Antineoplastic agents. 193. Isolation and structure of the cyclic peptide hymenistatin 1

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The Western Pacific Ocean sponge *Hymeniacidon* sp. was found to contain a cyclo-octapeptide designated hymenistatin 1 (**1**) active against the P388 leukemia cell line (ED_{50} 3.5 μg/mL). Structural determination was accomplished utilizing high field NMR (400 MHz) and mass spectral techniques (FAB MS/MS) followed by chiral gas chromatographic analysis to establish the absolute configuration (all *S*-amino acids).

Key words: cytostatic, *Hymeniacidon*, hymenistatin, cyclo-octapeptide, P388 leukemia.

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On a trouvé que l'éponge *Hymeniacidon* sp., trouvée dans l'ouest de l'océan Pacifique, contient un cyclo-octapeptide appelé hymenistatine 1 (**1**) qui est actif contre la ligne des cellules P388 de la leucémie (ED_{50} 3,5 μg/mL). On déterminé la structure à l'aide des techniques de la RMN à 400 MHz et de la spectrométrie de masse (FAB MS/MS) et, en se basant sur une analyse par chromatographie en phase gazeuse chirale, on a pu établir que tous les acides aminés sont de configuration *S*.

Mots clés : cytostatique, *Hymeniacidon*, hymenistatine, cyclo-octapeptide, P388 de la leucémie.

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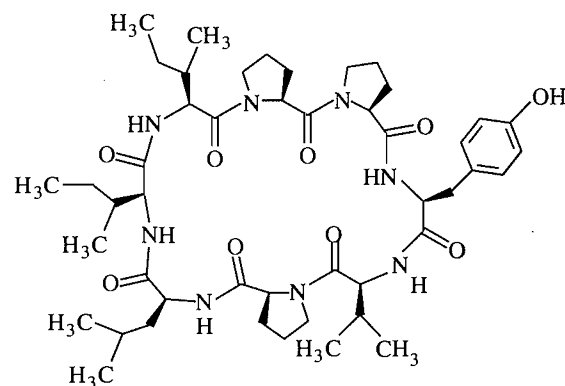
Early (2) investigations directed at isolation of marine sponge antineoplastic constituents led us initially to the geodiastatin proteins (3) and later to a series of pyrrolactams (4). Meanwhile, we have been pursuing other more promising leads in the Porifera phylum, including antineoplastic peptides. While a number of amino acids (5) have been isolated from marine sponges, only a small number of peptides (6) and antineoplastic sub[illegible]ces (7) have been isolated from these organisms. One of the *Hymeniacidon* sp. (Demospongiae Class) we collected in Palau in 1979 gave aqueous 2-propanol – methylene chloride extracts that led to a 30% life extension against the U.S. National Cancer Institute's murine P388 lymhocytic leukemia (PS system). Bioassay directed isolation using the PS leukemia led to isolation of a new cytostatic peptide designated hymenistatin (1).

After extraction and a preliminary solvent partition separation, a 1985 recollection (~ 250 kg wet wt.) of the sponge gave a PS active methylene chloride extract that was successively partitioned between 9:1 methanol–water and hexane, then diluted to 3:2 methanol–water and extracted with methylene chloride. Upon evaporation of the chlorocarbon an orange-colored solid was obtained, which significantly (PS ED_{50} 0.26 μg/mL) inhibited the PS leukemia. Initial gel-permeation chromatographic separation of the methylene chloride soluble fraction on Sephadex LH-20 and elution with 3:2 methylene chloride – methanol led to a further concentration of activity. A much better separation was achieved employing partition chromatography on Sephadex LH-20 with 3:1:1 hexane–toluene–methanol. The two principal active fractions from this chromatogram were combined and separated on a column of



Hymenistatin 1

(**1**)

Sephadex LH-20 in methanol. The nearly pure cytostatic constituent obtained by this means was chromatographed using a silica gel Lobar column. Elution with 97.5:2.5 methylene chloride – methanol afforded the cell growth inhibitory (PS ED_{50} 3.5 μg/mL) biosynthetic product we designated as hymenistatin 1 (**1**).

Mass spectral measurements of hymenistatin indicated the presence of eight nitrogen atoms, and that information combined with the eight amide carbonyls observed in the ^{13}C NMR spectrum pointed to an octapeptide. The relatively high intensity of the molecular ion (base peak) observed in the FAB-MS spectrum suggested that the presumed octapeptide might be cyclic. Extensive application of 2D NMR techniques was next used to determine the identity of the eight amino acid units. The 400 MHz ^{1}H NMR spectrum clearly showed the presence of only five amide protons. By following the spin systems of these protons using $^{1}H,^{1}H$-COSY, and $^{1}H,^{1}H$-relayed COSY (8),

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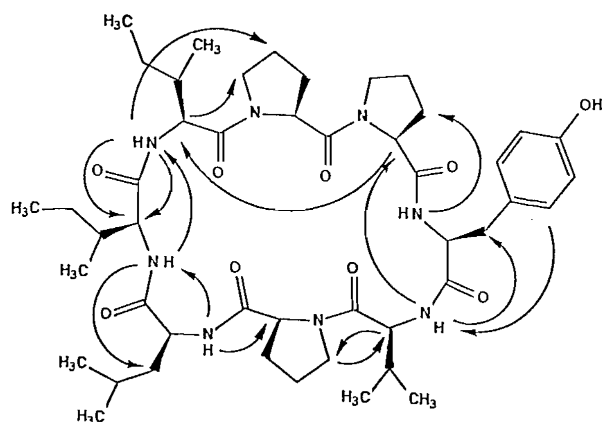


FIG. 1. Selected NOE's for hymenistatin **1** in deuteriochloroform.

cyclo [Pro^{a}-Pro^{b}-Tyr-Val-Pro^{c}-Leu-Ile-Ile]

Observed mass spectral fragmentation resulting from protonation at proline (indicated by a, b, or c):

a, ProH^{+}---Pro---Tyr---Val---Pro---Leu---Ile---Ile
m/z 195 358 457 554 667 780 — from ms of *m/z* 780

b, ProH^{+}---Tyr---Val---Pro---Leu---Ile---Ile---Pro
m/z 261 360 457 570 683 796 — from ms of *m/z* 796

c, ProH^{+}---Leu---Ile---Ile---Pro---Pro---Tyr---Val
m/z 211 324 437 534 794 — from ms of *m/z* 794

FIG. 2. Hymenistatin 1 peptide sequence determination by FAB MS/MS.

these amino acids were determined to be valine, leucine, tyrosine, and two isoleucine units. Utilization of ^{1}H,^{13}C-COSY, and ^{1}H,^{1}H,^{13}C-RELAY (9) showed that the remaining NMR signals consisted of three independent spin systems of the type X-CH-CH_2-CH_2-CH_2-X typical of proline (10). The eight amino acid units accounted for the observed accurate-mass molecular weight. Actual sequence of the amino acids was ascertained from 1D-NOE experiments as summarized in Fig. 1.

To further corroborate the proposed structure, positive ion FAB MS/MS was pursued. As shown in Fig. 2, protonation at each proline nitrogen afforded three different linear peptide ions in the mass spectrum noted as A, B, and C. All but one of the ions predicted from their fragmentation was also observed, thereby confirming the structure assigned hymenistatin (**1**).

The absolute configuration of each chiral center was assigned using chiral GC analysis (Chirasil-Val III column) of the peptide hydrolysate following preparation of the *N*-pentafluoropropionyl/isopropyl ester derivatives (11). All of the amino acid units were found to bear the L configuration. Discovery of hymenistatin 1 (**1**), a seemingly unexceptional (12) cyclic peptide, that has cell growth inhibitory properties provides a new insight into structure requirements for such biological activity. Further biological evaluations and total synthesis are in progress.

Experimental

General methods

Solvents used for chromatographic procedures were redistilled. The Sephadex LH-20 (25-100 μm) employed for gel permeation and partition chromatography was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Gilson FC-220 race track and F-80 microfractionators connected to Gilson HM UV–VIS Holochrome detectors were used for chromatographic fractionation experiments. Column chromatographic procedures with silica gel utilized the silica gel 60 prepacked "Lobar" columns supplied by E. Merck, Darmstadt, West Germany. The silica gel GF Uniplates for TLC were from Analtech Inc., Newark, Delaware. All TLC plates were viewed with UV light and (or) developed with a ceric sulphate – sulfuric acid spray (heating to approximately 150°C for 10 min).

The uncorrected melting points were observed using a Kofler-type melting point apparatus. The UV spectrum was recorded using a Hewlett–Packard 8450A UV–VIS spectrophotometer equipped with a HP7225A plotter. Optical rotation and IR spectral data were obtained using a Perkin–Elmer 241 polarimeter and a Nicolet MX-1 FTIR spectrophotometer, respectively. Mass spectra (70 eV and FAB) were recorded employing a Kratos MS-50 spectrometer. The NMR experiments were conducted with a Bruker WH-400 instrument and deuteriochloroform as solvent (TMS internal standard).

Animal collection and preliminary experiments

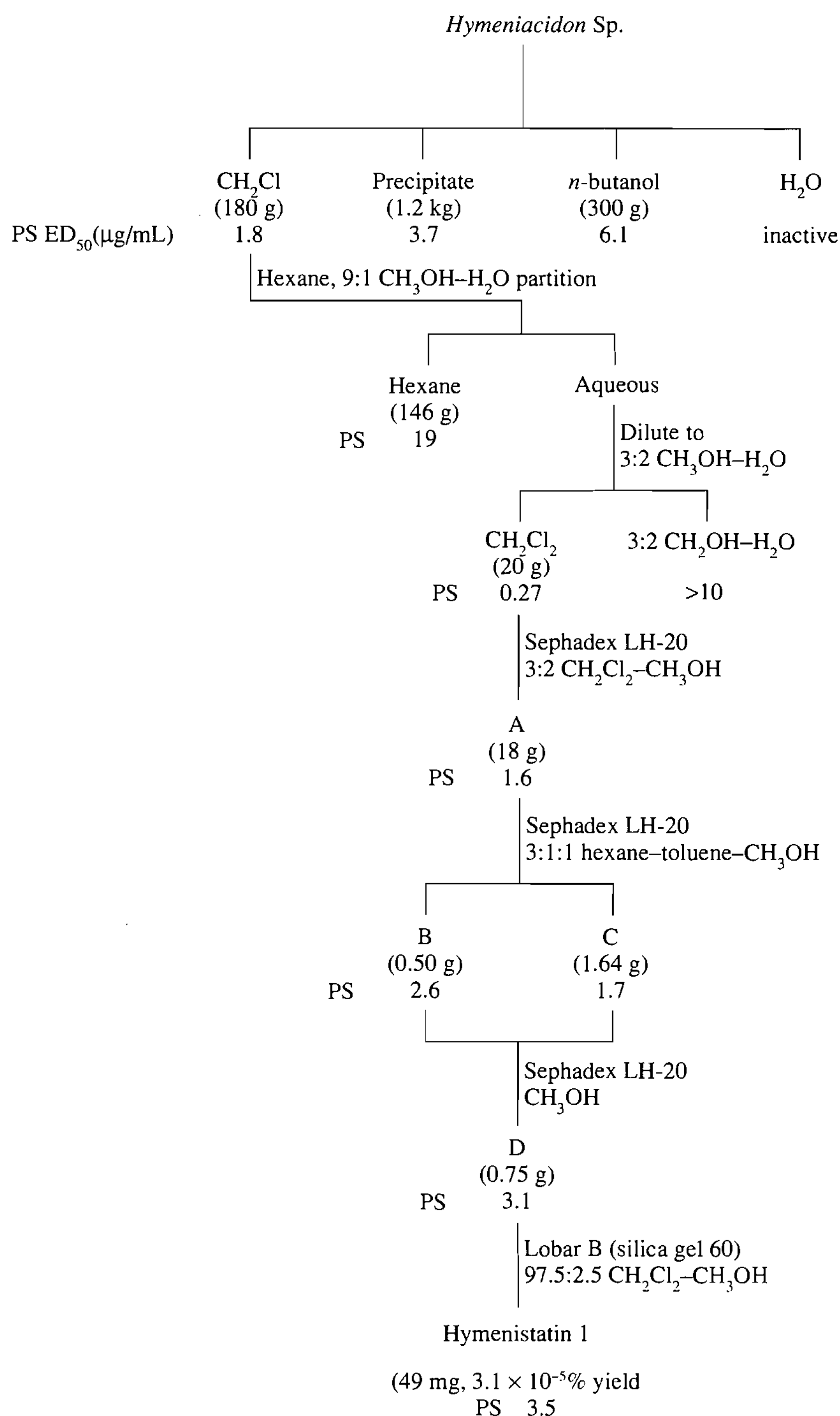
In early 1979, approximately 2 kg (wet wt) of the sponge *Hymeniacidon* sp. (Hymeniacidonidae family, Halichondrida Order, Ceratinomorpha Subclass, Demospongiae Class) was collected by scuba near Long Island (south side) in the Palau Archipelago, Western Caroline Islands. Taxonomic identification was conducted in the Smithsonian Institution where reference specimens are on file. The initial sample of Hymeniacidon sp. was preserved in 2-propanol. Removal of solvent gave an extract that reached a confirmed level of activity against the U.S. National Cancer Institute's murine P388 lymphocytic leukemia (PS system) with 30% life extension at 5.5 mg/kg.

Animal extraction and solvent partitioning

In March 1985, 250 kg (wet wt) of the *Hymeniacidon* sp. was recollected from the same area in Palau near the south side of Long Island and preserved in 2-propanol. The 2-propanol solution was decanted and the sponge was reextracted with the same alcohol. The extract was evaporated (*in vacuo*) to a 50 L water concentrate and found to contain a considerable amount (1.2 kg) of a pale brown suspension (PS T/C toxic at ≥ 50 mg/kg), which was removed by centrifugation (1000 *g*) and decantation. The cream-colored aqueous phase was successively partitioned between methylene chloride (90 L) and *n*-butanol (90 L). Evaporation of solvent from the chlorocarbon and *n*-butanol extracts gave, respectively, a very dark, brown, gel-like solid (180 g, PS ED_{50} 1.8 μg/mL and T/C 105 at 50 mg/kg) and a pale brown amorphous solid (300 g PS ED_{50} 6.1 μg/mL and T/C toxic at ≥ 50 mg/kg). The remaining aqueous extract was found to be PS inactive and was discarded. A solution of the methylene chloride fraction (180 g) in 9:1 methanol–water (1 L) was extracted with hexane (3 × 1 L). The methanol–water phase was diluted to 3:2 and extracted with methylene chloride. The resulting hexane (146 g), methylene chloride (20 g), and 3:2 methanol–water (14 g) fractions were concentrated and aliquots submitted for bioassay. Significant PS cytostatic activity (PS ED_{50} 0.26 μg/mL) was found to reside in the methylene chloride extract.

Isolation of hymenistatin 1 (1)

The 20 g sample of the PS active methylene chloride fraction in 3:2 methylene chloride – methanol was chromatographed on a column of Sephadex LH-20 (2.5 kg; 9 × 130 cm). While no pronounced separation of components was observed, further concentration to an active fraction designated A (elution volume 3.9 L, 18 g, PS ED_{50} 1.6 μg/mL) was realized. Further partition chromatography on Sephadex LH-20 (2.5 kg; 9 × 130 cm) and elution with 3:1:1 hexane–toluene–methanol gave active fractions B (elution vol. 500 mL, 0.50 g, PS ED_{50} 2.6 μg/mL) and C (elution vol. 480 mL, 1.64 g, PS ED_{50} 1.7 μg/mL)



SCHEME 1

among 11 distinct composite fractions. Fractions B and C were combined and further separated in methanol on a Sephadex LH-20 column (500 g, 3.5 × 120 cm). Among the ten fraction groups obtained, a fraction labeled D (at elution vol. 60 mL, 0.75 g, PS ED_{50} 3.1 μg/mL) showed an almost single spot on tlc. Final purification was achieved utilizing a medium pressure (to 50 psi; 1 psi = 6.9 kPa) liquid chromatograhy unit with a prepacked silica gel 60 column (2.5 × 30 cm) and elution with 97.5:2.5 methylene chloride – methanol. Hymenistatin 1 was isolated as a colorless, amorphous solid (49 mg, 3.1×10^{-5} % yield) melting at 180–182°C: $[\alpha]_D$ −8.6° (*c* 1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε): 222 (3.82), 278 (3.16) nm; IR (NaCl plate): 3320, 2960, 2920, 1680, 1617, 1517 cm^{-1}; MS (HR-FAB): 893.5505 $[M + H]^+$ for $C_{47}H_{73}N_8O_9$; calcd. 893.5510; NMR ($CDCl_3$), δ, **Proline-a** unit, ^{1}H: 4.20 (H-2), 3.72 (H-5a), 3.38 (H-5b), 2.15 (H-3a), 2.11 (H-4a), 1.95 (H-4b), 1.93 (H-3b); ^{13}C: 60.89 (C-2), 47.35 (C-5), 31.87 (C-3), 25.06 (C-4); **Proline-b** unit, ^{1}H: 4.10 (dd, *J* = 9.4, 3.5; H-2), 3.28 (H-5a), 3.21 (H-5b), 2.16 (H-3a), 1.77 (H-3b), 1.60 (H-4a), 0.85 (H-4b); ^{13}C: 59.19 (C-2), 46.94 (C-5), 28.52 (C-3), 21.11 (C-4); **Proline c** unit, ^{1}H: 3.92 (H-5a), 3.79 (t, *J* = 7.8; H-2), 3.68 (H-5b), 2.31 (H-3a), 2.08 (H-4a), 2.06 (H-4b), 1.91 (H-3b), ^{13}C: 63.11 (C-2), 48.55 (C-5), 30.00 (C-3), 24.75 (C-4); **Tyrosine** unit, ^{1}H: 8.70 (br s, OH), 7.03 (d, *J* = 8.1; H-5), 6.84 (d, *J* = 8.0; H-6), 4.22 (H-2), 3.29 (H-3a), 2.90 (t, *J* = 13.0, H-3b); ^{13}C: 156.56 (C-7), 129.67 (C-5, C-9), 127.00 (C-4), 115.94 (C-6, C-8), 58.13 (C-2),

36.67 (C-3); **Valine** unit, ^{1}H: 7.58 (d, J = 8.8; NH), 4.56 (t, J = 5.9, H-2), 1.95 (H-3), 0.98 (6H; H-4, H-5); ^{13}C: 56.29 (C-2), 31.67 (C-3), 19.29 (C-4), 18.19 (C-5); **Leucine** unit, ^{1}H: 6.25 (br s, NH), 3.98 (H-2), 1.96 (H-3a), 1.82 (H-3b), 1.55 (H-4), 0.93 (3H; d, J = 6.3, H-6), 0.88 (3H, H-5); ^{13}C: 55.79 (C-2), 39.27 (C-3), 25.31 (C-4), 22.95 (C-6), 21.27 (C-5); **Isoleucine-a** unit, ^{1}H: 7.70 (d, J = 8.5, NH), 4.40 (t, J = 8.5, H-2), 1.57 (H-3), 1.50 (H-4a), 1.15 (H-4b), 0.96, (3H; H-6), 0.87 (3H; H-5); ^{13}C: 60.52 (C-2), 38.26 (C-3), 24.95 (C-4), 15.47 (C-6), 10.64 (C-5); and **Isoleucine-b** unit, ^{1}H: 7.40 (d, J = 8.5, NH), 4.69 (t, J = 9.0, H-2), 1.76 (H-3), 1.53 (H-4a), 1.05 (H-4b), 0.82 (3H; H-6), 0.80 (3H; H-5); ^{13}C: 55.01 (C-2), 36.94 (C-3), 24.79 (C-4), 16.20 (C-6), 11.87 (C-5). Eight carbonyl ^{13}C resonances appear at 172.72, 172.29, 172.10, 171.49, 171.95, 171.18, 170.41, 170.35. Where no multiplicity is indicated it could not be determined due to overlapping signals.

Assigment of the hymenistatin (1) chiral centers

The cyclo-octapeptide was hydrolyzed with 1:1 propionic acid – 12 N hydrochloric acid at 160°C for 15 min (13). The corresponding amino acids were converted to *N*-pentafluoropropionyl/isopropyl ester derivatives and configurations established by chiral capillary chromatography as reported by Shaw and Cotter (11) using a Chirasil Val III column. Each amino acid component was found to belong to the *S*(L) series.

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