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Callyaerins A–F and H, new cytotoxic cyclic peptides from the Indonesian marine sponge *Callyspongia aerizusa*

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ABSTRACT

Bioassay guided fractionation of the EtOAc fraction of the sponge *Callyspongia aerizusa* yielded seven new cytotoxic cyclic peptides callyaerins A–F (**1**–**6**) and H (**8**). Their structures were determined using extensive 1D (^{1}H, ^{13}C and DEPT) and 2D (COSY, HMQC, HMBC, TOCSY, and ROESY) NMR and mass spectral (ESI and HRESI-TOF) data. All compounds were cyclic peptides containing ring systems of 5–9 amino acids and side chains of 2–5 amino acids in length. An unusual (*Z*)-2,3-diaminoacrylic acid unit provided the template for ring closure and afforded the linkage to the peptidic side chain which was always initiated with a proline moiety. All peptides contained three or more proline residues and the remaining residues were predominantly hydrophobic residues with all amino acids present in the L form. Callyaerins A–F (**1**–**6**) and H (**8**) showed biological activity in antibacterial assays and in various cytotoxicity assays employing different tumour cell-lines (L5178Y, HeLa, and PC12). Callyaerins E (**5**) and H (**8**) exhibited strong activity against the L5178Y cell line with ED_{50} values of 0.39 and 0.48 μM, respectively. On the other hand, callyaerin A (**1**) showed strong inhibitory properties towards *C. albicans*.

1. Introduction

Marine sponges are a rich source of bioactive cyclic peptides and depsipeptides with unique structures involving a wide variety of unusual amino acids and other building blocks.[1–4] Structure elucidation of such compounds can be quite challenging insofar as they defy traditional amino acid sequencing strategies. Many cyclic peptides isolated from marine sponges exhibit interesting biological properties including a reverse of multi-drug resistance in tumour cells,[5] cytotoxicity,[6] HIV inhibition,[7,8] and nematocidal activity.[9] The genus *Callyspongia* includes 182 species, of which around 15 species only have been investigated chemically. Sponges belonging to the genus have been known to yield biologically active polyacetylenic[10] and nitrotetradecenyl pyridine[11] compounds with different chain lengths and unsaturation positions. Nematocidal depsipeptides, phoriospongin A and B were isolated from the Australian marine sponge *Callyspongia bilamellata*.[9] To date only our previous work describing a preliminary investigation of the metabolic diversity of the Indonesian sponge *Callyspongia aerizusa* has provided an insight into the chemical potential of this sponge species. We were able to isolate a new cytotoxic cyclic peptide callyaerin G (**7**)[12] and found indications of a considerable number of other cyclic peptides present in the extract. These peptides were related analogues of callynormine A, a cyclic peptide isolated from the southern Kenyan sponge *Callyspongia abnormis*.[13] As a continuation of this work we have now completed a detailed investigation of the bioactive ethyl acetate fraction of this sponge. In addition to the callyaerin G (**7**) previously described by us,[12] here we describe the isolation, structure elucidation, and biological activities of a further seven callyaerin congeners of variable ring size and side-chain length. Most of the isolated compounds displayed strong activity against the tumour cell line L5178Y, and varying degrees of antibacterial and antifungal properties.

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2. Results and discussion

The EtOAc soluble extract of the sponge *C. aerizusa* was fractionated using silica gel column chromatography and the resulting fractions were subjected to MTT assays toward the tumour cell lines L5178Y, HeLa, and PC12. The active fractions were subjected to semi-preparative HPLC to afford fractions containing seven novel cyclic peptides that were sufficiently pure to allow structural studies. In all cases we have used a combination of homo- and heteronuclear NMR techniques, in combination with ESI-MS and amino acid analysis, to provide unambiguous structures of the various compounds. Initially we chose callyaerin E (**5**, Fig. 2) as a reference compound, since it was available in sufficient quantity to allow the acquisition of a complete set of 1D and 2D NMR spectra. These provided exemplary data from which we were able to develop a strategy to elucidate the structures of the remaining analogues some of which were isolated in low yields precluding the recording of a full set of spectra.

Table 1
Complete ^{1}H and ^{13}C NMR data of callyaerin E (**5**) (DMSO-d_6, 600 MHz)

A. A.	Position	δ_C	δ_H (J in Hz)	ROESY[a]	HMBC[a]
DAA	N*H*		8.36 s	1-α, 1-N*H*, 7-N*H*, 7-α, 7-γ, C1-α, C1-δ	7-α, 7-CO, DAA–CO
	CO	167.5 s			
	α	98.8 s			
	β	143.3 d	7.28 d (13.6)		1-α, DAA-α, DAA–CO
R1 Leu	N*H*		5.27 dd (13.3, 10.0)	DAA–N*H*	1-CO, DAA-α
	CO	170.8			
	α	57.9 d	4.36 m	2-α, 2-β, 2-δ, DAA–N*H*	1-CO, 8-α
	β	41.8 t	1.77 m, 1.23 m		1-CO
	γ	26.5 d	1.60 m		1-α
	δ, δ′	22.5 q, 23.2 q	0.96 d (6.0), 0.94 d (6.6)		1-β
R2 Pro	CO	171.9 s			
	α	63.9 d	4.01 dd (9.9, 5.5)	1-α, 3-α, 3-N*H*	1-CO, 2-CO, 2-γ
	β	29.1 t	2.16 m, 1.67 m	1-α, 3-N*H*	
	γ	24.9 t	2.06 m, 1.88 m	3-N*H*	
	δ	47.8 d	3.93 br t (8.8) 3.59 m		
R3 Phe	N*H*		7.73 d (6.7)	2-α, 2-β, 2-γ, 4-N*H*, 4-α	2-CO, 2-α, 3-α, 3-β, 3-CO
	CO	168.9 s			
	α	54.1 d	4.39 m	4-α, 4-N*H*	
	β	35.1 t	3.00 m		3-α, 3-CO, 1″, 2″
	Aromatic	126.3, 128.5, 138.3	7.17–7.32 m		
R4 Phe	N*H*		7.31 d (6.9)	3-N*H*	3-CO, 4-β, 4-CO
	CO	171.2 s			
	α	53.1 d	4.63 ddd (8.8, 6.9, 4.7)	3-α, 3-β, 5-δ	3-CO, 4-β, 4-CO
	β	36.1 t	3.00 m, 2.71 dd (13.3, 4.7)	5-α, 6-γ	5-α, 5-CO, 1′, 2′, 6′
	Aromatic	C1: 137.3 s, C2,6: 129.5 d, C3,5: 129.1 d, C4: 126.5 d 1	7.17–7.30 m		
R5 Pro	CO	171.3 s			
	α	62.8 d	4.41 m	6-α	4-CO
	β	28.4 t	2.15 m, 1.82 m		
	γ	24.5 t	1.93 m, 1.89 m	4-α	
	δ	47.2 d	3.49 br t (8.8), 3.42 m	3-α	4-CO
R6 Pro	CO	171.5 s			
	α	64.0 d	4.16 dd (7.9, 9.8)	5-α, 7-α, 7-N*H*	5-CO, 6-CO
	β	29.6 t	2.22 m, 1.41 m		
	γ	25.8 t	1.74 m, 1.59 m		
	δ	49.5 d	3.34 m, 2.48 m		5-CO
R7 Val	N*H*		7.03 d (10.1)	DAA–N*H*	6-α, 7-α, 7-β, 7-CO
	CO	171.2 s			
	α	56.5 d	4.56 dd (10.1, 3.9)	6-α, 6-β, 7-γ, DAA–N*H*	7-β, 7-γ, 7-CO
	β	28.8 d	2.33 m		
	γ	18.4 q, 18.3 q	1.06 d (6.9), 0.97 d (6.8)		
C1 Pro	CO	172.4 s			
	α	59.5 d	4.36 m	2-α, 2-N*H*	2-α
	β	26.6 t	2.22 m, 1.57 m	2-α, 2-N*H*	
	γ	23.8 t	1.78 m, 1.68 m		
	δ	45.8 d	3.57 m, 3.21 m	2-α, 2-N*H*, DAA–N*H*	1-β, 1-CO, 1-γ
C2 Ile	N*H*		7.62 d (7.9)	1-α, 1-β, 1-δ, 3-α, 3-N*H*	1-α, 1-CO, 2-α, 2-CO
	CO	171.1 s			
	α	58.3 d	3.99 dd (7.9, 7.3)	1-α, 1-β, 1-δ, 3-α, 3-N*H*	2-β, 2-CO, 2-γ
	β	35.3 d	1.95 m		
	γ	24.6 t	1.45 m, 1.21 m		
	γδ	15.9 q, 11.4 q	0.88 d (6.9), 0.85 t (6.9)		
C3 Ile	N*H*		7.37 d (8.0)	2-N*H*, 2-α, 2-δ, 2-γ, 4-N*H*	2-α, 3-α, 3-CO
	CO	170.8 s			
	α	57.8 d	4.08 t (7.6)	2-α, 2-NH, 2-β, 2-γ, 4-N*H*	3-β, 3-δ, 3-γ, 3-CO
	β	34.2 d	1.82 m	4-N*H*	
	γ	25.1 t	1.39 m, 1.27 m		
	γδ	15.0 q, 10.8 q	0.87 d (6.9), 0.80 t (6.7),		
C4 Gly	N*H*		7.88 t (6.0)	Terminal-NH_2, 3-N*H*, 3-α, 3-β	3-α, 3-CO, 4-CO, 4-α
	CO	170.9 s			
	α	42.3 t	3.72 dd (16.7, 6.2)	3-α, 3-δ	4-CO
			3.57 dd (16.7, 5.9)		
	NH_2		7.04 br s, 6.97 br s	4-N*H*, 4-α	4-CO, 4-α

[a] The numbering refers to residues in the ring for R-residues and in the chain for C-residues.

		Ring	Sidechain
		R1 R2 R3 R4 R5 R6 R7 R8	C1 C2 C3 C4 C5
1	A	Ile Hyp Val Ile Leu Pro Pro Leu	Pro Ile Phe Gly
2	B	Ile Hyp Ile Ile Leu Pro Pro Leu	Pro Ile Ile
3	C	His Hyp Leu Leu Pro Pro Val	Pro Leu Phe Gly
4	D	Ile Ile Phe Pro Hyp Pro Leu	Pro Ile Asn Ala Ile
5	E	Leu Pro Phe Phe Pro Pro Val	Pro Ile Ile Gly
6	F	Val Pro Val Phe Pro	Pro Leu Phe Ile
7	G	Leu Pro Pro Pro Pro Leu	Pro Phe Phe Phe
8	H	Val Pro Val Phe Pro Pro Leu	Pro Ile

Footnote: Hyp is γ-hydroxyproline.

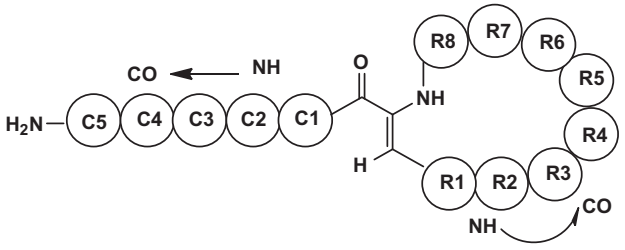

Figure 1. Structure of all the compounds isolated from *Callyspongia aerizusa*. The variable size of the ring and side-chain residues of the cyclic peptides necessitated the use of the nomenclature shown where R refers to ring residues and C to side-chain residues. The structure elucidation of callyaerin G has been reported by us elsewhere.[12]

Callyaerin E (**5**) was obtained as a white amorphous powder. Positive mode ESIMS showed pseudo-molecular ion peaks at *m*/*z* 1262 $[M+H]^+$ and 1284 $[M+Na]^+$, which were consistent with the molecular formula $C_{66}H_{95}N_{13}O_{12}$ as confirmed by HRESI-TOFMS. Extensive analysis of 2D NMR data (TOCSY and COSY) disclosed the presence of eleven spin systems which could be assigned to eleven amino acid residues. Seven characteristic spin systems in the TOCSY spectrum were detected to low field (7.9–5.2 ppm) and originated from the amidic protons of one Gly, one Leu, two Ile, two Phe and one Val. [See Figs. S1–S3 of Supplementary data.] Close inspection of the high field region indicated the presence of four Pro units. In addition, the signals at δ_H 8.36 (s, NH) and 7.28 (d, J = 13.6 Hz, β-H) in association with their HMBC correlations to δ_C 167.7 (s), 98.8 (s), and 143.3 (d) indicated the presence of an unusual (*Z*)-2,3-diaminoacrylic acid (DAA) moiety.[12,13] Two singlet signals at δ_H 7.04 (br s) and 6.97 (br s) were assigned to a terminal NH_2 group of the peptidic side chain, as there were no Gln or Asn units present.

In the ^{13}C spectrum the presence of 12 carbonyls at δ_C 167.7–172.7 ppm and 11 α-carbons of amino acids in the region δ_C 42.3–58.3 further confirmed a compound featuring 11 amino acids along with a DAA unit. Thus, 25 of the 26 double bond equivalents required by the molecular formula were accounted for by the carbonyl groups, proline rings, DAA, and aromatic rings of Phe, indicating **5** was a cyclic peptide. The sequence of amino acids and the location of DAA were established by a detailed examination of the ROESY and HMBC spectra (Fig. 3). In the ROESY spectrum diagnostic NOE correlations (Table 1) were observed between H_α and NH of adjacent residues for residues R2–R4, R6–DAA and C1–C4, and between NH and NH of adjacent residues for residues R7–DAA–R1, R3–R4, and C2–C4. The positions of the Pro residues R2 and R5 were apparent from the correlations of H_α of the preceding residue with H_δ's of these Pro units. The position of both the closure of the ring system and the attachment of the side chain followed from the correlations of the NH of DAA (Table 1) to both H_α and NH of R1 and to H_α and H_δ of C1, respectively. The location of the side chain terminal NH_2 was determined from the correlations of NH_2 to NH and H_α of C4. Finally the sets of NOE correlations between signals of R1 and C1 with those of DAA defined the (*Z*)-configuration of the DAA unit (Fig. 1) which was the same as that found in the X-ray structure of callynormine A.[13]

The sequence established from the through-space homonuclear NMR data was confirmed from the through-bond heteronuclear correlations observed in the HMBC spectra (Table 1). In particular the two substructures Leu-Pro-Phe-Phe-Pro-Pro-Val (R1–R7) and Pro-Ile-Ile-Gly (C1–C4) were corroborated by HMBC correlations of NH and H_α of each amino acid to the amide carbonyl carbons through $^2J_{CH}$ and $^3J_{CH}$. In the case of the ring Pro-units the respective H_α always correlated with the carbonyl carbon of the preceding unit and in most cases a correlation with the same carbonyl carbon was observed for H_δ (Table 1). The position of the DAA unit was confirmed from HMBC correlations of β-H (DAA) to C_α (R1) and in reverse NH (R1) to C_α (DAA). A complete assignment of the ^1H and ^{13}C NMR data is given in Table 1. Initially it was particularly difficult to distinguish Leu and Ile as these occurred in considerable numbers in all compounds. However a careful inspection of the multiplicity of H_α in the well resolved 1D ^1H spectra and the characteristic ^{13}C chemical shifts of the γ- and δ-methyl groups afforded unambiguous assignments.

The base peak at *m*/*z* 865 $[M-(Pro+2Ile+Gly+NH_2)]^+$ for compound **5**, arises from cleavage of the side chain. As is usual with this type of molecule the fragmentation patterns in the ESI-MS and HRESI-TOFMS spectra are of limited use in providing sequence information as internal fragmentation of the ring system is difficult

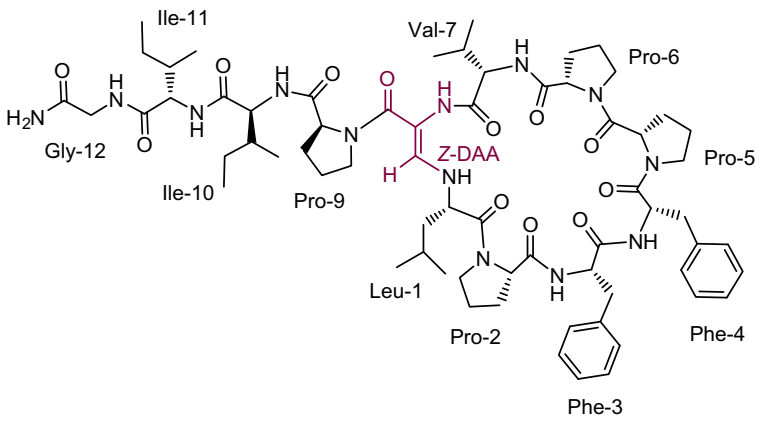

Figure 2. Structure of callyaerin E (**5**).

Table 2
^{1}H and ^{13}C NMR data of Callyaerin A (**1**) (DMSO-d_6, 600 MHz).

A. A.	Position	δ_C	δ_H (J in Hz)
DAA	N*H*		8.29 s
	CO	167.7 s	
	α	98.2 s	
	β	143.4 d	7.35 d (13.2)
R1 Ile	N*H*		5.87 dd (13.1, 10.2)
	CO	172.0 s	
	α	64.4 d	4.08 m
	β	37.8 d	1.41 m
	γ	24.0 t	1.41 m, 0.79 m
	γ′δ	14.2 q, 10.5 q	0.79 m, 0.42 t (7.3)
R2 Hyp	CO	173.3 s	
	α	55.6 d	4.26 m
	β	37.7 t	2.08 m, 1.89 m
	γ	68.7 d	4.41 m
	δ	56.7 t	3.77 br d, 3.70 dd (11.4, 3.7)
	OH		5.36 d (3.1)
R3 Val	N*H*		9.03 br s
	CO	172.0 s	
	α	66.1 d	2.98 dd (10.7, 7.1)
	β	27.1 t	2.65 m
	γ	19.2 q, 19.5 q	0.83 d (6.6), 0.82 d (7.0)
R4 Ile	N*H*		8.81 br s
	CO	171.2 s	
	α	59.3 d[a]	3.79 m
	β	36.1 d	1.49 m
	γ	[d]	1.40 m, 1.13 m
	γ′δ	15.2 q, 10.2 q	0.81 d (6.9), 0.78 t (7.4)
R5 Leu	N*H*		7.44 d (7.0)
	CO	172.3 s	
	α	49.1 d[b]	4.61 m
	β	41.1 t	1.77 m, 1.25 m
	γ	[d]	1.62 m
	δ, δ′	21.0 q[c], 21.0 q[c]	0.87 d (6.5), 0.86 d (6.6)
R6 Pro	CO	171.4 s	
	α	64.0 d	4.05 m
	β	26.2 t	2.30 m, 1.91 m
	γ	[e]	1.99 m, 1.91 m
	δ	46.1 t	3.61 m, 3.47 m
R7 Pro	CO	171.4 s	
	α	62.5 d	4.27 m
	β	28.5 t	2.25 m, 1.61 m
	γ	[e]	1.94 m, 1.86 m
	δ	47.0 t	3.59 m, 3.31 m
R8 Leu	N*H*		6.72 d (10.0)
	CO	172.4 s	
	α	50.0 d[b]	4.61 m
	β	40.5 t	1.89 m, 1.57 m
	γ	[d]	1.65 m
	δ, δ′	22.8 q[c], 23.2 q[c]	0.96 d (6.6), 0.85 d (6.6)
C1 Pro	CO	172.7 s	
	α	61.9 d	4.25 m
	β	29.4 t	2.26 m, 1.50 m
	γ	[e]	1.83 m, 1.72 m
	δ	48.7 t	3.51 m, 3.21 m
C2 Ile	N*H*		7.47 m
	CO	171.1 s	
	α	58.6 d[a]	3.79 m
	β	34.9 d	1.67 m
	γ	[d]	1.17 m, 1.05 m
	γ′δ	14.9 q, 11.0 q	0.39 d (6.8), 0.70 t (7.3)
C3 Phe	N*H*		7.27 d (8.6)
	CO	171.5 s	
	α	59.3 d	4.27 m
	β	39.9 t	3.09 dd (13.9, 2.9), 2.63 dd (14.0, 12.1)
	Others	C1: 138.0 s, C2,6: 129.0 d, C3,5: 127.7 d, C4: 126.1 d	7.19–7.11 m
C4 Gly	N*H*		8.15 dd (6.8, 5.5)
	CO	170.8 s	
	α	42.0 t	3.91 dd (16.5, 7.0), 3.48 dd (16.5, 5.3)
	NH_2		7.24 br s, 6.97 br s

[a,b,c] Interchangeable.
[d] Overlapping signals at 24.6, 24.7, 24.8, 24.9.
[e] Overlapping signals at 25.0, 25.5, 25.8 ppm.

to interpret unambiguously a priori. With the planar structure in hand it was possible to match the observed fragmentation patterns with the sequence of amino acids deduced by NMR spectroscopy, as exemplified by the MS spectra of callyaerin B (**2**), (see Fig. S9, Supplementary data). No attempt was made to analyse such data for the other compounds.

The stereochemistry of the various amino acid residues was determined following acid hydrolysis of the parent peptide and subsequent derivatization according to the advanced Marfey's method.[14] Comparison of the resulting (*N*-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide) amino acid derivatives with those of appropriate standard amino acids using LC–MS indicated all amino acids were of the L-form.

An identical approach was used to establish the structures of callyaerins A (**1**), B (**2**), C (**3**) and H (**8**). The fully assigned ^{1}H and ^{13}C NMR data are given in Tables 2–4 and 6 as well as a representative schematic of the sequence correlations in the ROESY and HMBC spectra of callyaerin H (**8**) is shown in Figure 3. For the remaining two compounds, callyaerins D (**4**) and F (**6**), there was insufficient material available to record heteronuclear NMR spectra. However, in both cases there were sufficient sequence correlations in the ROESY spectra to afford a complete assignment of the ^{1}H NMR data (Table 5), that unambiguously establish their structures as shown, for example, by the correlations of Figure 4 for callyaerin F (**6**). In all cases, the linear peptide chains of the callyaerins originated with a proline attached to DAA via an amidic bond and consequently showed a base peak in the ESI-MS through cleavage of this bond to form a stable fragment of the cyclic peptide moiety. This furnished immediate confirmatory evidence of the number and nature of the amino acid residues in the side chain. In all cases only L-amino acids were detected after acidic hydrolysis and derivatization (see Table S2, Supplementary data).

The peptides are unusual in containing a considerable number of proline (and in four cases γ-hydroxyproline) residues, of which one proline is always positioned at the beginning of the side chain, while all others are found in the ring system. The remaining residues are predominantly hydrophobic amino acids such as, Ile, Leu, and Phe. The basic structural unit of the callyaerins comprises a cyclic peptide with a linear peptide side chain, both of variable size, linked through a non-proteinogenic (*Z*)-2,3-diaminoacrylic acid (DAA) functional group. Apart from our previous report of callyaerin G (**7**)[12] there is only one other example of a compound containing the DAA group from marine sources, namely callynormine A isolated from the marine sponge *C. abnormis*.[13] It was speculated that DAA biogenetically arises from oxidation of a serine or cysteine unit to give a formyl glycine which will undergo Schiff base formation with the amino group of the next amino acid in the ring, followed by double bond migration.[13] Thus such an arrangement of a peptide fused with the DAA group affords a novel class of marine natural products that are currently restricted to the sponge genus *Callyspongia*. Interestingly, the DAA function is encountered in the structures of antibiotics from actinobacteria, including capreomycins and viomycins from *Streptomyces* spp. as well as tuberactinomycins from *Nocardia* spp. which are used to treat tuberculosis.[16] However, since these structures are otherwise only distantly

Table 3
^{1}H and ^{13}C NMR data of callyaerin B (2) (DMSO-d_6, 600 MHz)

A. A.	Position	δ_C	δ_H (J in Hz)
DAA	N*H*		8.28 br s
	CO	167.2 s	
	α	97.9 s	
	β	142.6 d	7.04 d (13.6)
R1 Ile	N*H*		5.70 dd (13.6, 10.8)
	CO	172.1 s	
	α	64.6 d	3.94 t (9.8)
	β	37.9 d	1.45 m
	γ	29.1 t	1.40 m, 1.00 m
	γ′δ	[b]	0.85[a], 0.76 t (7.5)
R2 Hyp	CO	173.4 s	
	α	59.2 d	4.24 m
	β	37.5 t	2.10 m,1.89 m
	γ	68.7 d	4.40 br s
	δ	56.7 t	3.67 m, 3.80 m
	OH		5.40 d (2.4)
R3 Ile	N*H*		9.00 br s
	CO	172.1 s	
	α	64.2 d	3.07 m
	β	32.4 d	2.55 m
	γ	24.5 t	1.40 m, 1.10 m
	γ′δ	[b]	0.82[a] t, 0.78[a]
R4 Ile	N*H*		8.86 br s
	CO	171.3 s	
	α	57.8 d	3.81 (8.8)
	β	36.2 d	1.55 m
	γ	25.5 t	1.45 m, 1.15 m
	γ′δ	[b]	0.84[a], 0.81[a] t
R5 Leu	N*H*		7.37 d (6.6)
	CO	171.4 s	
	α	49.1 d	4.62 q (~6.7)
	β	41.1 t	1.76 m, 1.30 m
	γ	24.7 d	1.60 m
	δ, δ′	[b]	0.88[a]
R6 Pro	CO	172.3 s	
	α	63.9 d	4.03 t (8.2)
	β	24.9 t	2.30 m, 1.90 m
	γ	29.1 t	2.00 m, 1.90 m
	δ	46.1 t	3.60 m, 3.48 m
R7 Pro	CO	171.4 s	
	α	61.3 d	4.25 m
	β	25.7 t	2.20 m, 1.82 m
	γ	29.7 t	1.90 m, 1.50 m
	δ	47.0 t	3.55 m, 3.35 m
R8 Leu	N*H*		6.63 (10.0)
	CO	172.7 s	
	α	50.0 d	4.55 ddd (10.7, 10.4, 3.7)
	β	40.5 t	1.70 (m), 1.60 (m)
	γ	24.6 d	1.47(m)
	δ, δ′	[b]	0.88[a], 0.81[a]
C1 Pro	CO	171.2 s	
	α	62.5 d	4.25 m
	β	26.2 t	2.55 m, 1.80 m
	γ	29.7 t	1.70 m, 1.45 m
	δ	48.6 t	3.52 m, 3.15 m
C2 Ile	N*H*		7.58 d (10.4)
	CO	172.1 s	
	α	57.8 d	4.10 dd (9.4, 9.2)
	β	36.4 d	1.82 m
	γ	25.0 t	1.45 m, 1.15 m
	γ′δ	[b]	0.86[a], 0.82[a] t
C3 Ile	N*H*		7.23 d (9.5)
	CO	172.7 s	
	α	57.4 d	4.15 dd (9.5, 5.0)
	β	36.2 d	1.90 m
	γ	23.5 t	1.25 m, 1.00 m
	γ′δ	[b]	0.78[a], 0.71 t (7.5)
	NH_2		7.17 s, 6.97 s

[a] Signal overlap prevents determination of couplings.
[b] Specific assignments of the methyl signals are not possible. However the relative assignments are 23.1, 22.7, 22.1, 20.8 (δ-Leu), 15.7 (×2), 15.3, 15.2, 14.4 (γ-Ile), and 11.4, 11.0, 10.7, 10.3, 9.8 ppm (δ-Ile).

Table 4
^{1}H and ^{13}C NMR data of callyaerin C (3) (DMSO-d_6, 600 MHz)

A. A.	Position	δ_C	δ_H (J in Hz)
DAA	N*H*		8.57 s
	CO	167.8 s	
	α	99.1 s	
	β	143.3 d	7.65 d (13.7)
R1 His	N*H*		5.64 dd (13.7, 9.9)
	CO	172.3 s	
	α	59.7 d	4.48 ddd (10.5, 9.9, 4.0)
	β	34.3 t	2.33 m, 2.20 m
	Others	2: 134.9 d, 4: 114.6 d	NH1/3: 12.03 s, 2H: 7.39 s, 4H: 6.01 s
R2 Hyp	CO	171.7 s	
	α	61.6 d	4.14 dd (9.2, 4.3)
	β	37.9 t	2.07 m, 1.64 m
	γ	68.5 d	4.06 m
	δ	54.3 t	3.54 m, 1.81 m
	OH		5.09 d (3.8)
R3 Leu	N*H*		9.84 d (7.7)
	CO	172.5 s	
	α	52.2 d	4.16 ddd (11.4, 7.6, 3.9)
	β	39.4 t	1.59 m, 1.51 m
	γ	[a]	1.73 m
	δ	20.7 q +[b]	0.86 d (6.6), 0.82 d (6.5)
R4 Leu	N*H*		7.57 d (6.3)
	CO	169.9 s	
	α	49.2 d	4.42 ddd (9.1, 6.1, 5.1)
	β	39.7 t	1.63 m, 1.22 m
	γ	[a]	1.47 m
	δ	b × 2	0.89 d (6.6), 0.89 d (6.5)
R5 Pro	CO	172.0 s	
	α	62.9 d	4.30 dd (9.0, 8.0)
	β	29.2 t	2.34 m, 1.58 m
	γ	[a]	1.92 m
	δ	47.2 t	3.67 m, 3.19 m
R6 Pro	CO	172.0 s	
	α	63.9 d	4.39 dd (11.4, 7.2)
	β	26.8 t	2.20 m, 1.89 m
	γ	[a]	2.00 m
	δ	46.1 t	3.56 m
R7 Val	NH		7.32 d (10.3)
	CO	171.8s	
	α	56.6 d	4.67 dd (10.2, 4.4)
	β	29.3 d	2.54 m
	γ	17.4 q, 18.9 q	1.11 d (7.0), 0.97 d (6.9)
C1 Pro	CO	173.7 s	
	α	62.3 d	4.32 dd (10.8, 7.0)
	β	29.1 t	2.29 m, 1.53 m
	γ	[a]	1.88 m, 1.77 m
	δ	48.6 t	3.48 m, 3.28 m
C2 Leu	N*H*		7.90 d (6.2)
	CO	171.9 s	
	α	52.6 d	3.90 ddd (11.1, 7.2, 3.8)
	β	38.4 t	1.64 m, 1.16 m
	γ	[a]	1.64 m
	δ	20.7 q + b	0.87 d (6.5), 0.75 d (6.5)
C3 Phe	N*H*		7.80 d (9.3)
	CO	171.0 s	
	α	55.1 d	4.24 ddd (12.1, 9.3, 3.1)
	β	37.5 t	3.30 m, 2.98 dd (13.3, 12.0)
	others	2,6: 129.6 d, 3,5: 127.9 d, 4: 126.4 d	2,6: 7.35 m, 3,4,5: 7.15 m
C4 Gly	N*H*		7.74 dd (7.1, 5.1)
	CO	170.7 s	
	α	42.1 t	3.88 dd (17.0, 7.2), 3.54 dd (16.8, 5.0)
	NH_2		7.20 br s, 7.08 br s

[a] Interchangeable signals at 24.4 (x2), 24.5, 24.6, 25.5, 25.9.
[b] Interchangeable Me signals at 21.4, 22.8, 23.0, 23.4.

related, it does not seem justified to speculate on the microbial origins of the callyaerins.

Callyaerins A–F (**1**–**6**) and H (**8**) showed biological activity in various assays. Their cytotoxicity activity (Table 7) was assessed against the tumour cell line L5178Y (mouse lymphoma), and in part against HeLa (human cervix carcinoma) and PC12 (rat brain

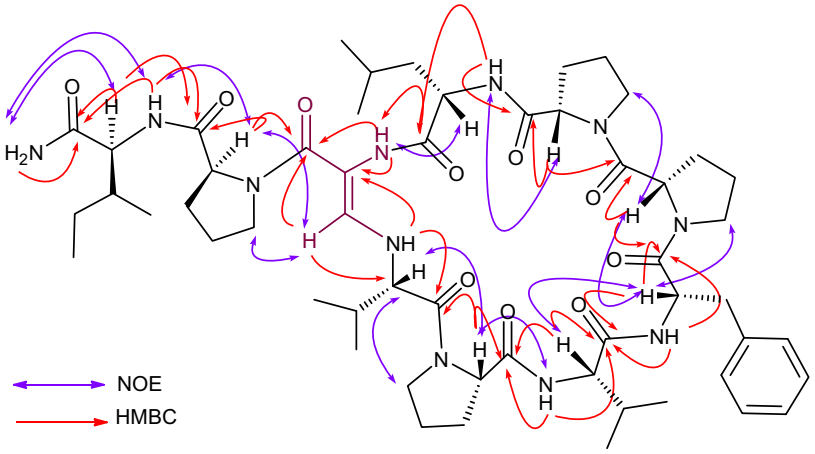

Figure 3. Schematic of the ROESY and HMBC correlations of H (**8**).

tumour), Callyaerins E (**5**) and H (**8**), in particular, exhibited strong activity against the L5178Y cell line with ED_{50} values of 0.39 and 0.48 μM, respectively, similar to those of callyaerin G (**7**) reported previously.[12] The remaining congeners were less active against the L5178Y cell line with ED_{50} values ranging from 2.92 to 4.14 μM, apart from F which was inactive. Callyaerin E (**5**) was the most active compound in the three cell lines tested, while callyaerin F (**6**) showed the least activity. In addition, callyaerin A (**1**) exhibited strong inhibitory properties towards *C. albicans* and moderate activity against the gram negative *Escherichia coli* while it was only mildly active or inactive against the gram positive *Staphylococcus aureus* and *Bacillus subtilis*, respectively (Table 8). Callyaerin E (**5**) displayed strong antimicrobial activity towards *C. albicans* and *B. subtilis* but was only mildly active against the two remaining bacterial strains. Some general conclusions regarding a structure–activity relationship (SAR) was apparent according to the bioassay results, which indicated that increasing the number of proline residues in the cyclic moiety enhanced the cytotoxicity, while replacement of a proline with a hydroxyproline reduced the cytotoxicity. Reduction of the cyclic moiety to a six-membered ring as in callyaerin F caused a total loss of activity against all the tumour cell lines tested. Proline-rich cyclic peptides have been described for their medicinal properties, particularly their antimicrobial activity[17a] while compounds of marine origin such as stylopeptide,[17b] wainunuamide,[17c] axinellins,[18] and phakellistatins[19] have been reported to be cytotoxic. Proline residues in these peptides have been linked to conformational control in a molecule by providing rigidity due to the restricted dihedral angles in proline which reduces other conformation possibilities in a cyclic structure.[20]

3. Conclusion

Investigation of the Indonesian *Callyspongia aerisuza* sponge yielded novel proline-rich peptides, the callyaerins. The analogues showed biological activity in a number of assays and indicated their cytotoxicity increased with the number of proline residues present in the molecule. This study adds considerably to our knowledge of the secondary metabolites produced by the genus *Callyspongia* which has been predominated until now by polyacetylenic[10] and nitrotetradecenyl pyridine[11] metabolites.

4. Experimental

4.1. General experimental procedures

The UV spectra were determined using a Perkin–Elmer double beam spectrophotometer Model 550 S, attached to a Hitachi recorder Model 561, using 1 cm quartz cell. Optical rotations were measured on a Perkin–Elmer Model 341 LC polarimeter. ESI- and LC–MS spectra were obtained with a Thermofinnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector. HRESIMS were determined on a Micromass Q-Tof 2 mass spectrometer. Column chromatography was performed on Silica Gel 60 (0.04–0.063 mm, Merck) and RP-18 Lobar columns (40–63 μm, 25 mm id −310 mm, Merck, Darmstadt, Germany). Pre-coated Silica Gel 60 F_{254} plates (Merck) were used for TLC analyses.

1D (^{1}H and ^{13}C) and 2D (TOCSY, ROESY, HMBC and HMQC) nuclear magnetic resonance spectra were recorded on a Bruker AVANCE DMX600 NMR spectrometer (apart from some 1D ^{13}C spectra on a Bruker ARX 400 instrument) using DMSO-d_6 as solvent and internal reference (^{1}H: 2.50 ppm, ^{13}C: 39.5 ppm). ^{1}H chemical shifts and coupling constants were evaluated from ^{1}H spectra after Gaussian multiplication. In cases of signal overlap chemical shifts were evaluated from cross peaks in the 2D TOCSY spectrum. ^{13}C shifts were from the 1D spectrum or from the 2D correlations.

HPLC separation was performed on a semi-preparative HPLC system consisting of a Lachrom-Merck Hitachi L-7100 pump and a L-7400 UV detector using a C-18 column (300 × 8 mm id, prefilled with Eurospher 100, Knauer, Berlin, Germany), with a flow rate of 5.0 mL/min. Solvents were distilled prior to use and spectral grade solvents were used for spectroscopic measurements.

4.2. Animal material

The sponges were collected by scuba diving at a depth of 4–5 m at Ambon in Indonesia in 1996. Freshly collected sponges were frozen immediately after collection and then freeze-dried. A voucher specimen has been deposited in the Zoological Museum, University of Amsterdam, under the registration no. ZMAPOR 17717.

4.3. Extraction and isolation

The sponge sample was freeze-dried (300 g) and was repeatedly extracted with MeOH (2 L × 4). The total MeOH extract was evaporated under reduced pressure to dryness (310 mg) and subjected to vacuum liquid chromatography (VLC) using hexane, CH_2Cl_2, EtOAc, and MeOH. The preliminary screening for cytotoxic activity of the different fractions revealed that the EtOAc fraction showed strong activity with 100% mortality at concentrations of 20 and 50 μg/mL in the brine shrimp test. The bioactive EtOAc fraction

Table 5
^{1}H NMR data of callyaerin D (**4**) and F (**6**) (600 MHz, DMSO-d_6)

Callyaerin D (**4**)				Callyaerin F (**6**)			
A. A.	Position	δ_H (J in Hz)	Sequential NOEs	A. A.	Position	δ_H (J in Hz)	Sequential NOEs
DAA	NH	8.42 br s	1-N*H*, 7α	DAA	N*H*	8.25 s	1-N*H*
	α				α		
	β	7.38 d (8.8)			β	7.25 d (14.0)	
R1 Ile	NH	5.30 t (10.7)	DAA–N*H*	R1 Val	N*H*	5.14 dd (10.1, 13.7)	
	α	4.41 m			α	4.38 br d (9.8)	2-δ
	β	1.98 m	2-N*H*		β	2.31 m	
	γ	0.94 m, 1.20 m	2-N*H*		γ	0.87 d (6.9), 0.66 d (6.9)	
	γ′δ	0.83[a], 0.84[a]		R2 Pro	α	3.95 dd (10.3, 6.7)	3-N*H*
R2 Ile	NH	7.64 d (4.9)	1-β, 1-γ		β	2.17 m, 1.81 m	
	α	4.00 dd(4.9, 3.5)	3-N*H*		γ	2.03 m, 1.84 m	
	β	1.81 m			δ	3.86 m, 3.74 m	
	γ	1.25 m, 1.30 m		R3 Val	N*H*	7.54 d (5.1)	
	γ′δ	0.80[a], 0.80[a]			α	3.86 m	4-N*H*
R3 Phe	NH	7.50 d (6.0)	2-α, 4-δ		β	2.11 m	
	α	4.61 m	4-α, 4-δ		γ	0.93 d (6.9), 0.86 d (6.9)	
	β	3.06 dd (13.1, 9.7), 2.77 dd (13.1, 3.5)		R4 Phe	N*H*	7.50 d (6.1)	5-δ
	Others	7.25 d (8.8), 7.23 d (6.9), 7.21 d (7.6)			α	4.54 ddd (9.3, 6.1, 3.5)	5-δ
R4 Pro	α	4.17 t (7.1)	3-α		β	3.01 dd (12.9, 9.7)	
						2.71 dd (12.8, 3.1)	
	β	1.40 m,1.70 m			others	7.14–7.28 m	
	γ	1.42 m,1.66 m		R5 Pro	α	4.28 m	DAA–N*H*
	δ	2.23 m, 3.27 m	3-N*H*		β	2.09 m, 1.80 m	
R5 Hyp	α	4.40 m			γ	1.91 m, 1.83 m	
	β	2.00 m, 2.21 m			δ	3.58 m, 3.35 m	
	γ	1.91 m		C1 Pro	α	4.26 m	2-NH
	δ	3.65 m, 3.93 t (6.3)			β	2.19 m, 1.42 m	
R6 Pro	α	4.30 t (8.6)			γ	1.73 m, 1.45 m	
	β	1.75 m, 2.17 m			δ	3.26 m, 2.31 m	
	γ	1.60 m, 1.70 m		C2 Leu	N*H*	7.66 d (9.6)	
	δ	3.30 m, 3.53 t (8.8)			α	4.62 td (10.1, 3.9)	3-N*H*
R7 Leu	NH	7.54 d (9.5)	DAA–N*H*		β	1.93 m, 1.67 m	
	α	4.47 td (9.4, 5.5)	DAA–N*H*		γ	1.72 m	
	β	1.85 m, 1.71 m			δ	0.94 d (6.9), 0.84 d (6.9)	
	γ	1.70 m		C3 Phe	N*H*	6.17 d (7.8)	
	δ	0.80 m, 0.82 m			α	4.42 td (7.6, 4.5)	4-N*H*
C1 Pro	α	4.32 m	2-α, 2-N*H*		β	2.93 m	
	β	1.70 m, 2.16 m			others	7.14–7.28 m	
	γ	1.80 m, 1.90 m		C4 Ile	N*H*	7.52 d (8.5)	
	δ	3.61 m, 3.38 m	2-α, 2-N*H*		α	3.98 dd (8.3, 6.1)	Terminal-NH_2
C2 Ile	NH	7.76 d (6.3)	1-α		β	1.75 m	
	α	3.93 t (6.3)	3-N*H*		γ	1.29 m, 1.10 m,	
	β	1.90 m			γ′δ	0.76 d (6.8), 0.77 t (7.3)	
	γ	1.40 m, 1.28 m			NH_2	7.04 br s, 6.95 br s	
	γ′δ	0.80[a], 0.85[a]					
C3 Asn	NH	7.88 d (7.6)	2-α				
	α	4.55 q (~7)	4-N*H*				
	β	2.69 dd (15.1, 6.9), 2.35 dt (15.1, 6.9)					
	NH_2	6.93 s, 7.31 s					
C4 Ala	NH	7.57 d (6.9)	3-α				
	α	4.16 m	5-N*H*				
	β	1.25 d (6.9)					
C5 Ile	NH	7.34 d (6.7)	4-β				
	α	4.05 dd (8.9, 6.7)	Terminal-NH_2				
	β	1.77 m					
	γ	1.12 m, 1.45 m					
	γ′δ	0.85[a], 0.87[a]					

[a] Signal overlap prevents determination of couplings.

(120 mg) was chromatographed over a silica gel column using a CH_2Cl_2/MeOH gradient to give five fractions. Fraction-1 (30 mg) was submitted to semi-preparative HPLC (HPLC gradient program: 60:40 MeOH/H_2O at 0 and 5 min; 100:0 MeOH/H_2O at 38 and 45 min and a flow rate 5.0 mL/min) to yield callyaerins C (5.5 mg) and E (7.5 mg). Fraction-2 was subjected to RP-18 reversed phase column chromatography using a MeOH/H_2O gradient to give callyaerin H (6 mg). Fraction-3 was purified via semi-preparative HPLC (HPLC gradient program: 60:40 MeOH/H_2O at 0 and 5 min; 100:0 MeOH/H_2O at 38 and 45 min and a flow rate 5.0 mL/min) to afford callyaerin F (2 mg). Fraction-4 was purified over a silica gel column using CH_2Cl_2/MeOH (85:15) and then by semi-preparative HPLC (HPLC gradient program: 60:40 MeOH/H_2O at 0 and 5 min; to 100 MeOH at 38 min, held until 45 min with a flow rate of 5.0 mL/min) to yield callyaerins A (6 mg) and B (8 mg). Fraction-5 was subjected to RP-18 reversed phase column chromatography using MeOH/H_2O (85:15) to afford callyaerin D (3 mg).

Table 6
^{1}H and ^{13}C NMR NMR data of callyaerin H (**8**) (600 MHz, DMSO-d_6)

A.A.	Position	δ_C	δ_H (J in Hz)
DAA	N*H*		8.47 br s
	CO	167.2 s	
	2	98.8 s	
	3	143.3 d	7.23 d
R1 Val	N*H*		5.26 dd (13.7, 10.0)
	CO	172.4 s	
	α	62.7 d	4.42 dd (10.0, 1.4)
	β	29.3 d	2.35 m
	γγ′	15.6 q, 21.6 q	0.68 d (6.7), 0.89 d (6.9)
R2 Pro	CO	172.4 s	
	α	59.8 d	3.97 dd (10.5, 6.8)
	β	26.8 t	2.03 m, 1.82 m
	γ	24.3 t	2.18 m, 1.82 m
	δ	46.2 d	3.82 m, 3.75 m
R3 Val	N*H*		7.59 d (5.1)
	CO	170.7 s	
	α	61.0 d	3.86 t (4.7)
	β	28.8 d	2.10 m
	γγ′	18.4 q,18.5 q	0.93 d (7.0), 0.86 d
R4 Phe	N*H*		7.51 d (5.8)
	CO	168.9 s	
	α	53.5 d	4.57ddd (9.6, 5.9, 3.8)
	β	36.2 t	3.03 dd (13.1, 9.7), 2.77 dd (13.1, 3.6)
	others	1: 137.9 s, 2,6: 128.9 d, 3,5: 128.1 d, 4: 126.5 d	7.2–7.3 m
R5 Pro	CO	171.5 s	
	α	63.2 d	4.34 dd (11.4, 7.5)
	β	29.5 t	1.80–2.10 m
	γ	24.3 t	1.80–2.10 m
	δ	46.4 d	3.63 m, 3.35 m
R6 Pro	CO	171.3 s	
	α	63.6 d	4.17 dd (9.3, 7.7)
	β	29.5 t	2.21 m, 1.69 m
	γ	25.5 t	1.45 m
	δ	46.5 d	3.27 m
R7 Leu	N*H*		7.54 d (9.6)
	CO	172.2 s	
	α	50.4 d	4.51 ddd (9.8, 9.8, 4.3)
	β	41.0 t	1.77 m
	γ	24.7 d	1.85 m
	δ, δ′	19.9 q, 22.6 q	0.86 d, 0.82 d
C1 Pro	CO	171.9 s	
	α	63.6 d	4.34 dd (9.6, 7.8)
	β	29.5 t	2.16 m, 1.72 m
	γ	25.6 t	1.81 m, 1.55 m
	δ	48.5 d	3.48 br t (8.0), 3.24 m
C2 Ile	N*H*		7.50 d (9.1)
	CO	173.2 s	
	α	57.2 d	4.01 dd (9.2, 6.1)
	β	35.5 d	1.92 m
	γ	28.6 t	1.21 m, 1.40 m
	γ′δ	15.3 q, 11.3 q	0.85 d (6.6), 0.82 t (7.3)
	NH_2		6.96 br s, 6.89 br s

4.3.1. Callyaerin A (1)

White amorphous powder; $[\alpha]_D$ −80 (*c* 0.12, MeOH); UV λ_{max} (MeOH) 207 and 281 nm. (+) ESI-MS (rel. int.%): *m/z* 1357.6 $[M+H]^+$ (80), 1379.8 $[M+Na]^+$ (50). HRESI-TOFMS: *m/z* 1357.821 (calcd for $C_{69}H_{109}N_{14}O_{14}$, 1357.8248). ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) data, see Table 2.

4.3.2. Callyaerin B (2)

Yellowish-white amorphous powder, $[\alpha]_D$ −89 (*c* 0.2, MeOH). UV λ_{max} (MeOH) 210 and 284 nm. (+) ESI-MS (rel. int.%): *m/z* 1280.6 $[M+H]^+$ (15), 1302.8 $[M+Na]^+$ (100); FAB-MS$^+$ 940.2. HRES-I-TOFMS: *m/z* 1280.839 (calcd for $C_{65}H_{110}N_{13}O_{13}$, 1280.8268). ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) data, see Table 3.

4.3.3. Callyaerin C (3)

White amorphous powder, $[\alpha]_D$ −52 (*c* 0.15, MeOH). UV λ_{max} (MeOH) 208 and 283 nm. (+) ESI-MS (rel. int.%): *m/z* 1268.5 $[M+H]^+$ (100), 1290.7 $[M+Na]^+$ (45). HRESI-TOFMS: *m/z* 1268.718 (calcd for $C_{63}H_{94}N_{15}O_{13}$, 1268.7156). ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) data, see Table 4.

4.3.4. Callyaerin D (4)

White amorphous powder, $[\alpha]_D$ −49 (*c* 0.2, MeOH). UV λ_{max} (MeOH) 205 and 285 nm. (+) ESI-MS (rel. int.%): *m/z* 1386.5 $[M+H]^+$ (100), 1408.8 $[M+Na]^+$ (90), 861 (35) $[M-(Ile+Ala+Asn+Ile+Pro+NH_2)]^+$; FAB-MS$^+$ 861.5. HRESI-TOFMS: *m/z* 1386.817 (calcd for $C_{69}H_{108}N_{15}O_{15}$, 1386.8071). ^{1}H NMR (600 MHz, DMSO-d_6) data, see Table 5.

4.3.5. Callyaerin E (5)

White amorphous powder, $[\alpha]_D$ −68 (*c* 0.25, MeOH). UV λ_{max} (MeOH): 206 and 282 nm. (+) ESI-MS (rel. int.%): *m/z* 1262.5 $[M+H]^+$ (95), 1284.7 $[M+Na]^+$ (90), 865 $[M-(Pro+2Ile+Gly+NH_2)]^+$ (100). HRESI-TOFMS: *m/z* 1284.710 (calcd for $C_{66}H_{95}N_{13}O_{12}Na$, 1284.71209), 1300.6880 (calcd for $C_{66}H_{95}N_{13}O_{12}K$, 1300.6860). ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) data, see Table 1.

4.3.6. Callyaerin F (6)

White amorphous powder, $[\alpha]_D$ −32 (*c* 0.15, MeOH). UV λ_{max} (MeOH): 204 and 279 nm. (+) ESI-MS (rel. int.%): *m/z* 548.3, 1095 $[M+H]^+$ (75), 1117 $[M+Na]^+$ (100). (−) ESI-MS *m/z* (rel. int.%): 1093 $[M-H]^-$ (40) and 1140 $[M+HCOOH]^-$ (100). HRESI-TOFMS: *m/z* 1094.6380 (calcd for $C_{58}H_{84}N_{11}O_{10}$, 1094.6324), 1116.620 (calcd for $C_{58}H_{83}N_{11}O_{10}Na$, 1116.6222). ^{1}H NMR (600 MHz, DMSO-d_6) data, see Table 5.

4.3.7. Callyaerin H (8)

White amorphous powder, $[\alpha]_D$ −93 (*c* 0.3, MeOH). UV λ_{max} (MeOH): 208 and 284 nm. (+) ESIMS (rel. int.%): *m/z* 1044 $[M+H]^+$ (75), 1066 $[M+Na]^+$ (100), and 817 $[M-(Pro+Ile+NH_2)]^+$ (90). HRES-I-TOFMS: *m/z* 1066.608 (calcd for $C_{54}H_{81}N_{11}O_{10}Na$, 1066.6066), 1082.581 (calcd for $C_{54}H_{81}N_{11}O_{10}K$, 1082.5805). ^{1}H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) data, see Table 6.

4.4. Acid hydrolysis and absolute configuration of component amino acids using LC–MS analysis of the Marfey derivatives[14]

Each of the isolated peptides (1.0 mg) was treated separately with 2 mL 6 N HCl (pa) and heated at 110 °C for 24 h in ampoules previously sealed under N_2 gas. The resulting solutions were concentrated, with consecutive addition of H_2O (5 mL each) to ensure complete elimination of HCl. To 50 μL of each acid hydrolysate (or authentic amino acid standard at comparable concentration), 100 μL FDNPL (1% *N*-(5-flouro-2,4-dinitrophenyl)-L-leucinamide in acetone) and 20 μL 1 M $NaHCO_3$ were added. The mixture was heated over a hot plate at 40 °C for 1 h with frequent mixing. After cooling, 10 μL of 2 M HCl was added and then concentrated to dryness before dissolving in 1000 μL MeOH. L and D amino acid standards were treated separately with FDNPL in the same manner. The FDNPL derivatives were analysed using LC–MS by comparison

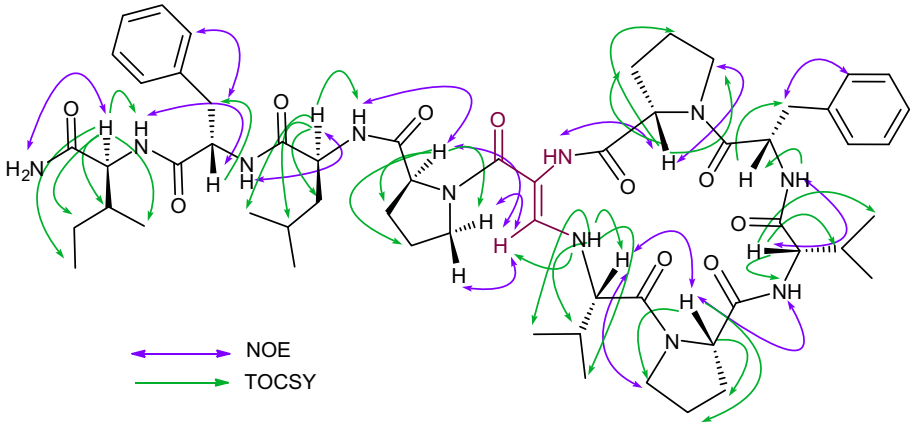

Figure 4. Schematic of the TOCSY and ROESY sequential correlations of callyaerin F (**6**).

Table 7
Cytotoxicity test of the isolated callyaerins

Compound	Brine shrimp assay mortality (%)		% Growth inhibition ED_{50} (μM)		
	20 μg/mL	50 μg/mL	L5178Y	Hela cell	PC12 cell
Total extract	100	100	n.t.	n.t.	n.t.
Ethyl acetate extract	95	100	n.t.	n.t.	n.t.
Hexane extract	30	50	n.t.	n.t.	n.t.
Callyaerin A	n.t.	n.t.	3.61	n.t.	n.t.
Callyaerin B	15	35	4.14	>8	>8
Callyaerin C	n.t.	n.t.	2.92	n.t.	n.t.
Callyaerin D	n.t.	n.t.	3.03	n.t.	n.t.
Callyaerin E	45	70	0.39	3.4	3.8
Callyaerin F	n.t.	n.t.	>9	>9	>9
Callyaerin G[a]			0.41	4.43	>8
Callyaerin H	30	55	0.48	n.t.	n.t.

n.t. not tested.
[a] From our previous work.[12]

Table 8
Antibiotic assay of the isolated callyaerins

Compound	Zone of inhibition (mm)			
	SA 5–10 μL	BS 5–10 μL	EC 5–10 μL	CA 5–10 μL
Callyaerin A	9–9	0–0	10–15	25–30
Callyaerin B	11–11	0–0	7–10	15–15
Callyaerin C	0–0	7–10	0–0	0–0
Callyaerin D	0–0	12–12	0–0	0–7
Callyaerin E	9–10	15–17	9–11	20–20
Callyaerin F	0–7	0–9	0–0	0–0
Callyaerin H	n.t.	n.t.	n.t.	n.t.

SA = *Staphylococcus aureus*, BS = *Bacillus subtilis*, EC = *Escherichia coli*, CA = *Candida albicans*, n.t. not tested.

of the retention time and molecular weight with those of standard amino acids FDNPL derivatives.

4.5. Cytotoxicity and antimicrobial studies

Cytotoxicity and antimicrobial assays were done following previously described protocols.[15]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.012.

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