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Novel sesterterpenoid and norsesterterpenoid RCE-protease inhibitors isolated from the marine sponge *Hippospongia* sp.

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Abstract—Barangcadoic acid A (**1**) and rhopaloic acids D (**5**) to G (**8**), novel terpenoids with RCE protease inhibitory activity, have been isolated from the marine sponge *Hippospongia* sp. collected in Indonesia. The structures of **1** and **5–8** were elucidated by analysis of spectroscopic data. © 2002 Elsevier Science Ltd. All rights reserved.

Ras is a membrane bound G protein that functions as a molecular switch in a network of signaling pathways controlling cell differentiation and proliferation.[1–3] In cancer cells, unregulated cell signaling and proliferation may occur through overexpression or mutation of proto-oncogenes. Mutated Ras genes, which constitutively encode activated Ras proteins, have been identified in approximately 30% of all human cancers. As a result, the Ras signaling pathway has been identified as an important target for the development of anticancer drugs.[1–3]

Ras must be membrane bound to be active. A series of post-translational modifications at the C terminus CAAX motif of Ras increases its membrane affinity. First the protein is prenylated at the cysteine residue with either farnesyl or geranylgeranyl residues, then a specific protease cleaves the –AAX tripeptide from the protein, and finally the cysteine carboxyl group is methylated. Each processing step significantly increases the affinity of farnesylated proteins for membranes. To date, most approaches to therapeutic intervention in the Ras signaling pathway have focused on development of farnesyl transferase (Ftase) inhibitors that block the lipid modification needed for proper Ras membrane localization. Ras proteolysis also represents a promising target for disrupting Ras signaling. The recent characterization of hRCE1 (human Ras-converting enzyme),[2] which is responsible for proteolytic processing of Ras, led to the development of an assay to screen for RCE protease inhibitors.[3] Crude EtOH extracts of the Indonesian sponge *Hippospongia* sp. showed promising activity in the RCE protease assay. Bioassay guided fractionation of the extracts led to the identification of barangcadoic acid A (**1**) and rhopaloic acids A (**2**) to G (**8**), a family of terpenoids with RCE protease inhibitory activity. Details of the isolation, structure elucidation, and biological activities of the novel terpenoids **1** and **5–8** are presented below.

Hippospongia sp., was harvested by hand using SCUBA on the inner reef at Barangcadi Island, Ujung Pandang, Sulawesi, Indonesia. Freshly collected sponge (60 g) was repeatedly extracted with EtOH. The combined extracts were concentrated in vacuo, and the resulting aqueous suspension was partitioned between H_2O and EtOAc. Bioassay guided fractionation of the RCE-protease inhibitory EtOAc soluble material by sequential application of Sephadex LH20 chromatography (eluent: 80% MeOH/CH_2Cl_2), reversed-phase flash gradient column chromatography (eluent: 100% H_2O to 100% MeOH) and reversed-phase HPLC (eluent: 65% MeCN/(0.05%TFA/H_2O)) yielded pure barangcadoic

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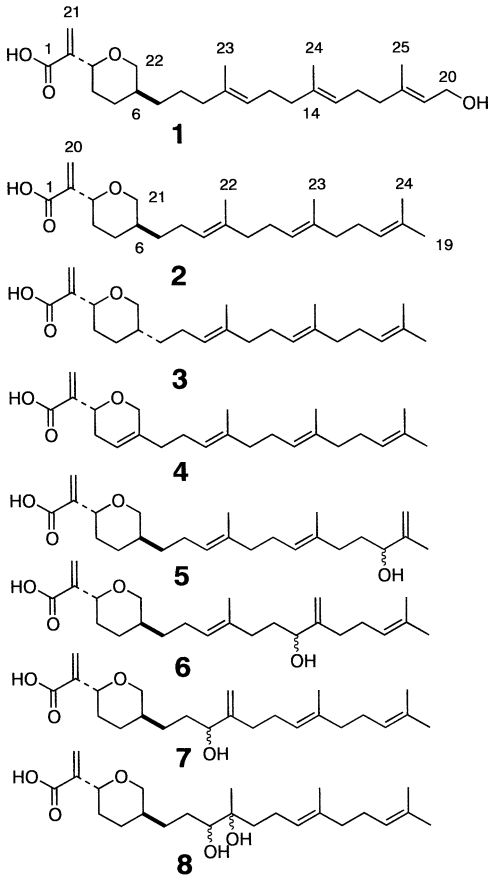
acid A (**1**) and rhopaloic acids A (**2**) to G (**8**) as clear oils.



Barangcadoic acid A (**1**) (35 mg) gave a $[M–H]^-$ ion at m/z 403.2846 in the negative ion HRFABMS spectrum that was appropriate for a molecular formula of $C_{25}H_{40}O_4$. The ^1H, ^{13}C, COSY, HMQC, and HMBC NMR data[4] obtained for **1** readily identified a carboxyl group (^{13}C δ 169.7), an olefinic methylene (^1H δ 6.36, bs, H-21; 5.92, ds, H-21′: ^{13}C δ 141.0 (C-2): 126.7 (C-21)), three trisubstituted olefins (^1H δ 5.40, t, $J=6.3$ Hz, H-19; 5.08, m, H-15; 5.06, m, H-11: ^{13}C δ 140.0 (C-18), 135.3 (C-14), 134.7 (C-10), 124.4 (C-11), 123.9 (C-15), 123.2 (C-19)), an oxygenated methine carbon (^1H δ 4.10, d, $J=10.7$ Hz, H-3: ^{13}C δ 75.9 (C-3), two oxygenated methyl residues (^1H δ 4.15, d, $J=6.3$ Hz, H-20/H-20′; 4.02, ddd, $J=11.4$, 2.7, 0.8 Hz, H-22_{eq}; 3.13, t, $J=11.4$ Hz, H-22_{ax}: ^{13}C δ 74.0 (C-22), 59.4 (C-20) and three allylic methyls (^1H δ 1.66, s, Me-25; 1.58, s, Me-24; 1.55, s, Me-23: ^{13}C δ 16.3 (C-25), 10.0, 15.9 (C-23/C-24)). These functionalities accounted for only five of the six sites of unsaturation required by the molecular formula, indicating that **1** also contained a ring.

HMBC correlations observed between a ^1H resonance at δ 4.10 (H-3) and ^{13}C resonances assigned to the carboxyl (δ 169.7: C-1) and olefinic methylene (δ 141.0: C-2 and 126.7: C-21) groups, and between the olefinic methylene protons at δ 6.36 (H-21) and 5.92 (H-21′) and ^{13}C resonances at δ 169.7 (C-1), 141.0 (C-2), and 75.9 (C-3) established that the carboxyl group (C-1) and the oxygenated methine carbon (C-3) were geminal substituents on the fully substituted carbon (C-2) of the olefinic methylene fragment. HMQC, COSY, and HMBC data routinely established the connectivity from C-3 to C-6 and C-22. HMBC correlations between H-3 (δ 4.10) and the oxygenated methyl carbon resonating at δ 74.0 (C-22) and between the geminal protons at δ 4.02 and 3.13 (H-22_{eq} and H-22_{ax}) and the C-3 resonance at δ 75.9 established the presence of a six-membered ether ring.

COSY correlations observed between the olefinic proton resonance at δ 5.40 (t, $J=6.9$ Hz; H-19) and both the methylene proton resonance at δ 4.15 (d, $J=6.9$ Hz; H-20/H-20′) and a methyl resonance at δ 1.66 (bs: Me-25) showed that the second oxygenated methyl (C-20) and another methyl (Me-25) were vicinal substituents on a trisubsituted olefin. HMBC correlations observed between the olefinic resonance at δ 5.40 (H-19) and carbon resonances at δ 59.4 (CH_2; C-20), 16.3 (CH_3; C-25) and 39.7 (CH_2; C-17) confirmed this assignment and identified an aliphatic methylene carbon (C-17) as the third substituent on the trisubstituted olefin. Two overlapping olefinic resonances at δ 5.06 (H-11) and 5.08 (H-15), which both appeared as broad triplets ($J\approx7$ Hz), showed COSY correlations to olefinic methyl resonances at δ 1.66 (Me-23) and 1.58 (Me-24), respectively, and to the clusters of allylic methylene proton resonances at δ 1.92–2.07 and δ 2.01–2.12. These COSY correlations identified two additional trisubstituted olefins, each having one methyl and two aliphatic methylene carbons as substituents. HMBC and COSY correlations confirmed that the three trisubsituted olefins comprised the regular terpenoid fragment extending from C-9 to C-20.

A well resolved methylene proton resonance at δ 1.07 (H-7) showed HMBC correlations to carbon resonances at δ 76.0 (C-22), 35.5 (C-6), 30.3 (C-5), 24.8 (C-8), and 39.6 (C-9) demonstrating that the tetrahydropyran and terpenoid fragments were linked via the ethane fragment C-7/C-8 to give the regular sesterterpenoid skeleton shown in **1** for barangcadoic acid A. The H-8/H8′ resonance at δ 1.38 showed HMBC correlations to carbon resonances at δ 35.5 (C-6), 31.8 (C-7), 39.6 (C-9) and 134.7 (C-10) in agreement with the proposed structure.

Sequential selective irradiation of the Me-25 (δ 1.66), Me-24 (δ 1.58), and Me-23 (δ 1.55) resonances in a series of 1D NOESY experiments resulted in enhancement of the H-20 (δ 4.15), H-16 (δ 2.08), and H-12 (δ 2.10) resonances, respectively, establishing the *E* configuration for the $\Delta^{10,11}$, $\Delta^{14,15}$ and $\Delta^{18,19}$ olefins in **1**. The H-3 resonance was a broad doublet with $J=10.7$ Hz indicating that it was axial. Similarly, H-22_{ax} appeared as a triplet with $J=11.4$ Hz indicating that H-6 was also axial and, therefore, C-2 and C-7 were *trans* to each other as shown in **1**.

Rhopaloic acid A (**2**) (105 mg) gave an $[M–H]^-$ ion at m/z 373.2743 in the negative ion HRFABMS spectrum

appropriate for a molecular formula $C_{24}H_{38}O_3$. Comparison of its NMR and optical rotation data with literature values showed that it was identical to **2** previously isolated from the sponge *Rhopaloeides* sp.,[5a] and subsequently synthesized by several groups.[5b,c] Rhopaloic acids B (**3**) and C (**4**) were similarly identified by comparison of their spectroscopic data with literature values for compounds **3** and **4** also isolated from the same *Rhopaloeides* sp.[6]

Rhopaloic acids D (**5**) and E (**6**) were obtained in very low yield as an inseparable mixture (0.2 mg) in the ratio $\approx$6:4 (**5**:**6**). The mixture of compounds gave a $[M+H]^+$ ion at m/z 391.2852 in the CIMS appropriate for a molecular formula of $C_{24}H_{38}O_4$, which contained one more oxygen atom than the molecular formula of rhopaloic acid (**2**). Examination of the NMR data (C_6D_6) obtained for the mixture of **5** and **6**[7,8] showed that both molecules contained the substituted tetrahydropyran fragment found in rhopaloic acid A (**2**) but differed from **2** in the C-7 to C-19 fragment. A pair of singlet olefinic resonances at δ 4.77 (H-24) and 4.94 (H-24′), that each integrated for $\approx$0.5H and were correlated to each other in the COSY spectrum and to a common olefinic carbon resonance at δ 110.6 (C-24) in the HMQC spectrum, showed that the major compound in the mixture contained an olefinic methylene in the C-7 to C-19 fragment. HMBC correlations were observed from the two olefinic methylene resonances to a carbinol methine resonance at δ 75.4 (C-17) and to an olefinic methyl resonance at δ 17.5 (C-19). COSY correlations were observed between both of the olefinic resonances at δ 4.77 (H-24) and 4.94 (H-24′) and an olefinic methyl resonance at δ 1.62 (Me-19), and between the δ 4.94 (H-24′) resonance and a carbinol methine resonance at δ 3.89 (H-17). The above data identified a disubstituted olefin with geminal methyl and carbinol methine carbon substituents. This substructure could only be accommodated at the terminus of the C-7 to C-19 fragment as shown in the proposed structure **5** for rhopaloic acid D.

A second pair of olefinic methylene proton resonances that also integrated for $\approx$0.5H each, were found at δ 4.88 (H-23) and 5.07 (H-23′) in the ^{1}H spectrum of the mixture of **5** and **6**.[7,8] Arguments based on COSY and HMBC data allowed a tentative placement of an olefinic methylene at C-14/C-23 and an allylic alcohol at C-13 in rhopaloic acid E as shown in **6**.

Rhopaloic acid F (**7**), obtained in only very small amounts, gave a $[M+H]^+$ ion at m/z 391.2839 in the CIMS appropriate for a molecular formula of $C_{24}H_{38}O_4$, making it isomeric with rhopaloic acids D (**5**) and E (**6**). Examination of its NMR data[9] showed that it also contained an olefinic methylene (C_6D_6; δ 4.85 (H-22), 4.99 (H-22′), 109.2 (C-22)) and adjacent allylic alcohol (δ 3.78 (H-9), 75.1 (C-9)) unit identical to the fragment found in the side chain of **6**. The small amount of **7** that was available made it impossible to use NMR data to provide rigorous location of the olefinic methylene and adjacent secondary alcohol functionalities. However, since **7** was isomeric with **5** and **6** the only remaining position for the olefinic methylene was at C-10/C22 and the COSY data precluded the possibility of the carbinol methine being at C-11, leading to the proposed structure **7** for rhopaloic acid F.

Rhopaloic acid G (**8**) (0.1 mg) gave a $[M+NH_4]^+$ ion at 426.3219 in the CI MS appropriate for a molecular formula of $C_{24}H_{40}O_5$, which required only five sites of unsaturation. Examination of the NMR data obtained for **8**[10] showed that it was simply an analog of rhopalpoic acid A (**2**) in which one of the internal olefins in the side chain had been converted to a vicinal diol. The ^{1}H NMR spectrum of **8** contained only three olefinic methyl (C_6D_6; δ 1.56, 1.63, 1.68) and two olefinic methine (δ 5.23, 5.29) resonances in addition to aliphatic methyl (δ 0.98) and carbinol methine (δ 3.08) resonances that could be assigned to the C-7 to C-19 fragment. The methyl resonance at δ 0.98 (Me-22) showed HMBC correlations to carbon resonances at δ 74.4 (C), 78.7 (CH), and 36.4 (CH_2) demonstrating that it was one of the internal olefins that had been converted to a diol. The base peak in the CI spectrum appeared at m/z 214.1198 ($C_{11}H_{18}O_4$), consistent with α cleavage of a C-9/C-10 diol resulting in the charge residing on the C-1 to C-9 tetrahydropyran containing fragment. The remainder of the NMR data was consistent with the proposed structure **8** for rhopaloic acid G.

Barangcadoic acid A (**1**) has a regular sesterterpenoid skeleton where the carbons C-1/C-2/C-21 form the head of the starter unit in the assembly of the five isoprene units. Rhopaloic acids A (**1**) to G (**8**) are nor-terpenoids and on first analysis, because they contain the same substituted tetrahydropyran fragment, they appear to be related to the sesterterpenoid skeleton of barangcadoic acid A simply by loss of the C-20 carbon in **1**. However, the placement of the olefins in **2** to **8** suggests that the head of the starter unit in the assembly of their terpenoid skeletons should be the carbons C-18/C-19/C-24. Resolution of this interesting biogenetic contradiction will likely have to await future biosynthetic feeding experiments.

Barangcadoic acid A and rhopaloic acids A to D/E all had IC_{50} values of $\approx$10 μg/mL in the RCE-protease assay. Two colon tumor cell lines were used for in vitro cytotoxicity testing. The LoVo cell line has a mutated K-Ras and activated Ras pathway and consequently it is quite sensitive to Ras pathway inhibitors. CaCo has normal Ras and is generally resistant to Ras pathway inhibitors. All of the compounds **1** to **5**/**6** had IC_{50} values $\approx$1–2 μg/mL against the LoVo cell line and 3–4-fold less activity against the CaCo cells, the expected trend for RCE protease inhibitors. However, the compounds are more active in the cell-based assays ($IC_{50}\approx$1 μg/mL) than in the enzyme assays ($IC_{50}\approx$10 μg/mL), suggesting that the cytotoxic effect of the compounds might result from hitting more than one molecular target. Terpenoids **1** to **5**/**6** are apparently the first reported natural product inhibitors of the RCE-protease.[11]

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4. Barangcadoic acid A (**1**): $[\alpha]_D^{28}$ +34.9 (*c* 3.80, CH_2Cl_2); negative ion HRFABMS (thioglycerol matrix) $[M-H]^-$ *m*/*z* 403.2846 ($C_{25}H_{39}O_4$, calcd 403.2850); ^{1}H NMR ($CDCl_3$): δ 6.36 (bs, H-21), 5.92 (bs, H-21), 5.40 (t, 6.6 Hz, H-19), 5.08 (m, H-15), 5.06 (m, H-11), 4.15 (d, 6.6 Hz, H-20), 4.10 (d, 10.7 Hz, H-3_{ax}), 4.02 (ddd, 11.4, 2.7, 0.8 Hz, H-22_{eq}), 3.13 (t, 11.4 Hz, H-22_{ax}), 2.12–2.01 (m, H-12/H-16), 2.07–1.92 (m, H-9/H-13/H-17), 1.94 (m, H-4), 1.92 (m, H-5_{eq}), 1.66 (s, H-25), 1.58 (s, H-24), 1.58 (H-6_{ax}), 1.55 (s, H-23), 1.38 (m, H-8), 1.31 (m, H-4), 1.19 (m, H-5_{ax}), 1.07 (m, H-7) ppm; ^{13}C NMR ($CDCl_3$): δ 169.7 (C-1), 141.0 (C-2), 140.0 (C-18), 135.3 (C-14), 134.7 (C-10), 126.7 (C-21), 124.4 (C-11), 123.9 (C-15), 123.2 (C-19), 75.9 (C-3), 74.0 (C-22), 59.4 (C-20), 39.7, 39.7, 39.6 (C-9/C-13/C-17), 35.5 (C-6), 32.0 (C-4), 31.8 (C-7), 30.3 (C-5), 26.5, 26.4 (C-12, C-16), 24.8 (C-8), 16.3 (C-25), 16.0, 15.9 (C-23/C-24) ppm.
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7. Rhopaloic acid D (**5**): ^{1}H NMR (C_6D_6): δ 6.35 (bs, H-20), 5.91 (bs, H-20), 5.26 (m, H-13), 5.14 (m, H-9), 4.94 (bs, H-24), 4.78 (bs, H-24), 4.08 (d, 11.3 Hz, H-3_{ax}), 3.92 (H-21_{eq}), 3.88 (H-17), 2.88 (H-21_{ax}), 1.62 (s, H-19), 1.54 (s, H-22/H-23) ppm; ^{13}C NMR (C_6D_6): δ 168.2 (C-1), 148.1 (C-18), 141.8 (C-2), 135.1 (C-10/C-14), 125.9 (C-20), 124.8 (C-9/C-13), 110.6 (C-24), 76.2 (C-3), 75.4 (C-17), 73.8 (C-21), 36.0 (C-6), 32.6 (C-7), 32.3 (C-4), 30.4 (C-5), 17.7 (C-19), 16.0 (C-23/C-24) ppm.
8. Rhopaloic acid E (**6**): ^{1}H NMR (C_6D_6): δ 6.35 (bs, H-20), 5.91 (bs, H-20), 5.21 (m, H-17), 5.13 (m, H-9), 5.07 (bs, H-23), 4.88 (bs, H-23), 4.08 (d, 11.3 Hz, H-3_{ax}), 3.94 (H-13), 3.92 (H-21_{eq}), 2.88 (H-21_{ax}), 2.22 (m, H-16), 2.20 (m, H-15), 2.11 (m, H-12), 1.66 (s, H-19), 1.55, (s, H-24), 1.54 (s, H-22) ppm, A); ^{13}C NMR (C_6D_6): δ 168.2 (C-1), 152.2 (C-14), 141.8 (C-2), 135.1 (C-10), 131.6 (C-18), 124.8 (C-9), 124.7 (C-17), 109.4 (C-23), 76.2 (C-3), 75.0 (C-13), 73.8 (C-21), 40.0 (C-11), 36.1 (C-12), 36.0 (C-6), 32.6 (C-7), 32.3 (C-4), 31.8 (C-15), 30.4 (C-5), 25.7 (C-19), 17.7 (C-24), 16.0 (C-22) ppm.
9. Rhopaloic acid F (**7**): ^{1}H NMR (C_6D_6): δ 6.33 (bs, H-20), 5.86 (bs, H-20), 5.29 (t, 6.5 Hz, H-13), 5.23 (t, 6.1 Hz, H-17), 4.99 (bd, 4 Hz, H-22), 4.85 (bs, H-22), 4.03 (d, 10.6 Hz, H-3_{ax}), 3.85 (dm, 10.3 Hz, H-21_{eq}), 3.78 (t, 5.7 Hz, H-9), 2.85 (t, 10.3 Hz, H-21_{ax}), 2.23 (m, H-12), 2.18 (m, H-16), 2.09 (m, H-15), 1.68 (s, H-19), 1.61 (s, H-23), 1.56 (s, H-24), 1.32 (m, H-6_{ax}/H-8) ppm; ^{13}C NMR (C_6D_6): δ 135.6 (C-14), 131.1 (C-18), 124.5 (C-17), 124.2 (C-13), 109.3 (C-22), 76.1 (C-3), 75.1 (C-9), 73.7 (C-21), 39.9 (C-15), 35.7 (C-6/C-8), 32.6 (C-4), 29.7 (C-6/C-8), 26.8 (C-16), 25.6 (C-19), 17.5 (C-24), 15.9 (C-23) ppm.
10. Rhopaloic acid G (**8**): ^{1}H NMR (C_6D_6): δ 6.35 (bs, H-20), 5.88 (bs, H-20), 5.29 (t, 6 Hz, H-13), 5.23 (t, 7 Hz, H-17), 4.09 (d, 10.4 Hz, H-3_{ax}), 3.89 (m, H-21_{eq}), 3.09 (m, H-9), 2.90 (t, 10.3 Hz, H-21_{ax}), 2.19 (m, H-16), 2.10 (m, H-15), 2.04 (m, H-12), 1.90 (m, H-4), 1.68 (s, H-19), 1.64 (s, H-23), 1.61 (m, H-6), 1.56 (s, H-24), 1.35 (m, H-11), 1.31 (m, H-7), 1.22 (m, H-4′), 1.18 (m, H-8), 1.07 (m, H-8′), 0.98 (s, H-22), 0.90 (m, H-5) ppm; ^{13}C NMR (C_6D_6): δ 168.0 (C-1), 141.8 (C-2), 135.3 (C-14), 131.4 (C-18), 125.6 (C-20), 125.3 (C-13), 124.7 (C-17), 78.6 (C-9), 76.2 (C-3), 74.4 (C-10), 73.7 (C-21), 40.2 (C-15), 36.3 (C-11), 32.3 (C-4), 30.1 (C-7), 28.2 (C-8), 27.2 (C-16), 25.9 (C-19), 23.5 (C-22), 17.7 (C-24), 16.1 (C-23) ppm.
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