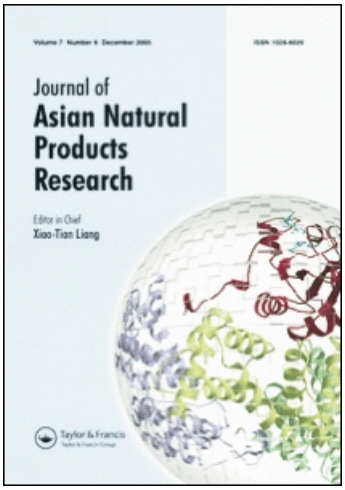

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ORIGINAL ARTICLE

Minor constituents from the tubers of *Gymnadenia conopsea*

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Four new minor constituents including two cyclodipeptides (**1** and **2**) and two cyclopentene derivatives (**3** and **4**), together with four known cyclodipeptides, have been isolated from an ethanolic extract of the tubers of *Gymnadenia conopsea*. Their structures including absolute configurations were determined by spectroscopic data interpretation combined with chemical methods. Among them, compound **1** contains an abnormal *S*-(4″-hydroxybenzyl)cysteine residue, **3** and **4** possess [(4-methylcyclopentyl)methyl]benzene and (4-hydroxymethylcyclopentyl)benzene carbon skeletons, respectively, both of which are first found from the natural source.

Keywords: *Gymnadenia conopsea*; Orchidaceae; cyclodipeptides; cyclopentene derivatives

1. Introduction

Gymnadenia conopsea R. Br. is a plant belonging to the Orchidaceae family and is distributed in Tibet, Xinjiang, Inner Mongolia, Sichuan, Qinghai, and the Gansu Provinces of China. The dried tubers of this plant, other species of this genus, and the genus *Coeloglossum*, known as 'Wangla' (Chinese name), have long been used as a traditional Tibetan remedy to treat coughs, asthma, and syndromes, and as a tonic in Chinese folk medicine, with multiple indications such as invigorating vital energy, promoting the production of body fluids, having a tranquilizing effect, and enhancing intelligence [1–3]. We previously reported that an ethanolic extract of the tubers of *G. conopsea* collected in Sichuan Province was active in improving impaired memory in mice caused by scopolamine and cycloheximide, and from the extract, 20 glycosidic constituents including four new 4-diglycosyloxybenzyl alcohol derivatives and six new bis- or mono-(4-glycosyloxybenzyl)-2-isobutyltartrate derivatives were isolated [4]. Continuing our investigation of less polar fractions from the same material, we report herein the isolation and structural elucidation of four new minor constituents including two cyclodipeptides (**1** and **2**) and two cyclopentene derivatives (**3** and **4**), together with four known cyclodipeptides identified as cyclo(L-Leu-L-Tyr) [5], cyclo(L-Leu-L-Pro) [6], cyclo(L-Val-L-Tyr), and cyclo(L-Ala-D-Phe) [7] by comparing with corresponding literature data. Among them, compound **1** contains an

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abnormal *S*-(4″-hydroxybenzyl)cysteine residue, **3** and **4** possess [(4-methylcyclopentyl)methyl]benzene and (4-hydroxyme thylcyclopentyl)benzene carbon skeletons, respectively, both of which are first found from the natural source (Figure 1).

2. Results and discussion

The ethanolic extract of the dried tubers of *G. conopsea* was suspended in water and then partitioned with EtOAc. The aqueous solution was chromatographed successively over macroporous resin, silica gel, and reversed-phase medium-pressure liquid chromatography and preparative HPLC to yield **1**–**4**.

Compound **1**, a white amorphous powder with $[\alpha]_D^{20}$ +35.7 ($c = 0.56$, MeOH), showed the presence of hydroxyl and/or amino (3340 and 3215 cm^{-1}), amide carbonyl (1677 cm^{-1}), and aromatic ring (1610, 1596, and 1515 cm^{-1}) functional groups in its IR spectrum. The (+)-ESI-MS displayed a quasi-molecular ion peak at *m/z* 289 $[M + Na]^+$, and (+)-HR-ESI-MS at *m/z* 289.06232 $[M + Na]^+$ indicated the molecular formula of **1** as $C_{12}H_{14}N_2O_3S$, with seven degrees of unsaturation. The ^1H NMR spectrum in MeOH-d_4 showed characteristic resonances due to a 4-hydroxyphenyl unit at δ 7.08 (2H, d, $J = 8.4$ Hz, H-2″ and H-6″) and 6.66 (2H, d, $J = 8.4$ Hz, H-3″ and H-5″). In addition, it displayed two AB spin coupling systems assignable to two isolated and deshielded methylenes at δ 3.68 and 3.63 (each 1H, d, $J = 12.8$ Hz, H-7″a and H-7″b) and δ 4.06 and 3.83 (each 1H, d, $J = 18.0$ Hz, H-2a and H-2b), as well as an ABX spin coupling system attributed to a tri-substituted ethyl unit (CH_2CH) at δ 2.97 (1H, dd, $J = 14.4$, 4.4 Hz, H-3′a), 2.76 (1H, dd, $J = 14.4$, 3.6 Hz, H-3′b), and 4.12 (1H, dd, $J = 4.4$, 3.6 Hz, H-2′). In addition to carbon resonances corresponding to the above units (see Section 3), the ^{13}C NMR and DEPT spectra of **1** showed resonances due to two amide carbonyls at δ 169.4 (C-1′) and 168.7 (C-1) which were consistent with the strong IR absorption at 1677 cm^{-1}. Combined analysis of these spectroscopic data suggested that **1** was a cyclodipeptide containing glycine, cysteine, and 4″-hydroxybenzyl units, which was further supported by the amino acid analysis indicating the presence of glycine and cysteine in the acid hydrolyzate of **1**. The structure of **1** was finalized by the 2D NMR spectroscopic data analysis. The resonances of the protonated carbons and their corresponding protons in the NMR spectra were assigned unambiguously by the HSQC spectroscopic data analysis of **1**. In the HMBC spectrum, correlations of H_2-2/C-1,

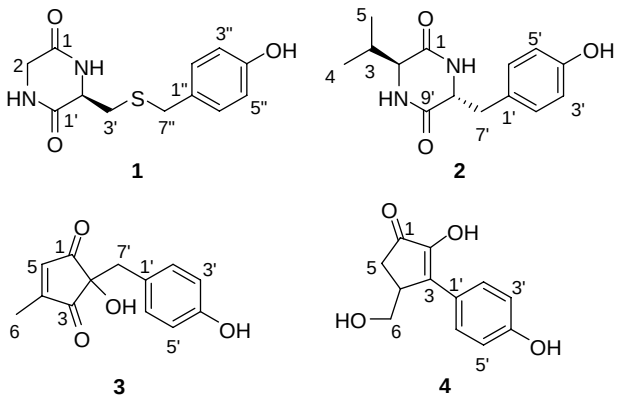

Figure 1. The structures of compounds **1**–**4**.

H_2-3′/C-1′ and C-2′, H-2′/C-1′, H-2″ and H-6″/C-4″ and C-7″, H-3″ and H-5″/C-1″, and H_2-7″/C-2″ and C-6″ (Figure 2), in combination with the shifts and coupling patterns of these protons and carbons, confirmed the presence of glycine, cysteine, and 4″-hydroxybenzyl residues in **1**. In addition, correlations of H_2-3′ with C-7″ and H_2-7″ with C-3′ indicated unequivocally that 4″-hydroxybenzyl was located at the sulfur atom of the cysteine unit to form an abnormal amino acid residue, *S*-(4″-hydroxybenzyl)cysteine. Meanwhile, correlations of C-1 with H-2′ and C-1′ with H_2-2 revealed a cyclodipeptide connection of the glycine and *S*-(4″-hydroxybenzyl)cysteine residues. By comparing the $[\alpha]_D^{20}$ value of **1** with those of 3-[1-(4-methoxybenzylsulfanyl)-1-methylethyl]piperazine-2,5-dione and cyclo(Gly-L-Cys) [8,9], the absolute configuration of the *S*-(4″-hydroxybenzyl)cysteine residue in **1** was proposed to be L. This was confirmed by Marfey's analysis of the acid hydrolyzate of **1** [10]. The acid hydrolyzate of **1** was treated with Marfey's reagent, (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA), and the resulting FDAA derivatives were analyzed by reversed-phase HPLC. Peaks in the chromatogram were identified by comparing the retention times with those of the FDAA derivatives of authentic amino acids. Marfey's reagent derivative of the amino acids liberated from **1** showed peaks matching L-cysteine. Therefore, the structure of **1** was elucidated as cyclo[glycine-L-*S*-(4″-hydroxybenzyl)cysteine].

Compound **2** was obtained as a white amorphous powder with $[\alpha]_D^{20}$ +39.4 ($c = 0.22$, MeOH). The (+)-ESI-MS displayed a quasi-molecular ion peak at *m/z* 263 $[M + H]^+$. The ^{1}H NMR spectrum of **2** in DMSO-d_6 showed diagnostic resonances for a 4-hydroxyphenyl unit at δ 6.94 (2H, d, $J = 8.5$ Hz, H-2′ and H-6′) and 6.61 (2H, d, $J = 8.5$ Hz, H-3′ and H-5′), and an isopropyl unit at δ 1.40 (1H, m, H-3) and 0.59 (6H, d, $J = 7.0$ Hz, H_3-4 and H_3-5), in addition to resonances attributable to two nitrogen-bearing methines at δ 4.10 (1H, m, H-8′) and 3.54 (1H, m, H-2) and a deshielded methylene attached to one of the methines at δ 3.03 (1H, dd, $J = 14.0$, 4.0 Hz, H-7′a) and 2.72 (1H, dd, $J = 14.0$, 4.5 Hz, H-7′b). It also showed exchangeable resonances assignable to a phenolic hydroxyl group at δ 9.22 (1H, s) and two amide protons at δ 8.01 and 7.84 (each 1H, s). These data are very similar to those of the co-occurring cyclo(L-Val-L-Tyr) [7]. However, the chemical shifts for the two methyls of the valine residue of **2** were shifted significantly by $\Delta\delta$ −0.09 and +0.26 ppm, respectively, as compared

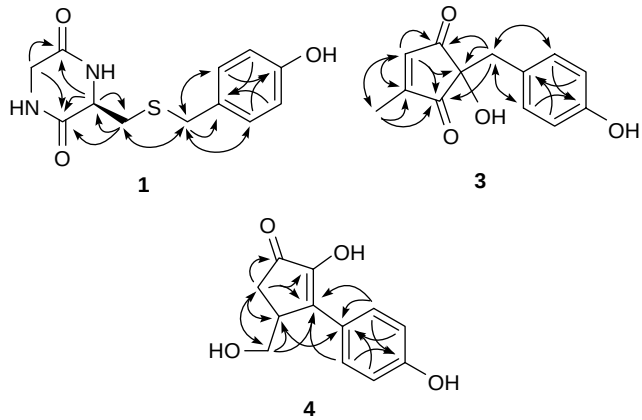

Figure 2. Important HMBC correlations (→) of compounds **1**, **3**, and **4**.

with those of cyclo(L-Val-L-Tyr). This indicated that compound **2** is a stereoisomer of cyclo(L-Val-L-Tyr), which was supported by the optical rotation data, and confirmed by Marfey's analysis of the acid hydrolyzate of **2** using the same procedure as described above. Marfey's reagent derivative of the amino acids liberated from **2** showed peaks matching L-valine and D-tyrosine, respectively. Thus, the structure of **2** was elucidated as cyclo(L-Val-D-Tyr).

Compound **3** was obtained as a yellowish gum with $[\alpha]_D^{20} \approx 0$ ($c = 0.12$, $CHCl_3$). The IR spectrum showed absorption bands for hydroxyl (3392 cm^{-1}), carbonyl (1750 and 1700 cm^{-1}), and aromatic ring (1612 and 1515 cm^{-1}) groups. The (+)-ESI-MS spectrum of **3** exhibited a quasi-molecular ion peak at *m/z* 255 $[M + Na]^+$, and (+)-HR-ESI-MS at *m/z* 255.0679 $[M + Na]^+$ indicated a molecular formula $C_{13}H_{12}O_4$ for **3**. The ^1H NMR spectrum of **3** in $CDCl_3$ displayed resonances attributed to a 4′-hydroxyphenyl group at δ 6.83 (2H, d, $J = 8.0$ Hz, H-2′ and H-6′) and 6.68 (2H, d, $J = 8.0$ Hz, H-3′ and H-5′), and a tri-substituted double bond at δ 6.82 (1H, br s, H-5). In addition, it showed two singlets ascribed to a methylene at δ 3.07 (2H, H_2-7′) and an olefinic tertiary methyl at δ 1.94 (3H, H_3-6), respectively, as well as two broadened exchangeable singlets due to two hydroxyl groups at δ 4.90 and 2.69 (each 1H, br s). In addition to the carbon signals corresponding to the above units (Table 1), the ^{13}C NMR and DEPT spectra indicated the presence of an oxygen-bearing quaternary carbon at δ 75.6 (C-2) and two conjugated carbonyls at δ 201.5 (C-1) and 203.1 (C-3) in **3**. Based on the above spectroscopic data, a structure of cyclopentenedione derivative with substituents of 4′-hydroxybenzyl, hydroxyl, and methyl groups was proposed for **3**. The structure of **3** was constructed finally by HSQC and HMBC data analysis. Furthermore, the resonances of the protonated carbons and their bonded protons in the NMR spectra (Table 1) were assigned unambiguously by the HSQC experiment. In the HMBC spectrum, two- and three-bond correlations of H-5 with C-1, C-2, C-3,

Table 1. ^1H and ^{13}C NMR spectroscopic data (δ) of compounds **3** and **4**[a].

	3		**4**	
Position	δ_H	δ_C	δ_H	δ_C
1		201.5		202.4
2		75.6		148.8
3		203.1		139.7
4		161.5	3.32 m	37.7
5	6.82 s	144.4	a: 2.46 dd ($J = 18.0, 6.0$) b: 2.25 d ($J = 18.0$)	36.5
6	1.94 s	11.5	a: 3.64 dd ($J = 10.5, 3.0$) b: 3.19 dd ($J = 10.5, 6.0$)	63.8
1′		125.2		125.1
2′	6.83 d ($J = 8.0$)	131.0	7.68 d ($J = 8.5$)	129.9
3′	6.68 d ($J = 8.0$)	115.6	6.81 d ($J = 8.5$)	115.6
4′		155.8		158.1
5′	6.68 d ($J = 8.0$)	115.6	6.81 d ($J = 8.5$)	129.9
6′	6.83 d ($J = 8.0$)	131.0	7.68 d ($J = 8.5$)	115.6
7′	3.07 s	41.3		
4′-OH	4.90 br s		9.60 br s	
2-OH	2.69 br s		4.65 br s	

Notes: [a] Data were measured at 500 MHz for ^1H NMR and at 125 MHz for ^{13}C NMR, in $CDCl_3$ for **3** and in DMSO-d_6 + D_2O for **4**. Proton coupling constants (J) in Hz are given in parentheses. The assignments were based on HSQC and HMBC experiments.

C-4, and C-6 and of H_3-6 with C-3, C-4, and C-5 (Figure 2), in combination with the chemical shifts of these protons and carbons, revealed that **3** had a parent structure moiety of 2-hydroxy-4-methylcyclopent-4-ene-1,3-dione. In addition, HMBC correlations of H-3′ and/or H-5′ with C-1′, C-2′, C-4′, and C-6′; H-2′ and/or H-6′ with C-1′, C-3′, C-4′, C-5′, and C-7′; and H_2-7′ with C-1′, C-2′, and C-6′, together with chemical shifts of these protons and carbons, proved the presence of the 4′-hydroxybenzyl group in **3**. Meanwhile, HMBC correlations of H_2-7′ with C-1, C-2, and C-3 demonstrated unequivocally that the 4′-hydroxybenzyl group was located at C-2 of the parent structure. Therefore, the structure of **3** was elucidated as 2-hydroxy-2-(4′-hydroxybenzyl)-4-methylcyclopent-4-ene-1,3-dione. Because of the optical inactive nature and no Cotton effect in the CD spectrum of **3**, it is considered to be a racemate.

Compound **4**, a yellowish amorphous powder, $[\alpha]_D^{20} \approx 0$ ($c = 0.17$, MeOH), showed IR absorption bands for hydroxyl (3365 cm^{-1}), conjugated carbonyl (1677 cm^{-1}), and aromatic ring (1605 and 1515 cm^{-1}) groups. The (−)-ESI-MS of **4** exhibited a quasi-molecular ion peak at *m/z* 219 $[M - H]^-$, and (−)-HR-ESI-MS at *m/z* 219.0687 $[M - H]^-$ indicated the molecular formula of **4** as $C_{12}H_{12}O_4$. The ^{1}H NMR spectrum of **4** in DMSO-d_6 showed resonances assignable to a methine at δ 3.32 (1H, m, H-4), which was coupled with two methylenes at δ 2.46 (1H, dd, $J = 18.0$, 6.0 Hz, H-5a) and 2.25 (1H, d, $J = 18.0$ Hz, H-5b) and at δ 3.64 (1H, dd, $J = 10.5$, 3.0 Hz, H-6a) and 3.19 (1H, dd, $J = 10.5$, 6.0 Hz, H-6b). In addition, the ^{1}H NMR spectrum showed resonances attributed to a 4′-hydroxyphenyl unit at δ 7.68 (2H, d, $J = 8.5$ Hz, H-2′ and H-6′) and 6.81 (2H, d, $J = 8.5$ Hz, H-3′ and H-5′), along with resonances for three exchangeable hydroxyl protons at δ 9.60 (2H, br s) and 4.65 (1H, br s). In addition to the carbon signals corresponding to the above units, the ^{13}C NMR and DEPT spectra of **4** displayed a pair of olefinic quaternary carbons at δ 148.8 (C-2) and 139.7 (C-3) and a conjugated carbonyl carbon at δ 202.4 (C-1). The above spectroscopic data indicated that **4** was a cyclopentenone derivative substituted by hydroxyl, hydroxymethyl, and 4′-hydroxyphenyl groups. The structure of **4** was constructed further by HSQC and HMBC data analysis. In particular, in the HMBC spectrum of **4**, two- and three-bond correlations of H-4 with C-1, C-2, C-3, C-5, and C-6; H_2-5 with C-1, C-2, C-3, C-4, and C-6; and H_2-6 with C-3, C-4, and C-5 (Figure 2), together with the chemical shifts of these protons and carbons, demonstrated a parent structure of 2-hydroxyl-4-hydroxymethylcyclopent-2-enone for **4**. Meanwhile, HMBC correlations of H-2′ and H-6′ with C-3 and C-4′ and H-3′ and H-5′ with C-1′ and C-4′, in combination with their chemical shifts, revealed the presence of the 4′-hydroxyphenyl located at C-3 of the parent structure. Therefore, the structure of **4** was elucidated as 2-hydroxy-4-hydroxymethyl-3-(4′-hydroxyphenyl)cyclopent-2-enone. Because of optical inactivity and no Cotton effect observed in the CD spectrum of **4**, it is also considered to be a racemate.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a PE model 343 polarimeter. UV spectra were measured on a Cary 300 spectrometer. IR spectra were recorded on a Nicolet 5700 FT-IR microscope spectrometer (FT-IR microscope transmission). 1D and 2D NMR spectra were obtained at 400 or 500 MHz for ^{1}H, and 100 or 125 MHz for ^{13}C, respectively, on INOVA 400 and 500 MHz spectrometers in MeOH-d_4, $CDCl_3$, DMSO-d_6, or DMSO-d_6 + D_2O with solvent peaks as references. ESI-MS data were measured with a Q-Trap LC/MS/MS (Turbo Ionspray source) spectrometer. HR-ESI-MS data were measured

using an AccuToFCS JMS-T100CS spectrometer. Column chromatography was performed with silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, China) and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden). HPLC separation was performed on an instrument consisting of a Waters 600 controller, a Waters 600 pump, and a Waters 2487 dual λ absorbance detector, with an Alltima (250 mm × 10 mm i.d.) preparative column packed with C_{18} (5 μm). TLC was carried out with precoated silica gel GF254 glass plates. Spots were visualized under UV light or by spraying with 7% H_2SO_4 in 95% EtOH followed by heating. Amino acids were analyzed using a Hitachi model L-8500 amino acid analyzer equipped with a column (number 2622: 4.6 × 40 mm; Hitachi, Tokyo, Japan). Marfey's analysis was performed on an Agilent HP1100 HPLC system, with an Alltima (250 × 4.6 mm i.d.) column packed with C_{18} (5 μm).

3.2 Plant materials

The tubers of *G. conopsea* were collected at Ganzi, Sichuan Province, China in September 2005. The plant identification was verified by Prof. Lin Ma (Institute of Materia Medica). A voucher specimen (No. 05916) has been deposited at the Herbarium of the Department of Medicinal Plants, Institute of Materia Medica.

3.3 Extraction and isolation

The air-dried tubers of *G. conopsea* (16.2 kg) were powdered and extracted with 20.0 liters of 75% EtOH at room temperature for 4 × 42 h. The EtOH extract was evaporated under reduced pressure at <40°C to yield a residue (618.2 g). The residue was suspended in H_2O (2000 ml) and then partitioned with EtOAc (5 × 1200 ml). The aqueous phase was applied to an HP-20 macroporous adsorbent resin (1500 g) column. Successive elution of the column with H_2O, 15% EtOH, 40% EtOH, and 95% EtOH (5000 ml each) yielded four fractions after removing the solvents. The active fraction (100.2 g) eluted by 40% EtOH was suspended in H_2O (500 ml) and then partitioned with *n*-BuOH (5 × 400 ml) to give H_2O and *n*-BuOH fractions. The *n*-BuOH fraction (50 g) was separated by medium-pressure liquid chromatography over reversed-phase silica gel eluting with a gradient of increasing MeOH (0–90%) in H_2O to give five fractions (A–E) on the basis of TLC analysis. Fraction E (2.05 g) was separated by normal-phase silica gel column chromatography, eluting with a gradient of increasing MeOH (0–100%) in $CHCl_3$, afforded nine subfractions (E_1–E_9). Subfraction E_7 (153 mg) was subjected to column chromatography over Sephadex LH-20, using petroleum ether–$CHCl_3$–MeOH (5:5:1) as the eluting solvent, to afford **1** (21 mg) and **2** (2.1 mg). Separation of fraction E_1 (272 mg) on normal-phase silica gel column chromatography, eluting with petroleum ether–EtOAc (3:1), afforded **3** (37 mg). Subfraction E_4 (353 mg) was chromatographed over silica gel column chromatography using $CHCl_3$–MeOH (20:1) as the eluting solvent and purified further by Sephadex LH-20, eluting with $CHCl_3$–MeOH (1:1), to afford **4** (46 mg).

3.3.1 *Cyclo[glycine-L-S-(4″-hydroxybenzyl)cysteine] (**1**)*

A white amorphous powder; $[\alpha]_D^{20}$ +35.7 ($c = 0.56$, MeOH); UV (MeOH) λ_{max} (log ε): 229 (3.59), 279 (0.69) nm; IR ν_{max}: 3340, 3215, 3074, 2915, 1677, 1610, 1595, 1515, 1470, 1332, 1262, 1237, 1071, 816 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4): δ 7.08 (2H, d, $J = 8.4$ Hz, H-2″ and H-6″), 6.66 (2H, d, $J = 8.4$ Hz, H-3″ and H-5″), 4.12 (1H, dd, $J = 4.4$ and 3.6 Hz, H-2′), 4.06 (1H, d, $J = 18.0$ Hz, H-2a), 3.83 (1H, d, $J = 18.0$ Hz, H-2b), 3.68 (1H, d, $J = 12.8$ Hz, H-7″a) and 3.63 (1H, d, $J = 12.8$ Hz, H-7″b), 2.97 (1H, dd,

$J = 14.4$ and 4.4 Hz, H-3′a), 2.76 (1H, dd, $J = 14.4$ and 3.6 Hz, H-3′b); ^{13}C NMR (100 MHz, MeOH-d_4): δ 169.4 (s, C-1′), 168.7 (s, C-1), 157.7 (s, C-4″), 131.3 (d, C-2″), 131.3 (d, C-6″), 129.9 (s, C-1″), 116.3 (d, C-3″), 116.3 (d, C-5″), 56.5 (d, C-2′), 45.7 (d, C-2), 37.6 (t, C-7″), 37.0 (t, C-3′); (+)-ESI-MS: *m/z* 267 $[M + H]^+$ and 289 $[M + Na]^+$; (+)-HR-ESI-MS: *m/z* 289.06232 $[M + Na]^+$ (calcd for $C_{12}H_{14}NaN_2O_3S$, 289.06228).

3.3.2 Cyclo(L-Val-D-Tyr) (2)

A white amorphous powder; $[\alpha]_D^{20}$ +39.4 ($c = 0.22$, MeOH); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.22 (1H, s, HO-4′), 8.01 (1H, s, NH), 7.84 (1H, s, NH), 6.94 (2H, d, $J = 8.5$ Hz, H-2′ and H-6′), 6.61 (2H, d, $J = 8.5$ Hz, H-3′ and H-5′), 4.10 (1H, m, H-8′), 3.54 (1H, m, H-2), 3.03 (1H, dd, $J = 14.0$, 4.0 Hz, H-7′a), 2.72 (1H, dd, $J = 14.0$, 4.5 Hz, H-7′b), 1.40 (1H, m, H-3), 0.59 (6H, d, $J = 7.0$ Hz, H_3-4 and H_3-5); (+)-ESI-MS: *m/z* 263 $[M + H]^+$.

3.3.3 2-Hydroxy-2-(4′-hydroxybenzyl)-4-methylcyclopent-4-ene-1,3-dione (3)

A yellowish gum; $[\alpha]_D^{20} \approx 0$ ($c = 0.12$, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 227 (4.37), 270 (0.75) nm; IR ν_{max}: 3392, 2920, 1750, 1700, 1670, 1612, 1515, 1441, 1377, 1313, 1236, 1174, 1114, 1070, 1028, 930, 896, 836, 790, 754 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data: see Table 1; (+)-ESI-MS: *m/z* 255 $[M+Na]^+$; (+)-HR-ESI-MS: *m/z* 255.0679 $[M+Na]^+$ (calcd for $C_{13}H_{12}NaO_4$, 255.0633).

3.3.4 2-Hydroxy-4-hydroxymethyl-3-(4′-hydroxyphenyl)cyclopent-2-enone (4)

A yellowish amorphous powder; $[\alpha]_D^{20} \approx 0$ ($c = 0.17$, MeOH); UV (MeOH) λ_{max} (log ε): 227 (0.66), 325 (1.52) nm; IR ν_{max}: 3365, 1677, 1605, 1515, 1444, 1386, 1275, 1244, 1176, 1129, 1048, 1025, 1000, 827, 765 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6 + D_2O) data: see Table 1; positive-mode ESI-MS: *m/z* 243 $[M + Na]^+$; (−)-ESI-MS: *m/z* 219 $[M - H]^-$; (−)-HR-ESI-MS: *m/z* 219.0687 $[M - H]^-$ (calcd for $C_{12}H_{11}O_4$, 219.0657).

3.3.5 Acid hydrolysis of 1 and 2, and Marfey's analyses of the hydrolyzates

Compound **1** (1.6 mg) or compound **2** (0.9 mg) was dissolved in 6 mol/l HCl (0.5 ml), and heated at 110°C in a sealed vial for 13 h. The cooled reaction mixture was evaporated to dryness under reduced pressure, and HCl was removed from the residue of the acid hydrolyzate by repeated evaporation from frozen water (1.5 ml). The amino acid mixture was dissolved in water (40 μl), and then treated with 1% FDAA in acetone (100 μl) and 6% triethylamine (Et_3N) in 50 μl of water at 40°C for 1 h. After cooling to room temperature, the derivative and the standard amino acid samples were derivatized in the same manner as the acid hydrolyzate above (1% FDAA and 6% Et_3N), were filtered, and the filtrates were analyzed by HPLC (Alltima RP-C_{18}, 5 μm, 250 × 4.6 mm, flow rate: 1 ml/min, UV detection at 340 nm, mobile phase: 35% CH_3CN/H_2O + 1% HOAc). The FDAA derivatives of amino acids liberated from **1** showed a peak at 9.21 min, matching the retention time of FDAA L-cysteine (9.17 min; 12.14 min for D−); those liberated from **2** showed peaks at 14.81 and 12.79 min, matching the retention times of FDAA L-valine (14.86 and 26.83 min for D−) and D-tyrosine (12.85 and 10.24 min for L−).

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