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New Cytotoxic Manzamine Alkaloids from a Palaun Sponge

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Abstract—A crude extract of a marine sponge showed initial inhibitory bioactivities in a yeast assay for inhibitors of methionine aminopeptidase-2 (Met AP-2). Bioassay-directed fractionation indicated that the activity was concentrated in the CH_2Cl_2-soluble fraction, and chromatography on silica gel led to the isolation of the two new bioactive alkaloids *N*-methyl-*epi*-manzamine D **1** and *epi*-manzamine D **2**. The structures of the *epi*-manzamines were assigned by ^1H and ^{13}C NMR, DEPT, HMQC, and HMBC spectroscopy, and by comparison with the spectra of related compounds, and the structure of **1** was confirmed by X-ray structure analysis. Neither of the two isolated compounds showed selectivity in the yeast assay for inhibitors of Met AP-2, but both compounds were cytotoxic to HeLa and B16F10 mammalian cells, with compound **1** showing strong activity against the B16F10 cell line.

Angiogenesis, or the development of new blood vessels, is an essential requirement of solid tumor growth, and it has been proposed to be the rate-limiting factor for tumor growth; angiogenesis inhibitors could thus play an important role in cancer chemotherapy.The antiangiogenic agent fumagillin has been shown to target methionine aminopeptidase type 2 (Met AP-2); the related enzyme Met AP-1 is not affected by fumagillin.[1,2] Yeast strains are available with the genes for both Met AP-1 and Met AP-2 deleted;[3] compounds that act in the same way as fumagillin will be selective inhibitors of Met AP-2, and will thus show a greater growth inhibition for yeasts lacking Met AP-1 than for those lacking Met AP-2.[1] The differential sensitivity of Δmap1 and Δmap2 strains of yeast thus provides a tool for screening for angiogenesis inhibitors in plant extracts.

As part of a random screen of plants and marine organisms for potential anticancer constituents,[4] an extract of a marine sponge was subjected to investigation. The crude extract showed weakly selective activity against the Δmap1 yeast strain in our antiangiogenesis bioassay (Table 1), and it was thus selected for fractionation for isolation of its bioactive compounds.

Results and Discussion

Partitioning of the crude extract between aqueous MeOH and various organic solvents led to concentration of its bioactivity in the CH_2Cl_2 fraction. The active CH_2Cl_2 fraction was purified by bioactivity-directed fractionation using column chromatography and TLC on silica gel to give compounds **1** (120 mg, 7.3%) and **2** (21 mg; 1.3%). Both compounds showed non-selective activity against the yeast strains (Table 1).

Table 1. Bioactivity of initial extract and isolated manzamines in cytotoxicity, DNA-damaging, and MetAP inhibition bioasssays

Fraction or compound	Inhibition of MetAP IC_{50} (μg/mL)			Cytotoxicity IC_{50} (μg/mL)	
	Δmap1Δerg6	Δmap1	Δmap2	HeLa	B16F10
Crude extract	6.1	13.9	36.4	5.0	3.0
N-Methyl *epi*-manzamine D (**1**)	0.68	2.1	2.6	1.0	0.1
epi-Manzamine D (**2**)	0.87	2.8	4.7	3.0	1.0

Keywords: biologically active compounds; alkaloids; marine metabolites; sponges; X-ray crystal structures.
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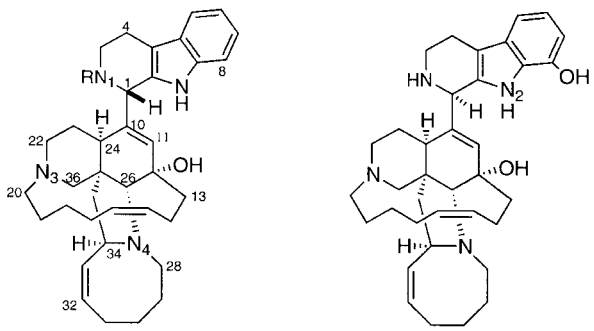

1 R = CH_3 N-Methyl-*epi*-manzamine D **3** 8-Hydroxymanzamine D
2 R = H *epi*-Manzamine D

Compound **1**, orthorhombic crystals from MeOH, had the composition $C_{37}H_{50}N_4O$ as indicated by HREIMS data, and gave a positive Dragendorff test. Its UV spectrum [λ_{max} (log ϵ) 223 (4.24) and 281 (3.74)] suggested the presence of an indole moiety.[5] Its ^1H NMR, ^{13}C NMR, and DEPT spectra indicated the presence of an N-CH_3 group (δ_H 2.30 ppm (3H, s) and δ_C 44.22 ppm), together with signals for an indole moiety, sixteen –CH_2– groups, four –CH– groups, two quarternary carbons, two –CH=CH– groups, and one CH=C– group. A comparison of these data with the literature data for 8-hydroxymanzamine D[6] (**3**) indicated that compounds **1** and **3** had similar structures; in particular the ^{13}C NMR signals for C-11 to C-37 in both compounds were essentially identical, indicating that they have the same skeleton and stereochemistry. A negative Cotton effect ($\Delta\epsilon$ −13.7 at 223.5 nm) in the CD spectrum of **1** indicated that the stereochemistry at C-1 is *S*,[7] in contrast to the *R* stereochemistry at this position in 8-hydroxymanzamine D. Therefore, compound **1** is the previously unreported *N*-methyl-*epi*-manzamine D. The assignments of its NMR data by DQCOSY, DEPT, HMQC and HMBC spectra are indicated in Table 2.

The structure of compound **1** was confirmed by X-ray analysis as described in the Experimental section; a SHELXTL-PC graphical structure of **1** is shown in Fig. 1.

Compound **2**, amorphous powder, had the molecular formula $C_{36}H_{48}N_4O$ as indicated by its HREIMS data. Its UV spectrum [λ_{max} MeOH nm (log ϵ): 225 (4.23) and 281

Table 2. NMR data ($CDCl_3$) of compounds **1**, **2** and **3**

Atom	Multiplicities[a]	**1**			**2**	**3**[b]
		δ_c	δ_H	HMBC	δ_c	δ_c
1	–CH–	69.22	3.97 (s)	1-H, –N–CH_3	61.31	60.8
3	–CH_2–	53.02	2.49 (m), 3.03 (ddd, 12.94, 4.74, 1.45)		43.71	43.9
4	–CH_2–	22.00	2.73 (ddd, 14.96, 12.94, 1.53), 2.89 (m)		22.21	22.3
4a	–C=	109.41		–NH, 1-H, 3-H, 4a-H, 4b-H	109.42	110.3
4b	–C=	136.52		5-H, 6-H, –NH–	136.22	129.5
5	–CH=	117.39	7.43 (d, 7.61)	6-H	117.39	109.5
6	–CH=	120.81	7.09 (d, 7.61)	5-H	120.80	119.9
7	–CH=	118.55	7.02 (dt, 7.61, 1.17)	8-H	118.51	106.8
8	–CH=	111.88	7.59 (d, 7.61)	7-H	111.99	143.0[c]
8a	–C=	126.71		7-H, 8-H, –NH–	126.99	125.1
9a	–C=	143.27		1-H, –NH–	132.49	144.8
10	–C=	143.27		1-H, 3-H, 24-H	144.09	132.8
11	–CH=	133.39	5.94 (s)	24-H, 26-H	132.45	132.7
12	–C–	70.98			70.71	70.6
13	–CH_2–	39.64	1.75 (m), 1.92 (m)	26-H	39.48	39.6
14	–CH_2–	20.64	2.17 (m)	15-H, 16-H	20.64	20.6
15	–CH=	127.04	5.59 (dt, 3.10, 7.48)	14-H	127.16	127.6
16	–CH=	132.85	5.54 (dt, 10.65, 4.01)	14-H	132.76	131.7
17	–CH_2–	24.88	1.59 (m), 2.41 (m)		24.86	24.9
18	–CH_2–	26.40	1.15 (m), 1.45 (m)		26.38	26.4
19	–CH_2–	24.46	1.40 (m), 1.72 (m)		24.45	24.4
20	–CH_2–	53.21	2.26 (m), 2.53 (dd, 12.28, 5.72)		53.33	53.3
22	–CH_2–	49.31	2.76 (m)	36a-H, 36b-H	49.11	49.1
23	–CH_2–	33.60	1.43 (m), 2.16 (m)		33.91	33.7
24	–CH–	37.51	1.84 (dd, 7.02, 11.94)	1-H, 35-H, 36-H	37.13	37.5
25	–C–	46.86		26-H, 34-H, 35a-H, 35b-H, 36-H	46.81	46.7
26	–CH–	78.78	3.53 (br. s)	11-H, 13-H, 24-H, 34-H, 36a-H, 36b-H	78.77	78.9
28	–CH_2–	53.32	3.12 (dd, 9.61, 11.44), 3.94 (m)	26-H	53.26	53.3
29	–CH_2–	26.21	1.91 (m), 2.48 (m)		26.21	26.3
30	–CH_2–	24.35	1.42 (m), 1.97 (m)		24.37	24.0
31	–CH_2–	28.30	2.34 (m)	32-H	28.27	28.6
32	–CH=	141.82	6.18 (dt, 9.92, 7.18)	31a-H, 34-H	141.92	142.5
33	–CH=	124.11	5.21 (dd, 9.92, 9.77)	34-H, 35a-H, 35b-H	124.06	123.6
34	–CH–	57.16	4.68 (m)	32-H, 33-H, 35b-H	57.14	57.5
35	–CH_2–	42.57	1.26 (dd, 12.51, 1.37), 1.69 (m)	32-H	43.06	43.2
36	–CH_2–	71.07	2.09 (d, 11.30), 2.76 (d, 12.30)	24-H, 26-H, 35a-H	70.83	70.9
37	N_1–CH_3	44.22	2.30 (3H, s)	1-H		
	N_2–H		10.63 (br. s)			

[a] The multiplicities of all carbons were determined by DEPT spectra.
[b] Data from Ref. 6.
[c] The multiplicity of this signal is –C=.

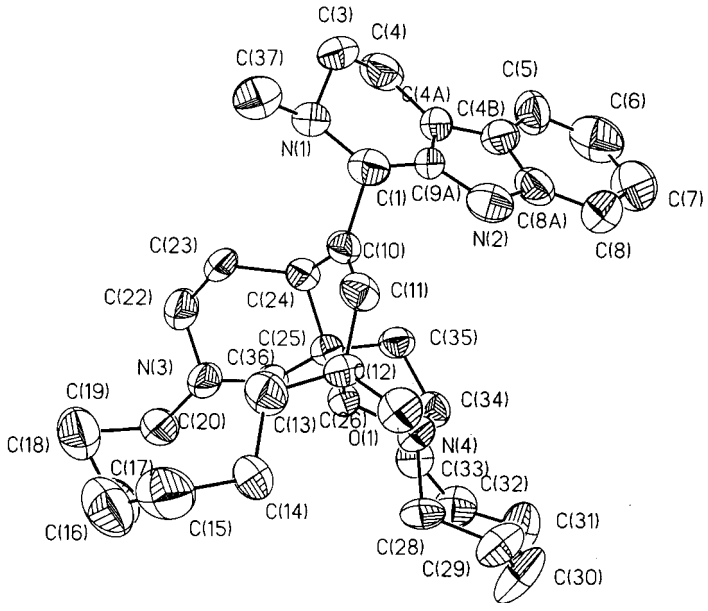

Figure 1. SHELXTL-PC graphical structure of *N*-methyl-*epi*-manzamine (**1**).

(3.79)] suggested the presence of an indole moiety, and the compound gave a positive Dragendorff test. Its ^{1}H and ^{13}C NMR spectra were very similar to those of compound **1**, except that it lacked the signals for an *N*-methyl group. A negative Cotton effect ($\Delta\epsilon$ −14.3 at 221.5 nm) in the CD spectrum of **2** indicated that the stereochemistry of C-1 is also *S*,[7] and this information thus indicated that **2** is *epi*-manzamine D. We assigned the ^{1}H and ^{13}C NMR spectra of **2** with the aid of DQCOSY, DEPT, HMQC and HMBC spectra; the assigned spectroscopic data are reported in Table 2. The only significant differences in the ^{13}C NMR chemical shifts between **1** and **2** were at carbons 1 (δ_C 69.22 ppm in **1** and δ_C 61.31 ppm in **2**) and 3 δ_C 53.02 ppm in **1** and δ_C 43.71 ppm in **2**). These differences are readily explicable by the *N*-methyl group present in **1**.

Since manzamine D is a known compound,[8] and since compound **2** differs from manzamine D only at a doubly allylic position, we considered the possibility that compounds **1** and **2** could have been formed by epimerization of manzamine D or its *N*-methyl analog. We thus tested the stability of the allylic proton to epimerization by heating compound **1** in deuterated methanol (CD_3OD). No changes in the ^{1}H NMR spectrum of **1** were observed even after boiling the solution for 32 h; longer times resulted in decomposition of the sample. Since some deuterium exchange would have been expected if compound **1** and *N*-methylmanzamine D were interconvertible, we thus conclude that **1** (and by logical extension compound **2** also) are natural products, and are not artifacts caused by the extraction or storage conditions.

The bioactivities of **1** and **2** are reported in Table 1. Although our initial interest in the extract was due to its potential antiangiogenic activity, the differential activity observed between the Δmap1 and Δmap2 yeast strains was not significant for either compound **1** or **2**, and we thus conclude that neither compound has antiangiogenic activity. Both compounds **1** and **2** did, however, show cytotoxic activity against HeLa and B16F10 cell lines (Table 1), consistent with previous reports for other alkaloids of the manzamine class.[8b] The greatest potency (IC_{50} 0.1 μg/mL) was observed for *N*-methyl-*epi*-manzamine D (**1**) against the B16F10 cell line.

Experimental

General experimental procedures

Optical rotations were recorded with a Perkin–Elmer 241 Polarimeter. NMR spectra were recorded in $CDCl_3$ on a JEOL Eclipse 500 instrument at 500.1624 MHz for ^{1}H and 125.7778 MHz for ^{13}C, and on a Varian Unity 400 NMR instrument at 399.951 MHz for ^{1}H and 100.578 MHz for ^{13}C, using standard pulse sequence programs. Exact mass measurements were obtained on a VG 7070E-HF mass spectrometer. UV spectra were measured on a Shimadzu UV1201 spectrophotometer. Other conditions were as previously described.[4]

Animal material

A marine poriferan was collected in Palau in January 1993 by Dr Brad Carté and designated PAL93-055. The collection was a massive nondescript branching mound sponge resembling a potato sponge, approximately 10×8 cm^2, with a crumbly and easily broken texture. Both exterior and interior coloration was light brown. The surface was rough, with visible oscules 3–5 mm in size, and having a mucous exudate.

Isolation of compounds 1 and 2

The crude methanol extract (UM 2056 M, 1.65 g) of the sponge was partitioned between *n*-hexane and 60% aq. MeOH. The aq. MeOH fraction was then diluted to 50% MeOH and partitioned with CH_2Cl_2. The bioactive CH_2Cl_2

fraction (249 mg, 16.2%) was subjected to column chromatography on Si gel eluted with CH_2Cl_2:MeOH, 20:1, and collection of 60 fractions of 10 mL each. The fractions were checked by TLC in CH_2Cl_2:MeOH (7:3), and similar fractions were combined; the combined fractions 5–10 and 11–24 were found to be bioactive. Combined fractions 5–10 were further purified by column chromatography on Si gel with the same solvents used previously, and compound **1** (120 mg, 7.3% from crude extract) was isolated as the major component by recrystallization from MeOH. Fractions 11–24 gave compound **2** (21 mg, 1.3% from crude extract) by PTLC on Si gel with the solvent CH_2Cl_2:MeOH (15:1).

Compound 1. Orthorhombic crystals, mp 185–188°C; $[\alpha]_D^{23}=+91.4°$ (c 0.27, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 223 (4.24) and 281 (3.74); CD (MeOH) $\lambda_{ext.}$ nm ($\Delta\epsilon$) 205 (+26.8), 223.5 (−13.7), 229 (sh, −12.2), 271 (+6.3), 295 (+3.4); IR (KBr) ν_{max} 3200–3500 (br), 3004, 2937, 1651, 1616, 1454, 1070, 1060 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HREIMS m/z (M^+) 566.3974 (calcd for $C_{37}H_{50}N_4O$, 566.3984).

Compound 2. Amorphous powder, $[\alpha]_D^{23}=+77.3°$ (c 0.165, $CHCl_3$), UV (MeOH) λ_{max} (log ϵ) 225 (4.23) and 281 (3.79); CD (MeOH) $\lambda_{ext.}$ nm ($\Delta\epsilon$) 204 (+22.1), 221.5 (−14.3), 226 (sh, −13.7), 269.5 (+11.7), 291.5 (+6.8); IR (KBr) ν_{max} 3200–3600 (br), 3004, 2931, 1650, 1620, 1454, 1071 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2. The NMR data of compound **2** were similar to those of a spectrum of manzamine D,[8] except for minor differences attributable to the their stereochemical difference. HREIMS m/z (M^+) 552.3830 (calcd for $C_{36}H_{48}N_4O$, 552.3828).

X-Ray crystallography of compound 1

Clear colorless orthorhombic crystals (~1 mm×1 mm×0.3 mm) were obtained from methanol. A selected crystal was cut (~0.5 mm×0.5 mm×0.3 mm), mounted on a glass fiber with epoxy, and centered on the goniometer of a Siemens (Bruker) P4 diffractometer. Unit cell parameters were determined by least squares refinement of 36 reflections that had been automatically centered on the diffractometer. Intensity data were collected, processed by the XSCANS v2.1 program, (Siemens Analytical X-ray Instruments, Madison, WI, 1994), and corrected for absorption. The structure was solved by direct methods and refined using the SHELXTL NT v5.10 program package.[9] The Laue symmetry and systematic absences were consistent with the orthorhombic space groups $P2_12_12_1$. The final refinement involved an anisotropic model for all non-hydrogen atoms and fixed positions for the hydrogen atoms. Solvent disorder was modeled as a water molecule at ~80% occupancy, hydrogen-bonded to O1 (3.16 Å), N2 (3.25 Å) and N4 (3.15 Å). Attempts were made to model a disordered methanol molecule in a larger spatial void, but were abandoned due to very low occupancies and very large thermal parameters. The program package SHELXTL-PC was used for the ensuing molecular graphics generation.

$C_{37}H_{50}N_4O_2$ $0.79H_2O$ (595.37), temperature 293(2) K, wavelength 0.71073 Å, orthorhombic, space group $P2(1)2(1)2(1)$, a=10.8154(14) Å (α=90°) b=13.474(2) Å (β=90°) c=24.728(3) Å (γ=90°), V=3603.4(9) $Å^3$, Z=4, $density_{calc.}$=1.097 g/cm^3, absorption coefficient: 0.069 mm^{-1}, $F(000)$=1289, crystal size 0.5×0.5×0.3 mm^3, theta range for data collection 1.65 to 22.49°, index ranges $-11\le h\le 11$, $-14\le k\le 14$, $-26\le l\le 26$, reflections collected 5349, independent reflections, 4431 unique [R(int)=0.0401], completeness to theta=22.49°=100.0%, absorption correction empirical, max. and min. transmission 0.9529 and 0.9028, refinement method Full-matrix least-squares on F^2, data/restraints/parameters 4431/0/391, goodness-of-fit on F^2 1.002, final R indices [$I>2\sigma(I)$], $R(F)$=0.0870, R_wF^2=0.2512; R indices (all data) $R(F)$=0.1391, R_wF^2=0.2740, absolute structure parameter −5(4), extinction coefficient 0.021(3), largest difference between peak and hole 1.112 and $-0.254e$ $Å^{-3}$.

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