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Isolation and structural elucidation of callipeltins J–M: antifungal peptides from the marine sponge *Latrunculia* sp.

Maria Valeria D'Auria,[a] Valentina Sepe,[a] Rosa D'Orsi,[a] Filomena Bellotta,[a] Cécile Debitus[b] and Angela Zampella[a,*]

[a]*Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli 'Federico II', via D. Montesano 49, 80131 Napoli, Italy*
[b]*IRD (ex ORSTOM), Centre de Nouméa, B.P. A5 Nouméa Cedex, New Caledonia*

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Abstract—Continued investigation of the polar extracts of the marine sponge *Latrunculia* sp. has resulted in the discovery of callipeltins J–M. The new structures were determined by interpretation of their NMR and MS data. The stereochemistry of the unusual 3-methylglutamine residue in callipeltins J and K was secured by stereoselective synthesis and Marfey's LC–MS analysis.
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1. Introduction

Callipeltins are a group of marine peptides with unusual structural features and remarkable biological properties, isolated from the sponges *Callipelta* sp. and *Latrunculia* sp.[1–4] From a structural point of view the most distinctive feature of callipeltins is the presence of several non-proteinogenic units; from a biological point of view, callipeltin A displays a broad range of biological activities, ranging from antiviral activity, cytotoxic activity against several human tumour cell lines and regulatory activity of the myocardial force of contraction.[5,6] The unusual structural features of these peptide metabolites and the interesting biological activities have aroused considerable interest among the synthetic chemistry community. As a result, all the non-proteinogenic units in callipeltins, namely (3*S*,4*R*)-3,4-dimethyl-L-glutamine (diMeGln),[7–9] (3*S*,4*R*)-3,4-dimethyl-L-pyroglutamic acid (the N-terminus unit in callipeltin B),[9] (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (AGDHE),[10–13] (2*R*,3*R*,4*R*)-3-hydroxy-2,4,6-trimethylheptanoic acid[14–17] linked to the N-terminus of callipeltins A, C, D and F–I; and (*R*)-β-methoxy-D-tyrosine (β-OMeTyr)[18–20] were obtained in a stereoselective manner. The availability of synthetic standards allowed the assignment of the stereochemistry of β-OMeTyr residue,[20] left unassigned in the original paper due to the decomposition of the above residue under standard acidic hydrolysis of the parent peptide, the revision of the stereochemistry of 3-hydroxy-2,4,6-trimethylheptanoic acid[16] and confirmation of the stereochemistry of diMeGln and AGDHE residues. In addition, by employing quantum molecular calculation of coupling constants and LC–MS Marfey's analysis, we found that both of the threonine residues in callipeltin A have the D-*allo* configuration, consistent with what was independently found in the structurally related cyclic depsipeptide neamphamide A.[21] All these structural and synthetic studies have led to a complete stereochemical assignment of callipeltin A and its congeners that recently received indirect support by the total synthesis of callipeltins D and E.[22,23]

Apart from synthetic studies, further knowledge on the structure–activity relationship studies could arise from the chemical and biological evaluations of new natural analogues of the parent callipeltin A. In this respect the sponge *Latrunculia* sp., collected at the Vanuatu Island, was proved to be a rich source of new linear callipeltin derivatives structurally related to callipeltin C (**1**),[2] which in turn represents the acyclic counterpart of callipeltin A. Continuing investigation of the polar extracts of this sponge afforded four new callipeltins, named callipeltins J–M (**2**–**5**), the structural elucidation of which is the subject of the present paper.

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* Corresponding author. Tel.: +39 081678525; fax: +39 081678552; e-mail: azampell@unina.it

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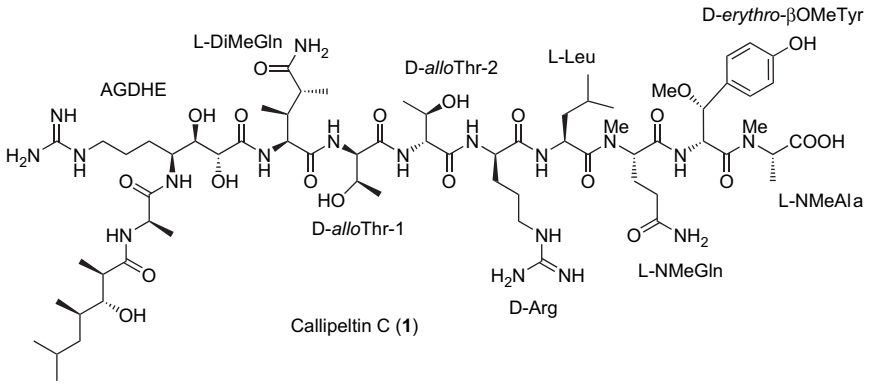

2. Results and discussion

The lyophilised sponge was extracted with methanol and the crude methanolic extract was subjected to a modified Kupchan's partitioning procedure. Fractionation of the butanol-soluble material (ca. 4 g) by DCCC (Droplet Counter Current Chromatography, $CHCl_3$/MeOH/H_2O, 7:13:8 ascending mode) followed by repeated reversed phase HPLC afforded pure callipeltins J–M (**2**–**5**).

The molecular formula of callipeltin J (**2**) was established as $C_{31}H_{58}N_8O_{11}$ by HR ESIMS calculation of the $[M+H]^+$ molecular ion at *m*/*z* 719.4297 (calcd for $C_{31}H_{59}N_8O_{11}$: 719.4303). Extensive interpretation of 2D NMR data obtained by the gradient-enhanced versions of COSY, TOCSY, HSQC, HMBC spectra (see Table 1), by comparison with those of callipeltin D, disclosed the presence of one residue each of alanine (Ala), 4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (AGDHE), threonine (Thr) and 3-hydroxy-2,4,6-trimethylheptanoic acid. In addition, the presence of a 3-methylglutamine residue, not previously found in other callipeltin derivatives, was easily inferred from the analysis of COSY, TOCSY, HMBC and MS data. Starting from the α-proton at δ 4.46, this was found to be coupled to a methine proton at δ 2.21 in turn coupled with a methyl group at δ 1.04 and to an allylic methylene at δ 2.39 and 2.17. HMBC data confirmed the assignment, whereas the identity of glutamine rather than glutamic was deduced from MS data.

The amino acid sequence of **2** and placement of acyl substituent were assigned from the analysis of the fragmentation pattern in the ESIMS/MS mass spectrum (Fig. 1). The alignment of four amino acid residues in **2** was the same as that of callipeltin D, with 3-methylglutamine in **2** replacing the 3,4-dimethylglutamine in callipeltin D.

The absolute stereochemistry of Ala, Thr and AGDHE residues in callipeltin J was determined by complete acid hydrolysis of **2** and Marfey's analysis of the resulting amino acids. The acid hydrolysate was derivatised with (1-fluoro-2,4-dinitrophenyl)-5-L-alaninamide (L-FDAA),[24] and then the LC–MS comparison of the derivatives from **2** with the FDAA derivatives of appropriate standards established the presence of D-Ala, D-*allo*Thr and (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic acid.

The molecular formula of callipeltin K (**3**) was established as $C_{67}H_{116}N_{18}O_{21}$ by HR ESIMS. The NMR and MS data for **3** were consistent with those of callipeltin C, except for

Table 1. ^1H and ^{13}C NMR data (500 MHz, CD_3OD) for compounds **2**–**5**

aa	**2**		**3**		aa	**4**		aa	**5**	
	δ_H[a]	δ_C	δ_H[a]	δ_C		δ_H[a]	δ_C		δ_H[a]	δ_C
TMHEA					TMHEA					
1		177.8		177.8	1		179.4			
2	2.61 m	45.6	2.61 m	44.4	2	2.61 m	44.5			
3	3.50 m	79.9	3.51 dd (8.5, 2.4)	79.4	3	3.51 dd (9.3, 3.1)	79.4			
4	1.79 m	33.8	1.79 m	33.1	4	1.76 m	33.8			
5	1.20 m	39.8	1.20 m	39.0	5	1.20 m	39.3			
6	1.67 m	26.5	1.67 m	25.9	6	1.67 m	25.5			
7	0.95 d (6.4)	24.7	0.95 d (6.2)	24.7	7	0.95 d (6.2)	23.9			
8	1.07 d (7.1)	14.4	1.07 d (6.5)	14.6	8	1.10 d (6.0)	14.6			
9	0.98 d (6.4)	17.5	0.98 d (6.9)	17.5	9	0.99 d (6.7)	17.5			
10	0.89 d (6.4)	22.0	0.88 d (6.9)	22.0	10	0.89 d (6.4)	21.6			
Ala					Ala					
α	4.31 q (7.1)	50.9	4.32 ovl	51.2	α	4.32 ovl	51.6			
β	1.39 d (7.1)	17.6	1.42 d (7.5)	17.7	β	1.42 d (7.2)	17.5			
CONH		175.9	8.27[b] br s	175.9	CONH	—	175.1			
AGDHE					AGDHE					
α	3.98 d (6.4)	72.7	3.99 d (6.6)	72.7	α	3.98 d (7.6)	72.8			
β	3.75 dd (6.4, 2.6)	75.3	3.69 dd (6.6, 2.2)	75.3	β	3.73 dd (7.6, 3.5)	75.0			

(*continued*)

Table 1. (*continued*)

aa	**2**		**3**		aa	**4**		aa	**5**	
	δ_H[a]	δ_C	δ_H[a]	δ_C		δ_H[a]	δ_C		δ_H[a]	δ_C
γ	4.15 m	50.7	4.15 m	50.9	γ	4.15 m	50.6			
δ	1.66, 1.30 m	26.4	1.63, 1.30 m	26.4	δ	1.63, 1.41 m	25.9			
ε	1.66 m	29.5	1.68 m	29.5	ε	1.68 m	29.5			
ζ	3.18 m	42.0	3.18 m	41.7	ζ	3.21 m	41.7			
CO		175.9		175.9	CO		175.4			
Guan	—	157.8	7.26[b] br s	157.8	Guan	—	na			
NH-4	—		7.74[b] br s		NH-4	—				
3-MeGln					diMeGln			diMePyroGlu		
α	4.46 ovl	58.1	4.46 ovl	58.2	α	4.43 d (8.5)	57.0	α	3.80 d (3.3)	63.4
β	2.21 m	39.7	2.21 m	39.7	β	2.25 m	39.3	β	2.59 m	39.7
βMe	1.04 d (6.8)	17.2	1.04 d (6.8)	17.2	βMe	1.02 d (7.0)	13.7	βMe	1.12 d (7.2)	14.7
γ	2.39, 2.17 m	40.2	2.39, 2.17 m	39.4	γ	2.66 m	41.3	γ	2.68 m	39.7
					γMe	1.18 d (6.7)	15.6	γMe	1.07 d (7.3)	10.0
CONH		173.1		173.1	CONH		172.6	CONH		182.0
$CONH_2$	7.61 br s	180.8	7.61[b] br s	180.8	$CONH_2$	—	179.3	CONH	—	174.5
Thr					Thr			Thr		
α	4.47 d (6.3)	60.0	4.36 d (8.1)	60.1	α	4.30 d (7.0)	60.0	α	4.33 d (6.7)	60.4
β	4.15 m	69.0	4.13 m	68.4	β	4.11 m	68.0	β	4.08 m	68.0
γ	1.24 d (7.1)	20.1	1.26 d (6.5)	20.4	γ	1.26 d (6.1)	20.2	γ	1.26 d ovl	20.1
CONH	—	172.7	8.43[b] br s	172.7	CONH	—	172.6	CONH	—	172.1
Thr					Thr			Thr		
α			4.22 d (6.4)	60.9	α	4.24 d (7.1)	60.7	α	4.22 d (7.0)	60.4
β			4.06 m	68.4	β	4.06 m	68.4	β	4.02 m	68.0
γ			1.31 d (6.5)	20.6	γ	1.31 d (7.3)	20.3	γ	1.26 d ovl	20.1
CONH			8.18[a] br s	172.8	CONH	—	171.9	CONH	—	171.6
Arg					Arg			Arg		
α			4.32 ovl	54.0	α	4.35 ovl	53.9	α	4.35 ovl	53.9
β			1.97, 1.67 m	29.5	β	1.98 m	29.5	β	1.93, 1.68 m	29.5
γ			1.68, 1.92 m	25.5	γ	1.68, 1.92 m	25.9	γ	1.67 m	26.2
δ			3.19 m	41.7	δ	3.19 m	41.7	δ	3.18 m	42.2
CONH			7.71[b] br s	na	CONH	—	174.7	CONH	—	173.8
Guan			7.40[b] br s	157.8	Guan	—	157.8	Guan	—	na
Leu					Leu			Leu		
α			4.73 dd (10.2, 4.6)	50.1	α	4.83 m	49.8	α	4.76 m	48.9
β			1.67, 1.45 m	40.7	β	1.67, 1.45 m	40.6	β	1.70, 1.40 m	40.6
γ			1.67 m	25.8	γ	1.74 m	26.0	γ	1.70 m	25.7
Me-γ			0.92 d (6.1)	21.4	Me-γ	0.99 d (7.2)	22.2	Me-γ	0.94 d (7.4)	21.6
Me-ψ			0.95 d (6.5)	23.5	Me-ψ	0.99 d (7.0)	22.2	Me-ψ	0.94 d (7.4)	23.9
CONH			7.99[b] br s	172.9	CONH	—	174.2	CONH	—	na
NMeGln					NMeGln			NMeGln		
α			4.93 dd (10.4, 4.5)	57.3	α	5.26 dd (9.7, 3.1)	56.4	α	4.90 dd (10.1, 4.7)	56.9
β			1.70 m	26.4	β	2.20 m	24.2	β	1.94, 1.70 m	24.6
γ			1.98, 1.92 m	32.4	γ	2.19, 2.22 m	31.8	γ	1.94 m	32.4
NMe			3.09 s	32.7	NMe	2.99 s	31.7	NMe	2.80 s	31.3
CO				na	CO		169.9	CO		169.7
$CONH_2$			na	176.9	$CONH_2$	—	176.9	$CONH_2$	—	na
β-OMeTyr					β-OTyr			β-OMeTyr		
α			5.23 d (8.4)	54.4	α	4.35 br d (2.6)	60.3	α	5.23 d (8.1)	54.1
β			4.33 d (8.4)	85.2	β	6.17 br d (2.6)	77.7	β	4.32 d (8.1)	85.0
C4				129.7	C4		128.7	C4		128.7
C5			7.21 d (7.9)	130.6	C5	7.16 d (8.4)	126.0	C5	7.21 d (8.1)	129.6
C6			6.82 d (7.9)	116.0	C6	6.84 d (8.4)	116.0	C6	6.79 d (8.1)	116.1
C7				157.9	C7		158.7	C7		158.5
OMe			3.14 s	56.7						
CONH			7.71[b] br s	174.9	CONH	—	164.8	CONH	—	172.1
MeAla					MeAla			MeAla		
α			5.14 q (7.1)	53.3	α	3.92 q (6.8)	56.6	α	5.14 q (6.5)	53.9
β			1.41 d (7.1)	14.7	β	1.53 d (6.8)	18.6	β	1.42 d (6.5)	14.7
NMe			2.83 s	31.4	NMe	2.98 s	32.3	NMe	3.10 s	32.6
COOH				174.2	COOH		169.4	COOH		173.3

ovl: overlapped.

[a] Coupling constants are in parentheses and given in hertz. ^1H and ^{13}C assignments aided by COSY, TOCSY, HMQC and HMBC experiments.

[b] Data recorded in CD_3OH.

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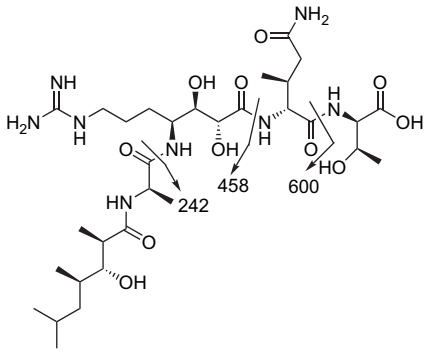


Figure 1. Callipeltin J (**2**) with ESI MS/MS fragmentations.

the presence of the signals of the spin system of 3-methylglutamine in the place of 3,4-dimethylglutamine in callipeltin C. The sequence of amino acid units in callipeltin K was deduced from inter-residue NOE interactions NH/CHα, acquired in CD_3OH, and HMBC correlations as shown in Figure 2. The absolute configurations of D-Ala, D-Arg, L-MeAla, L-Leu, L-MeGln, two D-*allo*Thr and (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic acid residues were determined by LC–MS analysis of the acid hydrolysate derivatised with Marfey's reagent and comparison with appropriate amino acid standards. To define the chirality of β-OMeTyr residue, a sample of **3** was ozonised and hydrolysed and then subjected to Marfey's analysis. Ion selective monitoring of L-FDAA-OMeAsp (*m*/*z* 416) showed a peak at t_R 17.3 min corresponding to (2*R*,3*S*)-β-OMeAsp, indicating a (2*R*,3*R*)-stereochemistry of β-OMeTyr residue.[20]

To determine the stereochemistry of the 3-glutamine residue in callipeltins J and K, both diastereoisomers of 3-methylglutamic acid were prepared through diastereoselective synthesis (Scheme 1).

The introduction of the β-methyl group in the above amino acid was achieved by diastereoselective methylation of a suitable L-aspartic acid derivative. Thus, the perbenzylated L-aspartate **6** was enolised with KHMDS and then alkylated with CH_3I affording an unseparable 3:2 mixture of *syn*/*anti* adducts. To assure the separation of the two diastereomers and the regioselective deprotection of the β-carboxyl group, the fully deprotected aspartate derivatives **8** were transformed into the cyclic anhydrides **9**. The alcoholysis of the latter with 2-propanol gave exclusively the diastereomeric mixture of α-ester β-acids **10a** and **10b** that were easily separated by HPLC. Each β-carboxylic acid was converted into the corresponding diazo ketone by way of the β-acid chloride (oxalyl chloride) and the obtained diazo ketones **11a** and **11b** were subjected to Wolff rearrangement followed by methanolysis to afford the protected 3-methylglutamic derivatives **12a** and **12b**. Concomitant removal of the protecting groups through acidic hydrolysis gave the required (2*S*,3*S*)- and (2*S*,3*R*)-3-methyl glutamic acids, **13a** and **13b**.[25] A small sample (100 μg) of the two synthetic 3-methyl glutamates was derivatised with both enantiomers of Marfey's reagent.[26]

The L- and D-FDAA derivatives **14a**–**14d** were analysed using ESI LC–MS in the positive ion mode. By monitoring for FDAA-β-MeGlu at *m*/*z* 414, they were detected as separate

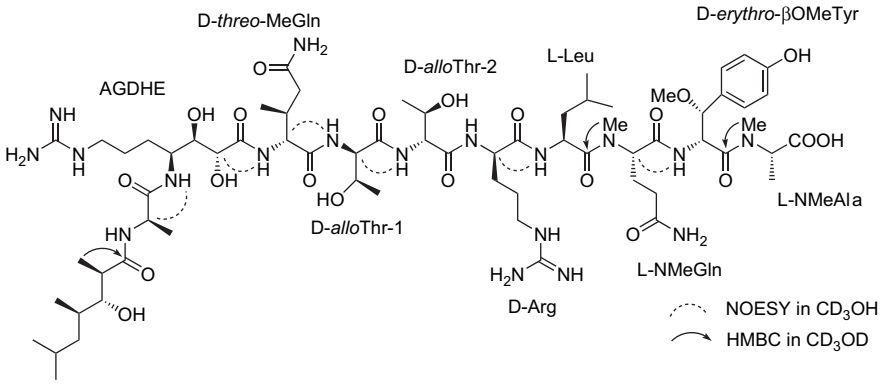
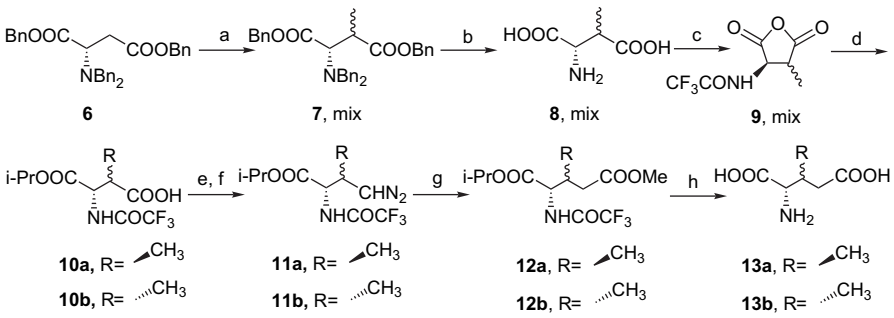


Figure 2. Callipeltin K (**3**) with NOESY and HMBC correlations.



Scheme 1. (a) CH_3I, KHDMS, THF, −78 °C, 89%; (b) H_2, $Pd(OH)_2$, EtOH, Parr apparatus, 80%; (c) TFAA, THF, 0 °C→rt; (d) ^{i}PrOH, rt, 82% over two steps; (e) $(ClCO)_2$, DMF, CH_2Cl_2, rt; (f) CH_2N_2, ether, 0 °C, 62% over two steps; (g) $PhCO_2Ag$, Et_3N, MeOH/THF, −15 °C→rt, 57%; (h) 6 N HCl, 160 °C.

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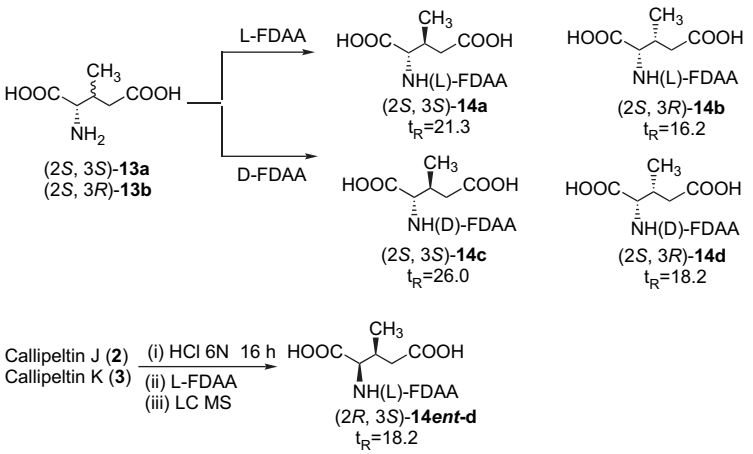

Scheme 2. HPLC retention times (t_R) of FDAA derivatives of synthetic (2*S*,3*S*)- and (2*S*,3*R*)-3-methyl-glutamic acid and configurational assignment of 3-MeGlu in callipeltins J and K.

peaks at 21.3, 16.2, 26.0, 18.2 min, respectively (Scheme 2). The L-FDAA derivative of β-MeGlu unit in callipeltins J and K was co-eluted with the D-FDAA derivative of (2*S*,3*R*)-3-methyl glutamic acid. Thus, the (2*R*,3*S*) configuration for the β-MeGln residue in **2** and **3** was unambiguously established (Scheme 2).

As concerning the absolute configuration of some non-proteinogenic units in callipeltins there are some apparent discrepancies that should be mentioned. The β-OMeTyr residue has the (2*R*,3*R*) configuration in callipeltins and in papuamide B[27] and the (2*S*,3*R*) configuration in neamphamide A,[27] despite the high degree of structural homology with callipeltin A. The 3,4-dimethylglutamine has a (2*S*,3*S*,4*R*) configuration in callipeltins and in papuamide, whereas a (2*R*,3*S*) configuration was found for the 3-methylglutamine residue in callipeltins J and K. The *anti*-dehydration of D-*allo*-Thr would afford the *E*-dAbu residue whereas in callipeltins H and I a *Z*-dAbu residue was found.[4] These findings confirm the important role of the epimerase domains in non-ribosomal peptide synthetases (NRPSs) that catalyse the conversion of a PCP bound, L/D-amino acid (in initiation modules) or L/D-peptidyl (in elongation modules) moiety by de- and re-protonating the Cα atom of the substrate.[28]

In the ^{1}H NMR spectrum of callipeltin L (**4**), the signal assigned to the β-methoxy group of the tyrosine residue (δ_H 3.15–3.20, 3H, s) was absent. The analysis of the 1D and 2D NMR data and comparison with those of callipeltin C disclosed the presence of the same residues of callipeltin C with a modification of the NMeGln-Tyr-NMeAla C-terminus moiety in callipeltin L, as evidenced by the strong perturbation of all the resonances of these residues (see Table 1). In particular, in the TOCSY spectrum an α-aminoacyl proton at δ 4.35 (δ_C 60.3) was found to be coupled with a broad doublet (J=2.6 Hz) carbinol proton at δ_H 6.17 (δ_C 77.7). The chemical shift of this proton and HMBC cross peak correlations with signals at δ 126.0 and 128.7 were consistent with the linkage of a *p*-hydroxyphenyl ring to the β-carbon of the spin system. In addition, the proton at δ_H 6.17 was found to correlate with the C1 carbon (δ_C 169.4) of the residue of NMeAla indicating that callipeltin L features an unusual ε-lactone system, likely arising from the nucleophilic displacement of the methoxy group at Cβ of the β-OMeTyr in callipeltin C by the carboxyl group of NMeAla C-terminus. As a consequence, the stereochemistry of the Cβ of tyrosine unit in callipeltin L is inverted with respect to **1** as suggested from the coupling constants' analysis (Table 1). Callipeltin L showed a high instability even in neutral conditions and at low temperature. In the ESIMS spectrum no peaks relative to the molecular ion were observed, whereas a peak at *m*/*z* 1245.7 corresponding to the loss of dipeptide azepan-2-one subunit was present. ESIMS/MS analysis of the peak at *m*/*z* 1245.7 revealed a fragmentation pattern compatible with the structure in Figure 3 and superimposable to those reported for callipeltin G.[4] The

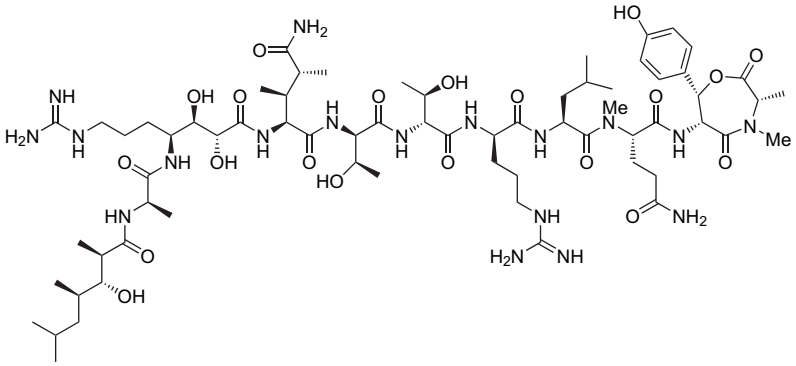

Figure 3. Callipeltin L (**4**).

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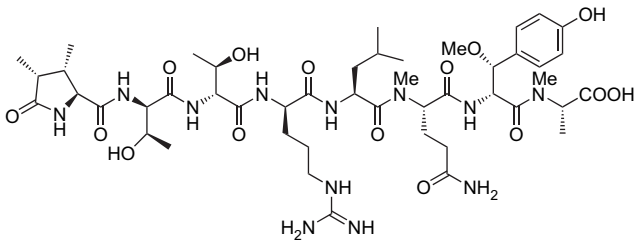
degradation of the natural metabolite hampered any stereochemical investigation. We, therefore, assumed that the configuration of all residues was the same as in callipeltin C.

Callipeltin M (**5**) is related to callipeltin B. In the ESIMS spectrum, the molecular ion peak at *m/z* 1049 is 18 mass units higher than in callipeltin B. The analysis of the 1D and 2D NMR spectra suggested callipeltin M to be the acyclic callipeltin B. In particular, the absence of a macrocyclic lactone closure was clearly indicated by the NMR resonances of the β centres in Thr residues (Table 1), whereas the cyclisation of 3,4-dimethylglutamine N-terminus as 3,4-dimethylpyroglutamic was evidenced mainly by the downfield shift of the Cα to 63.4 compared with the value of 57.0–57.7 of the same carbon in 3,4-dimethylglutamine residue in other callipeltin derivatives.



Callipeltin M (**5**)

The stereochemistry of the amino residues was determined to be D-*allo*-Thr, D-Arg, L-Leu, L-MeGln, L-MeAla, (2*S*,3*S*,4*R*) diMeGln, (2*R*,3*R*)-β-OMeTyr by Marfey's analysis.

Callipeltins K (**2**) and J (**3**) inhibit the growth of *Candida albicans* (ATCC 24433) in the standard disk assay with MIC values of ca. 10^{-4} M.

3. Experimental

3.1. General experimental procedures

Specific rotations were measured on a Perkin–Elmer 243 B polarimeter. High-resolution ESIMS spectra were performed with a Micromass QTOF Micromass spectrometer. ESIMS experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. NMR spectra were obtained on a Varian Mercury-400 and Inova-500 NMR spectrometers (^{1}H at 400 and 500 MHz, ^{13}C at 100 and 125 MHz, respectively) equipped with a Bruker X-32 hardware, δ (ppm), J in hertz, spectra referred to CD_2HOD as internal standards (δ_H=3.30). HPLC was performed using a Waters Model 6000 A pump equipped with U6K injector and a differential refractometer, model 401.

All reagents were commercially obtained (Aldrich, Fluka) at highest commercial quality and used without further purification except where noted. Dichloromethane, ether, tetrahydrofuran and triethylamine were distilled from calcium hydride immediately prior to use. All reactions were monitored by TLC on silica gel plates (Macherey, Nagel). Crude products were purified by column chromatography on silica gel 70–230 mesh. All reactions were carried out under argon atmosphere using flame-dried glassware.

3.2. Sponge material and separation of individual peptides

Latrunculia sp. (Class Demospongiae, Order Poecilosclerida, Family Latrunculidae) was collected at a depth of 15–20 m at Emae, Vanuatu South Pacific, in June 1996. The samples were frozen immediately after collection and lyophilised to yield 800 g of dry mass. Taxonomic identification was performed by Prof. John Hooper of Queensland Museum, Brisbane, Australia and reference specimens are on file (R1642) at the ORSTOM Centre of Noumea. Preliminary tests of bioactivity on polar extracts showed antifungal activity against *C. albicans* and cytotoxic activity against L16 cells (10 μg/mL, 100% inhibition).

The lyophilised material (800 g) was extracted with methanol (4×2.5 L) at room temperature and the crude methanolic extract (80 g) was subjected to a modified Kupchan's partitioning procedure as follows. The methanol extract was dissolved in a mixture of MeOH/H_2O containing 10% H_2O and partitioned against *n*-hexane. The water content (%, v/v) of the MeOH extract was adjusted to 20 and 40%, and partitioned against CCl_4 and $CHCl_3$, respectively. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH. The butanol-soluble material (ca. 4 g) was chromatographed by DCCC in five runs ($CHCl_3$/MeOH/H_2O, 7:13:8, ascending mode) and fractions of 4 mL were collected.

Fraction 10 was purified by HPLC on a Vydac C18 column (10μ, 250×10 mm, 4 mL/min) with 28% CH_3CN/H_2O (0.1% TFA) as eluent to give 16.8 mg of pure callipeltin K **3** (t_R=5.4 min). The additional peaks at t_R=2.4 and 3.1 min were further individually purified by HPLC on a Thermo-Hypurity column (5μ, 250×4.6 mm) eluting with 28% CH_3CN/H_2O containing 0.05% TFA (flow rate 1.2 mL/min) to give 6.9 mg of pure callipeltin M **5** (t_R=3.4 min) and 1.2 mg of pure callipeltin J **2** (t_R=5.4 min), respectively.

Fractions 11 and 12 were purified by HPLC on a Vydac C18 column (10μ, 250×10 mm, 4 mL/min) with 28% CH_3CN/H_2O (0.1% TFA) as eluent to give a peak at t_R=10.6 min containing mainly callipeltin L (**4**) that was further purified by HPLC on a Thermo-Hypurity column (5μ, 250×4.6 mm) eluting with 27% CH_3CN/H_2O containing 0.05% TFA (flow rate 1.2 mL/min) to give 5.0 mg of pure **4** (t_R=4.2 min).

3.3. Characteristic data for each compound

3.3.1. Callipeltin J (2). White amorphous solid; $[\alpha]_D^{25}$ −1.2 (*c* 0.09, methanol); IR (KBr) 3300, 1760, 1650 cm^{-1}; ^{1}H and ^{13}C NMR data in CD_3OD given in Table 1. ESIMS: *m/z* (%) 719.6 (25) $[M+H]^+$, 360.2 (100) $[M+2H]^{++}$; HRMS (ESI) calcd for $C_{31}H_{59}N_8O_{11}$: 719.4303; found: 719.4297 $[M+H]^+$.

3.3.2. Callipeltin K (3). White amorphous solid; $[\alpha]_D^{25}$ −7.0 (*c* 1.12, methanol); IR (KBr) 3340, 3080, 1760, 1625 cm^{-1};

^{1}H and ^{13}C NMR data in CD_3OD given in Table 1. ESIMS: *m/z* (%) 1509.7 (25) $[M+H]^+$, 754.5 (100) $[M+2H]^{++}$; HRMS (ESI) calcd for $C_{67}H_{117}N_{18}O_{21}$: 1509.8641; found: 1509.8635 $[M+H]^+$.

3.3.3. Callipeltin L (4). White amorphous solid; ^{1}H and ^{13}C NMR data in CD_3OD given in Table 1. ESIMS: *m/z* (%) 1245.7 (100) $[M+H]^+$.

3.3.4. Callipeltin M (5). White amorphous solid; $[\alpha]_D$ −1.9 (*c* 0.38, methanol); IR (KBr) 3340, 3080, 1760, 1625 cm^{-1}; ^{1}H and ^{13}C NMR data in CD_3OD given in Table 1. ESIMS: *m/z* (%) 1049.5 (30) $[M+H]^+$, 525 (100) $[M+2H]^{++}$; HRMS (ESI) calcd for $C_{47}H_{77}N_{12}O_{15}$: 1049.5631; found: 1049.5623 $[M+H]^+$.

3.4. Determination of absolute stereochemistry

3.4.1. Peptide hydrolysis. Peptide samples (200 μg) were dissolved in degassed 6 M HCl (0.5 mL) in an evacuated glass tube and heated at 160 °C for 16 h. The solvent was removed in vacuo and the resulting material was subjected to further derivatisation.

3.4.2. LC–MS analysis of Marfey's (FDAA) derivatives. A portion of the hydrolysate mixture (800 μg) or the amino acid standard (500 μg) was dissolved in 80 μL of a 2:3 solution of TEA/MeCN and treated with 75 μL of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) in 1:2 MeCN/acetone. The vials were heated at 70 °C for 1 h, and the contents were neutralised with 0.2 M HCl (50 μL) after cooling to room temperature. An aliquot of the L-FDAA derivative was dried under vacuum, diluted with MeCN/5% HCOOH in H_2O (1:1) and separated on a Vydac C18 (25×1.8 mm i.d.) column by means of a linear gradient from 10 to 50% aqueous acetonitrile containing 5% formic acid and 0.05% trifluoroacetic acid, over 45 min at 1 mL/min. The RP-HPLC system was connected to the electrospray ion source by inserting a splitter valve and the flow going into the mass spectrometer source was set at a value of 100 μL/min. Mass spectra were acquired in positive ion detection mode (*m/z* interval of 320–900) and the data were analysed using the suite of programs Xcalibur (ThermoQuest, San José, California); all masses were reported as average values. Capillary temperature was set at 280 °C, capillary voltage at 37 V, tube lens offset at 50 V and ion spray voltage at 5 V.

Retention times of authentic FDAA-amino acids (min): L-Thr (12.5), D-Thr (17.6), L-*a*Thr (13.1), D-*a*Thr (14.1), L-Ala (16.6), D-Ala (20.0), L-NMeAla (18.7), D-NMeAla (19.4), L-Arg (11.7), D-Arg (13.08), L-Leu (28.9), D-Leu (34.8).

To determine the absolute configuration of 3,4-diMeGln and 4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (AGDHE) an authentic sample of callipeltin C was used as standard. The hydrolysate of callipeltin C contained: (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (19.5), D-Arg (13.1), D-*a*Thr (14.0), (3*S*,4*R*)-3,4-diMe-L-Glu (17.7), L-NMeAla (18.5), D-Ala (20.1), L-Leu (28.7).

The hydrolysate of callipeltin J (**2**) contained: (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (19.5), D-Ala (20.5), D-*a*Thr (14.5), (2*R*,3*S*)-3-methyl-glutamic acid (18.2).

The hydrolysate of callipeltin K (**3**) contained: (2*R*,3*R*,4*S*)-4-amino-7-guanidino-2,3-dihydroxyheptanoic acid (19.5), D-Ala (20.5), D-*a*Thr (14.5), (2*R*,3*S*)-3-methyl-glutamic acid (18.2), D-Arg (13.0), L-Leu (29.3), L-NMeAla (18.5).

The hydrolysate of callipeltin M (**5**) contained: D-*a*Thr (14.5), (3*S*,4*R*)-3,4-diMe-L-Glu (17.7), D-Arg (13.0), L-Leu (29.3), L-NMeAla (18.5).

3.4.3. Determination of the absolute stereochemistry of β-OMeTyr residue in callipeltins K (3) and M (5). A stream of ozone in O_2 was bubbled through a cooled solution of callipeltin K (0.5 mg), callipeltin M (0.5 mg) or of all four diastereomers of β-OMeTyr (1 mg)[20] in MeOH (0.5 mL) at −78 °C for 1 h. Hydrogen peroxide (35%, 10 drops) was added to the reaction mixture and then allowed to stand at room temperature overnight. The solvent was removed under a stream of N_2 and the resulting β-methoxyaspartates were immediately subjected to Marfey's derivatisation. The ozonolysis products of callipeltins K and M were then dissolved in degassed 6 M HCl (0.5 mL) in an evacuated glass tube and heated at 160 °C for 16 h. The solvent was removed in vacuo and the resulting material was subjected to Marfey's derivatisation and LC–MS analysis.

Retention times of authentic L-FDAA-β-OMeAsp (min): (2*S*,3*S*)-β-OMeAsp (10.2), (2*S*,3*R*)-β-OMeAsp (16.1), (2*R*,3*R*)-β-OMeAsp (9.9), (2*R*,3*S*)-β-OMeAsp (17.3). The hydrolysate of ozonolysis products of **3** and **5** contained: (2*R*,3*S*)-β-OMeAsp (17.3).

3.5. Synthetic procedures for L-β-methyl-glutamic acids

3.5.1. L-*N*,*N*-Dibenzyl-β-methylaspartic acid dibenzyl ester 7. A solution of L-*N*,*N*-dibenzyl-aspartic acid dibenzyl ester **6** (2.0 g, 4.06 mmol) in dry THF (15 mL) was cooled to −78 °C under argon and stirred. Iodomethane (1.72 g, 12.1 mmol) was added followed by a slow addition of a solution of KHDMS (17.8 mL of a 0.5 M solution in toluene, 8.9 mmol). After the addition, the mixture was stirred at −78 °C for 30 min. A saturated solution of NH_4Cl (5 mL) was added and the mixture was warmed slowly to room temperature. The organic layer was separated and the aqueous layer was extracted several times with AcOEt. The collected organic layers were washed with saturated $NaHCO_3$ and brine. After evaporation of the solvent, silica gel chromatography (hexane/AcOEt, 95:5) gave **7** as an approximately 3:2 mixture of diastereoisomers (1.83 g, 89%). White amorphous solid; IR (KBr) 1790, 1310 cm^{-1}; HRMS (ESI) calcd for $C_{33}H_{34}NO_4$: 508.2488; found: 508.2466 $[M+H]^+$.

NMR data for major diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 1.21 (3H, d, *J*=7.1 Hz), 3.06 (1H, m), 3.37 (2H, d, *J*=13.5 Hz), 3.55 (2H, d, *J*=9.0 Hz), 4.00 (1H, d, *J*=11.7 Hz), 4.93 (2H, m), 7.2–7.4 (20H, m); ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 15.4, 39.6, 55.1, 66.4, 66.5, 128.5, 129.4, 129.5, 138.9, 139.3, 171.4, 171.7.

NMR data for minor diastereoisomer: ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 1.06 (3H, d, *J*=7.2 Hz), 3.24 (1H, m), 3.28 (2H,

d, J=13.5 Hz), 3.53 (2H, d, J=9.0 Hz), 3.66 (1H, d, J=11.2 Hz), 5.11 (2H, m), 7.2–7.4 (20H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.3, 35.4, 57.7, 66.4, 66.5, 127.4, 128.8, 129.2, 136.3, 137.6, 175.3, 176.8.

3.5.2. L-β-Methylaspartic acid 8. The mixture **7** (1. 0 g, 2.83 mmol) was dissolved in EtOH (20 mL) and the solution was poured in the pressure bottle of a Parr hydrogenation apparatus. $Pd(OH)_2$ Degussa E101 (100 mg) was added and the mixture was shaken under 6 atm of H_2 for 12 h. The bottle was degassed, the catalyst was filtered and washed several times with MeOH. The solvent was evaporated and the crude product was purified by RP-18 eluting with water to give **8** as an approximately 3:2 mixture of diastereoisomers (451 mg, 80%). White amorphous solid; HRMS (ESI) calcd for $C_5H_{10}NO_4$: 148.1367; found: 148.1355 $[M+H]^+$; IR (KBr) 3350, 1760 cm^{-1}.

NMR data for major diastereoisomer: ^{1}H NMR (400 MHz, D_2O) δ: 1.08 (3H, d, J=10.8 Hz), 2.94 (1H, m), 3.76 (1H, d, J=4.4 Hz); ^{13}C NMR (100 MHz, D_2O) δ: 14.2, 57.1, 65.1, 174.7.

NMR data for minor diastereoisomer: ^{1}H NMR (400 MHz, D_2O) δ: 1.01 (3H, d, J=7.6 Hz), 2.90–2.99 (1H, m), 3.88 (1H, d, J=3.3 Hz); ^{13}C NMR (100 MHz, D_2O) δ: 11.5, 51.8, 61.0, 172.7.

3.5.3. Isopropyl L-3-methyl-*N*-trifluoroacetylaspartates 10a and 10b. Trifluoroacetic anhydride (22.2 mg, 0.11 mmol) was added to a stirred suspension of 3-methyl-aspartic acids **8** (420 mg, 2.11 mmol) in dry THF (10 mL) at 0 °C over 10 min under nitrogen. The reaction mixture was then allowed to warm to room temperature and stirred until dissolution was complete (ca. 1.5 h). The mixture was evaporated under reduced pressure and the residue was dried for 3–4 h under high vacuum. Dry ^{i}PrOH (10 mL) was then added to the residue and the solution was left at room temperature overnight. ^{i}PrOH was removed under reduce pressure to give 389 mg (82%) of crude product. HPLC (Macherey, Nagel; SP 250/10 Nucleosil 50-7, 10μ, 250×10 mm, flow 5 mL/min) purification using hexane/AcOEt (8:2, with 0.05% TFA) as eluent afforded compounds **10a** (106.8 mg, t_R=10 min) and **10b** (188.7 mg, t_R=13 min).

3.5.3.1. Isopropyl (2*S*,3*R*)-3-methyl-*N*-trifluoroacetyl-aspartate 10a. White amorphous solid; HRMS (ESI) calcd for $C_{10}H_{15}F_3NO_5$: 286.0902; found: 286.0911 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.26 (3H, d, J=6.2 Hz), 1.28 (3H, d, J=6.2 Hz), 1.33 (3H, d, J=7.2 Hz), 3.43 (1H, dq, J=3.6, 7.2 Hz), 4.84 (1H, dd, J=3.6, 9.0 Hz), 5.08 (1H, m), 7.29 (1H, d, J=9.0 Hz), 7.69 (1H, br s); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.5, 21.7, 21.8, 41.9, 54.4, 71.4, 168.6, 178.2.

3.5.3.2. Isopropyl (2*S*,3*S*)-3-methyl-*N*-trifluoroacetyl-aspartate 10b. White amorphous solid; HRMS (ESI) calcd for $C_{10}H_{15}F_3NO_5$: 286.0902; found: 286.0905 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.26 (3H, d, J=6.3 Hz), 1.29 (3H, d, J=6.3 Hz), 1.33 (3H, d, J=7.4 Hz), 3.08 (1H, dq, J=4.2, 7.4 Hz), 4.81 (1H, dd, J=4.2, 8.3 Hz), 5.10 (1H, m), 7.33 (1H, d, J=8.3 Hz), 8.63 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.5, 21.7, 41.9, 54.5, 71.4, 168.6, 178.3.

3.5.4. Isopropyl (2*S*,3*R*)-5-diazo-4-oxo-*N*-trifluoroacetyl-isoleucinate 11a. Carboxylic acid **10a** (90 mg, 0.32 mmol) was dissolved in 5 mL of CH_2Cl_2 to which was added $(ClCO)_2$ (1.6 mL, 2 M in CH_2Cl_2) followed by the addition of 4 μL of DMF (0.05 mmol). After stirring for 15 min at room temperature the reaction mixture was concentrated in vacuum and the resulting residue was dissolved in ether and the resulting solution was added dropwise to an ethereal solution of CH_2N_2 (ca. 20 equiv) at 0 °C. The cloudy yellow mixture was stirred for 45 min and then concentrated under reduce pressure to give an oil, which was purified on silica gel (8% EtOAc/hexane) affording 58.4 mg of **11a** as yellow oil (62% yield).

HRMS (ESI) calcd for $C_{10}H_{15}F_3N_3O_3$: 282.1066; found: 286.1055 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.18 (6H, d, J=6.8 Hz), 1.20 (3H, d, J=7.4 Hz), 3.23 (1H, m), 4.61 (1H, dd, J=3.1, 7.5 Hz), 4.97 (1H, m), 5.29 (1H, br s), 7.71 (1H, d, J=7.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.7, 22.9, 23.1, 46.4, 54.8, 60.5, 69.1, 169.0, 179.0.

3.5.5. Isopropyl (2*S*,3*S*)-5-diazo-4-oxo-*N*-trifluoroacetyl-isoleucinate 11b. The title compound **11b** was prepared following the same procedure described for compound **11a**. HRMS (ESI) calcd for $C_{10}H_{15}F_3N_3O_3$: 282.1066; found: 286.1072 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.27 (6H, d, J=6.0 Hz), 1.34 (3H, d, J=7.3 Hz), 2.98 (1H, m), 4.58 (1H, dd, J=4.4, 7.4 Hz), 5.10 (1H, m), 5.38 (1H, s), 7.38 (1H, br s); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.5, 21.8, 21.9, 46.2, 54.9, 55.4, 70.7, 168.6, 171.3.

3.5.6. Isopropyl (2*S*,3*S*)-3-methyl-*N*-trifluoroacetyl-glutamate 12a. In a two-necked round-bottomed flask containing **11a** (55 mg, 0.2 mmol) were added MeOH (2.8 mL) and dry THF (2 mL). The solution was magnetically stirred under argon with exclusion of light and cooled at −15 °C. A solution of silver benzoate (4.58 mg, 0.02 mmol) in anhydrous triethylamine (83.6 μL, 0.6 mmol) was added and the reaction mixture was allowed to slowly warm up to room temperature and stirred for 3 h. The solvents were evaporated in vacuum and the residue was taken up in EtOAc. The organic solution was successively extracted with saturated aqueous solutions of $NaHCO_3$, NH_4Cl and NaCl, dried over $MgSO_4$, filtered and evaporated under vacuum. The crude product was purified by HPLC (Macherey, Nagel; ET 200/4 Nucleosil 100-5, 5μ, 250×4.6 mm) with hexane/AcOEt (75:25, with 0.05% TFA) to give pure **12a** (t_R= 10 min, 35.7 mg, 57%). White amorphous solid; HRMS (ESI) calcd for $C_{12}H_{19}F_3NO_5$: 314.1215; found: 314.1225 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.04 (3H, d, J=7.0 Hz), 1.29 (6H, d, J=6.3 Hz), 2.33 (1H, dd, J=6.8, 16.0 Hz), 2.47 (1H, dd, J=5.2, 16.0 Hz), 2.62 (1H, m), 3.70 (3H, s), 4.55 (1H, dd, J=5.2, 8.2 Hz), 5.09 (1H, m), 7.55 (1H, d, J=8.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 16.6, 21.9, 32.8, 37.5, 52.2, 57.1, 70.6, 94.6, 172.8, 175.7.

3.5.7. Isopropyl (2*S*,3*R*)-3-methyl-*N*-trifluoroacetyl-glutamate 12b. The title compound **12b** was prepared following the same procedure described for compound **11b**. White amorphous solid; HRMS (ESI) calcd for

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$C_{12}H_{19}F_3NO_5$: 314.1215; found: 314.1207 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ 1.07 (3H, d, J=7.0 Hz), 1.29 (6H, d, J=6.3 Hz), 2.1 (1H, dd, J=6.7, 16.5 Hz), 2.5 (1H, dd, J=8.2, 16.5 Hz), 2.84 (1H, m), 3.71 (3H, s), 4.22 (1H, d, J=7.5 Hz), 5.11 (1H, sep, J=6.3 Hz), 5.9 (1H, br s); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.9, 22.0, 22.1, 29.9, 32.9, 37.9, 60.2, 69.5, 170.5, 177.4.

3.5.8. (2*S*,3*S*)-3-Methyl-glutamic acid 13a. Protected glutamic acid **12a** (4 mg) was dissolved in degassed 6 M HCl (0.5 mL) in an evacuated glass tube and heated at 160 °C for 16 h. The solvent was removed under vacuum and an aliquot (100 μg) was subjected to Marfey's derivatisation[26] with L-FDAA and D-FDAA and LC–MS analysis.

White amorphous solid; HRMS (ESI) calcd for $C_6H_{12}NO_4$: 162.0766; found: 162.0755 $[M+H]^+$; ^1H NMR (400 MHz, D_2O) δ: 0.97 (3H, d, J=6.6 Hz), 2.38 (1H, dd, J=7.7, 15.6 Hz), 2.46 (1H, m), 2.58 (1H, dd, J=4.5, 15.6 Hz), 3.78 (1H, d, J=3.9 Hz); ^{13}C NMR (100 MHz, D_2O) δ: 14.4, 31.1, 37.6, 58.1, 163.5, 178.2.

3.5.9. (2*S*,3*R*)-3-Methyl-glutamic acid 13b. Protected glutamic acid **12b** (4 mg) was dissolved in degassed 6 M HCl (0.5 mL) in an evacuated glass tube and heated at 160 °C for 16 h. The solvent was removed under vacuum and the resulting material was subjected to Marfey's derivatisation[26] with L-FDAA and D-FDAA and LC–MS analysis. HRMS (ESI) calcd for $C_6H_{12}NO_4$: 162.0766; found: 162.0772 $[M+H]^+$; ^1H NMR (400 MHz, D_2O) δ: 1.01 (3H, d, J=6.6 Hz), 2.40 (1H, dd, J=7.0, 16.3 Hz), 2.57 (1H, dd, J=6.6, 16.3 Hz), 2.63 (1H, m), 4.03 (1H, d, J=3.9 Hz); ^{13}C NMR (100 MHz, D_2O) δ: 15.0, 31.0, 37.8, 58.2, 169.3, 176.4.

Retention times of L-FDAA-methyl-glutamic acid (min): (2*S*,3*S*)-3-methyl-glutamic acid (21.3), (2*S*,3*R*)-3-methyl-glutammic acid (16.2).

Retention times of D-FDAA-methyl-glutamic acid (min): (2*S*,3*S*)-3-methyl-glutamic acid (26.0), (2*S*,3*R*)-3-methyl-glutamic acid (18.2).

3.6. Antifungal tests

The broth macrodilution test was performed by using the NCCLS standard reference method for broth dilution antifungal susceptibility testing of yeasts.[29] Stock solutions of callipeltins F–J (10^{-3} M) were prepared, divided into aliquots and stored at −80 °C. A new aliquot was thawed on each day of use. Before testing, *C. albicans* (ATCC 24433) were maintained on Sabouraud's agar slants and periodically transferred to Sabouraud's agar plates and incubated for 48 h at 28 °C. To prepare stationary growth phase yeast, a colony was taken from the agar plate and transferred into 30 mL Sabouraud-dextrose broth (DIFCO laboratories, Detroit, MI) and incubated for 72 h at 35 °C. Cells were centrifuged at 1.000×g for 10 min and the pellet was washed twice with distilled water. Cells were counted and suspended in RPMI 1640 plus 0.165 M MOPS buffer at a density of 5000 CFU/mL. Yeast suspension (100 μL) was transferred into control wells or wells containing the callipeltins 10^{-4}–10^{-8} M final concentrations. The plates were incubated in air at 35 °C without agitation for 48 h. The experiments were run in triplicate. Negative growth corresponded to no visible growth in the well.

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