VOLUME 71, NUMBER 9

JOC Articles

APRIL 28, 2006

IB-01212, a New Cytotoxic Cyclodepsipeptide Isolated from the Marine Fungus *Clonostachys* sp. ESNA-A009

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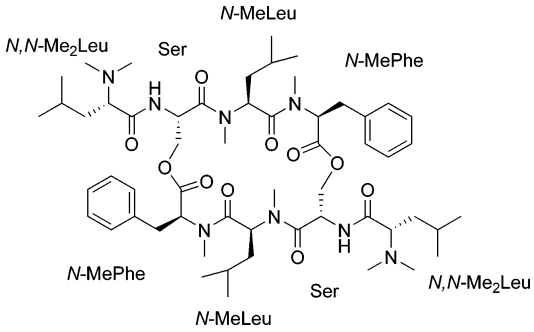
Received July 31, 2005



IB-01212, a new cytotoxic cyclodepsipeptide featuring C_2 symmetry, was isolated from the mycelium extract of *Clonostachys* sp. ESNA-A009. The amino acid sequence of the compound was determined by spectroscopy techniques. The absolute configuration of the amino acids was determined by a combination of the Marfey and menthol methods. The structure, which was confirmed by comparison of the analytical data for the natural product with a sample obtained by solid-phase peptide synthesis, was revealed to be a cyclic dimer formed by two chains of L-*N*,*N*-Me$_2$Leu-L-Ser-L-*N*-MeLeu-L-*N*-MePhe bound by the two esters formed between the carboxylic acid of the L-*N*-MePhe and the hydroxyl function of the L-Ser. IB-01212 is highly cytotoxic to different tumor cell lines.

Introduction

Historically, terrestrial fungi have been a rich source of pharmaceutically important compounds.[1] More recently, marine fungal species have been exploited as a source of novel bioactive metabolites.[2,3] Furthermore, fungi are a well-known source of cyclic peptides and depsipeptides, many of which contain unusual amino acid residues and exhibit biological activity.[4] However, few reports on the chemistry or biological activity members of marine fungi, specifically, those from the genus *Clonostachys*, can be found in the literature.

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10.1021/jo051600p CCC: $33.50
Published on Web 11/30/2005

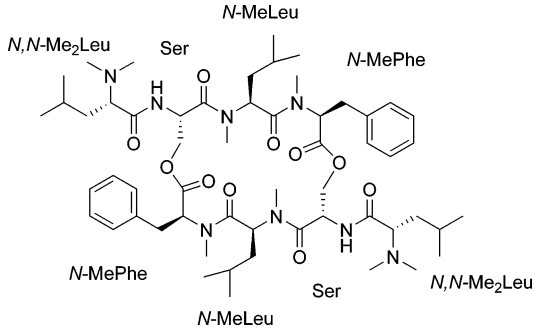

FIGURE 1. Cyclodepsipeptide (IB-01212) isolated from the fermentation broth of a fungal strain *Clonostachys* sp. ESNA-A009.

In the course of ongoing research aimed at exploring the biosynthetic potential of rare marine microorganisms to produce cytotoxic metabolites, we have isolated a new cyclodepsipeptide, IB-01212, from the culture broth of the marine fungus *Clonostachys* sp. ESNA-A009 (Figure 1). In this paper, we describe the isolation, structure elucidation, and biological activity of IB-01212. The synthesis of the compound is described in the following paper.[5]

Results and Discussion

The filamentous fungus *Clonostachys* sp. ESNA-A009 was isolated from an unidentified marine sponge collected in Japan using oatmeal agar supplemented with 100% seawater. A culture of the strain has been deposited in the "Colección Española de Cultivo Tipo" at the University of Valencia, Spain, under the accession number CECT 20477. The organism grew rather slowly, reaching 35 mm in diameter in 10 days at 24 °C on oatmeal, potato dextrose, and malt agar. All media were supplemented with 50% artificial seawater. The strain was able to grow between 24 and 28 °C, and the resulting white colonies were hairy or cottony and formed dark green sporodochia after 20 days of incubation. No soluble pigments were observed in any of the media tested. Spore suspensions of a pure culture of *Clonostachys* sp. ESNA-A009 was kept frozen at −70 °C in 20% glycerol. Fermentation was carried out as described in Experimental Section.

IB-01212 was isolated as a colorless amorphous powder from the mycelium extract of *Clonostachys* sp. ESNA-A009 by extraction with $CHCl_3$/MeOH/H_2O. The organic phase was purified by silica gel chromatography and by reverse phase HPLC using a octadecylsilica (C_{18}) column. The molecular formula of IB-01212 was determined by EI-MS to be $C_{56}H_{88}N_8O_{10}$ *m*/*z* 517.18 $(M + 2H)^+$ and in HRFAB-MS the compound gave an $(M + H)^+$ ion of *m*/*z* 1033.67 (calcd 1032.66 for $C_{56}H_{89}N_8O_{10}$). The IR spectrum of **1** showed absorptions at 3324, 1735, and 1642 cm^{-1}, which indicated peptide linkages.[6] The ^1H and ^{13}C NMR spectra data for IB-01212 are shown in Table 1.

Since the molecular formula of IB-01212 has twice the number of the carbon and proton atoms observed in the NMR spectra, we deduced that the compound must be a symmetrical dimer. Following the interpretation of COSY, GHSQC, and GHMBC spectra, the natural amino acid Ser, and the unnatural *N*-MeLeu, *N*,*N*-Me_2Leu, and *N*-MePhe were readily identified

TABLE 1. ^1H and ^{13}C NMR Data for Natural IB-01212 in $CDCl_3$[a]

residue	^{13}C (δ)	^1H (δ)
	N-MePhe	
N-CH_3	30.4	2.82 (s)
αCH	61.5	4.59 (dd, 3.5, 11.9)
βCH_2	35.8	3.05 (dd, 11.9, 14.5)
		3.64 (dd, 3.5, 14.5)
1-C	136.1	
2,6-CH	129.4	7.29 (m)
3,5-CH	129.1	7.29 (m)
4-CH	127.3	7.24 (m)
CO	170.5	
	N-MeLeu	
N-CH_3	30.9	2.88 (s)
αCH	52.6	4.65 (t, 6.9)
βCH_2	38.2	1.08 (m)
		1.55 (m)
γCH	24.7	0.94 (m)
δCH_3	22.9	0.55 (d, 6.6)
δCH_3	23.0	0.63 (d, 6.6)
CO	170.1	
	Ser	
NH		7.73 (d, 8.0)
αCH	47.7	5.16 (d, 8.0)
βCH_2	66.4	3.95 (dd, 2.2, 11.6)
		4.52 (dd, 2.5, 11.4)
CO	168.0	
	N,*N*-DiMeLeu	
N-CH_3	42.4	2.26 (s)
N′-CH_3	42.4	2.26 (s)
αCH	67.2	2.94 (dd, 4.8, 8.6)
βCH_2	36.1	1.40 (m)
		1.57 (m)
γCH	26.1	1.65 (m)
δCH_3	22.3	0.90 (d, 6.4)
δCH_3	23.6	0.93 (d, 6.4)
CO	173.2	

[a] NMR spectra were acquired on a 400 MHz instrument at the Instituto Biomar. All spectra were registered in $CDCl_3$ at 25 °C.

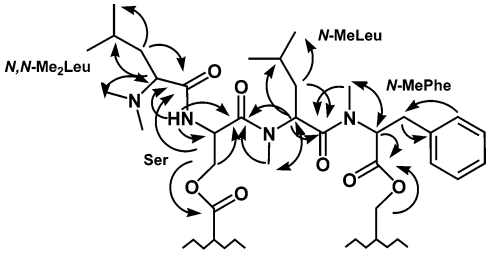

FIGURE 2. NMR analysis of the interactions that led to the elucidation of the structure of IB-01212.

by their respective spin systems. GHMBC experiments were used to unequivocally establish the amino acid sequence, and the long-range couplings gave the following evidence: (i) the long-range couplings from the CH_3 (δ 2.82) of *N*-MePhe to the C(α) (δ 61.5) of *N*-MePhe and the CO (δ 170.1) of *N*-MeLeu, and from the H(α) (δ 4.59) of the *N*-MePhe and H(β) (δ 3.95, 4.52) of Ser to the CO (δ 170.5) of *N*-MePhe evidenced the attachment of the two amino acids and the site of dimerization; (ii) the long-range couplings from the CH_3 (δ 2.88) of the *N*-MeLeu to C(α) (δ 52.6) of the *N*-MeLeu and the CO (δ 168.0) of the Ser and from H(α) (δ 4.65) of the *N*-MeLeu to the CO (δ 168.0) of Ser established their connection; (iii) the long-range couplings from the NH (δ 7.73) of Ser to the C(α) (δ 47.7) and the CO (δ 168.0) of Ser, and the CO (δ 173.2) of *N*,*N*-Me_2Leu demonstrated the connection between these two amino acid residues. These interactions, illustrated in Figure 2, led to the elucidation of the structure of IB-01212.

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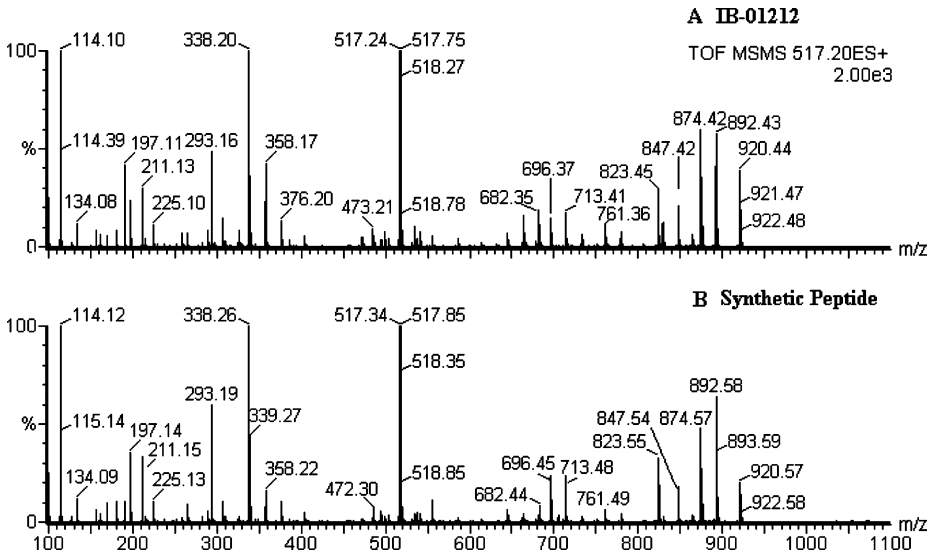

FIGURE 3. NanoESI-MS/MS ion spectra of (A) IB-01212 and (B) the synthetic peptide.

The stereochemistry of the amino acids in IB-01212 was determined by a combination of the Marfey and the 1-menthol methods.[7,8] Standard amino acid samples, L- and D-Ser, L- and D-*N*-MeLeu, and L- and D-*N*-MePhe, as well as an acid-hydrolyzed sample of the peptide, were individually derivatized with Marfey's reagent [N^{α}-(2,4-dinitro-5-fluorophenyl)-L-Ala amide, FDPAA] and subjected to reversed-phase HPLC analysis. Thus, by comparison of the retention times and coelution, the three amino acids were confirmed to be L-Ser, L-*N*-MeLeu, and L-*N*-MePhe (Figure 1, Supporting Information).

The absolute configuration of the *N*,*N*-Me_2Leu cannot be determined by the Marfey method because of its nonreactive α-amino group. In this case, the 1-menthol method was used, a technique that enables determination of the absolute configuration of compounds that contain free carboxyl groups, such as *N*,*N*′-Me_2Leu. Thus, samples of L- and D-*N*,*N*-Me_2Leu were prepared in solution by catalytic reductive condensation of L- and D-Leu-OH with formaldehyde, using the method developed by Ikutani.[9] The standard and hydrolyzed cyclodepsipeptide samples were derivatized with 1-menthol. The stereochemistry of the *N*,*N*-Me_2Leu was clearly established to be L by comparison of the retention times and coelution in HPLC (Figure 2, Supporting Information).

To confirm the proposed structure of IB-01212, the peptide was synthesized on solid phase.[5] The synthetic cyclodepsipeptide and an authentic sample of IB-01212 coeluted by HPLC (Figure 3, Supporting Information). Furthermore, the ^{1}H and ^{13}C NMR spectra [(400 MHz, $CDCl_3$), ^{1}H, ^{13}C, TOCSY (70 ms), GHSQC, and GHMBC of the synthesized peptide (Table 1, Supporting Information), acquired at the Barcelona Science Park NMR facilities, did not exhibit any significant differences compared to those of authentic IB01212 (Table 1). In addition, molecular fragmentation using tandem nano ESI-MS/MS[10] showed similar degradation patterns for the synthetic and natural compounds (Figure 3). The characterization of the main fragments were obtained by MS/MS (Table 2, Supporting Information).

The cytotoxic activity of IB-01212 was then evaluated against a panel of 14 different human tumor cell lines. The compound was most active against LN-caP (prostate cancer), SK-BR3 (breast cancer), HT29 (colon cancer), and HELA (cervix cancer), with an GI_{50} (growth inhibition) on the order of 10^{-8} M.

In conclusion, a new cytotoxic cyclodepsipeptide, IB-01212, has been isolated from the mycelium extract of *Clonostachys* sp. ESNA-A009. Structural determination by NMR and MS/MS revealed that the compound includes *N*-MeLeu, *N*-MePhe, and the rare amino acid *N*,*N*-Me_2Leu. ^{1}H and ^{13}C NMR spectra of IB-01212 contain only half of the expected signals, indicating that the peptide has C_2 symmetry. Chemical analysis of the natural compound via the Marfey and 1-menthol methods indicated that all of its constituent amino acids are in the L configuration. Finally, coelution of a synthetic peptide with an authentic sample of IB-01212 confirmed the structure proposed for the natural product.

Experimental Section

Fermentation, Extraction, and Isolation. A frozen culture or a well grown agar culture of *Clonostachys* sp. ESNA-A009, which was kept frozen at −70 °C in 20% glycerol, was used to inoculate 50 mL of a seed medium containing 0.4% glucose, 0.2% beef extract, 0.2% yeast extract, 2% starch, 0.4% tryptone, 0.5% NaCl, 0.02% KCl, 0.3% $MgCl_2$, and water in 250 mL shake flasks, which was then cultured at 24 °C on a rotary shaker at 150 rpm. The flasks were incubated for 48 h and used as a first stage inoculum. The same medium (300 mL) was added to 2 L Erlenmeyer flasks and inoculated with 10% of the first stage inoculum. The fermentation lasted for 5 days at 24 °C on a rotary shaker at 150 rpm.

Whole harvested broth (2.5 L) was filtered to separate the biomass and other solids. The mycelial cake was extracted twice (1.2 L) with $CHCl_3$/MeOH/H_2O (2:1:1), and the activity was concentrated in the lower layer. The organic solvent was concentrated and evaporated to dryness in vacuo to yield a crude extract (1.3 g). The extract purified by silica gel VFC (vacuum flash chromatography) using a mixture of *n*-hexane/EtOAc and EtOAc/MeOH as eluents. The fractions containing IB-01212 (120 mg) with antitumor activity were eluted with EtOAc/MeOH (1:1), EtOAc/

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MeOH (1:3), and pure MeOH. The active fractions were purified on a silica gel column using $CHCl_3$/MeOH mixtures as eluents. Cytotoxic activity was detected in fractions eluted with $CHCl_3$/MeOH (95:5) (28 mg). Further purification by RP-HPLC afforded pure compound IB-01212 (20 mg), which was eluted with MeOH/H_2O (9:1).

Samples were run on a Waters system equipped with a Symmetry C_{18} column (4.6 mm × 150 mm, 5 μm), using 0.1% aqueous TFA (Solvent A) and 0.1% TFA in acetonitrile (ACN) (Solvent B) in a linear gradient (60% to 15% A, 25 min) at a flow rate of 0.5 mL/min and UV detection at 220 nm. Under these conditions, the peak eluting at 13.3 min clearly contained IB-01212, as confirmed by HPLC-ESI-MS analysis and ^{1}H NMR: colorless powder; $[\alpha]_D$ −106 (*c* 1.00, $CHCl_3$); IR ν_{max} (KBr) 3324 br, 2873, 1735, 1642, 1466, 1250, 1093 cm^{-1}; HRFABMS *m*/*z* 1033.6713 $(M + H)^+$, calcd for $C_{56}H_{89}N_8O_{10}$, 1032.66. The ^{1}H and ^{13}C NMR data ($CDCl_3$), see Table 1.

Derivatization of Amino Acid Standards with Marfey's Reagent. Each amino acid standard solution (200 μL, 10 mM, 0.1 M $NaHCO_3$) was added to 200 μL of freshly prepared 10 mM FDAA in acetone, and the mixture was kept at 40 °C for 1 h. The reaction mixture was then cooled and acidified with 0.2 N HCl (100 μL). After filtration, each derivative was analyzed by RP-HPLC.

Hydrolysis of IB-01212 and Subsequent Derivatization with Marfey's Reagent. Sample of IB-01212 (0.25 mg) was dissolved in 6 N HCl and hydrolyzed in a vacuum-sealed ampules at 110 °C for 20 h. It was then concentrated to dryness. The residue was dissolved in 0.1 M $NaHCO_3$ (200 μL) and to the resulting mixture was added 200 μL of freshly prepared 10 mM FDAA in acetone and treated as above.

Derivatization of L- and D-*N*,*N*-Me$_2$Leu with 1-Menthol. Solutions of L- and D-*N*,*N*-Me$_2$Leu (1 mg) were prepared by dissolving 1 mL of either amino acid in 1 mL of DCM. The solutions were then acidified with 5 drops of HCl (6 N) and then concentrated to dryness. The residues were treated with 1-menthol (1 mg) in toluene and a catalytic amount of *p*-toluenesulfonic acid and then refluxed for 72 h. The reaction mixtures were then washed with saturated $NaHCO_3$ (aqueous) and H_2O and dried. The resulting products were resuspended in 200 μL of ACN/H_2O (1:1, v/v) solution. After filtration, the L- and D-*N*,*N*-Me$_2$Leu derivatives were analyzed by RP-HPLC.

Hydrolysis of IB-01212 and Subsequent Derivatization with 1-Menthol. IB-01212 (0.5 mg) was dissolved in 6 N HCl and hydrolyzed in a vacuum-sealed ampules at 110 °C for 20 h. The mixture was then concentrated to dryness and lyophilized with ACN−H_2O (1:1, v/v). The residue was treated with 1-menthol (1 mg) in toluene and a catalytic amount of *p*-toluenesulfonic acid and treated as above.

HPLC Analysis of the Stereochemistry of IB-01212. The FDPAA derivatives of amino acid standard samples were individually subjected to RP-HPLC analysis. Samples were run on a Waters system equipped with a C_{18} Symmetry column (4.6 mm × 150 mm, 5 μm), using 0.045% aqueous TFA (Solvent A) and 0.036% TFA in ACN (Solvent B) in a linear gradient (20% to 40% B, 70 min) at a flow rate of 1 mL/min, and UV detection at 340 nm. Unreacted FDPAA appeared as a broad peak at 31.3 min. The retention times for the amino acid derivatives were as follows: 21.5 min (L-Ser), 22.2 min (D-Ser), 59.6 min (L-*N*-Me-Leu), 62.9 min (D-*N*-Me-Leu), 56.3 min (L-*N*-Me-Phe), and 57.2 min (D-*N*-Me-Phe).

The 1-menthol derivatives of L-*N*,*N*-Me$_2$-Leu and D-*N*,*N*-Me$_2$-Leu were run on a Waters system equipped with a C_{18} Symmetry column (4.6 mm × 150 mm, 5 μm), using 0.045% aqueous TFA (Solvent A) and 0.036% TFA in ACN (Solvent B) in a linear gradient (25% to 40% B, 70 min) at a flow rate of 1 mL/min, with UV detection at 260 nm. Under these conditions, the retention times of the standards were 36.2 min (D-*N*,*N*-Me$_2$-Leu) and 37.3 min (L-*N*,*N*-Me$_2$-Leu).

Preparation of *N*,*N*-Me$_2$Leu-OH. A suspension of H-Leu-OH (5 g) in H_2O (150 mL), 37% aqueous formaldehyde (15 mL), and 10% Pd/C (5 g) was stirred in an autoclave (200 mL capacity), which was then repeatedly filled with hydrogen over time. The mixture was shaken at 50 °C until the hydrogen had been exhausted (ca. 8 h). The mixture was then heated to boiling and filtered to remove any unreacted formaldehyde. The catalyst was then filtered off, and the filtrate was evaporated under reduced pressure. Residual paraformaldehyde was removed by treating the crude product with H_2O and re-evaporating. The resulting residue was resuspended in ACN/H_2O (1:1, v/v) and lyophilized. The solid product was crystallized in EtOH/acetone, filtered, liberally washed with acetone, and dried under reduced pressure to give the title compound (3.2 g, 53%) as white, very hygroscopic solid: ES-MS calcd for $C_8H_{17}NO_2$ 159.13 g/mol, found *m*/*z* 160.64 $[M + 1]^+$; mp 208.3−210.4; IR (KCl) υ_{max} 2954, 2926, 1626, 1374, 1344, 578; ^{1}H NMR (400 MHz, D_2O) δ 3.44 (1H, m, α-CH), 2.75 (6H, s, N(CH_3)$_2$), 1.57 (2H, m, β-CH_2), 1.52 (1H, m, γ-CH), 0.83 (6H, m, δ-(CH_3)$_2$).

Acknowledgment. The authors thank Mr. J. A. Alonso-García for technical assistance, Dr. D. Grávalos for kindly performing the cytotoxic bioassays, and Dr. C. Cuevas (PharmaMar, S.A.U.) for her encouragement to perform the work. This work was partially supported by PharmaMar, S.A.U., Generalitat de Catalunya [Grup Consolidat (1999 SGR 0042)] and Ministerio de Ciencia y Tecnología (BQU 2003-00089, PETRI 95-0658-OP).

Supporting Information Available: Experimental data corresponding to the chemical and RP-HPLC analysis of the natural compound using the Marfey and 1-menthol methods; data on ^{1}H and ^{13}C NMR spectra [(400 MHz, $CDCl_3$), ^{1}H, ^{13}C, TOCSY (70 ms), GHSQC, GHMBC of the synthesized peptide; comparison of the analytical data [RP-HPLC and NanoESI-MS/MS ion spectra] for the natural product with a sample obtained by solid-phase peptide synthesis.[5] This material is available free of charge via the Internet at http://pubs.acs.org.

JO051600P