Penaresidin A and B, Two Novel Azetidine Alkaloids with Potent Actomyosin ATPase-Activating Activity from the Okinawan Marine Sponge *Penares* sp.

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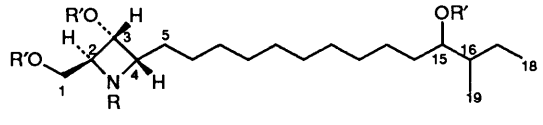
Two novel sphingosine-derived azetidine alkaloids, penaresidin A, **1a**, and B, **1b**, were isolated as potent actomyosin ATPase activators from the Okinawan marine sponge *Penares* sp. and the structures elucidated on the basis of spectral data, especially two-dimensional NMR spectra of their acetates.

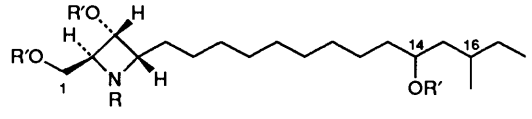
In our continuing studies on bioactive substances from marine organisms,[1] we have investigated extracts of numerous marine invertebrates collected in Okinawa, and the bioassay-guided purification resulted in the isolation of multifarious compounds with intriguing structures and interesting biological activities, some of which may have useful clinical applications[2] or be useful as chemical probes in the life sciences.[3] Recently we have examined extracts of the sponge *Penares* sp. and have isolated a novel antileukaemic triterpenoid, penasterol.[4] Further inquiry into the bioactive constituents of this sponge led to the isolation of two novel azetidine alkaloids, which we have named penaresidin A (compound**1a**) and B (compound **1b**), possessing potent actomyosin ATPase-activating activity. This is the first isolation of sphingosine-derived azetidine alkaloids from marine sources. In this paper we describe the isolation and structure elucidation of compounds **1a** and **1b**.

Results and Discussion

The methanol extract of the sponge *Penares* sp., collected at Unten Bay, Okinawa, was partitioned between toluene and water.[4] The aqueous layer was subsequently extracted with $CHCl_3$, EtOAc and Bu^{n}OH. The EtOAc-soluble fraction was subjected to column chromatography on Sephadex LH-20 ($CHCl_3$–MeOH) followed by silica gel column ($CHCl_3$–Bu^{n}OH–AcOH–water) to afford a *ca.* 1.5:1 mixture (**1**) of



1a R = R′ = H **2a** R = R′ = Ac **3a** R = Ac, R′ = H



1b R = R′ = H **2b** R = R′ = Ac **3b** R = Ac, R′ = H

penaresidin A, **1a** and B, **1b**, in 0.005% yield (wet weight). Since the ^{1}H NMR spectrum of the mixture **1** showed complex and indistinguishable signals, and since purification on silica gel and C_{18}-reversed-phase HPLC were ineffective, the mixture of products **1a** and **1b** was converted into the acetates, **2a** and **2b**, with acetic anhydride and pyridine. The mixture (**2**) of the acetates displayed relatively resolvable ^{1}H signals, and signals due to four pairs of acetyl methyls were observed, thus suggesting that compounds **2a** and **2b** are the tetraacetates of the penaresidins **1a** and **1b**, respectively, and exist as a 1.5:1 mixture, judging from the signal intensity. Structure determination was carried out mainly with the mixture **2**, since it was still difficult to separate tetraacetate **2b** from its isomer **2a** due to their having the same retention time on HPLC (silica gel or octadecylsilane) under several solvent systems.

The common molecular formula, $C_{27}H_{47}NO_7$, for compounds **2** was established by HR-EI-MS (*m/z* 497.3394, Δ −0.3 mmu). The IR spectrum exhibited an ester and an amide carbonyl absorption at 1740 and 1650 cm^{-1}, respectively, and no NH and/or OH absorption, indicating that the amide is tertiary. Four of five unsaturation degrees implied by the molecular composition can be accounted for by the four acetates. The remaining one has to come from a ring system, since no sp^2-carbons except acetate carbonyls were observed in the ^{13}C NMR spectrum. From the ^{1}H–^{1}H COSY spectrum the proton signals ranging from δ 4.2 to δ 5.2 were assigned to two partial structures: X^1–CH_2–$CH(X^2)$–$CH(X^3)$–$CH(X^4)$– [C-1–C-4] and $CH(X^5)$, where X^{1-5} = OAc or NAc. The ^{13}C chemical shift of the latter methine (δ_C 76.92 for **2a** and δ_C 72.67 for **2b**), based on the ^{1}H–^{13}C COSY spectrum, suggested that this methine bore an acetoxy group (X^5 = OAc). The ^{13}C NMR and DEPT spectra revealed that the rest of the molecule consisted of eleven methylenes, two methyls, one methine and no quaternary carbons. The remaining cyclic system, therefore, has to be constructed among four contiguous carbons [C-1–C-4] and one nitrogen atom. The presence of an azetidine ring was evidenced by the ^{1}H NMR spectrum of the monoacetate mixture (**3**) obtained by hydrolysis (NaOH–MeOH) of the tetraacetate mixture **2**: 1-H_2 and 3-H were shifted to higher field (**2a**: δ 4.33, 4.63 and 5.21; **3a**: δ 3.71, 3.87 and 3.67, respectively), while no notable change was observed for 2-H and 4-H (**2a**: δ 4.32 and 4.40; **3a**: δ 4.28 and 4.33, respectively). These results suggested that C-2 and C-4 should be connected to a nitrogen atom attached to an acetyl group unchanged on hydrolysis to generate the azetidine ring.

While the structure of the azetidine moiety was assigned, there remained one acetoxy methine, one sp^3-methine, two methyls and 11 methylenes, which are assembled into a side-chain attached to C-4. For the major component (compound **2a**) one methylene (δ_H 1.03 and 1.32) was shown to be adjacent to the terminal methyl by the ^{1}H–^{1}H COSY spectrum and these methylene protons (17-H_2) represented ^{1}H–^{13}C long-range connectivities to the two methines (δ_C 76.92 and 37.98), to which the acetoxy and the secondary methyl groups were attached,

Table 1 ^1H and ^{13}C NMR data for the tetraacetates **2a** and **2b** of penaresidin A and B

	2a			**2b**		
Position	^1H	*J*/Hz	^{13}C	^1H	*J*/Hz	^{13}C
1(a)	4.63 dd	15.2, 5.6	60.99 t	4.54 dd	15.2, 4.5	62.27 t
(b)	4.33 dd	15.2, 3.3		4.24 dd	15.2, 3.7	
2	4.32 m		65.05 d	4.28 ddd	4.5, 3.7, 4.2	66.62 d
3	5.21 dd	9.0, 5.2	66.45 d	5.09 dd	8.7, 4.2	67.41 d
4	4.40 m		63.19 d	4.40 m		64.81 d
5(a)	2.10 m		29.04 t	1.92 m		26.86 t
(b)	1.90 m			1.65 m		
14	1.45 m		43.35 t	4.91 ddt	10.7, 6.2, 4.8	72.67 d
15	4.81 dt	8.0, 4.7	76.92 d	1.47 m		34.93 t
16	1.50 m		37.98 d	1.45 m		24.62 d
17(a)	1.03 m		25.19 t	1.03 m		25.52 t
(b)	1.32 m			1.32 m		
18	0.82 t		13.89 q	0.83 t		11.65 q
19	0.84 d		23.11 q	0.85 d		22.16 q
1-AcO	2.03 s		20.53 q, 170.08 s	2.05 s		20.59 q, 170.25 s
N-Ac	2.07 s		20.62 q, 170.31 s	2.08 s		20.64 q, 170.36 s
3-AcO	1.84 s		20.74 q, 169.93 s	1.87 s		20.91 q, 169.97 s
15-AcO	1.98 s		21.07 q, 170.76 s	1.99 s		21.20 q, 170.92 s

HMBC correlations for major isomer **2a**: C-1/3-H, C-2/1-H^a, C-2/3-H, C-2/4-H, C-3/1-H^a, C-4/5-H_2, C-5/3-H, C-14/16-H, C-15/17-H_2, C-15/19-H_3, C-16/15-H, C-16/17-H_2, C-16/19-H_3, C-17/15-H, C-17/18-H_3, C-17/19-H_3, C-18/17-H_2 and C-19/16-H.

respectively, in the HMBC (^1H-detected heteronuclear multi-bond correlation) spectrum.[5] The acetoxy-bearing methine proton (δ_H 4.81), however, did not show vicinal coupling to the methylene (17-H_2) in the ^1H–^1H COSY spectrum. From these observations the acetoxy group was placed on C-15 and the methyl group on C-16 for compound **2a**.

The coupling pattern of the acetoxy-bearing methine of the minor component **2b** was different from that of **2a**. The ^1H–^1H COSY and *J*-resolved[6] correlations indicated that the acetoxy-bearing methine (δ_H 4.91) for compound **2b** was located between two methylenes. The ^1H–^1H COSY spectrum revealed that the secondary methyl group was on C-16 since the cross-peak for 17-H_2/16-H was observed. The acetoxy-bearing methine carbon (δ_C 72.67) showed prominent HMBC correlation with 16-H (δ_H 1.45), thus locating the acetoxy-bearing methine on C-14. The structure of compound **2b** was therefore concluded to be that of an acetoxy regioisomer of compound **2a**.

The coupling constants of 2-H/3-H (*J* 5.2 Hz) and 3-H/4-H (*J* 9.0 Hz) for compound **2a**, which was verified by decoupling experiments, indicated a 2-H/3-H *trans* and 3-H/4-H *cis* relationship.[7] Compound **2b** should possess the same relative configuration in the azetidine portion ($J_{2,3}$ 4.2, $J_{3,4}$ 8.7 Hz).*

Penaresidins seem to be biogenetically derived from sphingosine through cyclization of N-2 to C-4, an olefinic carbon of the latter, and the relative stereochemistry at C-2 and C-3 of sphingosine was retained in the penaresidins.[8] Such an azetidine alkaloid (aside from the β-lactams) is rare even in terrestrial materials and is apparently unprecedented in marine sources,[9] except for azetidine-2-carboxylic acid [10] or chartellines (indole–imidazole alkaloids with a β-lactam ring).[11] It is known that the actin–myosin system is involved in muscle contraction and many other cell-motility activities and the energy for the mobility events is provided by myosin ATPase. Penaresidins **1** elevated the ATPase activity of myofibrils from rabbit skeletal muscle[12] to 181% of the control value at 3×10^{-5} mol dm^{-3}. The tetraacetate mixture (**2**; 3×10^{-5} mol dm^{-3}) did not show activation of actomyosin ATPase. Penaresidins may become useful chemical tools for the study of molecular mechanisms in actin–myosin contractile systems, since there are very few substances which modulate the ATPase activities of myosin and actomyosin.

* The absolute stereochemistry of the azetidine portion, as well as the relative configuration of the substituents on the side-chain, remained to be defined.

Experimental

The IR spectra were recorded on a Hitachi 260-50 IR spectrophotometer. Optical rotations were measured on a JASCO DIP-360 polarimeter. ^1H and ^{13}C NMR spectra were recorded on Bruker AM-500, AM-400 and JEOL GX-500 spectrometers for solutions in $CDCl_3$ with internal $SiMe_4$ standard (0 ppm). Mass spectra were obtained on a JEOL HX-100 spectrometer operating at 70 eV for EI and using diethanolamine (DEA) as a matrix for FAB-MS.

Collection, Extraction and Separation.—The sponge *Penares* sp. was collected by netting at Unten Bay (−70 m), Okinawa island, in June 1987, and was frozen until used. The methanol (1500 cm^3 × 2) extract was dissolved in methanol–toluene (3:1; 200 cm^3) and then partitioned between toluene (1000 cm^3 × 2) and 1 mol dm^{-3} NaCl (1000 cm^3). The aq. layer was subsequently extracted with $CHCl_3$ (1000 cm^3 × 2), EtOAc (1000 cm^3 × 2) and Bu^{n}OH (1000 cm^3 × 2). The EtOAc-soluble fraction was subjected to column chromatography on Sephadex LH-20 (Pharmacia Fine Chemicals, 3.0 × 90 cm) with $CHCl_3$–MeOH (1:1), followed by rechromatography on silica gel (Wako gel C-300, Wako Chemical, 3.0 × 60 cm) with $CHCl_3$–Bu^{n}OH–AcOH–water (1.5:6:1:1) as eluent to give a mixture (**1**) of penaresidin A and B (0.005% yield by wet weight). The ratio of penaresidin A to B (1.5:1) was determined by analysis of the ^1H NMR spectrum of the tetraacetate mixture **2**.

Penaresidin A and B Tetraacetates **2**.—The mixture **1** was treated with excess of acetic anhydride (1.0 cm^3) and pyridine (1.0 cm^3). Evaporation of the organic solvents gave a residue, which was purified on a silica gel column (Wako gel C-300, 1.0 × 20 cm) with $CHCl_3$–MeOH (98:2) as eluent. The tetraacetate mixture **2** was obtained as an oil; $[\alpha]_D^{23}$ +47.9° (*c* 0.38, $CHCl_3$); ν_{max}(film)/cm^{-1} 2950, 2850, 1740, 1650, 1410, 1380, 1240, 1040 and 750; ^1H and ^{13}C NMR in $CDCl_3$ (Table 1); EI-MS *m/z* 497 (M^+), 482, 454, 437, 398 (100%), 378, 364, 335, 318, 292, 280, 238 and 198; FAB–MS *m/z* 603 (M + DEA +

H)$^+$ and 498 (M + H)$^+$ (Found: M^+ 497.3349. $C_{27}H_{47}NO_7$ requires M, 497.3352).

Hydrolysis of the Tetraacetates **2**.—To a solution of the tetraacetates **2** (2.0 mg) in MeOH (2 cm^3) was added 10% aq. NaOH (1 cm^3). The reaction mixture was stirred overnight and was then extracted with Et_2O. The organic layer was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave a residue, which was subjected to column chromatography on silica gel (Wako gel C-300, 0.5 × 8 cm) with $CHCl_3$–MeOH (99:1) to afford the monoacetate mixture **3** as an oil (1.4 mg); ν_{max}(film)/cm^{-1} 3400, 1610, 1560 and 1460; FAB-MS *m/z* 477 (M + DEA + H)$^+$ and 372 (M + H)$^+$; EI-MS *m/z* 371 (M$^+$) and 353 (Found: M^+ 371.3027. $C_{21}H_{41}NO_4$ requires M, 371.3036) δ($CDCl_3$) for **3a**: 4.33 (1 H, m, 4-H), 4.28 (1 H, m, 2-H), 3.86 (1 H, br d, 1-H^a), 3.71 (1 H, d, 1-H^b), 3.69 (1 H, m, 15-H), 3.67 (1 H, m, 3-H), 1.95 (3 H, s, Me-CO), *ca* 1.34 (br s, CH_2s), 0.90 (3 H, t, 18-H_3) and 0.87 (3 H, d, 19-H_3). For minor isomer **3b**, ^{1}H NMR signals were not clearly assigned because of the small amount of sample and the relatively low resolution of the spectrum.

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