Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron
Tetrahedron 64 (2008) 3147–3152
www.elsevier.com/locate/tet

Cyclic tetrapeptides from marine bacteria associated with the seaweed *Diginea* sp. and the sponge *Halisarca ectofibrosa*

Wimolpun Rungprom [a,b], Eric R.O. Siwu [c], Lynette K. Lambert [d], Chutiwan Dechsakulwatana [e], Michael C. Barden [f], Udom Kokpol [b], Joanne T. Blanchfield [f], Masaki Kita [g], Mary J. Garson [f,*]

[a] *Faculty of Science and Technology, Phra Nakorn Sri Ayutthaya Rajaphat University, Ayutthaya 13000, Thailand*
[b] *Natural Products Unit, Department of Chemistry, Chulalongkorn University, Bangkok 10330, Thailand*
[c] *Laboratory of Organic Chemistry, Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan*
[d] *Centre for Magnetic Resonance, The University of Queensland, Brisbane, QLD 4072, Australia*
[e] *Institute of Marine Science, Burapha University, Chonburi 20131, Thailand*
[f] *School of Molecular and Microbial Sciences, The University of Queensland, Brisbane, QLD 4072, Australia*
[g] *Laboratory of Bioorganic Chemistry, Department of Chemistry, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8571, Japan*

Received 30 October 2007; received in revised form 9 January 2008; accepted 25 January 2008
Available online 31 January 2008

Abstract

A *Pseudomonas* sp. was cultured which was associated with the Japanese seaweed *Diginea* sp. Crude extracts prepared from this bacterial culture were found to inhibit the growth of other marine bacterial strains. From this bacterial culture, two new peptides *cyclo*-[phenylalanyl-prolyl-leucyl-prolyl] (**3**) and *cyclo*-[isoleucyl-prolyl-leucyl-alanyl] (**4**) have been isolated together with two known peptides (**1**) and (**2**). The crude extract from a culture of *Pseudoalteromonas* sp. associated with the Thai sponge *Halisarca ectofibrosa* was found to inhibit the growth of *Bacillus subtilis* and *Vibrio anguillarum*. Isolation studies yielded a fraction containing two peptides that were identified as *cyclo*-[phenylalanyl-leucyl]$_2$ (**5**) and *cyclo*-[leucyl-isoleucyl]$_2$ (**6**) by means of LC-MS and 2D NMR data. Absolute stereochemistry was confirmed by the synthesis of *cyclo*-[L-phenylalanyl-L-leucyl]$_2$. Peptides (**1**)–(**3**) were also isolated from this bacterial strain. None of the individual peptides isolated in this study showed antibiotic activity.

Keywords: Peptides; NMR; Sponges; *Diginea*; *Halisarca*

1. Introduction

Medium ring-sized peptides containing the amino acids leucine, isoleucine, phenylalanine, valine and proline have been reported from diverse marine sources, including marine microorganisms.[1] The peptide *cyclo*-(L-Ile-L-Pro-L-Leu-L-Pro) was isolated from an actinomycete (*Nocardiopsis* sp.) collected from a Pacific deep-sea sediment.[2] Crews et al. isolated fenestin A (*cyclo*-(Pro-Pro-Leu-Ile)) and B (*cyclo*-(Pro-Val-Pro-Leu-Ile)) from the marine sponge *Leucophloeus fenestrata* and suggested that these metabolites might be of bacterial origin,[3] while three proline-containing cyclic peptides including *cyclo*-[Leu-Pro]$_2$ **1** and *cyclo*-[Phe-Pro]$_2$ **2** have been found in the didemnid ascidian *Cystodytes delle chiajei*.[4] Other examples of cyclic tetrapeptides have been isolated from bacteria associated with marine sponges.[5,6] In this paper we report the isolation and stereochemistry of six cyclic tetrapeptides isolated from two distinct bacterial strains. One bacterial strain was cultured from the seaweed *Diginea* sp., while the other strain was isolated from the Thai marine sponge *Halisarca ectofibrosa*. Both strains were found to inhibit the growth of other marine bacteria.

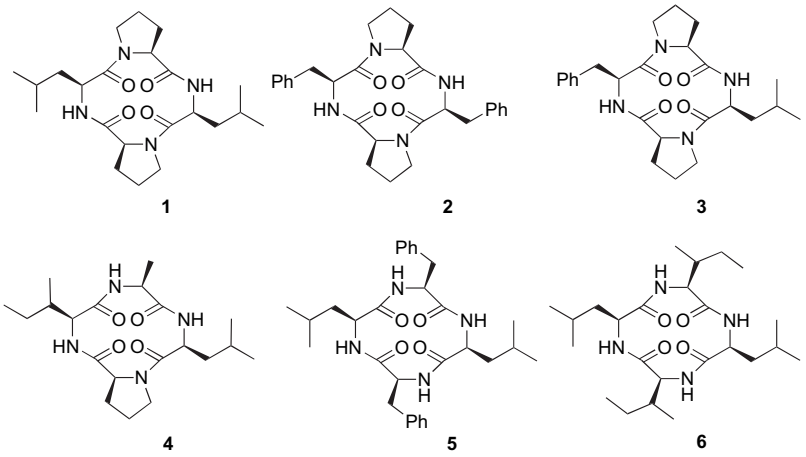
2. Results and discussion

A bacterial strain identified as a *Pseudomonas* sp. by 16S rRNA analysis was obtained from the Japanese seaweed

* Corresponding author. Tel.: +6133653605; fax: +6133654273.
E-mail address: m.garson@uq.edu.au (M.J. Garson).

0040-4020/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2008.01.089

Diginea sp., an alga which has a symbiotic relationship with dinoflagellates such as *Amphidinium* sp. The colonies inhibited the growth of other marine bacteria when grown on seawater-based agar medium. Purification by SiO_2 column chromatography and reversed-phase HPLC of the $CHCl_3$–MeOH-soluble fractions of the culture supernatant gave peptide-containing fractions that were analyzed by ^1H NMR and by LC-MS. Extensive 2D NMR analysis of the individual cyclopeptide components confirmed the isolation of the previously-characterized peptides **1** and **2**[4] in addition to the new peptides **3** and **4**. Peptides **1**–**3** were also produced by a bacterial strain, characterized as a *Pseudoalteromonas* sp. by 16S rRNA analysis, which was isolated from the Thai marine sponge *H. ectofibrosa*. In addition the Thai bacterial culture produced the new leucine-containing peptides **5** and **6**.



The ^1H NMR data of **1** and **2** were recorded in $CDCl_3$ at 600 MHz and showed significant chemical shift differences in the α proton region to the data reported by Aracil et al.[4] recorded at 360 MHz in $CDCl_3$. Differences in ^{13}C chemical shift values for the α carbons were also noted. Notably, all of the proton and carbon signals of **1** and **2** recorded at 600 MHz were observed in pairs, which suggested their C_2 symmetrical conformations.

Peptide **3** was isolated as an amorphous solid with $[\alpha]_D^{23}$ −21.6 (*c* 0.1, MeOH) and showed a molecular ion peak at *m/z* 477.2478 for the [M+Na] by HRESIMS consistent with the molecular formula $C_{25}H_{34}N_4O_4$. The peptide was not soluble in $CDCl_3$ and all NMR data were therefore acquired in CD_3OD. In the ^{13}C NMR (Table 1), in addition to signals corresponding to the side chains of the above amino acids, four carbonyl signals at δ_C 171.5, 170.0, 167.5 and 166.0 ppm could be assigned as the amide carbons of two Pro, one Leu and one Phe, respectively, by the $^3J_{CH}$ correlation from their β protons. The ^1H NMR (Table 1) of peptide **3** showed three α protons at δ_H 4.22, 4.19 and 4.10 while a fourth α proton was observed upfield at δ_H 2.59 ppm. The side chain signals for the individual amino acids were determined by DQFCOSY data and were fully consistent with the presence of Leu, Phe and two different Pro residues. The Pro unit with the α proton signal at δ_H 2.59 was placed adjacent to the Phe residue. The five bond correlation between this upfield signal and that at δ_H 4.19 (Phe), and the three bond correlation between δ_H 4.19 and δ_C 170.0 ppm assigned to the Pro carbonyl, further confirmed the placement of the Pro unit next to a Phe residue, as did NOESY correlations between the Pro α proton and the aromatic protons of Phe. Another correlation present in the DQFCOSY spectrum was between δ_H 4.10 (Leu) and δ_H 4.22 (Pro). In the MS, fragment ions at *m/z* 245 and 211 were consistent with Pro–Phe and Pro–Leu fragments, respectively. These data were consistent with *cyclo*-[Phe-Pro-Leu-Pro] or with *cyclo*-[Phe-Leu-Pro-Pro], however, by analogy with fenestin A,[3] the latter structure would be expected to show a fragment at *m/z* 195 for Pro–Pro in the MS. Further, the other diprolyl peptides isolated in this study all contained an alternate Pro-X–Pro-Y sequence rather than adjacent Pro–Pro units.

The second peptide (**4**) was isolated as an amorphous solid with $[\alpha]_D^{23}$ −13.0 (*c* 0.05, MeOH) and showed a molecular ion peak at *m/z* 417.2506 for the [M+Na] by HRESIMS consistent with the molecular formula $C_{20}H_{34}N_4O_4$. In the ^{13}C NMR ($CDCl_3$), in addition to signals corresponding to the side chains of the above amino acids, four carbonyl signals at δ_C 170.5, 170.2, 170.1 and 167.5 ppm could be assigned as the amide carbons of Ala, Leu, Pro and Ile, respectively, by the $^3J_{CH}$ correlation from their β protons. The ^1H NMR, also run in $CDCl_3$ showed four α protons at δ_H 4.21 (Pro), 3.97 (Ala), 3.93 (Leu) and 3.65 (Ile) ppm, with the side chain signals for the individual amino acids assigned by DQFCOSY data. The Ala α proton at δ_H 3.97 showed an HMBC correlation to the amide carbonyl of Leu, while the Ile α proton at δ_H 3.65 showed an HMBC correlation to the amide carbonyl of Ala. These data were fully consistent with *cyclo*-[Ile-Pro-Leu-Ala].

By LC-MS, the HPLC fraction containing the leucine-containing peptides **5** and **6** showed MH^+ ions at *m/z* 521 and 453 for *cyclo*-(Phe-Leu-Phe-Leu) and *cyclo*-(Leu-Ile-Leu-Ile). Ions at *m/z* 261 and 227 were consistent with Phe–Leu and Leu–Ile fragments, respectively. The ^1H NMR (CD_3OD; Table 2) showed four α protons, those at δ 4.31 and 3.65 ppm assigned to Phe and Leu in compound **5** and those at δ 3.94 and 3.84 ppm assigned to Leu and Ile in compound **6**, respectively.

Table 1
Proline-containing tetrapeptides isolated from marine bacteria

c-Leu-Pro-Phe-Pro **3**				*c*-Leu-Pro-Ile-Ala **4**			
Residue	Position	δ ^{1}H[a]	δ ^{13}C[b]	Residue	Position	δ ^{1}H[c]	δ ^{13}C[d]
Leucine	α	4.10, dd, 5.6, 6.4	53.3, d	Leucine	α	3.93, dd, 4.8, 8.0	53.3
	β	2.28, m	38.1, t		β	1.72, m	44.1
		1.88, m				1.61, m	
	γ	1.85, m	24.4, d		γ	1.85, m	24.7
	δ (CH_3)	0.92, d, 6.4	21.1		δ (CH_3)	0.93, d, 6.4	21.2
	δ (CH_3)	0.92, d, 6.4	22.3		δ (CH_3)	0.96, m, 6.4	22.6
	CO	—	167.5		CO	—	170.2
Proline-1	α	4.22, dd, 7.2, 8.8	58.9	Proline	α	4.21, dd, 7.2, 10.4	58.6
	β	2.28, m	27.7		β	2.33, m	29.1
		1.99, m				1.88, m	
	γ	2.01, m	21.9		γ	1.99, m	21.9
		1.99, m				1.87, m	
	δ	3.48, m	45.1		δ	3.58, m	45.9
		3.30, m				3.46, m	
	CO	—	171.5		CO	—	170.1
Phenylalanine	α	4.19, dd, 4.8, 5.6	58.5	Isoleucine	α	3.65, d, 6.4	62.7, d
	β	3.17, dd, 4.8, 13.6	39.6		β	1.84, m	39.9, d
		2.95, dd, 5.6, 13.6					
	1		135.4		γ	1.57, m	24.9, t
						1.21, m	
	2/6	7.20, d, 7.2	128.3		γ (CH_3)	0.99, d, 6.4	14.8
	3/5	7.35, dd, 5.6, 7.2	129.9		δ (CH_3)	0.92, t, 7.2	10.7
	4	7.35, t, 5.6	127.1		CO	—	167.2
	CO		166.0				
Proline-2	α	2.59, dd, 6.4, 11.2	57.7	Alanine	α	3.97, q, 7.2	50.8
	β	2.00, m	28.4		β	1.42, d, 7.2	19.9
		1.64, m					
	γ	1.89, m	21.1		CO	—	170.5
		1.60, m					
	δ	3.48, m	45.5				
		3.30, m					
	CO	—	170.0				

[a] Measured in CD_3OD relative to residual solvent at δ_H 3.31; 600 MHz.
[b] Measured in CD_3OD relative to residual solvent at δ_C 49.0; 125 MHz.
[c] Measured in $CDCl_3$ relative to residual solvent at δ_H 7.25; 600 MHz.
[d] Measured in $CDCl_3$ relative to residual solvent at δ_C 77.0; 125 MHz.

In CD_3OH, four NH signals were apparent, those at δ 8.20 and 8.14 ppm assigned to the Phe and Leu residues of **5** and those at δ 8.23 and 8.05 ppm assigned to the Leu and Ile residues of **6**. The side chain signals for the four individual amino acids were determined by the combination of DQFCOSY and TOCSY data which were fully consistent with the presence of Phe, Ile and two different Leu units. One of the two Leu units showed signals at δ_H 0.06 and 0.87 ppm assigned to the two diastereotopic β protons, suggesting that it could be adjacent to a Phe residue. The second Leu showed normal chemical shift values consistent with it being adjacent to an Ile residue. In the ^{13}C NMR, in addition to signals corresponding to the side chains of the above amino acids, four carbonyl signals at δ_C 171.8, 170.5, 169.5 and 168.8 ppm could be assigned as the amide carbons of Leu, Leu, Ile and Phe, respectively, by the $^{3}J_{CH}$ correlation from their β protons. Additionally, the $^{3}J_{CH}$ correlations between δ_H 4.31 and δ_C 170.5 ppm and between δ_H 3.65 and δ_C 168.8 ppm confirmed the placement of Phe next to a Leu unit. Correlations between δ_H 3.84 and δ_C 171.8 ppm and between δ_H 3.94 and δ_C 169.5 ppm confirmed the placement of the remaining Leu next to an Ile residue. In both DQFCOSY and TOCSY spectra, five bond correlations were apparent between the α protons of Phe and the unusual Leu residue, and between the normal Leu and Ile, while a NOESY spectrum showed correlations from the NH of the unusual Leu to the α proton of Phe, and from the NH of the normal Leu to the Ile α proton. Clearly, the two components of this fraction were *cyclo*-(Phe-Leu-Phe-Leu) and *cyclo*-(Leu-Ile-Leu-Ile). Owing to the small sample size, the two peptides were not further purified by reversed-phase HPLC.

To confirm its identity, peptide **5** was prepared by solid phase peptide synthesis using Kaiser's 4-nitrobenzophenone oxime resin.[7] The peptide was assembled by repeated coupling of Boc-protected amino acids in the following order: L-Phe, L-Leu, L-Phe and finally L-Leu. Cyclization of the peptide was achieved concomitantly with cleavage from the resin via treatment with diisopropylethylamine and acetic acid, a process characteristic of Kaiser resin.[8] The cleavage procedure yielded a mixture of peptide products from which *cyclo*-(L-Phe-L-Leu-L-Phe-L-Leu) was purified by reversed-phase HPLC. The sample of synthetic peptide showed identical ^{1}H and ^{13}C

Table 2
Symmetrical leucine-containing tetrapeptides isolated from *Pseudoalteromonas* sp.

c-Leu-Phe-Leu-Phe **5**					*c*-Leu-Ile-Leu-Ile **6**			
Residue	Position	δ ^{1}H[a]	δ ^{13}C[b]	δ ^{13}C[c]	Residue	Position	δ ^{1}H[a]	δ ^{13}C[b]
Leucine	α	3.65, ddd, 9.9, 4.4, 1.0	53.8, d	54.1	Leucine	α	3.94, ddd, 9.1, 4.4, 1.1	54.0, d
	β	0.06, m (a)	44.9, t	45.3		β	1.61, m (a)	44.5, t
		0.87, m (b)					1.75, m (b)	
	γ	1.42, m	24.4, d	24.7		γ	1.86, m	25.0, d
	δ (CH_3)	0.69, d, 6.6	23.1, q	23.3		δ (CH_3)	0.96, d, 6.8	21.6, q
	δ (CH_3)	0.73, d, 6.6	21.1, q	21.4		δ (CH_3)	0.97, d, 6.8	23.4, q
	CO	—	170.5	170.6		CO	—	171.8, s
	NH	8.14, s	—	—		NH	8.23, s	—
Phenylalanine	α	4.31, ddd, 4.8, 3.8, 1.0	57.2, d	57.4	Isoleucine	α	3.84, dd, 4.1, 1.1	60.7, d
	β	2.95, dd, 13.9, 4.8	39.9, t	40.3		β	1.93, m	40.3, d
		3.28, dd, 13.9, 3.8						
	1	—	136.6, s	136.8		γ	1.25, m (a)	
							1.54, m (b)	
	2/6	7.20, d, 8.4	131.5, d	131.8		γ (CH_3)	1.02, d, 7.1	15.4, q
	3/5	7.31, dd, 7.5, 8.4	129.3, d	129.6		δ (CH_3)	0.95, d, 7.6	11.9, q
	4	7.26, t, 7.5	128.2, d	128.5		CO	—	169.5
	CO	—	168.8, s	168.9		NH	8.05, s	—
	NH	8.20, s	—	—				

[a] Measured in CD_3OD or in CD_3OH relative to residual solvent at δ_H 3.31; at 750 MHz.
[b] Measured in CD_3OD relative to residual solvent at δ_C 49.0; at 500 MHz.
[c] Synthetic sample; at 750 MHz.

NMR data to the bacterial peptide component, including the characteristic upfield leucine signals.

The synthetic tetrapeptide **5** showed a negative optical rotation, as do the cyclic tetrapeptides reported from marine sources in which all the amino acid constituents are of L configuration.[2,5,6] In particular, the peptide (−)-*cyclo*-[Ile-Pro-Leu-Pro] isolated from *Nocardiopsis* sp. has been shown to contain all L amino acids.[2] The peptides **3** and **4** both showed a negative optical rotation consistent with L stereochemistry while Marfey analysis[9] of the HPLC fraction containing peptides **1**–**3** provided L amino acid components alone. There was insufficient of the natural peptides **5** and **6** to establish absolute configuration of these peptides, however, on biogenetic grounds an all L configuration is suggested for them also.

The methanol-soluble fractions of the *Pseudoalteromonas* culture supernatant showed antimicrobial activity when tested against *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Escherichia coli* or *Vibrio anguillarum.* However, neither the cyclopeptide-containing fractions nor the synthetic sample of peptide **5** showed any antibacterial activity when screened against common bacterial targets. Other researchers have also noted the absence of activity associated with cyclic tetrapeptide metabolites even when the metabolites have been isolated from extracts that show antimicrobial activity.[2] This may be ascribed to multiple ring conformations linked to the presence of trans or cis amide bonds,[10] particularly in proline-rich cyclopeptides.[11]

3. Conclusions

In conclusion, the methanolic extracts of two different marine bacterial strains have been investigated and shown to contain cyclic tetrapeptide components. When cultured, a *Pseudomonas* sp. associated with the Japanese seaweed *Diginea* sp. gave two new peptides *cyclo*-[phenylalanyl-prolyl-leucyl-prolyl] (**3**) and *cyclo*-[isoleucyl-prolyl-leucyl-alanyl] (**4**) together with two known peptides (**1**) and (**2**). Culture of a *Pseudoalteromonas* sp. associated with the Thai sponge *H. ectofibrosa* provided *cyclo*-[phenylalanyl-leucyl]$_2$ (**5**) and *cyclo*-[leucyl-isoleucyl]$_2$ (**6**) together with (**1**)–(**3**). Despite the crude extracts showing antibacterial activity, none of the individually-isolated peptides were active.

4. Experimental

4.1. General experimental procedures

One and two dimensional NMR spectra were acquired using Jeol JNM-A600, Bruker DRX-500, Bruker DMX-750 or JEOL JNM-ECA800 instruments. NMR spectra were obtained in CD_3OD or CD_3OH at room temperature. Samples were internally referenced to MeOH at δ_H 3.31 and δ_C 49.0. High resolution mass measurements (accuracy within 5 ppm) were obtained from a Finnigan MAT 900 XL-Trap electrospray (ESI) mass spectrometer with a Finnigan API III electrospray source. LC-MS data were obtained using either an Agilent 1100 series HPLC coupled to an PE Sciex API 3000 series mass spectrometer with positive ion electrospray (ES) or a PE Biosystems QSTAR mass spectrometer. Optical rotations were recorded on a Perkin–Elmer 241-MC polarimeter or a JASCO DIP-1000 polarimeter. Kaiser oxime resin, 1,3-diisopropylcarbodiimide (DIC) and diisopropylethylamine (DIEA) were purchased from Sigma–Aldrich Pty. Ltd. All Boc-protected amino acids, 2-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate (HBTU) and trifluroacetic acid (TFA) were purchased from Auspep Pty. Ltd, Australia. All solvents were distilled prior to use or were purchased of HPLC grade.

4.2. Biological material and cultivation of bacterial strains

A bacterial strain was isolated from the seaweed *Diginea* sp. collected from Ishigaki Island, Okinawa, Japan together with the dinoflagellate *Amphidinium* sp., by cultivation on 50% artificial seawater medium containing 0.5% proteose peptone and 0.1% yeast extract (pH 7.2), and was identified by 16S rRNA gene sequence analysis as a *Pseudomonas* sp. The sponge *H. ectofibrosa* (Demospongiae, family Halisarcidae) was collected near the Institute of Marine Science, Burapha, in the Gulf of Thailand. The sponge is characterized by the presence of irregular tubular chambers and the lack of any fibrous or mineral elements in the skeleton.[12] Voucher specimens and photographs of the sponge material are available from the authors. The bacterial strain (S-9) was isolated by cultivation on ORI medium containing 0.1% proteose peptone, 0.1% yeast extract, 0.05% phytone, 0.02% sodium thiosulphate, 0.005% sodium sulfite and 0.005% iron(II) sulfate. The strain was identified by 16S rRNA gene sequence analysis as a *Pseudoalteromonas* sp.

4.3. Extraction and isolation of peptides

The culture broth (1.5 L) of a bacterial strain no. 27 (*Pseudomonas* sp.) was centrifuged, and the bacterial cells were extracted with $CHCl_3$/MeOH (1:1, 500 mL). The combined extracts were concentrated, and the residue was partitioned with EtOAc and water. The EtOAc layer (152 mg) showed a potent antimicrobial activity against an orange-coloured unidentified bacterium associated with the same host (*Diginea* sp.). After further separation by Si gel chromatography and elution using a solvent gradient of hexanes/$CHCl_3$/EtOAc/MeOH, the fraction eluted in EtOAc/MeOH (1:1) was subjected to reverse phase HPLC using a MeOH/H_2O gradient from 50 to 100% MeOH and UV detection at 215 nm to give the tetrapeptides **1** (1.5 mg), **2** (1.5 mg), **3** (1.0 mg) and **4** (2.0 mg).

The combined culture broth (20 L) of bacterial strain S-9 (*Pseudoalteromonas* sp.) was centrifuged to separate bacterial cells from supernatant, and a portion of the supernatant (5 L) was passed through an Amberlite XAD-2 column and eluted with 100% MeOH, yielding an extract (60.5 mg) which displayed antimicrobial activity against *B. subtilis* and *V. anguillarum*. This extract was further fractionated on a C_{18} Sep Pak eluting with MeOH/H_2O 1:1 (5 mL), then with MeOH/H_2O 4:1 (5 mL) and finally 100% MeOH to give three fractions MB1 (25.5 mg), MB2 (13.5 mg) and MB3 (15.5 mg). Fraction MB2 was further fractionated by reverse phase HPLC using a MeOH/H_2O gradient from 50 to 100% MeOH and UV detection at 220 nm to give nine fractions, including MB2-4 (1.5 mg) containing peptides **1**–**3** and MB2-8 (2.0 mg) containing peptides **5** and **6**.

4.3.1. cyclo-[Leucyl-prolyl]$_2$ (**1**)[4]

^{1}H ($CDCl_3$, 600 MHz) δ 6.61 (2H, br s, N*H*), 4.09 (2H, t, J=7.7 Hz), 3.94 (2H, br s), 3.65 (2H, dt, J=12.1, 8.0 Hz), 3.55 (2H, td, J=9.2, 2.9 Hz), 2.63 (2H, m), 2.38 (2H, m), 2.04 (6H, m), 1.92 (2H, m), 1.78 (2H, m), 1.08 (6H, d, J=7.0 Hz), 0.92 (6H, d, J=7.0 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.9 (s, 2C), 169.0 (s, 2C), 59.2 (d, 2C), 54.1 (d, 2C), 47.1 (t, 2C), 38.2 (t, 2C), 29.6 (t, 2C), 25.1 (d, 2C), 24.2 (t, 2C), 23.0 (q, 2C), 21.9 (q, 2C).

4.3.2. cyclo-[Phenylalanyl-prolyl]$_2$ (**2**)[4]

^{1}H ($CDCl_3$, 600 MHz) δ 7.36 (4H, t, J=7.4 Hz), 7.30 (2H, t, J=7.0 Hz), 7.23 (4H, d, J=7.4 Hz), 5.64 (2H, br s), 4.28 (2H, br d, J=8.0 Hz), 4.09 (2H, br t, J=8.1 Hz), 3.68–3.56 (8H, m), 2.78 (2H, dd, J=14.6, 10.6 Hz), 2.34 (2H, m), 2.03 (4H, m), 1.91 (2H, m); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.8 (s, 2C), 165.0 (s, 2C), 139.5 (s, 2C), 129.3 (d, 2C), 129.1 (d, 2C), 127.5 (d, 2C), 59.1 (d, 2C), 56.1 (d, 2C), 45.4 (t, 2C), 36.8 (t, 2C), 28.3 (t, 2C), 22.5 (t, 2C).

4.3.3. cyclo-(Prolyl-leucyl-phenylalanyl-leucyl) (**3**)

$[\alpha]_D^{23}$ −21.6 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR (MeOH-d_4, 800 MHz), see Table 1; LC-MS 455 $[M+H]^+$, 245, 211; HRESIMS *m/z* 477.2480 (calcd for $C_{25}H_{34}NaN_4O_4$ $[M+Na]^+$, Δ +0.2 mmu).

4.3.4. cyclo-(Prolyl-leucyl-alanyl-isoleucyl) (**4**)

$[\alpha]_D^{23}$ −13.0 (*c* 0.05, MeOH); ^{1}H and ^{13}C NMR (MeOH-d_4, 800 MHz), see Table 1; LC-MS 395 $[M+H]^+$; HRESIMS *m/z* 417.2506 (calcd for $C_{20}H_{34}NaN_4O_4$ $[M+Na]^+$, Δ +2.8 mmu).

4.3.5. cyclo-[L-Phenylalanyl-L-leucyl]$_2$ (**5**)

^{1}H and ^{13}C NMR (MeOH-d_4, 750 MHz), see Table 2; LC-MS 521 $[M+H]^+$, 261; HREIMS *m/z* 543.2954 (calcd for $C_{30}H_{40}NaN_4O_4$ $[M+Na]^+$, Δ +0.7 mmu).

4.3.6. cyclo-[L-Leucyl-L-isoleucyl]$_2$ (**6**)

^{1}H and ^{13}C NMR (MeOH-d_4, 750 MHz), see Table 2; LC-MS 453 $[M+H]^+$, 227; HRESIMS *m/z* 475.3291 (calcd for $C_{24}H_{44}NaN_4O_4$ $[M+Na]^+$, Δ +3.1 mmu).

4.4. Marfey analysis[9]

Fraction MB2-4 (300 μg) containing peptides **1**–**3** was hydrolyzed with 0.5 mL of 6 M HCl in a sealed ampoule at 105 °C for 12 h. The remaining HCl was removed under a stream of N_2. The resulting hydrolyzate was resuspended in 0.1% of 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (50 μL) in acetone, then 0.1 M $NaHCO_3$ (100 μL) was added. The reaction mixture was heated at 80 °C for 3 min. After cooling to room temperature, the solution was neutralized with 0.2 M HCl (50 μL) and diluted with MeCN/H_2O/TFA(100 μL; 50:50:0.05). This solution was analyzed by reversed-phase HPLC (Altech™ Econosil C18; 40% MeCN in H_2O+0.05% TFA; 1.0 mL/min; UV detection at 340 nm). The retention times were 5.56 and 6.06 min for authentic L- and D-proline, 18.7 and 28.6 min for L- and D-leucine and 20.0 and 32.9 min for L- and D-phenylalanine, respectively. The retention times for the MB2-4 hydrolyzate at 5.54, 18.7 and 20.0 min, respectively, indicated the presence of L-proline, L-leucine and L-phenylalanine.

4.5. Peptide synthesis

Kaiser oxime resin (1 g) with a loading capacity of 1 mmol/g was swollen in DCM and Boc–Phe-OH (3.32 g, 12.5 mmol) was attached to the resin using DIC (0.969 mL, 6.25 mmol) in DCM as the coupling reagent and the coupling reaction allowed to proceed overnight. After thorough washing of the resin with DCM, MeOH and DMF, the Boc-protecting group was removed by treatment with TFA and the resin washed carefully and allowed to swell in DMF. The remaining amino acids (L-Leu, L-Phe, L-Leu) were conjugated to the growing peptide by stepwise coupling using standard Boc-protecting group, solid phase peptide synthetic protocols employing HBTU/DIEA as the activating agents and TFA/DCM for Boc-deprotection.[13] When all the amino acids were assembled and the final Boc group removed the peptidyl-resin was washed thoroughly with DCM, *i*-PrOH and DMF. Cleavage and cyclization of the peptide were achieved by treatment of the peptidyl-resin with a solution of DIEA (0.7 mL, 4 mmol), glacial acetic acid (0.23 mL, 4 mmol) in DMF (20 mL) for 24 h. The resin was then removed from the solution by filtration and the resin washed several times with DMF. The filtrate was lyophilized to yield a crude mixture of peptides from which a sample of the tetrapeptide (**3**) was purified by reverse phase HPLC using a MeOH/H_2O gradient and UV detection at 220 nm.

4.5.1. *cyclo-[L-Phenylalanyl-L-leucyl]$_2$ (**5**)*

$[\alpha]_D$ −92.2 (*c* 0.57, $CHCl_3$); ^1H and ^{13}C NMR (MeOH-d_4, 750 MHz), see Table 2; HREIMS *m/z* 543.2954 (calcd for $C_{30}H_{40}NaN_4O_4$ $[M+Na]^+$, Δ +0.7 mmu).

4.6. Antibacterial assays

Assays against the bacterial strains *S. aureus, M. luteus, B. subtilis, E. coli* and *V. anguillarum* were performed employing the standard agar diffusion assay.

Acknowledgements

We thank the Australia Research Council, the Royal Golden Jubilee Fund of Thailand, the Thailand Research Fund, and the Japan Society for Promotion of Sciences for financial support. We thank Dr. Sumaitt Putchakarn of the Institute of Marine Science, Burapha University for taxonomic identification of the sponge sample, Mr. Graham MacFarlane of the School of Molecular and Microbial Science, UQ for the high resolution mass spectrometric measurements. We thank Professor Daisuke Uemura, Nagoya University, for his interest and generous support of this work.

References and notes

1. Fenical, W. *Chem. Rev.* **1993**, *93*, 1673–1684.
2. Shin, J.; Seo, Y.; Lee, H.-S.; Rho, J.-R.; Mo, S. J. *J. Nat. Prod.* **2003**, *66*, 883–884.
3. Omar, S.; Tenenbaum, L.; Manes, L. V.; Crews, P. *Tetrahedron Lett.* **1988**, *29*, 5489–5492.
4. Aracil, J. M.; Badre, A.; Fadli, M.; Jeanty, G.; Banaigs, B.; Francisco, C.; Lafargue, F.; Heitz, A.; Aumelas, A. *Tetrahedron Lett.* **1991**, *32*, 2609–2612.
5. Mitova, M.; Tommonaro, G.; De Rosa, S. *Z. Naturforsch., C* **2003**, *58*, 740–745.
6. Mitova, M.; Popov, S.; De Rosa, S. *J. Nat. Prod.* **2004**, *67*, 1178–1181.
7. (a) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1980**, *45*, 1295–1300; (b) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, *47*, 3258–3261.
8. (a) Osapay, G.; Goodman, M. *J. Chem. Soc., Chem. Commun.* **1993**, 1599–1600; (b) Paulitz, C.; Steglich, W. *J. Org. Chem.* **1997**, *62*, 8474–8478.
9. Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591–596.
10. (a) Rich, D. H.; Jasensky, R. D. *J. Am. Chem. Soc.* **1980**, *102*, 1112–1119; (b) Loiseau, N.; Gomis, J.-M.; Delaforge, M.; André, F. *Biopolymers* **2003**, *69*, 365–385.
11. (a) Tabudravu, J. N.; Jaspars, M.; Morris, L. A.; Kettenes-Van Den Bosch, J. J.; Smith, N. *J. Org. Chem.* **2002**, *67*, 8593–8601; (b) Napolitano, A.; Bruno, I.; Riccio, R.; Gomez-Paloma, L. *Tetrahedron* **2005**, 6808–6815.
12. Bergquist, P. R.; Cook, S. de C. *Systema Porifera: A Guide to the Classification of Sponges*; Hooper, J. N. A., van Soest, R. V. M., Eds.; Plenum: New York, NY, 2002; pp 1077–1079.
13. Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180–193.