Boneratamides A−C, New Sesquiterpenoids Isolated from the Marine Sponge *Axinyssa aplysinoides*

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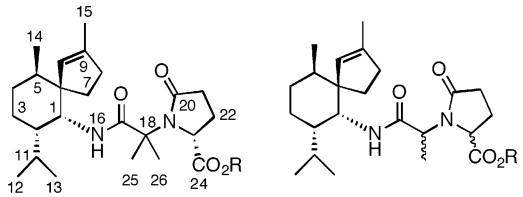
Three new sesquiterpenoids, boneratamides A (**1**)−C (**3**), have been isolated as their methyl esters **4**−**6** from extracts of the marine sponge *Axinyssa aplysinoides* collected in Indonesia. The structures of methyl esters **4**−**6** were elucidated by analysis of spectroscopic data and confirmed by single-crystal X-ray diffraction analysis of **4**.

Sponges continue to be the single richest source of new marine natural product structures reported in the literature,[1] which places them at the forefront of efforts to find new natural product lead compounds for anticancer drug development programs.[2] A small group of potently antimitotic metabolites isolated from sponges have received particular attention. Included in this group are the microtubule-stabilizing compounds discodermolide,[3] laulimalide,[4] and peloruside[5] along with the tubulin polymerization inhibitors belonging to the hemiasterlin,[2] spongistatin,[6] and halichondrin[7] families. As part of an ongoing program designed to uncover new antimitotic natural product chemotypes,[8] we have screened a library of crude MeOH extracts of marine sponges for activity in a cell-based antimitotic assay.[9] The extract from a specimen of *Axinyssa aplysinoides* collected in Indonesia showed promising activity in the assay. Fractionation of the extract led to the isolation of the methyl esters of three new sesquiterpenoids, boneratamides A (**1**), B (**2**), and C (**3**), and a very minor antimitotic metabolite, whose structure is still under investigation. The details of the isolation and structure elucidation of the boneratamides are presented below.

Specimens of *Axinyssa aplysinoides* (Dendy) (Demospongiae, order Halichondrida, family Halichondriidae) were harvested by hand using scuba on reefs off of Latondu Island, Taka Bonerate, Indonesia. Freshly collected sponge (50 g) was briefly immersed in EtOH immediately after collection in the field to inhibit bacterial growth until the specimens were deep frozen 48 h later. Frozen specimens were transported to Vancouver over dry ice, thawed, and exhaustively extracted with MeOH.

The combined MeOH extracts were concentrated in vacuo to give a brown gum that was partitioned between EtOAc and H_2O. Sephadex LH20 chromatography (eluent: 4:1 MeOH/CH_2Cl_2) of the EtOAc-soluble material (160 mg) gave a fraction exhibiting antimitotic activity in the cell-based assay.[9] The major components of this fraction gave very diffuse spots in both normal and reversed-phase TLC analysis, making it difficult to isolate pure compounds. Treatment of the active fraction with trimethylsilyldiazomethane sharpened the TLC spots, facilitating isolation of the boneratamides and the active component. Silica gel flash chromatography (eluent: step gradient from 19:1 hexanes/EtOAc to MeOH) of the methylated material gave a fraction (4.4 mg) eluting with 1:1 hexanes/EtOAc that was biologically active. Pure samples of boneratamide A methyl ester (**4**) (1.2 mg), boneratamide B methyl ester (**5**) (0.4 mg), and boneratamide C methyl ester (**6**) (0.2 mg) were obtained by final separation of the bioactive mixture using reversed-phase HPLC (eluent: 7:3 MeOH/H_2O). The boneratamide methyl esters **4**−**6** were not active in the antimitotic assay.



Boneratamide A methyl ester (**4**) was obtained as optically active colorless crystals that gave a $[M + H]^+$ ion at *m*/*z* 433.3099 in the HRESIMS appropriate for a molecular formula of $C_{25}H_{40}N_2O_4$ (calcd for $C_{25}H_{41}N_2O_4$, 433.3066), requiring seven sites of unsaturation. Twenty-five well-resolved resonances were observed in the ^{13}C NMR spectrum of ester **4** (Table 1, Supporting Information), and the HMQC data showed that there were 39 protons attached to carbon (6 × C; 5 × CH; 5 × CH_2; 8 × CH_3). A pair of resonances at δ 126.4 (C-10) and 143.6 (C-9) were assigned to a trisubstituted olefin, and resonances at δ 172.7 (C-17), 174.0 (C-20), and 175.5 (C-24) were assigned to ester or amide carbonyls, accounting for four of the seven sites of unsaturation required by the molecular formula of **4**. The absence of ^{13}C NMR evidence for additional unsaturated functionality indicated that boneratamide A methyl ester contained three rings.

An HMBC correlation between a methyl resonance at δ 3.17 and the carbonyl resonance at δ 175.5 (C-24) identified a methyl ester. The methyl ester along with the two additional carbonyl functionalities identified in the ^{13}C NMR data accounted for all four oxygen atoms in the molecular formula, indicating that the resonances at δ 172.7 (C-17) and 174.0 (C-20) had to be assigned to amides.

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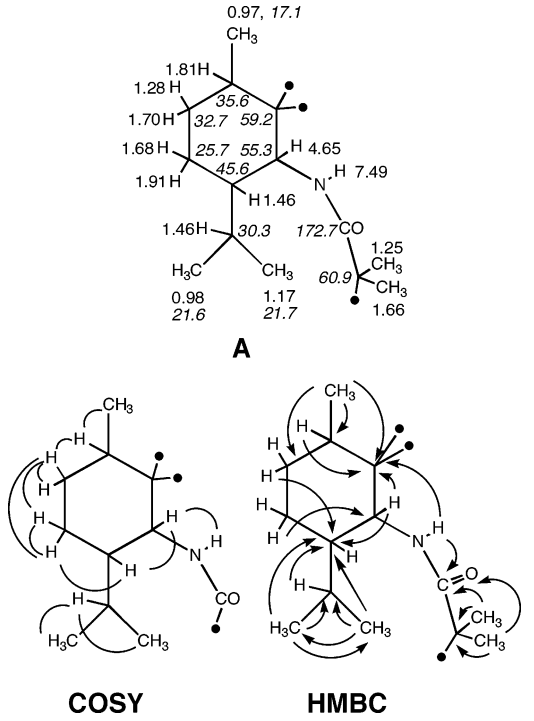

Figure 1. COSY and HMBC correlations in fragment **A**.

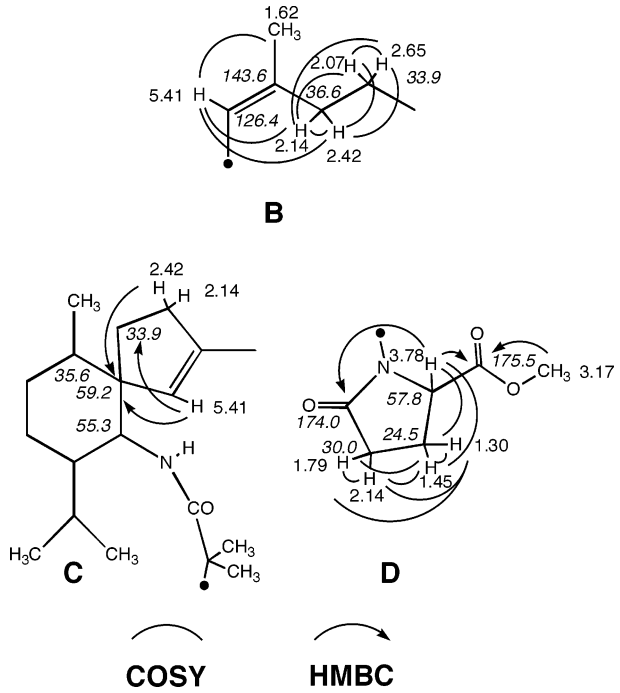

Figure 2. COSY and HMBC correlations in fragments **B**, **C**, and **D**.

A proton resonance at δ 7.49, which showed no $^1H/^{13}C$ HMQC correlations but did show an HMBC correlation to the carbonyl resonance at δ 172.7 (C-17), was assigned to an amide NH (H-16) (Figure 1). A pair of methyl singlets at δ 1.25 (Me-25) and 1.66 (Me-26) both showed HMBC correlations to a quaternary carbon at δ 60.9 (C-18) and to the amide carbonyl at δ 172.7 (C-17), demonstrating that methyls were geminal and the quaternary carbon bearing the methyls was attached to the amide carbonyl.

The amide NH (δ 7.49: H-16) resonance showed a COSY correlation to a methine resonance at δ 4.65 (H-1), and the methine resonance showed an HMBC correlation to the amide carbonyl resonance at δ 172.7 (C-17), establishing that the nitrogen atom (N-16) and methine carbon (C-1) were connected (Figure 1). HMQC data revealed that the methine carbon (C-1) had a chemical shift of δ 55.3, consistent with its attachment to nitrogen. Detailed analysis of the COSY, HMQC, and HMBC data, as illustrated in Figure 1, clearly showed that the nitrogen-bearing methine carbon was part of a cyclohexane ring, which also had isopropyl and methyl substituents.

An isolated proton spin system spanning the five-carbon fragment **B** shown in Figure 2 was readily identified from the COSY correlations indicated in the figure and the HMQC data for the protons (Table 1, Supporting Information). Both the olefinic methine resonance at δ 5.41 (H-10) and the methylene proton resonance at δ 2.42 (H-8) showed HMBC correlations to the quaternary carbon signal at δ 59.2 (C-6) in fragment **A**, and the olefinic methine resonance was further correlated to the methylene carbon resonance at δ 33.9 (C-7) in fragment **B**, indicating that both ends of fragment **B** were attached to the quaternary carbon (C-6) of fragment **A** to give a spiro system as shown in **C**.

The NMR data assigned to the remaining fragment of **4** were consistent with a 2-pyrrolidone-5-methyl carboxylate substructure **D** as shown in Figure 2. An HMQC correlation showed that the methine proton with a chemical shift of δ 3.78 (H-23) was attached to a carbon with a chemical shift of δ 57.8 (C-23), typical of an amino acid α carbon. HMBC correlations were observed between the methine resonance [δ 3.78 (H-23)] and both the ester carbonyl [δ 175.5 (C-24)] and amide carbonyl resonances [δ 174.0 (C-20)]. In the COSY spectrum, the methine resonance [δ 3.78 (H-23)] was correlated to a pair of methylene resonances at δ 1.30 (H-22) and 1.45 (H-22′), and these were in turn both correlated to methylene resonances at δ 1.79 (H-21) and 2.14 (H-21′). Fragments **C** and **D** accounted for all of the atoms and required sites of unsaturation in **4**, and simply joining them through the one remaining unsatisfied valence on each (C-18 and N-19) completed the constitution of boneratamide A methyl ester.

A series of 1D NOESY experiments established the relative stereochemisty of the sesquiterpenoid fragment of **4**. Irradiation of the NH-16 resonance at δ 7.49 induced an NOE in the H-5 resonance at δ 1.81, indicating that the C-1 amide substituent and H-5 were both axial. Irradiation of the H-10 olefinic methine resonance (δ 5.41) induced an NOE in the H-2 (δ 1.46) resonance, demonstrating that both C-10 and H-2 were axial as well. It was impossible to determine the configuration of C-23 relative to the terpenoid fragment by NMR, and the small sample size available precluded chemical degradation. Therefore, a single-crystal X-ray diffraction analysis was carried out on methyl ester **4**, and the ORTEP diagram is shown in Figure 3.[10] The X-ray analysis confirmed the structure assigned to **4** and showed that the relative stereochemistry is 1*R**,2*S**,5*R**,6*S**,23*R**.

Boneratamide B methyl ester (**5**) was isolated as an optically active solid that gave a $[M + H]^+$ ion at *m/z* 419.2911 in the positive ion HRESIMS appropriate for a molecular formula of $C_{24}H_{38}N_2O_4$ (calcd for $C_{24}H_{39}N_2O_4$, 419.2910), which differed from the formula of **4** simply by the loss of CH_2. Comparison of the ^1H and ^{13}C NMR data obtained for **5** with the data for **4** (Table 1, Supporting Information) showed that the two molecules were closely related. The major difference in the ^1H NMR data for **4** and **5** was the absence in the spectrum of **5** of the two singlets at δ 1.25 (Me-25) and 1.66 (Me-26) assigned to the geminal methyl groups attached to C-18 and the presence of a new methyl doublet at δ 1.37 (J = 7.2 Hz; Me-25),

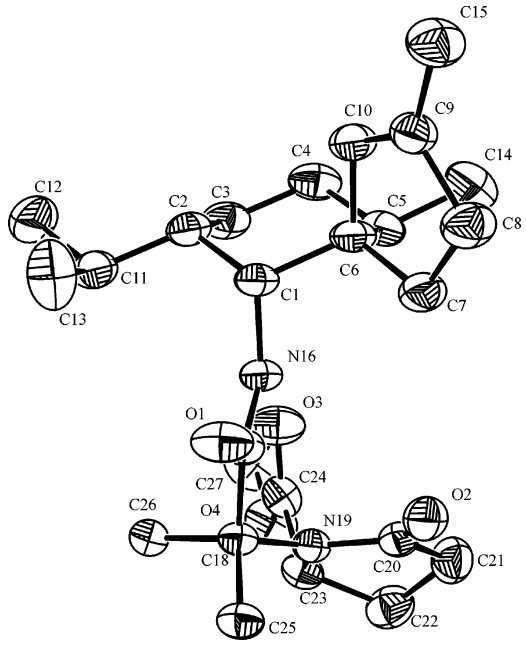

Figure 3. ORTEP diagram of methyl boneratamide A (**4**).

which showed a COSY correlation to a one-proton quartet at δ 4.46 (J = 7.2 Hz; H-18). In the HMBC spectrum of **5**, correlations were observed between the methine resonance (δ 4.46; H-18) and the two amide carbonyl resonances at δ 170.2 (C-17) and 175.6 (C-20) and between the methyl resonance at δ 1.37 (Me-25) and the amide carbonyl resonance at δ 170.2 (C-17). The above data showed that the constitution of boneratamide B methyl ester (**5**) differed from **4** simply by replacement of one of the methyl groups (Me-26) at C-18 in **4** with a proton in **5**.

Boneratamide C methyl ester (**6**) was isolated as an optically active solid that gave a $[M + H]^+$ ion at m/z 419.2899 in the HRESIMS consistent with a molecular formula of $C_{24}H_{38}N_2O_4$ (calcd for $C_{24}H_{39}N_2O_4$, 419.2910), which was identical to the formula of **5**. Analysis of the 1D and 2D NMR data obtained for **6** (Table 1, Supporting Information) showed that it had the same constitution as **5**, indicating that the two compounds were stereoisomers. It was apparent from the NMR data that the relative stereochemistry in the sesquiterpenoid fragments of **4**, **5**, and **6** was identical. Therefore, **5** and **6** differ in the relative configurations at either one or both of the C-18/C-23 stereogenic centers.

A ^{1}H NMR spectrum of the antimitotic Sephadex LH20 chromatography fraction before methylation clearly showed the signals for a mixture of boneratamides but did not show evidence for naturally occurring methyl esters. Therefore, the natural product boneratamides A−C exist as the free carboxylic acids **1**−**3**. Methyl ester **5** was hydrolyzed to the natural product boneratamide B (**2**), which was inactive in the antimitotic assay.

The boneratamides represent a new family of sesquiterpenoids that are notable because they are linked via an amide bond to a nitrogenous fragment that appears to have an amino acid origin. They show some resemblance to exigurin, recently isolated from *Geodia exigua* and reported to affect the development of fertilized sea urchin eggs.[11] The sesquiterpenoid fragment of the boneratamides is identical to the terpenoid portion of axisonitrile-3, and the amino group in the boneratamides is at the same position and has the same relative configuration as the isonitrile functionality in axisonitrile-3.[12]

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Supporting Information Available: Experimental section and a table of NMR data for esters **4**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 244783). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).
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