Cheilanthane Sesterterpenes, Protein Kinase Inhibitors, from a Marine Sponge of the Genus *Ircinia*

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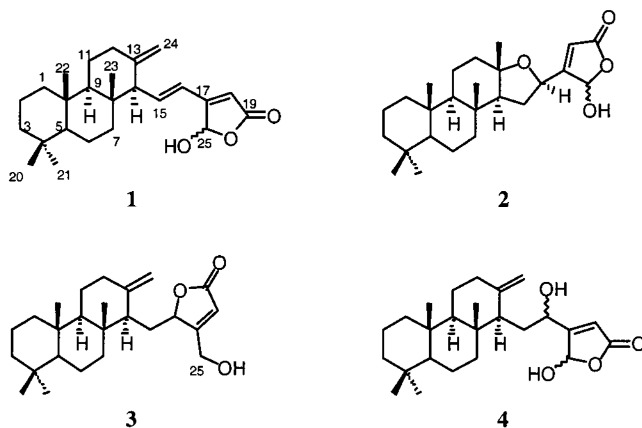
Four cheilanthane sesterterpenoids, 25-hydroxy-13(24),15,17-cheilanthatrien-19,25-olide (**1**), 13,16-epoxy-25-hydroxy-17-cheilanthen-19,25-olide (**2**), 25-hydroxy-13(24),17-cheilanthadien-16,19-olide (**3**), and 16,25-dihydroxy-13(24),17-cheilanthadien-19,25-olide (**4**), were isolated from the marine sponge *Ircinia* sp. Compounds **1**, **3**, and **4** are new natural products. The four compounds inhibit MSK1 (mitogen and stress activated kinase) and MAPKAPK-2 (mitogen activated protein kinase activated protein kinase), two protein kinases involved in mitogen and stress signal transduction.

In an effort to find selective inhibitors of protein kinase enzymes,[1,2] we have investigated the marine sponge *Ircinia* sp. Until now sponges of the genus *Ircinia* have yielded a number of terpenoids, in particular sesterterpenoids containing furanyl and/or butenolide groups,[3,4] but no cheilanthane sesterterpenoids have been described. Several cheilanthanes have however been isolated from other sources,[5,6] but these reports rarely mention any biological activity. Bioassay-guided purification afforded three new cheilanthane sesterterpenoids, **1**, **3**, and **4**, and one previously known, **2**. The isolation and structure elucidation of these compounds are reported, together with their protein kinase inhibitor activity.



Results and Discussion

Specimens of *Ircinia* sp. were freeze-dried, then pulverized to give 20.0 g of material, which was then extracted with CH_2Cl_2 (2.74 g) and subsequently partitioned between hexane and aqueous MeOH. The MeOH layer was passed through a reversed-phase C_{18} flash column. Final purification by reversed-phase C_{18} HPLC yielded four pure cheilanthane sesterterpene lactones, **1**−**4**, with retention times of 15.9, 13.8, 13.0, and 10.8 min, respectively.

The molecular formula of **1** was determined to be $C_{25}H_{36}O_3$ by HRESIMS (m/z 383.2603 $[M - H]^-$). IR absorptions at 3330 and 1748 cm^{-1} reveal that it contains hydroxyl and ester groups. The NMR resonances (Table 1) for a trisubstituted double bond [δ_C 160.8 (s) and 115.6 (d); δ_H 5.86 (s)], a carbonyl (δ_C 170.6), and a hemiacetal carbon (δ_C 97.3, δ_H 6.25) disclose a γ-hydroxy-γ-butenolide moiety and the signals δ_C 148.75 (s), 108.45 (t); δ_H 4.78 (s), 4.45

Table 1. ^{1}H (600 MHz), ^{13}C (150 MHz), and HMBC NMR Data for Compound **1** in $CDCl_3$[a,b]

position	^{13}C	^{1}H	HMBC
1α	40.1	0.85 (m)	
1β		1.70 (m)	
2α	18.6	1.43 (m)	
2β		1.62 (qt, J = 13.2, 3.6 Hz)	3
3α	42.0	1.15 (m)	
3β		1.40 (m)	
4	33.3		
5α	56.8	0.87 (m)	22
6α	18.8	1.55 (brd, J = 13.8 Hz)	
6β		1.33 (brq, J = 12.0 Hz)	
7α	42.59	1.15 (m)	
7β	42.49[c]	1.50 (m)	
8	39.95		
	40.01[c]		
9	59.3	1.06 (dd, J = 12.0, 2.4 Hz)	5, 10, 11, 12, 14, 23
10	38.0		
11α	22.1	1.70 (m)	
11β		1.40 (m)	
12α	36.5	2.06 (td, J = 18.0, 4.8 Hz)	5′, 9′, 10′
12β		2.45 (brd, J = 18.0 Hz)	
13	148.75		
	148.59[c]		
14α	62.55	2.47 (brd, J = 10.2 Hz)	13, 15, 16, 23
	62.49[c]		
15	143.89	6.60 (dd, J = 16.2, 10.2 Hz)	17
	143.82[c]	6.59 (dd, J = 16.2, 10.2 Hz)[c]	
16	122.63	6.31 (d, J = 16.2 Hz)	14, 15, 17, 18, 25
	122.56[c]		
17	160.8		
18	115.6	5.86 (s)	16, 17, 19, 25
19	170.6		
20	21.5	0.85 (s)	3,5
21	33.4	0.86 (s)	
22	16.4	0.88 (s)	
23	16.13	0.86 (s)	5, 7, 14
	16.07[c]		
24*E*	108.45	4.78 (s)	12, 14
24*Z*		4.45 (s)	
24*E*	108.14[c]	4.78 (s)[c]	
24*Z*		4.37 (s)[c]	
25	97.3	6.25 (brd, J = 7.8 Hz)	

[a] Assignments confirmed by two-dimensional experiments (COSY, ROESY, HSQC, and HMBC). [b]Mutiplicities were determined by DEPT experiments. [c]A second signal was observed for some nuclei at a ratio of ∼1:1 attributed to epimerizaion at C-25 of the γ-hydroxy butenolide moiety.

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Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data for Compounds **3** and **4** $CDCl_3$[a,b]

	3		**4**	
position	^{13}C	^{1}H	^{13}C	1H
1α	40.1	0.84 (m)	40.1	0.84 (m)
1β		1.68 (m)		1.66 (brd, J = 12.6 Hz)
2α	18.7	1.43 (m)	18.7	1.43 (brd, J = 14.0 Hz)
2β		1.64 (m)		1.61 (qt, J = 14.0, 3.6 Hz)
3α	42.0	1.16 (m)	42.0	1.15 (td, J = 14.0, 3.6 Hz)
3β		1.39 (m)		1.37 (m)
4	33.4		33.3	
5α	56.5	0.87 (m)	56.3	0.86 (m)
6α	19.1	1.62 (m)	19.1	1.62 (brd, J = 12.6 Hz)
6β		1.37 (m)		1.39 (m)
7α	41.0	1.17 (m)	40.82	1.10 (m)
7β		1.78 (m)	40.62c	1.80 (m)
8	40.3		40.2	
9α	60.3	1.08 (dd, J = 10.2, 2.0 Hz)	60.1	1.06 (m)
10	37.9		37.9	
11α	23.2	1.74 (m)	23.3	1.74 (brd, J = 13.2 Hz)
11β		1.33 (m)		1.32 (td, J = 13.2, 3.6 Hz)
12α	38.1	1.97 (td, J = 12.5, 4.5 Hz)	38.2	1.96 (m)
12β		2.42 (brd, J = 12.5 Hz)		2.41 (brd, J = 12.0 Hz)
13	148.0		149.44	
			149.08[c]	
14α	52.4	1.78 (m)	53.8	1.70 (m)
15	27.7	1.87 (ddd, J = 14.4, 7.8, 2.0 Hz)	30.19	1.94 (m, 2H)
		2.00 (m)	29.96[c]	
16	82.1	5.01 (brt, J = 6.0 Hz)	68.4	4.65 (brt, J = 6.0 Hz)
17	172.3		170.31	
			167.85[c]	
18	115.5	6.02 (brs)	118.92	6.00 (brs)
			117.83[c]	
19	172.3		170.51	
			170.11[c]	
20	21.5	0.81 (s)	21.5	0.81 (s)
21	33.5	0.87 (s)	33.3	0.87 (s)
22	15.2	0.70 (s)	16.2	0.81 (s)
23	16.3	0.82 (s)	15.5	0.70 (s)
24*E*	107.1	4.91 (s)	106.9	4.91 (s)
24*Z*		4.66 (s)		4.69 (s)
25	59.3	4.46 (d, J = 16.5 Hz)	98.13	6.24 (brs)
		4.62 (d, J = 16.5 Hz)	97.56[c]	6.15 (brs)[c]

[a] Assignments confirmed by two-dimensional experiments (COSY, ROESY, HSQC, and HMBC). [b]Mutiplicities were determined by DEPT experiments. [c]For compound **4** a second signal was observed for some nuclei at a ratio of ~1:1 attributed to epimerizaion at C-25 of the γ-hydroxy-γ-butenolide moiety.

(s) an exomethylene. The final olefinic resonances, δ_C 143.89 (d), 122.63 (d); δ_H 6.59 (dd, J = 16.2, 10.2 Hz), 6.31 (d, J = 16.2 Hz), belong to a trans double bond, and the UV absorption band at 265 nm indicates that this is conjugated to the butenolide moiety. The remaining signals are at higher field and correspond to four methyls, seven methylenes, three methines, and three quaternary carbons. These constitute a tricyclic ring system with the structures and relative stereochemistry of rings A and B clearly the same as several known marine sesterterpenes.[5−9] Furthermore, the HMBC correlations (Table 1) between H-24*Z*, H-24*E* and both C-12 and C-14 indicate that the third ring is six-membered with an exomethylene at C-13. This completes the tricyclic framework with trans A/B, trans B/C ring junctions, a *gem* dimethyl group at C-4, and axial methyl groups at C-8 and C-10. The three carbocyclic rings have regular chair conformations. This carbocyclic framework is the same as the spongiane diterpenes.[10] The resonance at δ_H 6.59 (dd, J = 16.2, 10.2 Hz) belonging to the trans disubstituted double bond shows allylic coupling to a methine at δ_H 2.47 (d, J = 10.2 Hz). This methine was assigned to H-14 from its correlations with C-13 and C-23 in the HMBC experiment. Thus, the β-substituted α,β-unsaturated γ-hydroxy-γ-butenolide side chain is attached at C-14 and is assigned a β configuration on the basis of the NOE between H-14 and H-9 in the ROESY experiment. There are several other correlations in the COSY, ROESY, and HMBC experiments that agree with this structure (Table 1). All the above evidence reveals that compound **1** is 25-hydroxy-13(24),15,17-cheilanthatrien-19,25-olide.

The next compound was found to have ^{1}H and ^{13}C NMR data identical with that of 13,16-epoxy-25-hydroxy-17-cheilanthen-19,25-olide (lintenolide C) (**2**), which has previously been isolated from the sponge *Cacospongia* cf. *linteiformis*.[11]

Compound **3**, which was obtained in the lowest yield, has the molecular formula $C_{25}H_{38}O_3$, as indicated by the HRESIMS (m/z 385.2745 $[M - H]^-$). Examination of the NMR data (Tables 1 and 2) shows that **3** has the same carbocyclic framework as **1** and differs only in the side chain attached at C-14. A butenolide moiety was again derived from the signals at δ_C 172.3 (s, 2C) and 115.5 (d); δ_H 6.02 (brs). The γ-butenolide is completed with an oxygen-bearing methine [δ_C 82.1; δ_H 5.01 (brt, J = 6.0 Hz)], the proton of which couples to methylene protons (δ_C 27.7; δ_H 2.00, 1.87) and has an allylic coupling to δ_H 6.02, as shown by the COSY experiment. The remaining resonances [δ_C 59.3 (t); δ_H 4.62 (d, J = 16.5 Hz), 4.46 (d, J = 16.5 Hz)] are characteristic of a vinylic primary alcohol, attached at C-17. This side chain moiety deduced for **3** is also a part structure in luffarin's K−M,[12] and a comparison of NMR data confirmed the above reasoning. As H-9 correlates with

Table 3. MSK1 and MAPKAPK-2 Inhibitory Activity for Compounds **1**−**4**

compound	MSK1 IC_{50} (μM)	MAPKAPK-2 IC_{50} (μM)
1	4	90
2	4	90
3	4	90
4	4	90

H-14 in the ROESY spectrum, the side chain at C-14 is assigned the β configuration. Compound **3** is thus 25-hydroxy-13(24),17-cheilanthadien-16,19-olide.

NMR data (Tables 1 and 2) again indicate that compound **4** has the same carbocyclic framework as both **1** and **3**, differing only at the C-14 side chain. Furthermore, it is obvious a γ-hydroxy-γ-butenolide also exists on the side chain moiety as in **1** and **2**. HRESIMS gives a molecular formula, $C_{25}H_{38}O_4$, for **4** (m/z 401.2681 $[M - H]^-$), which differs from that of **1** by the additional elements of H_2O. Compounds **1** and **4** are clearly similar. The difference is explained by secondary alcohol (δ_C 68.4; δ_H 4.65 (brt, J = 6.0 Hz) and methylene [δ_C 30.19; δ_H 1.94 (m, 2H)] groups in **4** replacing a trans disubstituted double bond in **1**. A ROESY correlation between H-9 and H-14 reveals the β stereochemistry of the side chain at C-14. Structure **4** is therefore assigned as 16,25-dihydroxy-13(24),17-cheilanthadien-19,25-olide.

The absolute configuration was not established for these compounds, and furthermore **1**, **2**, and **4** were isolated as inseparable 1:1 mixtures of the C-25 epimers.

MSK1 (mitogen and stress activated kinase) and MAPKAPK-2 (mitogen activated protein kinase activated protein kinase) are two serine protein kinases involved in signal transduction. Both of these enzymes are located in the nucleus and are thus late in the signal transduction pathway. Selective inhibitors of these enzymes will be most likely to exhibit highly specific cellular effects. As Table 3 reveals, cheilanthanes **1**−**4** contain inhibitor activity of these enzymes.

Experimental Section

General Experimental Procedures. Water was Millipore Milli-Q PF filtered, while all other solvents used were Omnisolv HPLC grade. The C_{18} packing used for flash column chromatography (15 cm × 5 cm i.d.) was Davisil 30−40 μm, 60 Å. A Hypersil BDS C_{18} 5 μm (10 mm × 250 mm i.d.) was used for semipreparative HPLC. A Waters 600 pump fitted with a 996 photodiode array detector and 717 plus Autosampler was used for the semipreparative separations. NMR spectra were recorded at 30 °C on a Varian Inova 600 MHz NMR spectrometer. Samples were dissolved in $CDCl_3$ (^1H δ 7.26 and ^{13}C δ 77.0 ppm). HRESIMS were measured on a Bruker BioAPEX 47e mass spectrometer equipped with a Bradford CT 06405 electrospray ion source. FTIR and UV spectra were recorded on a Perkin-Elmer 1725X spectrophotometer and a GBC UV/vis 916 spectrophotometer, respectively. Streptavidin-coated beads (scintillation proximity assay) were obtained from Amersham. The peptide was obtained from Neosystem Groupe SNPE, and recombinant MSK1 and MAPKAP-K2 were obtained from AstraZeneca.

Animal Material. The sponge was collected at a depth of 21 m from "Pacific Conquest", on the reef slope west side of Porpoise Cay, Wreck Reef (22.12.0′S; 155.19.9′E), Queensland, Australia, in January 1996. It was identified as belonging to the genus *Ircinia* {phylum Porifera, class Demospongiae, order Dictyoceratida, family Ircinidae} and is compressible and has a conulose surface and a brownish gray color. A voucher specimen, QMG306482, has been deposited at the Queensland Museum, South Brisbane, Queensland, Australia.

Extraction and Isolation. The marine sponge *Ircinia* sp. was freeze-dried and pulverized to give 20.0 g of material, which was then extracted with CH_2Cl_2. The resulting extract (2.74 g) was partitioned between hexane and aqueous MeOH {MeOH−H_2O (9:1)} and the later layer concentrated and passed through a flash C_{18} column using a H_2O−MeOH followed by MeOH−EtOAc gradient. The two fractions that eluted with 100% MeOH and MeOH−EtOAc (1:1) showed activity. The fraction MeOH−EtOAc (1:1) was purified by C_{18} HPLC isocratically using H_2O−MeOH (1:9). Four pure compounds were collected, **1**−**4**, with retention times of 15.9, 13.8, 13.0, and 10.8 min, respectively.

Scintillation Proximity Assay. MSK1 activity was determined with a scintillation proximity assay (SPA), which measured ^{33}P incorporation into a synthetic biotinylated peptide substrate (Biotinyl-Ahx-Lys-Lys-Leu-Asn-Arg-Thr-Leu-Ser-Val-Ala). The 60 μL assay was performed in 50 mM MOPS buffer (pH 7.4), which contained 10 mM $MgCl_2$, 0.001% Tween-20, 1.0 mM dithiothreitol (DTT), 50 μM ATP (0.125 μCi), 0.3 μM peptide substrate, and 9.0 mU MSK1 (specific activity 450 U/mg), at room temperature for 60 min. To stop the reaction, 200 μL of the stop solution was added. The stop solution was prepared in Ca^{2+}- and Mg^{2+}-free PBS pH 7.4, which contained 5 mM EDTA, 0.1% Triton X-100, and 0.5 mg/mL SPA beads. The sample plates were counted using the Wallac Trilux after allowing time for the beads to settle (typically 6 h).

Similar to MSK1, MAPKAPK-2 activity was determined with a SPA assay, which measured ^{33}P incorporation into a synthetic biotinylated peptide substrate (KKLNRTLSVA). The 25 μL assay was performed in 50 mM MOPS (pH 7.0), which contained 12.5 mM magnesium acetate, 0.25 mM EDTA, 0.0025% Brij 35, 0.05% bovine serum albumin (BSA), 0.125% β-mercaptoethanol, 50 μM ATP (1.0 μCi, ^{33}P), 3.75 μM peptide substrate, and 25 mU of MAPKAP-K2 (activated GST-MAPKAP-K2, specific activity 1590 U/mg), at room temperature for 40 min. To stop the reaction, 175 μL of the stop solution was added. The stop solution was prepared in Ca^{2+}- and Mg^{2+}-free PBS (pH 7.4), which contained 6.67 mM EDTA, 66.7 μM ATP, 0.13% Triton X-100, and 2.7 mg/mL SPA beads. The sample plates were counted using the Wallac Trilux after allowing time for the beads to settle (typically 6 h).

25-Hydroxy-13(24),15,17-cheilanthatrien-19,25-olide (1): amorphous, white solid (14 mg, 0.07% dry weight); $[\alpha]^{25}_D$ −36.09° (c 0.53 in $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 202 (3.56), 264 (3.79) nm; IR (film) ν_{max} 3330, 2924, 1760, 1748, 1733, 1645, 1456, 1387, 1130 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; negative-HRESIMS m/z 383.2603 (calcd for $[M - H]^-$, $C_{25}H_{35}O_3$ 383.2592).

25-Hydroxy-13(24),17-cheilanthadien-16,19-olide (3): amorphous, white solid (3 mg, 0.02% dry weight); $[\alpha]^{25}_D$ −8.53° (c 0.23 in $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 210 (3.47) nm; IR (film) ν_{max} 3368, 2925, 1750, 1717, 1694, 1682, 1588, 1386, 1208, 1137 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; negative-HRESIMS m/z 385.2745 (calcd for $[M - H]^-$, $C_{25}H_{37}O_3$ 385.2748).

16,25-Dihydroxy-13(24),17-cheilanthadien-19,25-olide (4): amorphous, white solid (25 mg, 0.13% dry weight); $[\alpha]^{25}_D$ −118.65° (c 0.44 in $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 202 (4.00) nm; IR (film) ν_{max} 3320, 2931, 1740, 1750, 1764, 1457, 1136 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; negative-HRESIMS m/z 401.2681 (calcd for $[M - H]^-$, $C_{25}H_{37}O_4$ 401.2697).

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