Diazepinomicin, a New Antimicrobial Alkaloid from a Marine *Micromonospora* sp.[†]

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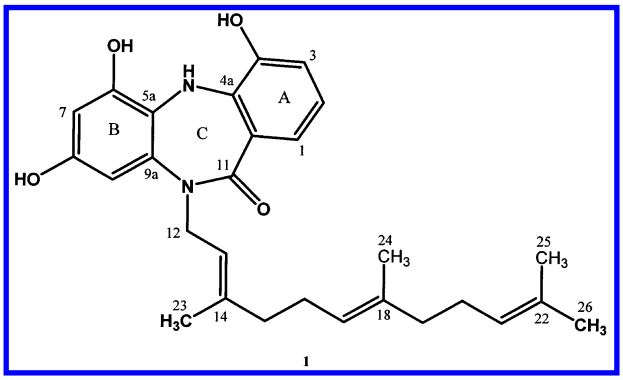
The structure of a new dibenzodiazepine alkaloid, diazepinomicin (**1**), isolated from the culture of a marine actinomycete of the genus *Micromonospora* was characterized using spectroscopic methods. Diazepinomicin represents a unique molecular class composed of a dibenzodiazepine core linked to a farnesyl side chain.

Owing to our continued interest in highly potent cytotoxic agents as potential "warheads" suitable for linkage to monoclonal antibodies targeting tumor-specific antigens,[1] we have continued to search for microbial sources of such compounds.[2] A recent report from the Fusetani group describing the exquisitely potent shishijimicins from the marine ascidian *Didemnum proliferum*[3] led us to begin an examination of the colonial animal as a source of the putative microbial producer of the enediynes. In the course of this work a unique actinomycete of the genus *Micromonospora* was isolated, which was designated DPJ12. Culture DPJ12 was grown on a variety of media under various conditions, some of which yielded potent antibacterial activity. Guided by this activity, a single antibiotic diazepinomicin (**1**) was isolated. In this report, we describe the isolation and structure determination of this unique natural dibenzodiazepine.



1

Micromonospora strain DPJ12, isolated from *Didemnum proliferum* Kott. collected by scuba at Shishijima Island, Japan, was fermented in the presence of HP20 resin for 9 days, at which time the cell mass and resin were collected by centrifugation. The collected solids were then extracted with methanol, and the new dibenzodiazepine component was isolated from this extract via solvent partitioning and chromatography. A 4-L fermentation yielded 4.8 mg of pure diazepinomicin (**1**), which was subsequently used for characterization by standard spectroscopic methods.

The molecular formula of **1** was established as $C_{28}H_{34}N_2O_4$ by high-resolution Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry (*m*/*z* 463.25815 [M + H]$^+$ calcd for $C_{28}H_{35}N_2O_4$ 463.25914), which required 13 degrees of unsaturation.

The ^{1}H and ^{13}C NMR in connection with APT and HSQC experiments (Table 1) of **1** indicated five aromatic and three olefinic methine, five methylene, and four methyl groups in addition to 11 quartenary carbon atoms that included one carbonyl group (δ_C 167.8). Combined, these data accounted for only 10 degrees of unsaturation. As the molecular formula required 13 degrees of unsaturation, we concluded that **1** must contain three rings.

Four hydrogen resonances (all singlets) lacked correlations in the HSQC spectrum of **1** and were therefore recognized as being located on heteroatoms. From their chemical shifts and HMBC data, three were assigned to phenolic hydroxyl protons [4-OH (δ_H 10.03), 6-OH (δ_H 9.93), and 8-OH (δ_H 9.03)], whereas the remaining hydrogen resonance at δ_H 6.72 was assigned to the 5-NH proton.

Information from the ^{1}H−^{1}H COSY and the HSQC spectra (obtained in DMSO-d_6) pointed to a three-proton spin system ranging from position 1 (δ_H 7.07, δ_C 122.2) over 2 (δ_H 6.70, δ_C 120.4) to position 3 (δ_H 6.83, δ_C 116.3), thus establishing half of ring A. The sequence of the carbon atoms in the remaining portion of ring A could be deduced from ^{1}H−^{13}C correlations in the HMBC experiment. Three-bond correlations were observed from H-1 and H-3 to C-4a (δ_C 141.1), from H-3 to C-1 (δ_C 122.2), as well as H-1 to C-3 (δ_C 116.3), and from H-2 to C-4 (δ_C 145.4) and C-11a (δ_C 124.8). Additional HMBC correlations from the hydroxyl proton (δ_H 10.03) to C-3, C-4, and C-4a and the chemical shifts indicated that C-4 carried the hydroxyl group. This firmly established the substitution pattern of ring A. A three-bond correlation from H-1 to C-11 (δ_C 167.8) suggested that the carbonyl carbon was bound to C-11a. The above results were corroborated by additional NMR data acquired on **1** in MeOH-d_4.

The substitution pattern of ring B was elucidated in a similar manner. The small coupling (J = 2.2 Hz) between the two aromatic protons at δ_H 6.19 (H-7) and δ_H 6.17 (H-9) placed them in meta positions flanking C-8 (δ_C 153.3), to which both protons showed two-bond HMBC correlations. The chemical shift of C-8 suggested that it carried a hydroxyl group, and that was readily confirmed by the HMBC correlations from the 8-OH (δ_H 9.03) to C-8, C-7 (δ_C 99.4) and C-9 (δ_C 100.4). Since C-7 also showed a HMBC correlation from the 6-OH proton (δ_H 9.93), it had to be located between these two hydroxyl groups. Further correlations from 6-OH to C-6 (δ_C 147.7) and C-5a (δ_C 124.8) established this sequence. The noted HMBC correlations from the proton on the secondary amine group (δ_H 6.72) to C-5a, C-6, C-9a (ring B) and C-4, C-4a, C-11a (ring A) secured the position of this amine bridging C-4a and C-5a. While HMBC correlations from H-9 to C-9a and C-8 were noted in DMSO-d_6, additional HMBC correlations from H-9

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Table 1. NMR Spectral Data for **1** Recorded in DMSO-d_6 and MeOH-d_4 at 400 MHz

	DMSO-d_6				MeOH-d_4	
pos.	δ_C	δ_H (mult., J = Hz)	COSY	HMBC	δ_C	δ_H (mult., J = Hz)
1	122.2	7.07 (dd, 7.0, 1.4)	H-2, H-3	C-3, C-4a, C-11	123.5	7.14 (dd, 8.0, 1.5)
2	120.4	6.70 (t, 7.9)	H-1, H-3	C-4, C-11a	122.1	6.75 (t, 7.8)
3	116.3	6.83 (dd, 7.2, 1.4)	H-1, H-2	C-1, C-4, C-4a	117.8	6.82 (dd, 7.8, 1.5)
4	145.4				147.0	
4a	141.1				143.0	
5		6.72 (s)		C-4, C-4a, C-5a, C-6, C-9a, C-11a	-	
5a	124.8				127.6	
6	147.7				149.4	
7	99.4	6.19 (d, 2.2)	H-9	C-5a,[a] C-6, C-8, C-9	101.0	6.20 (d, 2.4)
8	153.3				154.5	
9	100.4	6.17 (d, 2.2)	H-7	C-5a,[a] C-7,[a] C-8, C-9a	102.1	6.26 (d, 2.4)
9a	134.9				136.6	
11	167.8				171.3	
11a	124.8				126.1	
12	47.8	4.38 (d, 6.1)	H-13	C-9a, C-11, C-13, C-14	49.3	4.52 (d, 6.2)
13	121.6	5.26 (t, 5.7)	H-12, H-15, H-23	C-15, C-23	122.3	5.35 (t, 6.1)
14	136.8				139.4	
15	39.3	1.98 (m)	H-16	C-14, C-17	40.8	1.94 (m)
16	25.8	2.01 (m)	H-15, H-17	C-17, C-18	27.8	2.02 (m)
17	123.6	5.07 (m)	H-16, H-24	C-15, C-16, C-24	125.1	5.07 (m)
18	134.4				136.2	
19	39.3	1.92 (m)	H-20	C-17, C-18	40.6	2.08 (m)
20	26.2	1.98 (m)	H-19, H-21	C-21, C-22	27.3	2.02 (m)
21	124.2	5.05 (m)	H-20, H-25, H-26	C-19, C-20, C-25, C-26	125.4	5.04 (m)
22	130.5				131.9	
23	16.2	1.65 (br s)	H-13	C-13, C-14	16.6	1.71 (br s)
24	15.8	1.55 (br s)	H-17	C-17, C-18	16.1	1.57 (br s)
25	17.4	1.51 (br s)	H-21	C-21, C-22	17.7	1.54 (br s)
26	25.5	1.62 (br s)	H-21	C-21, C-22	25.9	1.63 (br s)
4-OH		10.03 (s)		C-3, C-4, C-4a		
6-OH		9.93 (s)		C-5a, C-6, C-7		
8-OH		9.03 (s)		C-7, C-8, C-9		

[a] Additional correlations noted in MeOH-d_4.

to C-7 (δ_C 101.0) and C-5a (δ_C 127.6) were observed when **1** was dissolved in MeOH-d_4 (Table 1). These data support the substructure of ring B, with the exception of identifying the substituent on C-9a.

According to the molecular formula, the remaining elements not accounted for include a single nitrogen atom and a $C_{15}H_{25}$ fragment. The hydrocarbon was identified as a farnesyl group (see below). Assignment of the nitrogen to an amide group including C-11 and bridging to C-9a was consistent with an IR absorption band at 1676 cm^{-1} and the chemical shifts of both C-9a (134.9) and C-11 (167.8). Direct evidence that rings A and B were fused via ring C, thus forming the dibenzodiazepine moiety, was deduced from important three-bond HMBC correlations to C-9a and C-11 from the protons of the first methylene group [δ_H 4.38, d, J = 6.1 Hz; δ_C 47.8 (t)] in the side chain.

Analysis of the COSY and HMBC data revealed that the remaining resonances assignable to the side chain originated from four methyls, five methylenes, and three methine groups constituting a farnesyl residue.[4] The structure elucidation of the farnesyl unit followed from HMBC correlations from the protons of the nitrogen-attached methylene group at δ_H 4.38 (H-12) to C-13 (δ_C 121.6) and C-14 (δ_C 136.8), supported by observed COSY correlations to H-13. Mutual HMBC correlations were noted between H-13 and C-23 (δ_C 16.2) and H-23 (δ_H 1.65) and C-13, with H-23 also correlating to C-14 (δ_C 136.8). In turn, HMBC correlations to C-14 were noted from the methylene protons at δ_H 1.98 (H-15), together establishing the first segment. The next segment of the farnesyl unit was confirmed on the basis of COSY correlations between H-15 and H-16 (δ_H 2.01) as well as from H-16 to the vinyl proton H-17 (δ_H 5.07). Vinylic coupling between H-17 and methyl proton 24 (δ_H 1.55) was evident in the COSY spectrum. HMBC correlations from H-17 to C-24 and from methyl 24 to C-17 and C-18 (δ_C 134.4) provided further support for this assignment. This partial structure was further extended on the basis of HMBC correlations from H-19 (δ_H 1.92) to C-17 and C-18. COSY correlations connected H-19 and H-20 (δ_H 1.98) with H-20 and H-21 (δ_H 5.05), which were located in the final segment of the farnesyl unit. The constitution of the final segment was further corroborated by HMBC correlations from H-20 to C-21 (δ_C 124.2) and from H-21 to C-19 (δ_C 39.3) and C-20 (δ_C 26.2). Vinylic coupling from H-21 to methyl 25 (δ_H 1.51) and methyl 26 (δ_H 1.62) as well as HMBC correlations from H-21 to C-25 (δ_C 17.5) and C-26 (δ_C 25.5) further corroborated this segment that terminated in methyl groups 25 and 26, both of which showed correlations to C-21 and C-22 (δ_C 130.5), thus supporting the assembly of the entire farnesyl side chain. The stereochemistry of the double bonds within the farnesyl moiety was established as all *E* on the basis of ^{13}C NMR chemical shifts.[4,5]

Further support for the structure of the diazepine ring as shown was derived from the analysis of fragment ions observed in FT-ICR IRMPD (infrared multiphoton dissociation), as shown in Figure 1. Fragment *a* represents excision of the amide bond from the diazepine ring, with the attached farnesyl group, substantiating the integrity of this structural unit (Table 2). Cleavage of the farnesyl unit adjacent to the amide nitrogen yields ion *b*, representing the dibenzodiazepine core. The base peak in the spectrum, *c*, is formed by α-cleavage of the farnesyl amido moiety. Another, structurally characteristic fragment ion, *d*, representing loss of farnesylamine, arises by cleavage of the amide bond and the N−C-9a bond.

Diazepinomicin showed modest antimicrobial activity against selected Gram-positive bacteria with MICs of about

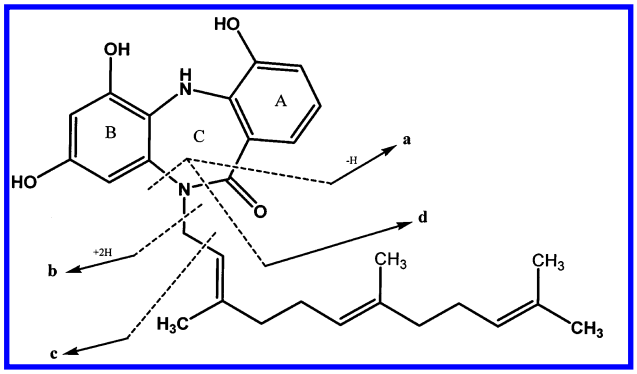

Figure 1. Proposed fragmentation pathway for the $[M + H]^+$ ions observed in the NanoESI FTMS IRMPD mass spectrum of **1**.

Table 2. MS Fragments of **1** Observed in the FTMS IRMPD (Infrared MultiPhoton Dissociation) Spectrum

ion	relative abundance (%)	*m/z*	composition	error (mmu)
a	17	214.04946	$C_{12}H_8NO_3$	−0.41
b	67	259.07093	$C_{13}H_{11}N_2O_4$	−0.40
c	100	271.07077	$C_{14}H_{11}N_2O_4$	−0.56
d	28	243.05217	$C_{13}H_9NO_4$	−0.44

32 μg/mL. The dibenzodiazepine core structure of diazepinomicin is exceptionally rare in nature, with the only other example of a natural substance cited in the patent literature.[6] Given the relative abundance of such compounds in the synthetic chemical literature, particularly derived from pharmaceutical research, an understanding of the biosynthesis of these compounds for potential directed biosynthetic experiments and genetic manipulation is warranted.

Experimental Section

General Experimental Procedures. UV data were obtained on a HP1100 HPLC system equipped with DAD. All 1D and 2D NMR spectra were recorded on a Bruker DPX-400 spectrometer at 400 and 100 MHz for ^{1}H and ^{13}C, respectively, using a 3 mm broadband probe. The number of attached protons for ^{13}C resonances was determined from an APT experiment. Proton detected heteronuclear correlations were measured using HSQC (optimized for $^1J_{C-H} = 140$ Hz) and HMBC (optimized for $^nJ_{C-H} = 8.3$ Hz) pulse sequences. IR spectra were obtained on a Nicolet Nexus 470 with SMART-Endurance Diamond ATR module. High-resolution mass spectra (HRMS) were obtained using a Bruker (Billerica, MA) APEXII FT-ICR mass spectrometer equipped with an actively shielded 7.1 T superconducting magnet (Magnex Scientific Ltd., UK), an external Bruker APOLLO ESI source, and a Synrad 50W CO_2 CW laser. The molecular ion at *m/z* 463 was isolated using correlated sweep and then dissociated using infrared multiphoton dissociation (IRMPD). All HPLC solvents were EM Omnisolv quality and used without further purification.

Isolation and Taxonomy of the Actinomycete Strain DPJ12. Strain DPJ12 was isolated from *Didemnum proliferum* Kott. collected by scuba at Shishijima Island, Japan. From a frozen sample, 120 mg of inner core tissue was macerated in 1 mL of sterile seawater. The resulting homogenized slurry was plated onto two different selective media. The plates were examined periodically for the presence of actinomycete colonies. Isolates were transferred to fresh agar media.

Chromosomal DNA was isolated from an agar-grown culture of DPJ12 by a simple Triton X-100, SDS cell lysis followed by a phenol/chloroform/isoamyl alcohol precipitation. The almost complete 16S rDNA (1442 bp) was PCR amplified using partial primers of 8FPL and 1492RPL.[7] The 16S rDNA of strain DPJ12 shares more than 99.5% identity with other *Micromonospora* species in the GenBank database. More specifically, the most closely related type strain is *Micromonospora chalcea*. Morphologically, DPJ12 produces orange substrate mycelia with occasional, sparse, white aerial mycelia. Spores are produced in a black spore mass and appear singly on sporophores. On the basis of morphological and phylogenetic evidence, the actinomycete strain DPJ12 was assigned to the genus *Micromonospora*.

Fermentation of DPJ12. For the first-stage seed, an agar grown culture of DPJ12 was inoculated into 10 mL of YPSS medium (4.0 g/L yeast extract, 0.5 g/L potassium phosphate dibasic, 10.0 g/L soluble starch, 500 mL of distilled water, and 500 mL of artificial seawater, pH 7.2). After 7 days incubation at 22 °C with agitation, the first stage was used to inoculate the production fermentation: 1 L of Bennett's broth made with 50% artificial seawater (10.0 g/L dextrose, 0.77 g/L beef extract, 1.0 g/L yeast extract, 2.0 g/L NZ-amine A, 50 g/L activated HP20 resin, 500 mL of distilled water, and 500 mL of artificial seawater, pH 7.3). The fermentation was incubated at 28 °C with agitation and harvested on the ninth day.

Isolation and Purification Procedures. The harvested fermentation broth (4 L) was centrifuged at 3800 rpm for 20 min, and the HP20 resin together with the cell mass was washed with deionized water (1 L) and then extracted with methanol (3 × 250 mL). The combined methanol extracts were concentrated in vacuo, and the aqueous suspension was extracted with ethyl acetate (4 × 60 mL). The ethyl acetate extract (278 mg) was purified by Sephadex LH-20 column chromatography (MeOH). Final purification of the alkaloid-rich fraction (10 mg) was achieved by reversed-phase HPLC (YMC ODS-A column, 10 × 250 mm, 5 μm) using a gradient of 0−90% acetonitrile/water containing 0.02% TFA over 30 min to give the new dibenzodiazepine alkaloid, diazepinomicin (**1**, 4.8 mg).

Diazepinomicin (1), 4,6,8-trihydroxy-10-(14,18,22-trimethyldodeca-13,17,21-trienyl)-5,10-dihydrodibenzo[*b*,*e*]-[1,4]diazepin-11-one: UV (MeOH/H_2O gradient with 0.025% formic acid, HP1100 photodiode array detector) λ_{max} (relative absorption) 212 (1), 230 (0.9), 298 (0.25) nm; IR (film) ν_{max} 3000−3500, 1676, 1604, 1575 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 463.25815 $[M + H]^+$ (calcd for $C_{28}H_{35}N_2O_4$, 463.25914).

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of diazepinomicin are available free of charge via the Internet at http://pubs.acs.org.

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