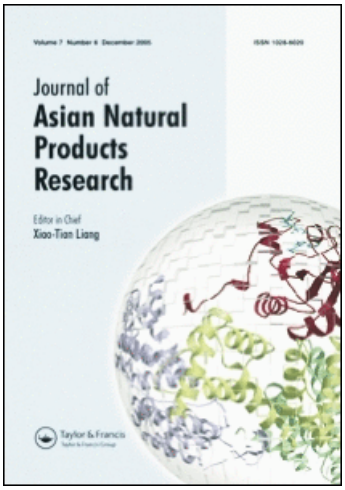

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TWO NEW METABOLITES FROM THE MANGROVE ENDOPHYTIC FUNGUS NO. 2524

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Two new metabolites, the cyclo-(L-Phe-L-Leu[1]-L-Leu[2]-L-Leu[3]-L-Ile) (**1**) and (3*S*,4*R*)-dihydroxy-(6*S*)-undecyl-α-pyranone (**2**) have been produced by the endophytic fungus no. 2524 isolated from a seed of mangrove *Avicennia marina* in Hong Kong. The structures have been elucidated by spectra including two-dimensional NMR, ESI tandem mass spectrometry and CD. These compounds show no activity toward human cancer cell lines Bel-7402, NCI-4460 and the normal human cell lines L-02.

Keywords: *Avicennia marina*; Endophytic fungus; Metabolites; Cyclo-(L-Phe-L-Leu[1]-L-Leu[2]-L-Leu[3]-L-Ile); (3*S*,4*R*)-Dihydroxy-(6*S*)-undecyl-α-pyranone

INTRODUCTION

The mangrove habitat has proved to be a rich source of new fungal species, and these now form the second largest ecological sub-group of marine fungi. However, mangrove endophytic fungi have been little investigated. Endophytes are defined as fungi colonizing healthy plant tissue without causing overt symptoms in or apparent injury to the host. Research on the metabolites of endophytes can reveal the relationship between them and their hosts. In our search for secondary metabolites of mangrove endophytic fungi, many cytotoxic and/or novel compounds have been isolated [1–4]. We report here the isolation and structures of two new metabolites from the fungus (strain no. 2524), which was an unidentified endophytic fungus separated from a seed of *Avicennia marina* (Forsk.) Vierh from the mangrove of Hong Kong. The endophyte was a nonsporulating isolate; various methods failed to induce sporulation. *A. marina* is a dominant mangrove plant in the subtropical mangroves in Hong Kong. Its fruits are used as a Chinese medicinal herb to treat diabetes and as supplementary sources of food for humans.

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RESULTS AND DISCUSSION

The fungus was grown in GPY broth (glucose 1%, peptone 0.5%, yeast extract 0.5%, pH 7.5, sea water 20%). The growth culture was filtered, and the mycelium and the culture filtrate were then extracted with methanol and ethyl acetate respectively. The mycelium extracts (66 g) were subjected to silica gel column chromatography followed by C-18 reversed-phase HPLC to give a new cyclic pentapeptide **1** (30 mg) and a new δ-lactone **2** (40 mg). The culture extracts (28 g) afforded eight known compounds: 2-butyl-3-methylsuccinic acid, triacetic acid lactone, glycerol monooleate, cyclo-(Tyr-Leu), cyclo-(Phe-Ala), cyclo-(Ala-Val), cyclo-(Pro-Gly) and 5-isobutyl-2,4-imidazolidinedione.

The cyclic pentapeptide **1** was obtained as a colourless amorphous power, $[\alpha]_D^{20} = -56$ (*c* 0.13, MeOH). The molecular formula was established as $C_{33}H_{53}N_5O_5$ by FAB-MS, LC-MS^n, elemental analysis and NMR data. The ^{1}H and ^{13}C NMR spectra for **1** have resonances typical of a cyclic peptide (Table I). In the ^{13}C NMR spectrum recorded in DMSO-d_6, five carbonyl resonances, at δ 171.6, 171.5, 171.4, 171.1 and 171.0, suggest that the molecule is a pentapeptide. The peptide nature of the molecule is further supported by the IR spectrum (3306, 1657 and 1635 cm^{-1}), the presence of five NH protons in the ^{1}H NMR spectrum (δ_H 8.58, 8.46, 8.33, 7.95, 7.23) and five α-CH signals (δ_C 62.8, δ_H 3.28; δ_C 52.7, δ_H 4.46; δ_C 52.1, δ_H 4.30; δ_C 51.9, δ_H 4.08; δ_C 51.8, δ_H 4.17).

Analysis of the NMR data clearly reveals one phenylalanine, one isoleucine and three leucine residues. The sequence of the cyclic peptide was established by analysis of HMBC data, which showed the correlations of the carbonyl carbons of each amino acid with the corresponding NH proton of the adjacent residue, e.g. the correlation of the carbonyl carbon (δ_C 171.4) at Leu^1 to the NH (δ_H 8.46) at Leu^2. Other principal HMBC correlations are: the carbonyl carbon (δ_C 171.5) at Leu^2 to the NH (δ_H 7.23) at Leu^3, the carbonyl carbon (δ_C 171.1) at Leu^3 to the NH (δ_H 8.33) at Ile, the carbonyl carbon (δ_C 171.0) at Ile to the NH (δ_H 7.95) at Phe, and the carbonyl carbon (δ_C 171.6) at Phe to the NH (δ_H 8.58) at Leu^1 (Fig. 1). Based on these observations, the structure of **1** was established as cyclo-(L-Phe-L-Leu^1-L-Leu^2-L-Leu^3-L-Ile) (Fig. 2).

The sequence was further confirmed by tandem mass spectrometry (ESI-MS^n) data (Fig. 3). The primary linear acylium ion, produced by the ring-opening of protonated cyclo-(L-Phe-L-Leu^1-L-Leu^2-L-Leu^3-L-Ile), was selected from the primary product-ion spectrum and subjected to multiple stages of collisionally activated decomposition (CAD). Amino-acid residues were sequentially removed, one at each stage of the CAD, from the C-terminus [5].

The absolute stereochemistry of the amino acids in **1** was determined by acid hydrolysis followed by chiral HPLC analysis. The hydrolysis sample gave three peaks with retention times of 5.59, 10.34 and 19.26 min respectively. When co-injected with appropriate standards, L-isoleucine, L-leucine and L-phenylalanine, these peaks and their retention times are unchanged. Furthermore, no more peaks appeared. In contrast, the retention times of D-isoleucine, D-leucine and D-phenylalanine are 4.83, 6.17 and 14.11, respectively. Thus, all the amino acids in compound **1** possess L-configurations. Therefore the structure of **1** is cyclo-(L-Phe-L-Leu^1-L-Leu^2-L-Leu^3-L-Ile).

Compound **2** was obtained as a colourless solid, $[\alpha]_D^{20} = -42$ (*c* 0.12; MeOH). The molecular formula was established as $C_{16}H_{30}O_4$ based on FAB-MS [M + 1 = 287], elemental analysis and NMR data. The IR spectrum suggests the presence of hydroxyl groups (3488, 3386 cm^{-1}). The IR and ^{13}C NMR spectra show the absorption signals of an ester carbonyl group (at 1726 cm^{-1} and δ 173.1). Two adjacent methines [δ C 74.3, δ H 3.96 (dd, 10.0, 1.5 Hz) and δ C 69.2, δH 4.03 (ddd, 10.5, 10.0, 3.5 Hz)] are assigned as bearing hydroxyl groups. In the ^{1}H–^{1}H COSY spectrum, the correlations between another methine

TABLE I NMR data of compound **1** in DMSO-d_6

Position	δ_C *(125 MHz)*	δ_H *(500 MHz)*	*HMBC*	^1H–^1H *COSY*
Phe				
1	171.6 (C)		H-(15), 3	
2	52.7 (CH)	4.64 (ddd, 9.0, 8.5, 7.0 Hz)	H-3	H-(8)
3	38.4 (CH_2)	a. 2.73 (dd, 13.5, 9.0 Hz)	H-2, aromatic	H-2, 3b
		b. 2.91 (dd, 13.5, 7.0 Hz)	H-2, aromatic	H-2, 3a
4	137.3 (C)		H-aromatic, 3	
5	127.9 (2 × CH)	7.18–7.23	H-aromatic, 3	
6	129.0 (2 × CH)	7.18–7.23	H-aromatic	
7	126.1 (CH)	7.18–7.23	H-aromatic	
(8)	NH	7.95 (d, 8.5 Hz)		H-2
Leu[1]				
9	171.4 (C)		H-(22),10, 11	
10	51.8 (CH)	4.17 (ddd, 7.5, 7.5, 6.5 Hz)	H-(15), 11	H-(15)
11	38.6 (CH_2)	1.38 (m)	H-10, 12, 13, 14	H-10, 12
12	24.0 (CH)	1.33 (m)	H-11, 13, 14	H-11, 13, 14
13	22.2 (CH_3)	0.77 (d, 6.0 Hz)	H-12, 14	H-12
14	22.3 (CH_3)	0.84 (d, 6.0 Hz)	H-12, 13	H-12
(15)	NH	8.58 (d, 6.5 Hz)		H-10
Leu[2]				
16	171.5(C)		H-17, 18, (29)	
17	51.9 (CH)	4.08 (ddd, 9.5, 8.0, 5.5 Hz)	H-18	H-(22)
18	39.9 (CH_2)	1.51 (m)	H-17, 19, 20, 21	H-17, 20, 21
19	24.3 (CH)	1.60 (m)	H-18, 20, 21	H-18, 20, 21
20	20.7 (CH_3)	0.79 (d, 6.0 Hz)	H-18, 21	H-21
21	23.0 (CH_3)	0.88 (d, 6.0 Hz)	H-20	H-20
(22)	NH	8.46 (d, 8.0 Hz)		H-17
Leu[3]				
23	171.1 (C)		H-(36), 24, 25.	
24	52.1 (CH)	4.30 (ddd, 7.5, 7.5, 7.5 Hz)	H-25, (29)	H-(29)
25	40.2 (CH_2)	1.55 (m)	H-26, 27, 28	H-24, 26
26	24.5 (CH)	1.46 (m)	H-25, 27, 28	H-25, 27, 28
27	22.5 (CH_3)	0.88 (d, 6.0 Hz)	H-26, 28	H-26
28	22.2 (CH_3)	0.91 (d, 6.0 Hz)	H-27	H-26
(29)	NH	7.23 (d, 7.5 Hz)		H-24
Ile				
30	171.0 (C)		H-(8), 31	
31	62.8 (CH)	3.28 (dd, 8.0, 3.5 Hz)	H-(36), 32	H-(36)
32	33.0 (CH)	2.23 (m)	H-33, 34, 35	H-31, 33, 35, (36)
33	25.0 (CH_2)	a. 1.01 (m)	H-32, 34, 35	H-33b, 34
		b. 1.41 (m)	H-32, 34, 35	H-33a, 34
34	9.8 (CH_3)	0.78 (t, 7.5 Hz)	H-33	H-33
35	15.1 (CH_3)	0.63 (d, 6.5 Hz)	H-32, 33	H-32
(36)	NH	8.33 (d, 8.0 Hz)		H-31

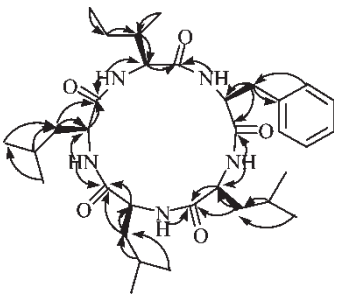
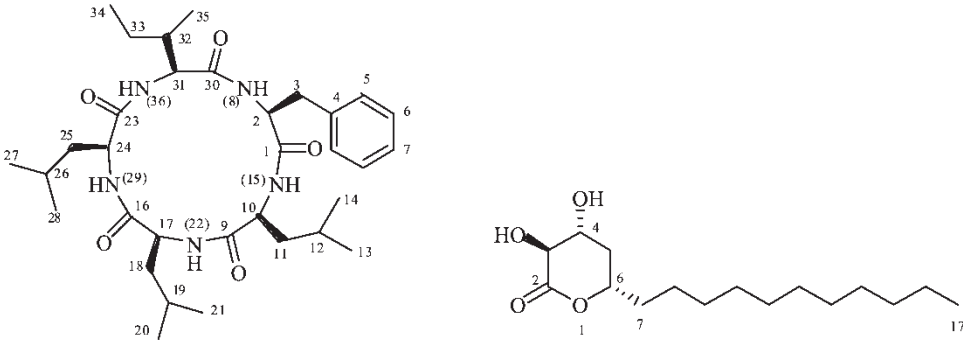


FIGURE 1 Key HMBC correlations of **1**.



1 **2**

FIGURE 2 Compounds **1** and **2**.

[δ C 78.6, δ H 4.32 (dddd, 11.5, 7.0, 5.0, 3.5 Hz)] and two methylenes were observed: CH_2 [a. 2.26 (ddd, 13.5, 3.5, 3.5 Hz, 1H); b. 1.81 (ddd, 13.5, 11.5, 10.5 Hz, 1H)] and CH_2 [a. 1.75 (dddd, 14.0, 7.0, 5.0, 3.5 Hz, 1H); b. 1.63 (dddd, 14.0, 10.0, 5.0, 5.0 Hz, 1H)]. HMBC and $^1H-^1H$ COSY spectra (Table II) completely established the planar structure of **2** as 3,4-dihydroxy-6-undecyl-α-pyranone. Protons at C-3, C-4, and C-6 in compound **2** are all shown to be axial by analysis of their coupling constants ($J_{3,4} = 10.0$ Hz; $J_{6,5b} = 11.5$ Hz). The stereochemistry of compound **2** was elucidated by the CD spectra combined with the CS Chem3D MM2 program.

The stereochemistry of δ-lactones is relatively simple and typical, and has been well studied. δ-Lactones have two conformations: half-chair and boat. The most likely conformation is the half-chair form. On analysis with MM2 in the Chem3D program, the steric energy for the half-chair conformation of compound **2** (29.963 kal mol^{-1}) is less than that of boat form (31.069 kal mol^{-1}). Beecham pointed out that if the β-C of a lactone is above the approximate plane formed with the lactone group —C—CO—O—C— then the lactone exhibits a positive Cotton-effect in the CD spectrum, otherwise a negative Cotton-effect is seen [6]. Based on Beecham's theory, the positive Cotton-effect compound **2** shows in its CD spectrum tentatively suggests that it has a stereochemistry of 3-*S*, 4-*R* and 6-*S* (Fig. 4).

In a preliminary investigation of cytotoxic activity the human cancer cell lines Bel-7402, NCI-4460 and the normal human cell line L-02 were used to examine the cell growth inhibitory properties of **1** and **2**. Compound **1** exhibits inhibitory activity against Bel-7402. The cellular livability was 67% at the dose of 15 μg mL^{-1}. However, no dose-related effects were observed for dosages between 15 and 500 μg mL^{-1}. Other results showed that these compounds do not exhibit significant cytotoxicity. Generally, most cyclopeptides or cyclic

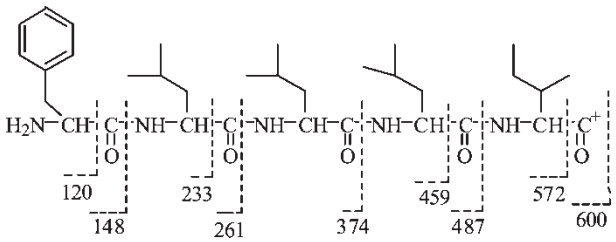


FIGURE 3 ESI-MS^n fragmentation patterns of **1**.

TABLE II NMR data of compound **2** in $CDCl_3$

Position	δ_C *(125 MHz)*	δ_H *(500 MHz)*	*HMBC*	$^1H-^1H$ *COSY*
1	O			
2	173.1 (C═O)		H-3	
3	74.3 (CH)	3.96 (dd, 10.0, 1.5 Hz)	H-5a, 5b	H-4
4	69.2 (CH)	4.03 (ddd, 10.5, 10.0, 3.5 Hz)	H-3, 5b	H-3, 5a, 5b
5	35.7 (CH_2)	a. 2.26 (ddd, 13.5, 3.5, 3.5 Hz)		H-3, 5b
		b. 1.81 (ddd, 13.5, 11.5, 10.5 Hz)		H-3, 5a
6	78.6 (CH)	4.32 (dddd, 11.5, 7.0, 5.0, 3.5 Hz)	H-5b	H-5b, 7a, 7b
7	35.7 (CH_2)	a. 1.75 (dddd, 14.0, 7.0, 5.0, 3.5 Hz)	H-5b	H-7b, 8a, 8b
		b. 1.63 (dddd, 14.0, 10.0, 5.0, 5.0 Hz)		H-7a, 8b
8	29.6 (CH_2)	a. 1.38 (m)		H-7a, 8b
		b. 1.49 (m)		H-7a, 7b, 8a
9–14	24.7 (CH_2)	1.26–1.34 (m)		
	29.2 (CH_2)			
	29.3 (CH_2)			
	29.4 (CH_2)			
	29.5 (CH_2)			
	29.6 (CH_2)			
15	31.9 (CH_2)		H-17	
16	22.7 (CH_2)		H-17	
17	14.1 (CH_3)	0.88 (t, 7.0 Hz)		
3-OH		3.28 (d, 1.5 Hz)		
4-OH		2.40 (br s)		

depsipeptides from microorganisms presented D-amino acids or unusual amino acids. They often possess unusual pharmacological properties, including as antibiotics, toxins, immunosuppressants and ion transport regulators. However, the cyclic peptide **1** is composed of only the usual L-amino acids residues with hydrophobic sides chains. Its simple architecture may explain the absence of potent cytotoxicity. The detailed relationship between its structural properties and biopharmacological activity is under investigation. Although endophytic fungus no. 2524 exhibits low biological relevance for the host, other mangrove endophytes are being investigated to find new natural molecules with diversified structures and bio-properties.

EXPERIMENTAL

General Experimental Procedures

The following instruments were used: Inova-500 NMR spectrometer, VGZAB mass spectrometer, Equinox 55-A590/3F-FTIR spectrophotometer, Varlan UV-VIS spectrophotometer, Elementar Vario EL CHNS-O elemental analyzer, Schmid + Haensch Polartronic HNQW5 optical rotations spectrometer, Therm Finnigan LCQ™ DECA XP LC-MS, Agilent 1100 HPLC using CROWNPAK CR (+) chiral column, JASCO (J810) CD spectrometer.

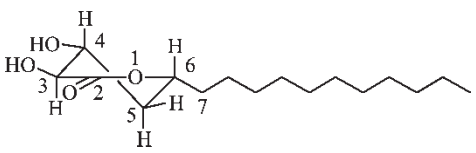


FIGURE 4 Conformation of compound **2**.

Fungal Strain

The endophytic fungus (strain no. 2524) were separated from a seed of *Avicennia marina* from a mangrove in Hong Kong and was unidentified. The endophyte was a non-sporulating isolate, and various methods failed to induce sporulation. Voucher specimens have been stored in the Department of Biology and Chemistry, City University of Hong Kong, Hong Kong, and the Department of Applied Chemistry, Zhongshan University, Guangzhou, China.

Culturing

Starter cultures (from Professors E. B. G. Jones and L. L. P. Vrijmoed) were maintained on potato dextrose agar. Plugs of agar supporting mycelial growth were cut and transferred aseptically to an Erlenmeyer flask (250 mL) containing 100 mL liquid medium (1.0% glucose, 0.5% peptone, 0.5% yeast extract, 20% seawater, pH 7.5) that was sterilized at 110°C for 20 min. The flask was then incubated at 28°C on a rotary shaker (rpm = 120) for 5–7 days. The mycelium was subsequently aseptically transferred to Erlenmeyer flasks (500 mL) containing 200 mL of the same liquid medium. The flasks were then incubated at 28°C on a rotary shaker (rpm = 120) for 20 days.

Extraction and Isolation of Metabolites

The growth culture (200 L) was filtered through cheesecloth and the separated mycelia were freeze-dried. Then, the dried mycelia (550 g) were extracted with methanol (3×2000 mL) for 7 days. The culture broth was subsequently concentrated and then extracted with ethyl acetate. Both extracts were concentrated by rotary evaporation. The mycelia extracts (66 g) were chromatographed on a silica gel column, with a light petroleum(b.p. 60–90°C) –EtOAc–MeOH gradient as eluent, followed by LC separations. The LC separations were performed with a Waters 515 HPLC pump equipped with a Waters 2487 dual λ absorbance detector and Nova-Pak® HR reverse-phase C-18 column (300 × 7.8 mm), with MeOH–H_2O (70:30 v/v) as the mobile phase at a flow rate of 2 mL min^{-1}. Compounds **1** (30 mg) and **2** (40 mg) were obtained from the mycelia extracts. The culture extracts (28 g) were repeatedly subjected to silica gel column chromatography with light petroleum (b.p. 60–90°C)–EtOAc–MeOH as eluent, to afford the eight known compounds.

Determination of Absolute Stereochemistry of Compound 1

Cyclo-(L-Phe-L-Leu^1-L-Leu^2-L-Leu^3-L-Ile) (2.5 mg) was dissolved in 6 M HCl (4 mL) and heated at 110°C for 24 h. The solvents and volatile residual acid were then removed under high vacuum to yield the hydrolysate, which was used without further purification.

Determination of absolute stereochemistry of amino acid residues was performed with an Agilent 1100 HPLC using a CROWNPAK CR (+) chiral column, and with DL- and L-leucine, isoleucine and phenylalanine as standards (Fluka). An optimized solvent system, aqueous perchloric acid (pH 1.5), was used as mobile phase at a flow rate of 0.8 mL min^{-1}, 15°C.

ESI-MS^n

All electrospray and MS^n experiments were performed on a Therm Finnigan LCQ™ DECA XP LC-MS. MS^n experiments used a sheath gas flow rate [arb]: 10; aux gas flow rate [arb]: 0; I spray voltage: 4.4 kV; spray current: 0.14 μA; capillary temp:

199°C; capillary voltage: 10 V; tube lens offset: 65 V. A solution of the cyclic peptide in MeOH–H_2O (80:20)–1% formic acid was injected into the mass spectrum *via* a 250 μL syringe at 2 μL min^{-1}.

Compounds Characterization

Compound **1** (30 mg) is a colourless amorphous power, mp >270°C, $[\alpha]_D^{25} = -56$ (*c* 0.13; MeOH); IR (KBr) $\nu(cm^{-1})$: 3306, 3065, 2960, 2873, 1657, 1635, 1536, 1465, 1383, 1283, 1152, 699, 654, 465; UV: λ_{max} (MeOH) 225 nm (ε 1397); ^1H NMR (DMSO-d_6), ^{13}C NMR (DMSO-d_6) and 2D NMR see Table I; FAB-MS: *m/z* 600 $[M+1]^+$, 374, 261, 242, 227, 208, 180, 165, 154, 120, 86, 77, 51; elemental analysis: found (%): C 65.88, H 8.89, N 11.56; calcd. for $C_{33}H_{53}N_5O_5$: C 66.11, H 8.85, N 11.69. CD spectrum (MeOH): CD (mdeg) +85.5 at λ 235 nm, −80.1 at λ 224 nm.

Compound **2** (40 mg), colourless amorphous power, mp. 105–107°C. $[\alpha]_D^{20} = -42$ (*c* 0.12; MeOH). FAB-MS: *m/z* 287 $[M+1]^+$, 257, 241, 223, 216, 205, 176, 165, 149, 137, 123, 69, 57; IR (KBr) ν (cm^{-1}): 3488, 3386, 2954, 2921, 2853, 1726, 1652, 1540, 1470, 1402, 1338, 1251, 1187, 1130, 1096, 937, 866, 721, 686, 646, 596; UV: λ_{max} (MeOH) 216 nm (ε 121). ^1H NMR ($CDCl_3$), ^{13}C NMR ($CDCl_3$) and 2D NMR see Table II. Elemental analysis: found (%): C 67.08, H 10.47, N 0.000; calcd. for $C_{16}H_{30}O_4$: C 67.13, H 10.48; CD spectrum (MeOH): CD (mdeg) +24.0 at λ 222 nm.

Acknowledgements

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