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BIOACTIVE MARINE METABOLITES, IV.[1] ISOLATION AND THE AMINO ACID COMPOSITION OF DISCODERMIN A, AN ANTIMICROBIAL PEPTIDE, FROM THE MARINE SPONGE *DISCODERMIA KIIENSIS*

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ABSTRACT.—An antimicrobial peptide, discodermin A, has been isolated from the marine sponge *Discodermia kiiensis*. The peptide showed antimicrobial activity as well as an inhibition of starfish embryo development. It appeared to be a tetradecapeptide consisting of D-Cys(O_3H), L-Asp, L-MeGlu, L-Thr (2 moles), Sar, D-Pro, D-Ala, D- and L-*t*-Leu, D-Leu, L-Phe, D-Trp, and L-Arg.

A large number of marine metabolites with diverse bioactivities have been isolated in the past twenty years. However, lipophilic cytotoxic peptides were not known until Ireland and Scheuer (2) discovered in 1980 from the tunicate *Lissoclinum patella* two antineoplastic thiazole-containing cyclic peptides, ulicyclamide and ulithiacyclamide. Recently, several congeners of these peptides have been found from some didemnids (3-5). Succeedingly, Rinehart *et al.* (6) obtained antineoplastic and antiviral depsipeptides, didemnins A, B, and C, from the Caribbean didemnid *Tridemnum* sp. and Pettit *et al.* (7, 8) obtained nine antineoplastic peptides, dolastatins 1-9, from the Indian Ocean sea hare *Dolabella auricularia*. Quite recently, Moore and co-workers (9) reported a cytotoxic cyclic peptide, majusculamide-C, which was isolated from the marine blue-green alga *Lyngbya majuscula*. Besides these, several toxic peptides were known from the freshwater blue-green alga (10-12). Strangely, no bioactive peptides have been reported from the sponge, though they are a rich source of bioactive metabolites. As a matter of fact, some peptides, including celenamides A and B (13) and others (14-17), were known, but their biological activities were not available.

In the course of our search for antimicrobial compounds from marine invertebrates, we found that the methanolic extract of the marine sponge *Discodermia kiiensis* Hoshino exhibited significant activities against fungi and Gram-positive and Gram-negative bacteria. The major active component, which was named discodermin A, has been isolated and shown to inhibit the development of starfish embryos as well as to have antimicrobial activity. It also appeared to be a tetradecapeptide with molecular weight of 1704. The isolation, biological properties, and amino acid composition of the peptide are described in this paper.

RESULTS

The aqueous EtOH extract of the frozen specimens of *D. kiiensis* gave a considerable amount of insoluble materials in the aqueous layer when partitioned with C_6H_6. The insoluble materials, which contained most of the activity, were collected by centrifugation and further purified by TSK G3000S, silica gel, and LiChroprep Si60 chromatographies, in order. Colorless solids thus obtained exhibited a single spot in tlc. However, reverse-phase hplc of this material gave several peaks, of which the major component was collected and named discodermin A. The antimicrobial activity of discodermin A is illustrated in Table 1. It also inhibited the development of starfish embryos (18) at a concentration of 2 μg/ml; most cells were lysed.

[1]For part III, see Fusetani, *et al.* (1).

TABLE 1. Minimal Inhibitory Concentration of Discodermin A

Test organism	*Minimal inhibitory concentration* (μg/ml)
Bacillus subtilis ATCC6633	3.13
Staphylococcus aureus 209P	>100
Staphylococcus aureus KC-11	>100
Staphylococcus epidermidis S-998	>100
Streptococcus faecalis CAYO4/1	>100
Streptococcus faecium CAYO9-1/2	>100
Mycobacterium smegmatis ATCC607	>100
Escherichia coli K-12	>100
Escherichia coli NIHJ	>100
Proteus mirabilis IFM-OM9	1.56
Proteus inconstans A-2	100
Proteus morganii 955-2	12.5
Serratia marcescens S-1007	>100
Pseudomonas aeruginosa NCTC10490	>100
Pseudomonas aeruginosa 99	>100
Pseudomonas aeruginosa 8689	>100
Candida albicans ATCC10231	>100
Saccharomyces sake	>100
Penicillium citrinum ATCC9849	>100
Aspergillus niger	>100

Discodermin A was soluble in MeOH, DMSO, and H_2O, but sparingly soluble or insoluble in EtOH, EtOAc, and $CHCl_3$. It gave purple color on tlc plates when sprayed with H_2SO_2 and was positive to Sakaguchi reagent, but negative to ninhydrin and Dragendorff reagents. The ir spectrum revealed the presence of peptide linkages (3400, 1650 cm^{-1}), which was supported by reaction with Cl_2/KI-starch reagent (19) on tlc plates, and an ester (1740 cm^{-1}). The uv spectrum showed λmax (MeOH) at 276, 282, and 292 nm (ε 6200, 6500, 5600), suggesting the presence of a tryptophanyl residue.

Amino acid analysis revealed the presence of one mole each of Asp, Sar, Pro, Ala, Leu, Phe, and Arg, and two moles of Thr. In addition, the chromatogram exhibited two unidentified peaks; a strongly acidic amino acid (designated as X_1) and a neutral amino acid having a retention time in amino acid analysis identical with that of Cys (designated as X_2). The presence of one mole of Trp was confirmed by the ^1H nmr (Figure 1) and the ^{13}C nmr (Figure 2) of the intact peptide. Gc/ms analysis of the acid hydrolysate mixture as their *N,O*-trifluoroacetyl *n*-butyl ester derivatives showed the presence of one more amino acid possessing the M^+ ion at *m/z* 369 (designated as X_3).

Qualitative cd analyses of the component amino acids (20), which were obtained from the acid hydrolysate by ion-exchange and gel chromatographies, allowed assignment of chirality of the amino acids as follows: L-Asp, L-Thr, D-Pro, D-Ala, D-Leu, L-Phe, D-Trp, and L-Arg. Two threonines were discriminated from *allo(a)*-Thr by silica gel tlc (21). Unknown amino acids X_1 and X_2 were isolated by the procedure above-mentioned, while X_3 was isolated by high voltage paper electrophoresis followed by gel filtration.

X_1 possessed the same mobilities as those of Cys (O_3H) in amino acid analysis and high voltage paper electrophoresis. The ^1H nmr, ^{13}C nmr, and fdms of X_1 were identical with those of the authentic Cys(O_3H). The cd spectrum showed a negative Cotton effect, suggesting the D-configuration.

The ^1H-nmr spectrum of X_2 contained a nine-proton singlet at δ 1.12 and a methine singlet at δ 3.50, which implied the presence of a *t*-butyl moiety. Fdms showed an intense fragment ion at *m/z* 57 (C_4H_9) in addition to the $(2M+H)^+$ peak at

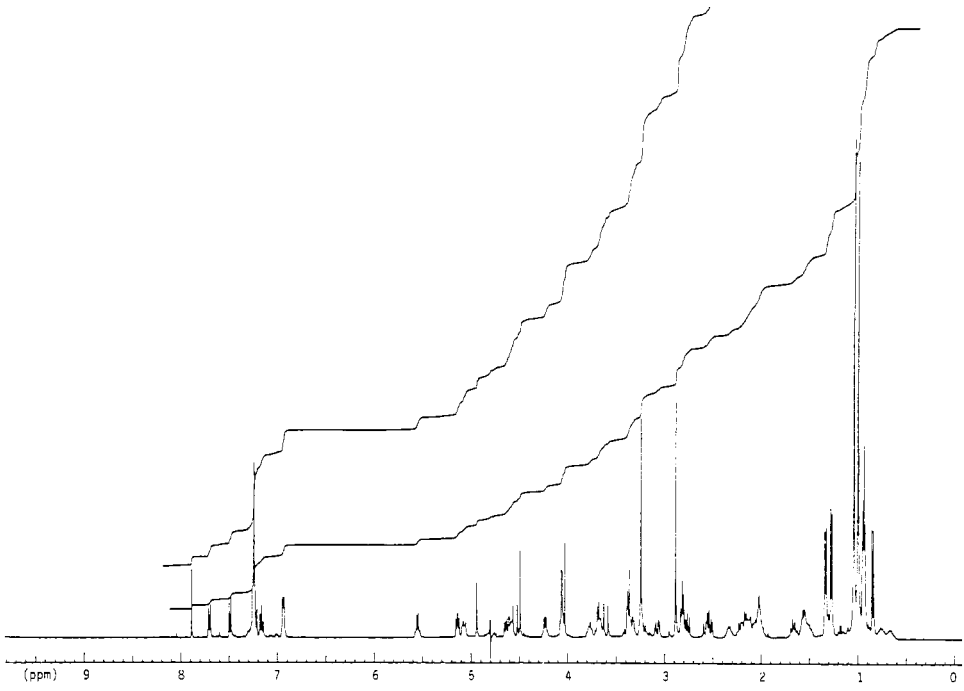

FIGURE 1. ^{1}H-nmr spectrum (400 MHz) of discodermin A in D_2O.

m/z 263 and the MH^+ peak at *m/z* 132. From these data, X_2 was assigned as *t*-Leu (22), which was sustained by the ^{13}C-nmr spectrum. The structure was secured by direct comparison with authentic *t*-Leu. As the cd spectrum did not show Cotton effect at around 200 nm, X_2 was believed to be a 1:1 mixture of L- and D-*t*-Leu.

X_3 was a dibasic carboxylic acid as judged from the gc/ms analysis: it gave the molecular ion peak at *m/z* 369 as *N,O*-trifluoro *n*-butyl ester derivative, while its *N,O*-trifluoro methyl ester derivative at *m/z* 285. The ^{1}H-nmr spectrum contained an *N*-methyl singlet at δ 2.70, a methine triplet at δ 3.60, and two contiguous methylenes at δ2.15 and 2.49. Above features and the fdms [*m/z* 162 (MH^+)] indicated that X_3 was *N*-methyl glutamic acid (MeGlu). Moreover, the compound was identical with authentic DL-MeGlu in the following properties: ^{1}H nmr, gc/ms, chromatographic and elec-

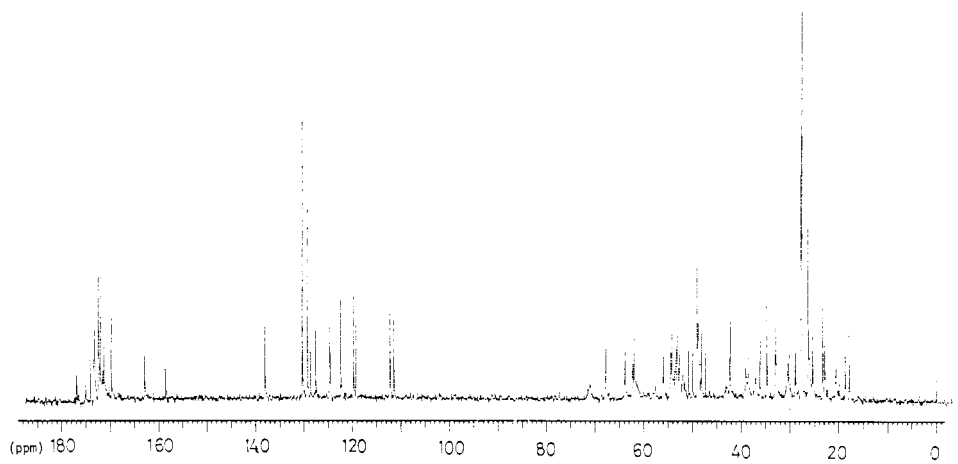

FIGURE 2. ^{13}C-nmr spectrum (25 MHz) of discodermin A in $^{12}CD_3OD$ at 70°.

trophoretic mobiities. As no data for cd spectrum of MeGlu was available, L-MeGlu was synthesized from N-acetyl L-Glu. Both the synthetic and the isolated MeGlu showed a positive Cotton effect at 210 nm, so that X_3 has the L-configuration.

The fab mass spectrum of discodermin A exhibited the $(M+H)^+$ ion at *m/z* 1705, which implied that it has the molecular weight of 1704. This was suported by the amino acid analysis and the ^{13}c-nmr spectrum. It was therefore concluded that discodermin A was a tetradecapeptide.[2]

Discodermin A was shown to be a peptide with unusual structural features. It contains two *t*-Leu residues which have been found only in the antibiotics bottromycins (23, 24) isolated from actinomycetes. Furthermore, half of its constituent amino acids possess the D-form. The sponge is known to contain bacteria and blue-green alga as symbionts (25), and some secondary metabolites of sponges are believed to be synthesized by their symbionts (26, 27). This may be true for discodermin A. Discodermin A inhibited the growth of microorganisms and the development of fertilized starfish embryos. It is likely that discodermin A takes part in the defense mechanism of the sponge.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All solvents used were analytical grade or hplc grade. Uv spectra were recorded on a Hitachi 330 spectrometer. Ir spectra were measured on a JASCO A-202 infrared spectrophotometer. ^{13}C-nmr spectra were recorded on a JEOL JNM-FX 100 nmr spectrometer. ^{1}H-nmr spectra were recorded on JEOL JNM-FX100 and JEOL JNM-FX400 nmr spectrometers. Ei and fd mass spectra were measured on a JEOL JMS-DX-300 mass spectrometer. Optical rotation was determined on a JASCO DIP-140 polarimeter. Each mp was obtained from a Yanagimoto micro melting point apparatus and was uncorrected. Cd spectra were recorded on a JASCO J-20 spectropolarimeter. Amino acid analyses were carried out with a Hitachi 835 amino acid analyzer. High voltage paper electrophoresis was run on a Camag HVE system. Silca gel Woelm for partition chromatography was purchased from M. Woelm. Lobar LiChroprep Si60 (size C) was purchased from E. Merck. TSK G3000S gel, LS 410 ODS column, and LS 110 resin were obtained from Toyo Soda Manufacturing Co., Ltd.

COLLECTION AND EXTRACTION.—Colonies of *D. kiiensis* were collected by snorkeling (-3 to -5 m) at Shikine Island of the Izu Archipelago in July 1981 and identified by Dr. T. Hoshino of the Mukaishima Marine Biological Station of Hiroshima University. The animal was frozen immediately after collection and kept frozen until extraction. Frozen sponge (1 kg) was homogenized in 70% EtOH (5 liters) and stirred for 30 min at room temperature. After filtration, the residue was extracted in the same manner with 70% EtOH (5 liters) and then with EtOH (3 liters). The combined extracts were concentrated in vacuo and the resulting aqueous suspension was extracted with C_6H_6 (2 liters, twice). The aqueous phase was centrifuged to yield yellowish precipitates (12 g).

ISOLATION OF DISCODERMIN A.—The precipitates dissolved in 50% EtOH were applied to a column of TSK G3000S (3×18 cm) and eluted with 600 ml each of 50% EtOH, EtOH, and Me_2CO, in that order. A half-portion of the 50% EtOH eluates (3.5 g) was applied to a column of silica gel (4×40 cm) and eluted with $CHCl_3$-MeOH-H_2O (83:25:4). The antimicrobial fractions (660-1660 ml, 1.5 g) were collected and then chromatographed on a LiChroprep Si60 column (size C) with $CHCl_3$-MeOH-H_2O (80:20:3). The 1400-2400 ml fractions, showing a single spot on silica gel tlc plate ($CHCl_3$-MeOH-H_2O, 70:30:5), gave colorless solids (1.4 g) upon evaporation. Final purification was carried out by reverse-phase hplc on an LS 410 ODS column (7.5 mm×30 cm) with 65% MeOH. A 50 mg portion of the sample was injected at a time, and a total of 880 mg of discodermin A was obtained.

Discodermin A: $[\alpha]^{22}D$ −6.3° (c 0.7, MeOH); mp 226-228°; ir (KBr) 3400, 3050, 2920, 1740 sh, 1650, 1630, 1520, 1190, 1035 cm^{-1}; uv λmax (MeOH) 276, 282, 292 nm (ε 6200, 6500, 5600).

AMINO ACID ANALYSIS.—A portion of discodermin A (1 mg) in a pyrex test tube was dissolved in 6*N* HCl (1 ml) and sonicated under reduced pressure. The test tube was sealed in vacuo and kept at 110° for 24 h. After evaporation, the residue was redissolved in 0.5 ml of 1% HCl and subjected to the amino acid analysis.

HIGH VOLTAGE PAPER ELECTROPHORESIS.—Paper electrophoresis was carried out either in buffer A, pyridine-HOAc-H_2O (1:2:47), or in buffer B, HCOOH-HOAc-H_2O (1:4:45).

[2]The structural study will be reported elsewhere.

ISOLATION OF THE CONSTITUENT AMINO ACIDS.—Discodermin A (400 mg) was hydrolyzed in 6 *N* HCl (40 ml) at 110° for 16 h in evacuated test tubes. After being freed from HCl in vacuo, the dried residue was applied to a column of Hitachi custom 2616 gel (2×50 cm, pyridinium form) and eluted successively with 0.1 *N* pyridine-formate (pH 2.6, 1 liter), 0.1 *N* pyridine-formate (pH 3.2, 800 ml), 0.2 *N* pyridine-formate (pH 3.2, 700 ml), 1 *N* pyridine-acetate (pH 5.1, 700 ml), and 2 *N* pyridine-acetate buffers (pH 5.1, 900 ml), in that order. The 20-ml fractions were collected and monitored by tlc on silica gel with $CHCl_3$-MeOH-H_2O (65:35:8). Fractions showing the same tlc pattern were combined and subjected to the amino acid analysis. Pure X_1 (yield, 26 mg), Ala (30 mg), X_2 (19 mg), Leu (22 mg), Asp (15 mg), Phe (42 mg), and Arg (18 mg) were obtained by this procedure. Further separation of Pro from Thr was performed on the same column (H^+ form) with gradient elution of HCl (0-0.9 *N*) to give 10 mg of Pro and 31 mg of Thr. Isolation of Trp and Arg was achieved on a column of LS 110 (2×2.5 cm). Arg was eluted by H_2O and Trp by MeOH; 31 mg of Trp was obtained.

X_1: ^{1}H nmr (100 MHz, D_2O) δ 4.60 (1H, X portion of an ABX system), 3.60 (2H, AB portion of an ABX system); ^{13}C nmr (25 MHz, D_2O) δ 168.3s, 48.6 d, 47.9 t; cd spectrum gave a negative Cotton effect at 198 nm; fdms *m/z* 339 $(2M+H)^+$, 170 $(MH)^+$.

X_2: ^{1}H nmr (400 MHz, D_2O) δ 3.50 (1H, s), 1.12 (9H, s); ^{13}C nmr (25 MHz, D_2O) δ 172.9s, 63.6d, 31.9s, 25.9q (3C); cd specrum did not exhibit Cotton effect; fdms *m/z* 263 $(2M+H)^+$, 132 $(MH)^+$, 86, 74, 59.

ISOLATION OF X_3.—Acid hydrolysate of discodermin A (87 mg) was subjected to high voltage paper electrophoresis (buffer A, 70 V/cm, 40 min). The band containing X_3 was eluted with 10% HOAc and gel-filtered on a column of Sephadex G-15 (1×27 cm) with water. Ninhydrin positive fractions (27-36 ml) were collected to give 2.3 mg of X_3.

X_3: ^{1}H nmr (100 MHz, D_2O) δ 3.60 (1H, t, J=7 Hz), 2.70 (3H, s), 2.49 (2H, m), 2.15 (2H, m); cd spectrum exhibited a positive Cotton effect at 210 nm; fdms *m/z* 162 $(MH)^+$.

DISTINCTION BETWEEN THR AND *A*-THR.—The Thr preparation isolated from discodermin A, authentic Thr, and *a*-Thr were co-chromatographed on a silica gel tlc plate with *n*-BuOH-Me_2CO-25% NH_4OH-H_2O (8:1:1:6, upper layer). The authentic Thr and the isolated preparation exhibited the identical Rf value (0.13), while *a*-Thr showed a different value (0.08).

SYNTHESIS OF MEGLU.—Methyl sulfinyl carbanion was prepared by heating NaH in freshly distilled DMSO (50 mg/ml) at 70° for 15 min. To a solution of *N*-acetyl L-Glu (500 mg, Aldrich) in dry DMSO (5 ml) was added the methyl sulfinyl carbanion solution in DMSO (8 ml, 14 m mol eq.). The mixture was stirred overnight at room temperature. To the reaction mixture was added methyl iodide (3 ml), and the mixture was stirred for 20 min at room temperature. After addition of H_2O and extraction with CH_2Cl_2, the organic phase was evaporated and hydrolyzed in 6 *N* NCl at 110° for 48 h. The hydrolysate was freed from HCl and a portion was purified by high voltage paper electrophoresis (buffer B, 100 V/cm, 40 min) followerd by gel filtration on Sephadex G-15 (1×27 cm) with water to obtain L-MeGlu (6 mg).

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