.88, 27.19, 24.64, 19.11, 11.55.

(*S*)-12-methyltetradecanoic acid (16). To a vigorously stirred solution of (*S*)-12-methyltetradec-9-enoic acid **15** (60 mg; 0.25 mmol) and flavine catalyst (10 mg; 25 μmol, 10 mol%) in EtOH (1 mL) under oxygen atmosphere, hydrazine hydrate (375 μL; 7.5 mmol) was added in one portion. Vigorous stirring was continued for 16 h and the mixture was acidified to pH = 1

by dilute aq. HCl and extracted with Et₂O (3 × 20 mL). The combined organic layers were dried and evaporated. The residual red liquid was purified by column chromatography (Et₂O : pentane; 1 : 4) and afforded **16** (50 mg, 83%) as a colorless thick liquid.

HRMS: calcd for C₁₅H₃₁O₂ [M + H]⁺ 243.231, found 243.233; [α]_D = +0.3 (*c* = 0.5 in CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ 2.34 (t, *J* = 7.5 Hz, 2H), 1.65–1.60 (m, 2H), 1.32–1.23 (m, 20H), 0.87–0.81 (m, 6H) ppm. ¹³C NMR (101 MHz, CDCl₃): δ 220.66, 36.62, 34.38, 34.07, 29.99, 29.66, 29.58, 29.48, 29.42, 29.23, 29.05, 27.09, 24.66, 19.20, 11.39.

Cyclo[-(Ter)-Orn-Thr-Hyp-hTyr-Orn-3-Hyp] (19). Peptide **19** was obtained according to the general procedure for the preparation of the caspofungin analogues using 39 μmol of resin bound peptide **17**. After lyophilization, peptide **19** (18 mg; 43%) was obtained as a colourless solid.

Purity was confirmed by analytical HPLC and was found to be higher than 95% (*R*_t = 21.55 min). ESI-MS calcd for C₅₈H₇₄N₈O₁₂: 1074.54, found: *m/z* 1075.85 [M + H]⁺; HRMS calcd for C₅₈H₇₄N₈O₁₂ [M + H]⁺ 1075.5504, found 1075.5529; ¹H-NMR (DMSO, 500 MHz): δ Orn-1: 8.59 (NH), 7.98 (εNH), 4.53 (αCH), 2.15/1.68 (βCH), 1.77 (γCH), 3.45/2.73 (δCH); Thr-2: 8.22 (NH), 4.76 (αCH), 4.39 (βCH), 1.13 (γCH); Hyp-3: 4.33 (αCH), 2.16/1.76 (βCH), 4.38 (γCH), 3.74/3.69 (δCH); hTyr-4: 7.55 (NH), 4.04 (αCH), 2.08/1.87 (βCH), 2.46/2.37 (γCH), 6.95/6.66 (Ar-H); Orn-5: 7.59 (NH), 4.70 (αCH), 1.84/1.69 (βCH), 1.59 (γCH), 2.88/2.83 (δCH); Hyp-6: 4.13 (αCH), 4.02 (βCH), 2.12/1.87 (γCH), 3.65 (δCH); Ter: 0.92 (CH₃), 1.37 (CH₂), 1.41 (CH₂), 1.75 (CH₂), 4.00 (OCH₂), 7.67/7.05 (Ar-H Ph-3), 7.83/7.75 (Ar-H Ph-2), 8.03/7.81 (Ar-H Ph-1) ppm. ¹³C-NMR (DMSO, 125 MHz): δ Orn-1: 51.1 (αCH), 25.2 (βCH), 22.7 (γCH), 35.1 (δCH); Thr-2: 55.5 (αCH), 65.6 (βCH), 18.7 (γCH); Hyp-3: 59.9 (αCH), 36.4 (βCH), 68.4 (γCH), 54.8 (δCH); hTyr-4: 51.4 (αCH), 32.5 (βCH), 30.2 (γCH), 128.5/114.1 (Ar-H); Orn-5: 48.2 (αCH), 28.0 (βCH), 22.1 (γCH), 37.9 (δCH); Hyp-6: 71.5 (αCH), 66.7 (βCH), 32.5 (γCH), 44.1 (δCH); Ter: 13.2 (CH₃), 21.1 (CH₂), 26.9 (CH₂), 27.6 (CH₂), 67.2 (OCH₂), 126.8/114.1 (Ar-H Ph-3), 125.3/125.8 (Ar-H Ph-2), 127.6/126.6 (Ar-H Ph-1) ppm.

Cyclo[-(DMT-Myr)-Orn-Thr-Hyp-hTyr-Orn-Hyp] (20). Peptide **20** was obtained analogously to peptide **4**, except for the amino acid sequence, using 31 μmol of resin bound peptide **17**. The first amino acid coupled was Fmoc-Hyp(*t*Bu)-OH instead of Fmoc-3-Hyp(*t*Bu)-OH. After lyophilization, peptide **20** (12 mg; 40%) was obtained as a colourless solid.

Purity was confirmed by analytical HPLC and was found to be higher than 99% (*R*_t = 23.25 min). ESI-MS calcd for C₅₀H₈₂N₈O₁₁: 970.61, found: *m/z* 971.75 [M + H]⁺; HRMS calcd for C₅₀H₈₂N₈O₁₁ [M + H]⁺ 971.6181, found 971.6123; ¹H-NMR (DMSO, 500 MHz): δ Orn-1: 8.07 (NH), 7.91 (εNH), 4.35 (αCH), 2.02/1.43 (βCH), 1.65 (γCH), 3.40/2.66 (δCH); Thr-2: 8.21 (NH), 4.74 (αCH), 4.38 (βCH), 1.07 (γCH); Hyp-3: 4.31 (αCH), 2.15/1.75 (βCH), 4.34 (γCH), 3.72 (δCH); hTyr-4: 7.53 (NH), 4.03 (αCH), 2.06/1.84 (βCH), 2.45/2.36 (γCH), 6.94/6.65 (Ar-H); Orn-5: 7.61 (εNH), 7.39 (NH), 4.66 (αCH), 1.79/1.59 (βCH), 1.55 (γCH), 2.79 (δCH); Hyp-6: 4.34 (αCH), 1.99/1.82 (βCH), 4.36 (γCH), 3.68/3.47 (δCH); Tail: 2.12 (C(O)CH₂),

1.47, 1.45/1.22, 1.39, 1.31/1.06, 1.26/1.21, 1.25/1.02, 1.19/0.89, 0.83 (CH₃), 0.82 (CH₃) ppm. ¹³C-NMR (DMSO, 125 MHz): δ Orn-1: 49.4 (αCH), 25.1 (βCH), 22.2 (γCH), 34.6 (δCH); Thr-2: 55.4 (αCH), 68.4 (βCH), 18.6 (γCH); Hyp-3: 59.9 (αCH), 36.4 (βCH), 68.0 (γCH), 54.7 (δCH); hTyr-4: 51.2 (αCH), 32.5 (βCH), 30.2 (γCH), 128.5/114.1 (Ar-H); Orn-5: 48.7 (αCH), 27.9 (βCH), 21.8 (γCH), 37.9 (δCH); Hyp-6: 58.1 (αCH), 36.8 (βCH), 65.5 (γCH), 55.0 (δCH); Tail: 43.3, 35.4, 34.3 (CHCH₂CH), 30.2, 28.6, 27.8, 27.6, 25.5, 24.6, 19.1 (CH₃), 10.2 (CH₃).

Cyclo[-(Myr)-Orn-Thr-Hyp-hTyr-Orn-Hyp] (21). Peptide **21** was obtained analogously to peptide **7**, except for the amino acid sequence, using 31 μmol of resin bound peptide **17**. The first amino acid coupled was Fmoc-Hyp(*t*Bu)-OH instead of Fmoc-3-Hyp(*t*Bu)-OH. After lyophilization, peptide **21** (6 mg; 20%) was obtained as a colourless solid.

Purity was confirmed by analytical HPLC and was found to be higher than 95% (*R*_t = 22.25 min). ESI-MS calcd for C₄₈H₇₈N₈O₁₁: 942.58, found: *m/z* 943.65 [M + H]⁺; HRMS calcd for C₄₈H₇₈N₈O₁₁ [M + H]⁺ 943.5868, found 943.5870; ¹H-NMR (DMSO, 500 MHz): δ Orn-1: 8.07 (NH), 7.91 (εNH), 4.29 (αCH), 1.96/1.37 (βCH), 1.58 (γCH), 3.34/2.60 (δCH); Thr-2: 8.21 (NH), 4.68 (αCH), 4.31 (βCH), 1.01 (γCH); Hyp-3: 4.24 (αCH), 2.09/1.69 (βCH), 4.27 (γCH), 3.66 (δCH); hTyr-4: 7.57 (NH), 3.97 (αCH), 2.39/2.30 (βCH), 2.00/1.78 (γCH), 6.88/6.69 (Ar-H); Orn-5: 7.65 (εNH), 7.39 (NH), 4.59 (αCH), 1.72/1.52 (βCH), 1.49 (γCH), 2.73 (δCH); Hyp-6: 4.27 (αCH), 1.93/1.76 (βCH), 4.30 (γCH), 3.62/3.41 (δCH); Tail: 2.06 (C(O)CH₂), 1.41, 1.20, 1.17/1.01, 1.14, 0.87 (CH₃), 0.80 (CH₃) ppm. ¹³C-NMR (DMSO, 125 MHz): δ Orn-1: 49.4 (αCH), 25.1 (βCH), 22.1 (γCH), 34.6 (δCH); Thr-2: 55.4 (αCH), 68.4 (βCH), 18.6 (γCH); Hyp-3: 59.9 (αCH), 36.4 (βCH), 67.9 (γCH), 54.7 (δCH); hTyr-4: 51.2 (αCH), 32.5 (βCH), 30.2 (γCH), 128.5/114.1 (Ar-H); Orn-5: 48.6 (αCH), 27.9 (βCH), 21.9 (γCH), 37.9 (δCH); Hyp-6: 58.0 (αCH), 36.8 (βCH), 65.5 (γCH), 55.1 (δCH); Tail: 34.3 (C(O)CH₂), 28.1, 27.5, 24.7, 21.3, 18.0 (CH₃), 13.2 (CH₃).

Biology

Candida MIC assay. Antifungal activity was evaluated by broth microdilution. The medium 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 of the compounds, to a concentration of 1 mg mL⁻¹. After dissolving each compound was diluted 20 times in YPD medium rendering a stock solution of 50 μg mL⁻¹. Caspofungin,³² purchased from Merck Sharp & Dohme B.V. (Haarlem, Netherlands), was included as a reference. Serial 2-fold dilutions of the test compounds in YPD medium were prepared as follows. 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 column 1. Then using an 8-channel pipet, compounds in column 1 were serially diluted 2-fold over the microtiter plate until column 11. The last column of the plate contained drug-

free wells dedicated for growth and sterility controls for each organism tested.

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 medium (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 medium and aerated for 24 h at 30 °C on a shaker set at 300 rpm. The broth cultures were diluted 10 times with the medium and the optical density of this suspension was measured at a wavelength of 600 nm. The suspension was further diluted to an OD₆₀₀ of 0.01 resulting in a concentration of (1–5) × 10⁶ cfu mL⁻¹. This suspension was further diluted 1 : 100 in YPD medium to yield (1–5) × 10⁴ cfu mL⁻¹. This final dilution was used for inoculating the plates. Plates containing the diluted compounds were inoculated with 100 μL per well of the appropriate microorganism using an 8-channel pipet. The final volume per well, including organism and compound, was 200 μL. Thus, the final number of cells per well was approximately 5–25 × 10³ cfu mL⁻¹.

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.

CD spectroscopy. CD spectra were recorded at 298 K on a JASCO J-810 spectrometer using 0.1 cm path length quartz cells. The CD spectra are an average of four scans, collected at 0.2 nm intervals between 190 and 250 nm. The peptides were prepared at concentrations of 0.1 mM in MeCN/H₂O (1/1, v/v) or 0.1 mM in TFE/H₂O (1/1, v/v). Ellipticity is reported as the mean residue ellipticity [*θ*] in degrees cm² dmol⁻¹.

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