Alternaramide, a Cyclic Depsipeptide from the Marine-Derived Fungus *Alternaria* sp. SF-5016

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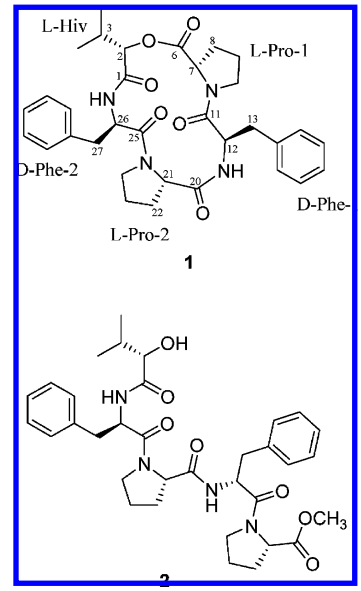
Alternaramide (**1**), a cyclic pentadepsipeptide, has been isolated from an EtOAc extract of the marine-derived fungus *Alternaria* sp. SF-5016 by various chromatographic methods. The structure of **1** was mainly determined by analysis of the NMR spectroscopic data, along with chemical methods such as Marfey's and Mosher's methods. Alternaramide (**1**) showed weak antibiotic activity against *Bacillus subtilis* and *Staphylococcus aureus*.

Marine microorganisms are recognized as an important source for structurally diverse bioactive secondary metabolites.[1,2] As a part of our ongoing studies on bioactive secondary metabolites from marine microorganisms from Korea, we have investigated the chemical constituents of the extracts obtained from cultures of the marine-derived fungus *Alternaria* sp. SF-5016. This paper describes the isolation, structure elucidation, and biological activity of the metabolite, named alternaramide (**1**), encountered in this investigation.

Alternaramide (**1**) was isolated as a white powder and analyzed for the molecular formula $C_{33}H_{40}N_4O_6$ by HRESIMS data (*m/z* 589.3024 $[M + H]^+$), which was fully supported by the ^1H and ^{13}C NMR data (Table 1). The ^1H NMR and DEPT spectra in $CDCl_3$ revealed the presence of two methyl groups, 10 aromatic methines, six sp^3 methines (one oxygenated), and eight methylene units. In addition to the signals corresponding to the above carbons, analysis of the ^{13}C NMR spectrum revealed the presence of five carbonyl and two quaternary aromatic carbons. Furthermore, the presence of characteristic signals for four α-amino carbons ($\delta_{C/H}$ 60.0/4.30, 52.2/4.99, 58.4/4.86, and 53.0/4.94) and five carbonyl/amide carbonyl carbons (δ_C 172.3−168.4) in the NMR spectra of **1** was indicative of a pentapeptide. The presence of one oxygen-bearing methine carbon ($\delta_{C/H}$ 76.8/5.35) in the NMR spectra further suggested that one of the carbonyl signals was from an ester moiety and that the molecule contained a hydroxy acid moiety, thus indicating a depsipeptidic nature of **1**. Analysis of the 2D NMR data (COSY, HMQC, and HMBC) allowed the identification of all of the amino and hydroxy acid residues in the molecule, revealing the presence of two phenylalanine (Phe), two proline (Pro), and one α-hydroxyisovaleric acid (Hiv) residue. Because two aromatic rings, two pyrrolidine rings, and five carbonyls accounted for 15 out of the 16 unsaturations in **1**, alternaramide was proposed to be a monocyclic depsipeptide. Interpretation of HMBC and NOESY data readily led to the construction of the sequence of the five residues in the molecule. Long-range correlations from H_2-27 and H-26 to C-25 revealed that this carbonyl carbon was positioned in Phe-2. HMBC correlations of H-26 and H-2 with C-1 then established the linkage of Phe-2 to Hiv. H-2 also showed a three bond correlation to C-6, which was positioned in the Pro-1 unit on the basis of HMBC correlations of H-7 and H-8 with C-6. Therefore, the further extended sequence of Phe-2 → Hiv → Pro-1 was unambiguously defined. A HMBC correlation of H-7 in the Pro-1 unit with C-11 and HMBC correlations of H_2-13 and H-12 with C-11 allowed the sequence of Pro-1 to Phe-1 to be established. HMBC correlations of H-12 to C-20 and of H-21 and H_2-22 to the same carbonyl carbon established the linkage of Phe-1 to Pro-2. Finally, the sequence of Pro-2 to Phe-2 was established on the basis of a NOESY correlation of H-26 with H_2-24, along with the necessity that the compound must have an additional ring to fulfill the unsaturations required by the molecular formula.



The absolute configurations of the chiral centers in **1** were determined by Marfey's method[3] and Mosher's method.[4] The absolute configurations of the amino acid units were established by acid hydrolysis (6 N HCl, 120 °C, 24 h) of **1**, followed by derivatization with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide)[3] and subsequent HPLC analysis. By comparing the retention times of authentic standards of L- and D-forms of Phe and Pro, the hydrolysate was identified to contain two units each of D-Phe and L-Pro. To establish the absolute configuration at C-2 of Hiv, alternaramide (**1**) was first converted to the linear peptidic methyl ester (**2**) by treatment of **1** with NaOMe in MeOH. Next, the *S*- and *R*-Mosher esters (**3a** and **3b**) of the linear peptide **2** were prepared.[4] Analysis of ^1H NMR and COSY spectra allowed the assignment of the proton chemical shifts for the two diastereomeric esters **3a** and **3b** in proximity of the esterified carbon (C-2). The differences in chemical shift values ($\Delta\delta = \delta_S - \delta_R$) for the two diastereomeric esters **3a** and **3b** were calculated in order to assign the absolute configurations at C-2 (Figure 1). Calculations for all of the relevant signals suggested the *S* absolute configuration at C-2. Taken together, the overall absolute configuration of alternaramide (**1**) could be assigned as 2*S*, 7*S*, 12*R*, 21*S*, and 26*R*.

Alternaramide (**1**) appears to have similar structural features to those of several depsipeptides isolated from marine-derived fungi, such as exumolides from *Scytalidium* sp.,[5] sansalvamides from

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Table 1. NMR Spectroscopic Data for Alternaramide (**1**) in $CDCl_3$

position	δ_C[a]	δ_H, mult. (J in Hz)[b]	key NOESY	HMBC (H→C#)
		L-Hiv		
1	168.4			
2	76.8	5.35, d (2.2)	H-5, H-26	1, 3, 4, 5, 6
3	29.7	2.53, m		
4	15.9	0.71, d (7.0)	H-24	2, 3, 5
5	19.2	0.82, d (7.0)		2, 3, 4
		L-Pro-1		
6	170.3			
7	60.0	4.30, dd (5.2, 8.8)		6, 8, 9, 10, 11
8	29.7	1.88, m 1.94, m		6, 7, 9, 10
9	24.9	1.46, m 1.75, m	H-12	7, 8, 10
10	47.0	2.51, m 3.43, m	H-12	8, 9
		D-Phe-1		
11	172.3			
12	52.2	4.99, m	H-10	11, 13, 20
13	40.5	3.00, m		11, 12, 14, 15/19
14	136.1			
15/19	129.4	7.18−7.33, m		
16/18	128.5	7.18−7.33, m		
17	126.6	7.18−7.33, m		
		L-Pro-2		
20	170.0			
21	58.4	4.86, d (7.3)	NH-20	20, 22, 24
22	24.55	1.65, m 2.41, m		20, 21, 23
23	24.57	1.87, m 2.00, m		24
24	46.0	2.90, dd (5.5, 13.2) 3.36, dd (9.5, 13.2)	H-26	25, 26, 28, 29
		D-Phe-2		
25	170.7			
26	53.0	4.94, m	H-24	1, 25, 27, 28
27	37.0	2.90, dd (5.5, 13.2) 3.36, dd (9.5, 13.2)		25, 26, 28, 29/33
28	137.6			
29/33	129.8	7.18−7.33, m		
30/32	128.7	7.18−7.33, m		
31	127.4	7.18−7.33, m		
NH-1		7.24[c]		1
NH-20		8.13, d (9.5)	H-12, H-13, H-21	20

[a] Recorded at 100 MHz. [b] Recorded at 400 MHz. [c] Observed in the COSY data.

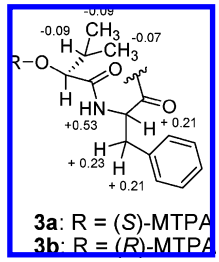

Figure 1. $\Delta\delta$ values [$\Delta\delta$ (in ppm) = $\delta_S - \delta_R$] obtained for the (*S*)- and (*R*)-MTPA esters of the methanolysis product (**3a** and **3b**, respectively).

Fusarium sp.,[6,7] and zygosporamide from *Zygosporium masonii*.[8] These peptides possess hydrophobic amino acid residues and an ester linkage. In addition, the presence of D-amino acid residues in this class of depsipeptide seems quite rare since zygosporamide is the only example among the class. It is noteworthy that the important role of a D-amino acid in the sansalvamide analogues has been indicated in extensive SAR studies of their antitumor activity.[9]

Alternaramide showed weak antimicrobial activity against *Bacillus subtilis* (KCTC 1021) and *Staphylococcus aureus* (KCTC 1928), affording zones of inhibition of 8 and 13 mm, respectively, at 400 μg/disk. However, **1** did not show antimicrobial activity against *Candida albicans* (KCTC 7965), *Proteus vulgaris* (KACC 12674), or *Filobasidiella neoformans* (KCTC 7003) at the same level. In addition, **1** weakly inhibited the activity of protein tyrosine phosphatase 1B (PTP1B),[10] showing 49% inhibition at the level of 150 μg/mL.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 341 digital polarimeter. UV spectra were recorded on a Biochrom 1300 UV/visible spectrophotometer. NMR spectra (1D and 2D) were recorded in pyridine-d_5 or $CDCl_3$ using a JEOL JNM ECP-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C), and chemical shifts were referenced relative to tetramethylsilane (δ_H/δ_C = 0). HMQC and HMBC experiments were optimized for $^1J_{CH}$ = 140 Hz and $^nJ_{CH}$ = 8 Hz, respectively. ESIMS data were obtained using a Q-TOF micro LC-MS/MS instrument (Waters, USA) at Korea University, Seoul, Korea. Solvents for extractions and flash column chromatography were reagent grade and used without further purification. Solvents used for HPLC were analytical grade. Flash column chromatography was carried out using YMC octadecyl-functionalized silica gel (C_{18}). HPLC separations were performed on a Shiseido Capcell Pak C_{18} column (10 × 250 mm; 5 μm particle size; 2 mL/min) or Agilent prep-C_{18} column (21.2 × 150 mm; 5 μm particle size; 5 mL/min). Compounds were detected by UV absorption at 210 nm.

Fungal Materials and Identification. *Alternaria* sp. SF-5016 (deposited at the College of Medical and Life Sciences fungal strain repository, Silla University) was isolated from a shoreline sediment sample collected from the shoreline in the Masan Bay area, Korea, in January 2006. The sediment sample was stored in a sterile plastic bag and transported to the laboratory, where it was kept frozen until processed. The sample was diluted 10-fold using sterile seawater. One milliliter of the diluted sample was processed utilizing the spread plate method in PDA medium (24 g of potato dextrose agar, 1 L of seawater) plates. The plate was incubated at 25 °C for 14 days. After purifying

the isolates several times, the final pure cultures were selected and preserved at −70 °C.

This fungus was identified on the basis of the analysis of the rRNA sequences. A GenBank search with the 28S rRNA gene of SF-5016 (Genbank accession number GQ865634) indicated *Alternaria alternata* (DQ678082), *Alternaria thalictrigena* (EU040211), and *Alternaria malorum* (AY251081) as the closest matches, showing sequence identities of 99.51%, 99.51%, and 99.14%, respectively. Therefore, the marine-derived fungal strain SF-5016 was characterized as *Alternaria* sp.

Fermentation, Extraction, and Isolation of Alternaramide (1). The fungal strain was cultured on 10 Petri-dish plates (90 mm), each containing 20 mL of potato dextrose agar media [0.4% (w/v) potato starch, 2% (w/v) dextrose, 3% (w/v) NaCl, 1.5% (w/v) agar]. Plates were individually inoculated with 2 mL of seed cultures of the fungal strain. Plate cultures were incubated at 25 °C for a period of 14 days. Extraction of the agar media with EtOAc (2 × 500 mL) provided an organic phase, which was then concentrated *in vacuo* to yield 352.2 mg of an extract. The EtOAc extract was subjected to C_{18} flash column chromatography (5 × 40 cm), eluting with a stepwise gradient of 20%, 40%, 60%, 80%, and 100% (v/v) MeOH in H_2O (500 mL each). The fractions eluted at 80% and 100% MeOH (49.6 mg) were combined and purified by semipreparative reversed-phase HPLC eluting with a gradient from 65% to 80% CH_3CN in H_2O (0.1% formic acid) over 35 min, then 100% CH_3CN for 20 min, to yield **1** (6.1 mg, t_R = 35.0 min).

Alternaramide (1): white powder; $[\alpha]^{25}_D$ −6 (*c* 0.53, MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.1); IR (neat) ν_{max} 3298, 3008, 2930, 1742, 1674, 1634, 1525, 1450, 1236, 750 cm^{-1}; ^1H, ^{13}C NMR data, Table 1; HRESIMS *m/z* 589.3024 $[M + H]^+$ (calcd for $C_{33}H_{41}N_4O_6$, 589.3026).

Preparation and Analysis of Marfey's Derivatives. Alternaramide (**1**, 0.6 mg) was hydrolyzed by heating in 6 N HCl (1 mL) at 110 °C for 24 h. After cooling, the solution was evaporated to dryness and redissolved in H_2O (50 μL). To the mixture from acid hydrolysis was added a 1% (w/v) solution (100 μL) of FDAA (Marfey's reagent; 1-fluoro-2,4-dinitrophenyl-5- L-alanine amide) in acetone. After the addition of $NaHCO_3$ solution (1 M, 20 μL), the mixture was incubated at 40 °C for 1 h. The reaction was stopped by the addition of HCl (2 N, 10 μL), the solvent was evaporated to dryness, and the resulting residue was dissolved in MeOH/H_2O (1:1; 1 mL). An aliquot of this solution (20 μL for **1** and 10 μL for the standards) was analyzed by HPLC [Capcell Pak C_{18} column, linear gradient from 30% to 60% CH_3CN in H_2O (0.1% formic acid) over 200 min; 1 mL/min; 25 °C; 340 nm]. Separately, L-Phe, D-Phe, L-Pro, and D-Pro were derivatized with FDAA in the same manner as that of **1**. Retention times (min) of the FDAA amino acid derivatives used as standards were as follows: L-Phe (101.5), D-Phe (119.0), L-Pro (53.5), D-Pro (57.7). Retention times (min) of the observed peaks in the HPLC trace of the FDAA-derivatized hydrolysis product of **1** were 53.5 and 119.0.

Methanolysis of 1. Alternaramide (**1**, 3.0 mg) was dissolved in 0.5 N NaOMe solution (1.5 mL of MeOH) and stirred at ambient temperature for 1 h. The mixture was neutralized by adding 1 N HCl and dried *in vacuo*. The residual material was partitioned with H_2O and EtOAc, and the resulting residue in EtOAc was purified by reversed-phase HPLC, eluting with a gradient from 65% to 80% MeOH in H_2O (0.1% formic acid) over 35 min, then 100% MeOH for 20 min, to yield the linear peptide **2** (2.1 mg, t_R = 35.0 min). The methanolysis product was confirmed by ESIMS (*m/z* 643 $[M + Na]^+$, molecular formula; $C_{34}H_{44}N_4O_7$) and ^1H NMR analysis. The linear peptide **2** showed the following ^1H NMR spectroscopic properties. ^1H NMR ($CDCl_3$, 400 MHz) δ (integration, multiplicity, *J* in Hz, assignment): δ 3.91 (1H, br s, H-2), 2.10 (1H, m, H-3), 0.81 (3H, d, 7.0, H_3-4), 0.99 (3H, d, 7.0, H_3-5), 4.28 (1H, dd, 8.1, 3.7, H-7), 1.90 (1H, m, H_2-8), 1.83 (1H, m, H_2-8), 1.78 (1H, m, H_2-9), 1.57 (1H, m, H_2-9), 3.50 (1H, m, H-10), 2.66 (1H, m, H_2-10), 4.89 (1H, m, H-12), 3.10 (1H, m, H_2-13), 3.02 (1H, m, H_2-13), 7.03−7.18 (10H, m, Ar-H), 4.48 (1H, br d, 5.8, H-21), 2.14 (1H, m, H_2-22), 1.65 (1H, m, H_2-22), 2.13 (1H, m, H_2-23), 1.84 (1H, m, H_2-23), 3.56 (1H, m, H_2-24), 2.74 (1H, m, H_2-24), 4.82 (1H, ddd, 13.9, 9.2, 5.5, H-26), 2.99 (1H, m, H_2-27), 2.94 (1H, m, H_2-27).

Preparation of Mosher Esters of the Linear Peptide (2). A sample of **2** (0.9 mg, 0.0014 mmol), (*R*)-MTPACl (7.5 mg, 0.029 mmol), and pyridine-d_5 (0.5 mL) were allowed to react in an NMR tube at ambient temperature for 24 h. The ^1H NMR data of the (*S*)-MTPA ester derivative (**3a**) were obtained directly on the reaction mixture by analysis of the ^1H NMR and COSY spectra: ^1H NMR (pyridine-d_5, 400 MHz) (integration, multiplicity, *J* in Hz, assignment): δ 5.55 (1H, d, *J* = 5.1, H-2), 2.43 (1H, m, H-3), 0.91 (3H, d, *J* = 7.0 Hz, H_3-4), 0.99 (3H, d, *J* = 7.0 Hz, H_3-5), 8.88 (1H, br s, NH-1), 5.30 (1H, m, H-26), 3.35 (1H, m, H_2-27), 3.22 (1H, m, H_2-27).

Similarly, the reaction mixture from another sample of **2** (0.8 mg, 0.0013 mmol), (*S*)-MPTACl (7.5 mg, 0.029 mmol), and pyridine-d_5 (0.5 mL) was processed as described above for **3a** to afford **3b**. ^1H NMR (pyridine-d_5, 400 MHz) (integration, multiplicity, *J* in Hz, assignment): δ 5.49 (1H, d, *J* = 4.4, H-2), 2.45 (1H, m, H-3), 1.00 (3H, d, *J* = 7.0 Hz, H_3-4), 1.08 (3H, d, *J* = 7.0 Hz, H_3-5), 8.77 (1H, NH-1), 5.28 (1H, m, H-26), 3.34 (1H, m, H_2-27), 3.16 (1H, m, H_2-27).

Purification of the (*R*)- and (*S*)-MTPA Ester Derivatives (3a and 3b). The reaction mixtures were recovered from the NMR tubes. After removal of solvents under vacuum, the residues were purified by reversed-phase HPLC (Agilent prep-C_{18}; 21.2 × 150 mm; 5 μm particle size; 5 mL/min) separately, using a gradient from 50% to 100% MeOH in H_2O (0.1% formic acid) over 35 min, then 100% MeOH for 20 min, to yield pure **3a** (1.2 mg; t_R = 41.6 min) or **3b** (0.7 mg; t_R = 41.0 min), respectively. ^1H NMR of **3a** ($CDCl_3$, 400 MHz, data were assigned on the basis of the correlations of $^1H-^1H$ COSY spectrum): δ 5.09 (1H, d, *J* = 4.4, H-2), 2.21 (1H, m, H-3), 0.87 (3H, d, *J* = 7.0 Hz, H_3-4), 0.85 (3H, d, *J* = 7.0 Hz, H_3-5), 7.20 (1H, NH-1), 4.87 (1H, m, H-26), 3.08 (1H, m, H_2-27), 2.98 (1H, m, H_2-27), 7.40 (1H, br s, NH-20), 4.82 (1H, m, H-12), 3.00 (1H, m, H-13), 2.97 (1H, m, H-13). ^1H NMR of **3b** ($CDCl_3$, 400 MHz, data were assigned on the basis of the correlations of $^1H-^1H$ COSY spectrum): δ 5.22 (1H, br s, H-2), 2.30 (1H, m, H-3), 0.96 (3H, d, *J* = 6.6 Hz, H_3-4), 0.92 (3H, d, *J* = 6.6 Hz, H_3-5), 6.67 (1H, br s, NH-1), 4.66 (1H, m, H-26), 2.87 (1H, m, H_2-27), 2.75 (1H, m, H_2-27), 7.30 (1H, br s, NH-20), 4.82 (1H, m, H-12), 2.97 (1H, m, H-13), 2.90 (1H, m, H-13).

Antimicrobial Assay Procedures. The bacterial strains were grown on nutrient agar [0.3% (w/v) beef extract, 0.5% (w/v) peptone, 1.5% (w/v) agar], and the yeast *Candida albicans* (KCTC 7965) and *Filobasidiella neoformans* (KCTC 7003) were grown on yeast mold agar [0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) peptone, 1% (w/v) dextrose, 2% (w/v) agar] and malt extract agar [1.275% (w/v) maltose, 0.275% (w/v) dextrin, 0.235% (w/v) glycerol, 0.078% (w/v) peptone, 1.5% (w/v) agar], respectively. Test compound was absorbed onto individual disks (6 mm diameter) at 400 μg/disk and placed on the surface of the agar. The assay plates were incubated at 25 °C for 48 h and examined for the presence of a zone of inhibition. Gentamicin was employed as the positive control and displayed 13 mm (at 50 μg/disk) and 16 mm (at 5 μg/disk) zones of inhibition against *Staphylococcus aureus* (KCTC 1928) and *Bacillus subtilis* (KCTC 1021), respectively.

PTP1B Assay Procedures. PTP1B (human, recombinant) was purchased from BIOMOL Research Laboratories, Inc. The enzyme activity was measured in a reaction mixture containing 2 mM *p*-nitrophenyl phosphate (*p*NPP) in 50 mM citrate, pH 6.0, 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT). The reaction mixture was placed in a 30 °C incubator for 30 min, and the reaction was terminated by the addition of 10 N NaOH. The amount of produced *p*-nitrophenol was estimated by measuring the increase in absorbance at 405 nm. The nonenzymatic hydrolysis of 2 mM *p*NPP was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme.

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Supporting Information Available: 1D- and 2D-NMR spectra for compounds **1**,**2**, **3a**, and **3b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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