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Cyclic heptapeptides from the soil-derived fungus *Clonostachys rosea*

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ABSTRACT

Three new cyclic heptapeptides (**1–3**) together with three known compounds (**4–6**) were isolated from a solid rice culture of the soil-derived fungus *Clonostachys rosea*. Fermentation of the fungus on white beans instead of rice afforded a new γ-lactam (**7**) and a known γ-lactone (**8**) that were not detected in the former extracts. The structures of the new compounds were elucidated on the basis of 1D and 2D NMR spectra as well as by HRESIMS data. Compounds **1** and **4** exhibited significant cytotoxicity against the L5178Y mouse lymphoma cell line with IC_{50} values of 4.1 and 0.1 μM, respectively. Compound **4** also displayed cytotoxicity against the A2780 human ovarian cancer cell line with an IC_{50} value of 3.5 μM. The preliminary structure-activity relationships are discussed.

1. Introduction

Soil-derived fungi are attracting continuous attention as sources of bioactive secondary metabolites.[1–5] *Clonostachys rosea* (syn. *Gliocladium roseum*) is an example of a soil-derived fungus that is of importance in agriculture as a biological control agent against a number of plant pathogenic fungi[6] as exemplified by *Botrytis cinerea* sporulation on rose debris[7] and strawberries.[8] *C. rosea* was also reported as an entomopathogenic fungus of two leafhoppers pest, *Oncometopia tucumana* and *Sonesimia grossa* in Argentina.[9] Furthermore, *C. rosea* showed significant inhibition against nematodes such as sheep nematodes.[10] Previous investigation of bioactive secondary metabolites of this fungus yielded verticillin-type epipolysulfanyldioxopiperazines,[11] an epidithiodioxopiperazine,[12] and bisorbicillinoids.[13] This provoked us to study the secondary metabolites of *C. rosea* which was isolated from a soil sample collected in Indonesia. In this study, three new cyclic heptapeptides (**1–3**) and three known compounds (**4–6**) were isolated from the fungal culture grown on rice medium while a new γ-lactam (**7**) and a known γ-lactone (**8**) were obtained when the fungus was grown on white beans instead (Fig. 1). The structure elucidation of the new compounds (**1–3**, and **7**) and cytotoxicity against a murine and a human cancer cell line are reported.

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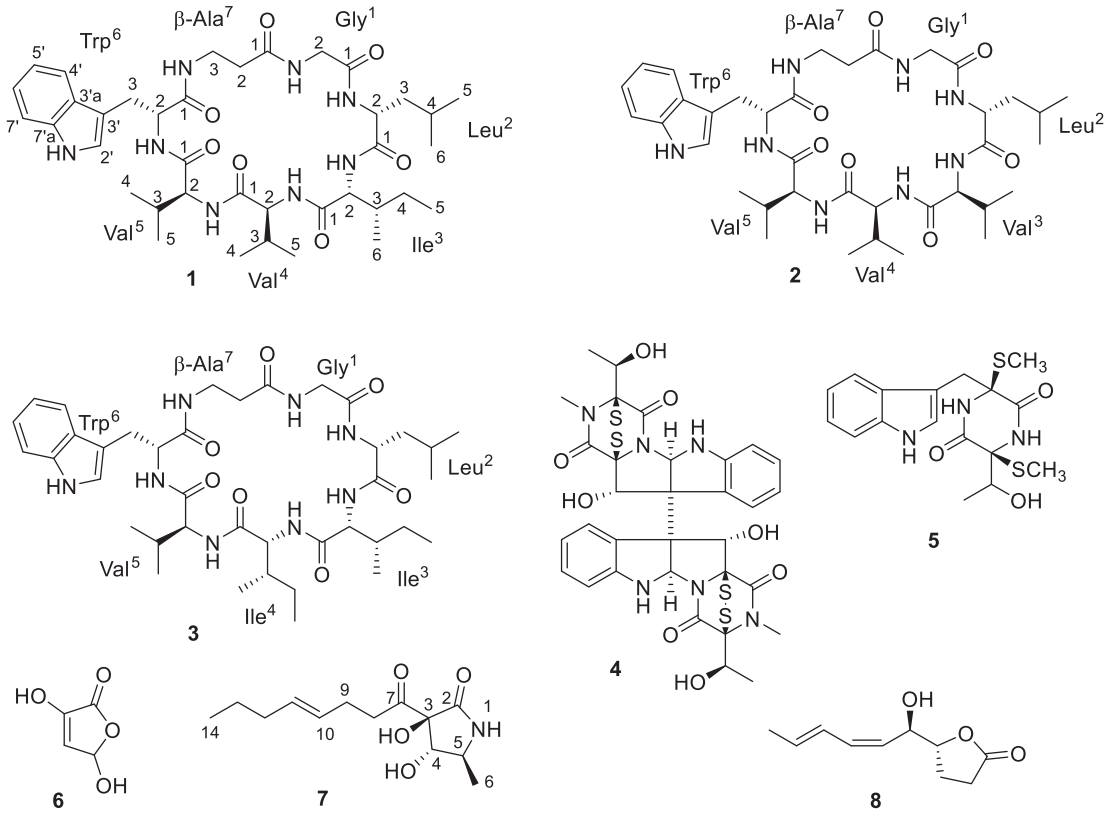

Fig. 1. Structures of compounds isolated from *C. rosea*.

2. Results and discussion

Compound **1** was isolated as an amorphous solid. Its molecular formula was deduced as $C_{38}H_{58}N_8O_7$ from the HRESIMS data. The peptide nature of **1** was established from the amide protons at the region δ_H 7.5–8.5 in addition to the characteristic α-protons between δ_H 3.5–4.5 (Table 1). Based on COSY, TOCSY, HSQC and HMBC spectra, seven amino acid residues were elucidated including a glycine (Gly), a leucine (Leu), an isoleucine (Ile), two valine (Val) units, a tryptophan (Trp) and a β-alanine (β-Ala) moiety.[14,15] Key HMBC correlations from the amide protons to the carbonyl carbons of the adjacent amino acid (from Ile^3-NH to Leu^2-CO, from Leu^2-NH to Gly^1-CO, from Gly^1-NH to β-Ala^7-CO, from β-Ala^7-NH to Trp^6-CO, and from Trp^6-NH to Val^5-CO) led to the construction of the partial structure Ile^3-Leu^2-Gly^1-β-Ala^7-Trp^6-Val^5 (Fig. 2). This was further confirmed by the ROESY correlations between amide protons and α-protons of the adjacent amino acid (Ile^3-NH/Leu^2-H-2, Leu^2-NH/Gly^1-H$_{ab}$-2, Gly^1-NH/β-Ala^7-H$_{ab}$-2, β-Ala^7-NH/Trp^6-H-2, and Trp^6-NH/Val^5-H-2). The substructure Ile^3-Val^4-Val^5 was assembled by key ROESY correlations between Val^4-NH/Ile^3-H-2 and between Val^5-NH/Val^4-H-2. Consequently, compound **1** was proved to be a new cyclic heptapeptide whose structure was elucidated as cyclo-(Gly-Leu-Ile-Val-Val-Trp-β-Ala) in accordance with the unsaturation index (14 DBE) as implied by the molecular formula. After acid hydrolysis of **1** and subsequent application of Marfey's derivatization method,[16] the amino acid residues were identified as D-Leu, D-*allo*-isoleucine, L-Val and D-Trp.

The HRESIMS data of compound **2** indicated the molecular formula $C_{37}H_{56}N_8O_7$, which is 14 amu smaller than that of **1**. Extensive analysis of ^{1}H NMR, COSY, HSQC and HMBC spectra of **2** revealed the replacement of the isoleucine (Ile) moiety by a valine (Val) unit in **2** compared to **1**. This finding was further confirmed by Marfey's reaction results of **2**, showing only D-Leu, L-Val and D-Trp. Key HMBC correlations from Gly^1-NH (δ_H 8.13) to β-Ala^7-CO (δ_C 171.7), from Leu^2-NH (δ_H 8.17) to Gly^1-CO (δ_C 168.9), from Val^3-NH (δ_H 7.81) to Leu^2-CO (δ_C 172.2), from Val^4-NH (δ_H 7.93) to Val^3-CO (δ_C 171.2), from Val^5-NH (δ_H 7.53) to Val^4-CO (δ_C 171.0), from Trp^6-NH (δ_H 8.21) to Val^5-CO (δ_C 170.6), and from β-Ala^7-NH (δ_H 8.24) to Trp^6-CO (δ_C 171.9) as well as ROESY correlations between Gly^1-NH/β-Ala^7-H$_{ab}$-2, Leu^2-NH/Gly^1-H$_{ab}$-2, Val^3-NH/Leu^2-H-2, Val^4-NH/Val^3-H-2, Val^5-NH/Val^4-H-2, Trp^6-NH/Val^5-H-2, and β-Ala^7-NH/Trp^6-H-2 determined the sequence of the

Table 1
^{1}H and ^{13}C NMR data of compound **1–3**.[a]

Unit	Position	**1**		**2**		**3**	
		δ_C, type	δ_H (J in Hz)	δ_C, type [b]	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
Gly[1]	NH		8.05, dd (5.8, 4.0)		8.13, dd (5.6, 4.9)		7.99, dd (5.9, 4.1)
	1	169.0, C		168.9, C		168.9, C	
	2	42.7, CH_2	3.83, dd (16.3, 5.8), 3.61, dd (16.3, 4.0)	42.6, CH_2	3.81, dd (16.1, 5.6), 3.61, dd (16.1, 4.9)	42.6, CH_2	3.85, dd (16.2, 5.9), 3.61, dd (16.2, 4.1)
Leu[2]	NH		8.18, d (6.7)		8.17, d (7.4)		8.15, d (7.1)
	1	172.2, C		172.2, C		172.2, C	
	2	51.5, CH	4.30, m	51.1, CH	4.30, m	51.5, CH	4.31, m
	3	39.5, CH_2	1.49, m	39.2, CH_2	1.50, m	39.6, CH_2	1.48, m
	4	24.1, CH	1.59, m	23.9, CH	1.60, m	24.1, CH	1.58, m
	5	22.8, CH_3	0.88, d (6.6)	22.7, CH_3	0.87, d (6.6)	22.8, CH_3	0.87, d (6.5)
	6	21.7, CH_3	0.83, d (6.6)	21.6, CH_3	0.83, d (6.6)	21.8, CH_3	0.83, d (6.5)
Val[3] or Ile[3]	NH		7.83, d (8.8)		7.81, d (8.6)		7.83, d (8.7)
	1	171.2, C		171.2, C		171.3, C	
	2	56.3, CH	4.33, m	58.0, CH	4.21, m	55.9, CH	4.35, m
	3	36.8, CH	1.84, m	30.1, CH	2.02, m	37.0, CH	1,82, m
	4	25.7, CH_2	1.29, m, 1.10, m	18.4, CH_3	0.84, d (6.8)	25.7, CH_2	1.29, m, 1.10, m
	5	11.5, CH_3	0.84, t (7.4)	17.1, CH_3	0.84, d (6.8)	11.6, CH_3	0.84, t (7.3)
	6	14.5, CH_3	0.81, d (7.2)			14.4, CH_3	0.80, d (6.9)
Val[4] or Ile[4]	NH		7.84, d (8.8)		7.93, d (8.8)		7.93, d (7.9)
	1	171.5, C		171.0, C		171.1, C	
	2	57.9, CH	4.19, m	57.8, CH	4.19, m	57.3, CH	4.19, m
	3	30.5, CH	2.04, m	30.2, CH	2.08, m	36.4, CH	1.80, m
	4	19.2, CH_3	0.83, d (6.8)	18.9, CH_3	0.85, d (6.8)	23.9, CH_2	1.31, m, 1.08, m
	5	17.7, CH_3	0.76, d (6.8)	17.2, CH_3	0.77, d (6.8)	10.8, CH_3	0.76, t (7.4)
	6					15.3, CH_3	0.81, d (6.7)
Val[5]	NH		7.64, d (5.5)		7.53, d (5.3)		7.55, d (5.0)
	1	170.7, C		170.6, C		170.6, C	
	2	58.9, CH	3.86, m	58.5, CH	3.89, m	58.5, CH	3.90, m
	3	29.3, CH	1.73, m	29.1, CH	1.76, m	29.5, CH	1.76, m
	4	18.9, CH_3	0.51, d (6.8)	18.6, CH_3	0.53, d (6.8)	19.0, CH_3	0.53, d (6.7)
	5	18.2, CH_3	0.57, d (6.8)	17.5, CH_3	0.57, d (6.8)	18.0, CH_3	0.53, d (6.7)
Trp[6]	NH		8.27, d (8.5)		8.21, d (8.6)		8.23, d (8.8)
	1	171.8, C		171.9, C		171.9, C	
	2	53.2, CH	4.53, m	53.1, CH	4.50, m	53.1, CH	4.55, m
	3	27.5, CH_2	3.24, dd (14.6, 3.3), 2.83, dd (14.6, 11.2)	27.3, CH_2	3.23, dd (14.6, 3.4), 2.84, dd (14.6, 11.2)	27.7, CH_2	3.21, dd (14.5, 3.5), 2.84, dd (14.5, 11.2)
	1′-NH		10.73, d (1.6)		10.74, d (1.8)		10.73, d (1.8)
	2′	123.9, CH	7.11, d (1.6)	123.8, CH	7.12, d (1.8)	124.0, CH	7.12, d (1.8)
	3′	110.3, C		109.8, C		110.2, C	
	4′	118.4, CH	7.61, d (7.9)	118.2, CH	7.63, d (7.9)	118.5, CH	7.63, d (7.9)
	5′	118.0, CH	6.95, t (7.9)	117.8, CH	6.95, t (7.9)	118.0, CH	6.95, t (7.9)
	6′	120.7, CH	7.02, t (7.9)	120.4, CH	7.02, t (7.9)	120.7, CH	7.02, t (7.9)
	7′	111.1, CH	7.28, d (7.9)	110.9, CH	7.28, d (7.9)	111.1, CH	7.28, d (7.9)
	3′a	127.0, C		126.7, C		127.0, C	
	7′a	136.1, C		135.9, C		136.1, C	
β-Ala[7]	NH		8.17, t (6.0)		8.24, t (5.9)		8.20, t (5.4)
	1	171.7, C		171.7, C		171.6, C	
	2	35.5, CH_2	2.51, m, 2.23, dt (14.9, 5.2)	35.4, CH_2	2.50, m, 2.25, dt (14.6, 5.1)	35.6, CH_2	2.50, m, 2.22, dt (14.9, 5.1)
	3	35.3, CH_2	3.33, m	35.2, CH_2	3.33, m	35.3, CH_2	3.33, m

[a] Recorded at 600 MHz for ^{1}H and 150 MHz for ^{13}C in DMSO-d_6.
[b] Data were extracted from HSQC and HMBC spectra.

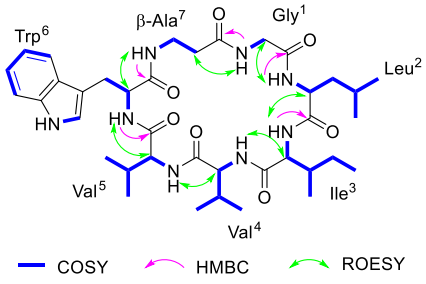

Fig. 2. Key COSY, HMBC and ROESY correlations of **1**.

amino acids as cyclo-(Gly-Leu-Val-Val-Val-Trp-β-Ala) in **2**.

The molecular formula of **3** was determined as $C_{39}H_{60}N_8O_7$ by HRESIMS data, suggesting the presence of an additional methylene group in **3** when compared to **1**. This was explained by replacement of a valine residue by an isoleucine residue in **3** based on 1D and 2D NMR data of **3**. The results of Marfey' reaction of **3** indicated a peptide consisting of D-Leu, L-Val, D-*allo*-isoleucine and D-Trp. The sequence of the amino acids was confirmed as cyclo-(Gly-D-Leu-D-*allo*-Ile-D-*allo*-Ile-L-Val-D-Trp-β-Ala) by the HMBC and ROESY data of **3** by applying the same strategy as described for **1** and **2**.

Compound **7** was obtained as a white powder. The HRESIMS data of **7** established the molecular formula $C_{13}H_{21}NO_4$ with four degrees of

Table 2
^{1}H and ^{13}C NMR data of compound **7**.[a]

position	δ_C, type	δ_H (J in Hz)
1-NH		8.03, s
2	171.9, C	
3	86.8, C	
3-OH		6.26, s
4	83.5, CH	3.62, dd (7.0, 4.7)
4-OH		5.86, d (4.7)
5	51.3, CH	3.19, dq (7.0, 6.2)
6	18.3, CH_3	1.11, d (6.2)
7	209.6, C	
8	39.1, CH_2	2.76, ddd (18.7, 8.8, 6.2)
		2.59, ddd (18.7, 8.8, 6.1)
9	25.3, CH_2	2.08, m
10	129.2, CH	5.39, dt (15.4, 5.2)
11	130.0, CH	5.37, dt (15.4, 5.2)
12	34.0, CH_2	1.91, m
13	22.1, CH_2	1.31, m
14	13.5, CH_3	0.84, t (7.4)

[a] Recorded at 600 MHz for ^{1}H and 150 MHz for ^{13}C in DMSO-d_6.

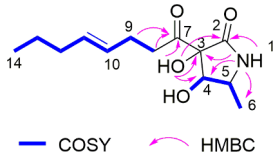

Fig. 3. Key COSY and HMBC correlations of **7**.

unsaturation. Interpretation of ^{1}H, ^{13}C NMR (Table 2) and HSQC spectra revealed the presence of two methyl, four methylene, and four methine groups (two olefinic methine and two oxygenated methine) in addition to three quaternary carbons (two carbonyl carbons and one oxygenated carbon). These data accounted for three degrees of unsaturation, suggesting the presence of a single ring system in **7**. The ^{1}H NMR spectrum showed three exchangeable protons including an amide proton at δ_H 8.03 (1-NH) and two hydroxy protons at δ_H 6.26 (3-OH) and 5.86 (4-OH). Analysis of ^{1}H-^{1}H COSY spectral data led to the construction of two spin systems from C-4 to C-6 and from C-8 to C-14 (Fig. 3). On the basis of the HMBC correlations from 1-NH to C-2 (δ_C 171.9), C-3 (δ_C 86.8), C-4 (δ_C 83.5) and C-5 (δ_C 51.3) and from 3-OH to C-2, C-3 and C-4, a γ-lactam moiety with a methyl and two hydroxy groups at C-5, C-4 and C-3 was established. In addition, the HMBC correlations from 3-OH, H-4 (δ_H 3.62), H_2-8 and H_2-9 to C-7 indicated attachment of the side chain (C-4 to C-7) at the C-3 position. Thus, the planar structure of **7** was elucidated as shown. The trivial name clonostalactam is suggested for this compound. The coupling constant (15.4 Hz) between H-10 and H-11 suggested the geometry of the double bond at C-11/C-12 to be *E*. The relative configuration of the γ-lactam moiety was evident from the ROESY spectrum of **7**. The ROESY correlations between 3-OH and H-4, and between H-4 and Me-6 (δ_H 1.11) indicated that these groups are on the same side of the lactam ring whereas the ROESY correlations between 4-OH and H-5 (δ_H 3.19) suggested they are on the opposite side of the lactam ring.

By comparing NMR and MS data with those reported in the literature, the known compounds were identified as verticillin D (**4**),[17] glioperazine (**5**),[18] 3,5-dihydroxyfuran-2(5*H*)-one (**6**),[19] and sapinofuranone B (**8**).[20]

The cytotoxicity of compounds **1**–**8** against the L5178Y mouse lymphoma cell line and against the A2780 human ovarian cancer cell line was investigated using the MTT assay. Cyclo-(Gly-D-Leu-D-*allo*-Ile-L-Val-L-Val-D-Trp-β-Ala) (**1**) and verticillin D (**4**) showed significant cytotoxicity against the L5178Y mouse lymphoma cell line with IC_{50} values of 4.1 and 0.1 μM, respectively. Verticillin D (**4**) also exhibited cytotoxicity against the A2780 human ovarian cancer cell line with an IC_{50} value of 3.5 μM. The remaining compounds proved to be inactive when tested at a dose of 10 μM. The presence of Ile^{3}-Val^{4} units in **1** is important for its cytotoxicity, when compared to **2** (no Ile unit) and **3** (two Ile units).

3. Experimental

3.1. General experimental procedures

Optical rotations were recorded utilizing a PerkinElmer-241 MC polarimeter. 1D and 2D NMR spectra were measured on Bruker ARX 300 or AVANCE DMX 600 NMR spectrometers. A FTHRMS-Orbitrap (Thermo Finnigan) mass spectrometer was used to record HRESIMS, while low resolution mass spectra were obtained from a Finnigan LCQ Deca XP Thermoquest spectrometer. Analytical HPLC analysis was performed using a Dionex P580 system with a photodiode array detector (UVD340S) and a Europhere 10 C18 column (125 × 4 mm, L × ID, Knauer, Germany). HPLC separation of Marfey's derivatives of amino acids was done using a Knauer Azura system coupled with a Knauer Smartline UV Detector 2600 and a EC 250/4.6 Nucleosil 120–5, C4 column (Macherey & Nagel). Semi-preparative HPLC separation was performed at a flow rate of 5 mL/min using a Lachrom-Merck Hitachi system coupled with a Eurosphere 100 C18 column (300 × 8 mm), a L7100 pump and a L7400 UV detector. Sephadex LH-20 and Merck MN silica gel 60 M (0.04–0.063 mm) were used as stationary phases for column chromatography. The resulting fractions from column chromatography were checked by TLC (thin layer chromatography) using pre-coated silica gel 60 F254 plates (Merck). The spots were visualized under 254 and 365 nm or by spaying with anisaldehyde reagent.

3.2. Fungal material

The fungal strain *Clonostachys rosea* was isolated from a soil sample collected in Banyumas, Indonesia in June 2016 using agar plates with isolation medium (7.5 g Bacto agar, 7.5 g malt extract, 0.125 g chloramphenicol, 500 mL demineralized water, adjust pH to 7.4–7.8). Purification of the initially obtained colony was done on the following medium (6.5 g Bacto agar, 10 g malt extract, 0.05 g yeast extract, 20 mL glycerin, 475 mL demineralized water, adjust pH to 7.4–7.8). DNA amplification and sequencing of the ITS region were carried out according to a previously described protocol to identify the fungal strain (GenBank accession number MF946558).[21] The strain was preserved at −80 °C in the laboratory of one of the authors (P.P.).

3.3. Cultivation and extraction

The fungal strain was grown on solid rice medium under static conditions at room temperature in ten 1 L Erlenmeyer flasks (each flask containing 100 g rice and 100 mL water followed by autoclaving) for 21 days. Exhaustive overnight extraction of the fungal culture with EtOAc (3 × 500 mL for each flask) followed by filtration and concentration of the obtained extract by evaporation of the solvent under reduced pressure yielded the crude EtOAc extract (9.8 g). Cultivation of the fungal strain on white beans was carried out in two 1 L Erlenmeyer flasks (each flask containing 100 g white beans and 100 mL water followed by autoclaving) at room temperature under static conditions for 21 days. Exhaustive overnight extraction with EtOAc (3 × 500 mL) followed by filtration and solvent evaporation under vacuum afforded the crude EtOAc extract (2.2 g).

3.4. Isolation of compounds

The crude EtOAc extract from rice cultures was subjected to liquid-liquid fractionation by suspending it in 90% MeOH and partitioning it against *n*-hexane to yield 7.3 g of *n*-hexane fraction, 1.4 g of MeOH fraction and a residue which was insoluble in both phases. Part of this residue was purified on a silica column using CH_2Cl_2-MeOH (95:5) as

solvent to afford **4** (6.4 mg). The MeOH fraction was fractionated by vacuum liquid chromatography (VLC) on silica gel using CH_2Cl_2-MeOH as solvent system with gradient elution to give nine fractions (Fr.R1–Fr.R9). Fr.R5 (99 mg) was further chromatographed on a Sephadex LH-20 column using MeOH as mobile phase followed by purification using semi-preparative HPLC to yield **5** (0.9 mg). Fr.R6 (160 mg) was purified by semi-preparative HPLC with a gradient of MeOH-H_2O as solvent system to afford **1** (6.6 mg), **2** (1.2 mg) and **3** (2.1 mg). Fr.R9 was purified by washing with MeOH several times to give **6** (0.7 mg) as a precipitate.

The crude EtOAc extract obtained from the beans culture was subjected to vacuum liquid chromatography (VLC) on silica gel using gradient elution of *n*-hexane-EtOAc followed by CH_2Cl_2-MeOH to afford seven fractions (Fr.B1–Fr.B9). Fr.B4 (308.7 mg) was subjected to a Sephadex LH-20 column using CH_2Cl_2-MeOH (1:1) as eluent and then separated by semi-preparative HPLC with MeOH-H_2O as mobile phase to give **7** (2.7 mg) and **8** (2.5 mg).

Cyclo-(Gly-D-Leu-D-*allo*-Ile-L-Val-L-Val-D-Trp-β-Ala) (**1**): brown amorphous solid; $[\alpha]_D^{23}$ −16 (c 1.0, MeOH); UV (MeOH) λ_{max} 281 and 220 nm; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS $[M + H]^+$ *m/z* 739.4510 (calcd for $C_{38}H_{59}N_8O_7$, 739.4501).

Cyclo-(Gly-D-Leu-L-Val-L-Val-L-Val-D-Trp-β-Ala) (**2**): brown amorphous solid; $[\alpha]_D^{23}$ −15 (c 0.1, MeOH); UV (MeOH) λ_{max} 281 and 219 nm;^{1}H and ^{13}C NMR data, see Table 1; HRESIMS $[M + Na]^+$ *m/z* 747.4165 (calcd for $C_{37}H_{56}N_8NaO_7$, 747.4164).

Cyclo-(Gly-D-Leu-D-*allo*-Ile-D-*allo*-Ile-L-Val-D-Trp-β-Ala) (**3**): brown amorphous solid; $[\alpha]_D^{23}$ −15 (c 0.5, MeOH); UV (MeOH) λ_{max} 281 and 219 nm; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS $[M + Na]^+$ *m/z* 775.4478 (calcd for $C_{39}H_{60}N_8NaO_7$, 775.4477).

Clonostalactam (**7**): white powder; $[\alpha]_D^{20}$ −31 (c 0.1, MeOH); UV (MeOH) λmax 232.0, 290.9(sh); ^{1}H and ^{13}C NMR data see Table 2; HRESIMS $[M + H]^+$ *m/z* 256.1548 (calcd for $C_{13}H_{22}NO_4$, 256.1543) and $[M + Na]^+$ *m/z* 278.1367 (calcd for $C_{13}H_{21}NNaO_4$, 278.1363).

3.5. Marfey's analysis

Acid hydrolysis of each of the isolated peptides (0.5 mg) was performed by adding 1 mL of 6 N HCl in addition to 0.4% β-mercaptoethanol (for protection of tryptophan residue) followed by heating at 110 °C for 24 h.[14,22] The resulting solutions were concentrated under vacuum with consecutive addition of H_2O (5 mL each) to ensure complete elimination of HCl. Each acid hydrolysate (25 μL) was treated with 50 μL of FDNPL (1% N-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide in acetone) and 10 μL of 1 M $NaHCO_3$. The mixture was heated for 1 h at 40 °C on a hot plate with frequent mixing. The resulting solutions were allowed to cool. 5 μL of 2 N HCl was added to each solution and then concentrated to dryness. The derivatized amino acid product was then dissolved in 1,000 μL MeOH and submitted for HPLC analysis. Derivatization of the standard amino acids (L- and D-forms) was performed following the same procedure. The retention times of the derivatized peptide hydrolysates were compared with those of standard derivatized amino acids using HPLC analysis.[14,23] For C18 HPLC analysis, a gradient of MeOH and 0.1% HCOOH in H2O [0 min (10% MeOH); 5 min (10% MeOH); 35 min (100% MeOH); 45 min (100% MeOH); 50 min (10%MeOH); 25 °C, 1 mL/min] was employed. To enhance the HPLC resolution of some amino acids residues, a C4 HPLC RP-column [(MeOH, 0.1% HCOOH in H_2O): 0 min (15% MeOH); 2 min (15% MeOH); 180 min (65%MeOH); 180.1 min (100% MeOH); 185 min (100% MeOH), 185.1 min (15% MeOH), 190 min (15% MeOH), 30 °C, 1 mL/min] was also used instead of the commonly used C18 column, in analogy to the C3 Marfey's method.[24]

3.6. Cytotoxicity assay

MTT method was used to test cytotoxicity of all isolated compounds against the L5178Y murine lymphoma cell line. Kahalalide F (IC_{50} 4.3 μM) obtained from *Elysia grandifolia* was used as positive control.[25] The compounds that displayed cytotoxic activity were then submitted to test their activity against the A2780 human ovarian cancer cell line according to the protocols described in the literature.[26] Cisplatin was used as positive control with an IC_{50} value of 2.0 μM.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2019.07.025.

References

1. Choochuay J, Xu X, Rukachaisirikul V, et al. Curvularin derivatives from the soil-derived fungus *Aspergillus polyporicola* PSU-RSPG187. *Phytochem Lett.* 2017;22:122–127.
2. Sun T, Zou J, Chen G, et al. A set of interesting sequoiatones stereoisomers from a wetland soil-derived fungus *Talaromyces flavus*. *Acta Pharm Sin B.* 2017;7:167–172.
3. Phainuphong P, Rukachaisirikul V, Tadpetch K, et al. γ-Butenolide and furanone derivatives from the soil-derived fungus *Aspergillus sclerotiorum* PSU-RSPG178. *Phytochemistry.* 2017;137:165–173.
4. Phainuphong P, Rukachaisirikul V, Saithong S, et al. Lovastatin analogues from the soil-derived fungus *Aspergillus sclerotiorum* PSU-RSPG178. *J Nat Prod.* 2016;79:1500–1507.
5. Akone SH, Rahn S, Henrich B, et al. 2-Pentenedioic acid derivatives from a soil-derived fungus *Gongronella butleri*. *Phytochem Lett.* 2014;10:184–188.
6. Roberti R, Veronesi A, Cesari A, et al. Induction of PR proteins and resistance by the biocontrol agent *Clonostachys rosea* in wheat plants infected with *Fusarium culmorum*. *Plant Sci.* 2008;175:339–347.
7. Morandi MA, Mattos LP, Santos ER, Bonugli RC. Influence of application time on the establishment, survival, and ability of *Clonostachys rosea* to suppress *Botrytis cinerea* sporulation on rose debris. *Crop Prot.* 2008;27:77–83.
8. Cota LV, Maffia LA, Mizubuti ES, Macedo PE, Antunes RF. Biological control of strawberry gray mold by *Clonostachys rosea* under field conditions. *Biol Control.* 2008;46:515–522.
9. Toledo A, Virla E, Humber R, Paradell S, Lastra CL. First record of *Clonostachys rosea* (Ascomycota: Hypocreales) as an entomopathogenic fungus of *Oncometopia tucumana* and *Sonesimia grossa* (Hemiptera: Cicadellidae) in Argentina. *J Invertebr Pathol.* 2006;92:7–10.
10. Baloyi M, Laing M, Yobo K. Use of mixed cultures of biocontrol agents to control sheep nematodes. *Vet Parasitol.* 2012;184:367–370.
11. Dong J-Y, He H-P, Shen Y-M, Zhang K-Q. Nematicidal epipolysulfanyldioxopiperazines from *Gliocladium roseum*. *J Nat Prod.* 2005;68:1510–1513.
12. Dong J-Y, Zhou W, Li L, Li G-H, Liu Y-J, Zhang K-Q. A new epidithiodioxopiperazine metabolite isolated from *Gliocladium roseum* YMF1. 00133. *Chin Chem Lett.* 2006;17:922–924.
13. Zhai M-M, Qi F-M, Li J, et al. Isolation of secondary metabolites from the soil-derived fungus *Clonostachys rosea* YRS-06, a biological control agent, and evaluation of antibacterial activity. *J Agric Food Chem.* 2016;64:2298–2306.
14. Bundi A, Wüthrich K. ^{1}H NMR parameters of the common amino acid residues measured in aqueous solutions of the linear tetrapeptides H-Gly–Gly–X–L-Ala–OH. *Biopolymers.* 1979;18:285–297.
15. Richarz R, Wüthrich K. Carbon-13 NMR chemical shifts of the common amino acid residues measured in aqueous solutions of the linear tetrapeptides H-Gly–Gly–X–L-Ala–OH. *Biopolymers.* 1978;17:2133–2141.
16. Marfey P. Determination of D-amino acids. II. Use of a bifunctional reagent, 1, 5-difluoro-2, 4-dinitrobenzene. *Carlsberg Res Commun.* 1984;49:591.
17. Joshi BK, Gloer JB, Wicklow DT. New Verticillin and glisoprenin analogues from *Gliocladium catenulatum*, a mycoparasite of *Aspergillus flavus* Sclerotia. *J Nat Prod.* 1999;62:730–733.
18. Zheng C-J, Kim Y-H, Kim W-G. Glioperazine B, as a new antimicrobial agent against *Staphylococcus aureus*, and glioperazine C: two new dioxopiperazines from *Bionectra byssicola*. *Biosci Biotechnol Biochem.* 2007;71:1979–1983.
19. Dixit P, Khan MP, Swarnkar G, Chattopadhyay N, Maurya R. Osteogenic constituents from *Pterospermum acerifolium* Willd. flowers. *Bioorg Med Chem Lett.* 2011;21:4617–4621.
20. Evidente A, Sparapano L, Fierro O, Bruno G, Motta A. Sapinofuranones A and B, two new 2 (3*H*)-dihydrofuranones produced by *Sphaeropsis sapinea*, a common pathogen of conifers. *J Nat Prod.* 1999;62:253–256.
21. Kjer J, Debbab A, Aly AH, Proksch P. Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. *Nat Protoc.* 2010;5:479.
22. Ng L, Pascaud A, Pascaud M. Hydrochloric acid hydrolysis of proteins and determination of tryptophan by reversed-phase high-performance liquid

chromatography. *Anal Biochem.* 1987;167:47–52.
23. Ibrahim SR, Min CC, Teuscher F, et al. Callyaerins A-F and H, new cytotoxic cyclic peptides from the Indonesian marine sponge *Callyspongia aerizusa*. *Bioorg Med Chem.* 2010;18:4947–4956.
24. Vijayasarathy S, Prasad P, Fremlin LJ, et al. C3 and 2D C3 Marfey's methods for amino acid analysis in natural products. *J Nat Prod.* 2016;79:421–427.
25. Ashour M, Edrada R, Ebel R, et al. Kahalalide derivatives from the Indian sacoglossan mollusk *Elysia grandifolia*. *J Nat Prod.* 2006;69:1547–1553.
26. Engelke LH, Hamacher A, Proksch P, Kassack MU. Ellagic acid and resveratrol prevent the development of cisplatin resistance in the epithelial ovarian cancer cell line A2780. *J Cancer.* 2016;7:353.