Phorbasterones A−D, Cytotoxic *Nor*-Ring A Steroids from the Sponge *Phorbas amaranthus*

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Received October 17, 2003

The sponge *Phorbas amaranthus* from Florida contains the new ring A-contracted steroids, phorbasterones A−D, and the known anthosterones A and B. The structures of phorbasterones A−D were determined by interpretation of their spectroscopic data. Phorbasterones show moderate cytotoxicity against HCT-116 tumor cells.

In a systematic survey of Caribbean sponges, Pawlik and co-workers found that extracts of the bright-red sponge *Phorbas amaranthus* deterred feeding by the bluehead wrasse *Thallasoma bifasciatum*.[1] The measured nutrient content of *P. amaranthus* was significant, but the tissue was exceptionally fragile (tensile strength) with no obvious physical defenses.[2] Therefore, the deterrent properties of the sponge are likely attributed to an undescribed "chemical defense". In our search for the antifeedant principles of *P. amaranthus* we isolated the known oxidized steroids anthosterones A (**1**) and B (**2**) and four new congeners, phorbasterones A−D (**3**−**6**). Steroids **1** and **2**, with contracted cyclopentane A-rings, were first described by Anderson, Clardy, and co-workers in 1988.[3] Phorbasterones comprise a family of homologues that differ from **1** and **2** by side-chain (C20−29) alkyl branching, isomerism, or oxidation level. In this report, we describe the isolation and structure elucidation of **3**−**6**. Metabolites from the genus *Phorbas* are rare. The only other natural products described from this genus are the alkaloids phorbazoles from a Red Sea species (*Phorbas* aff. *clathrata*),[4] the potent cytotoxic phorboxazoles A and B from a Western Australian *Phorbas* species,[5,6] the monocyclic diterpenoids phorbasins A and B from an Australian species,[7] and the gagunins, which are highly oxygenated verrucosane diterpenes.[8] Phorbasterones **3**−**6** were found to be cytotoxic to HCT-116 cells (IC_{50} 1−3 μg/mL).

Samples of *P. amaranthus* collected by hand (scuba) at Dry Rock, Key Largo, Florida, were immediately frozen and kept at −20 °C until needed. The $CHCl_3$-soluble fraction, obtained after preliminary methanol extraction of the sponge, was purified by sequential column chromatography (silica, EtOAc−CH_2Cl_2, MeOH−CH_2Cl_2 gradient), Sephadex LH20 chromatography (hexane−CH_2Cl_2, 1:3), and reversed-phase HPLC (C_8, Microsorb, MeOH−H_2O followed by C_{18}, Microsorb, CH_3CN−H_2O) to afford anthosterones A and B (**1** and **2**) and phorbasterones A−D (**3**−**6**). Compounds **1** and **2** were identified by comparison of ^1H and ^{13}C NMR data with those of reported values,[3] while the structures of **3**−**6** were derived as follows.

Anthosterones **1** and **2** and the phorbasterones share a characteristic ring A-contracted steroid nucleus of general structure **7** (Figure 1). The core ^{13}C NMR resonances (C1 to C19) were virtually identical for each compound (Table 1). Key ^1H NMR signals, including the isolated geminal signals H_2-1 (δ 2.08 , d, J = 13.3 Hz, 1H; 2.16, d, J = 13.3 Hz, 1H)[3] and H-6 (δ 6.72, t, J = 3.3 Hz, 1H), assigned to a highly polarized α,β-unsaturated cyclopentenone and the C18 and C19 angular methyl groups (δ 0.73, s, 3H; 1.21, s, 3H), confirmed the presence of an exocyclic double bond conjugated to the cyclopentanone ring. The structural differences between **1**, **2**, and the new compounds **3**−**6** could be attributed solely to differences in the side chains and assigned by examination of the ^1H and ^{13}C NMR spectra.

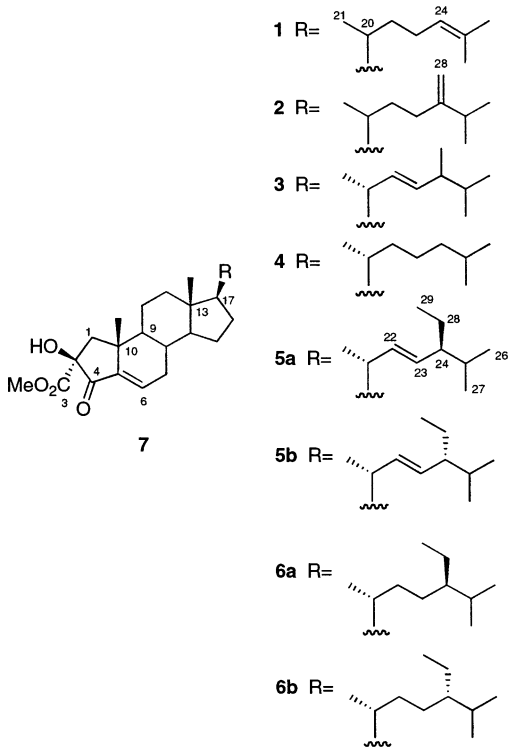

Figure 1.

Compound **3**, $C_{29}H_{44}O_4$, was isomeric with **2** by a desorption electron impact mass spectrum (DEI, *m/z* 456.3247 [M^+]). The ^1H NMR spectrum of **3** ($CDCl_3$) revealed signals due to a 1,2-disubstituted vinyl group (δ 5.15, m, 2H, H-22, H-23) and four methyl groups (δ 1.00, d, J = 6.6 Hz, 3H; 0.89, d, J = 6.9 Hz, 3H; 0.82, d, J = 6.9 Hz, 3H; 0.80, d, J = 6.6 Hz, 3H). Comparison of the ^{13}C NMR signals of **3** C20−C28 showed an almost perfect match for (*E*)-22-

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10.1021/np034037j CCC: $27.50
Published on Web 03/12/2004

Table 1. Selected ^{13}C NMR Chemical Shifts of **2**−**6** (δ, $CDCl_3$, 100 MHz)

#	**2**[a]	**3**[b]	**4**[c]	**5a**[d,h]	**5b**[e,h]	**6a**[g,h]	**6b**[f,h]
2	79.9	79.9	79.9	79.9	79.9	79.9	79.9
3	173.4	173.4	173.4	173.4	173.4	173.4	173.4
4	200.9	200.9	200.9	200.9	200.9	200.9	200.9
5	145.1	145.1	145.1	145.1	145.1	145.1	145.1
6	136.3	136.3	136.3	136.3	136.3	136.3	136.3
20	35.7	40.2	35.7	40.4	40.4	36.1	36.2
		(−0.1)	(0.0)	(−0.1)	(0.0)	(0.0)	(0.0)
21	21.9	21.0	18.7	21.1	20.9	18.8	18.8
		(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)
22	31.0	135.8	36.1	138.1	138.0	33.9	33.9
		(−0.2)	(0.0)	(−0.3)	(−0.2)	(0.0)	(0.0)
23	34.6	132.1	23.8	129.5	129.5	26.1	26.4
		(+0.2)	(0.0)	(+0.2)	(+0.2)	(0.0)	(0.0)
24	156.8	43.1	39.5	51.2	51.2	45.8	46.0
		(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)
25	33.8	33.2	28.0	31.9	31.8	29.1	28.9
		(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)
26	21.9	19.6[i]	22.5[i]	19.0[i]	18.9[i]	19.8	19.6
		(0.0)	(0.0)	(0.0)	(0.0)	(−0.1)	(0.0)
27	21.8	20.2[i]	22.8[i]	21.3[i]	21.2[i]	19.0[i]	19.0[i]
		(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)
28	106.0	18.0		25.4[i]	25.4[i]	23.0[i]	23.0[i]
		(0.0)		(0.0)	(0.0)	(−0.1)	(0.0)
29				12.2	12.4	12.3	12.3
				(0.0)	(0.0)	(0.0)	(0.0)
MeO	53.4	53.4	53.4	53.4	53.4	53.4	53.4

[a] The numbering for anthosterone B (ref 1) is changed here to be consistent with that for **3**−**6**. Differences in δ ($\Delta\delta$) for side-chain resonances (in parentheses) were computed with respect to the following reference compounds. [b] 22-Dehydrocampesterol. [c] 5α-Cholestan-3-one. [d] Stigmasterol. [e] Poriferasterol. [f] Clionasterol. [g] Sitosterol as follows: [$\delta_{\text{phorbasterone}} - \delta_{\text{standard sterol}}$]. [h] Isolated as a 1:1 mixture. [i] Interchangeable.

dehydrocampesterol,[9,10] which confirms the double-bond location (C22−C23) and configuration in **3**.

Phorbasterone B (**4**), $C_{28}H_{44}O_4$, is a lower homologue of **3** (DEI, *m*/*z* 444.3240 $[M]^+$) that contains one less double bond than **1**, as evidenced by lack of side-chain vinyl signals in the ^1H NMR spectrum of **4** ($CDCl_3$). Comparison of side-chain ^{13}C NMR signals, particularly of C20−C27, and the ^1H NMR methyl signals (δ 0.93, d, J = 6.6 Hz, 3H; 0.87, d, J = 6.6 Hz, 3H; 0.86, d, J = 6.6 Hz, 3H) matched the signals of a 5α-cholestan-3-one.[10,11] Thus, phorbasterone B (**4**) is 22,23-dihydroanthosterone A.

Using reversed-phase HPLC under varying conditions we effected a separation of phorbasterones C (**5**) and D (**6**), each as a mixture of epimers. Accurate mass measurement of **5** (DEI *m*/*z* 470.3382 $[M^+]$) revealed a formula $C_{30}H_{46}O_4$ that corresponds to an ethyl-branched homologue of **3**, while the formula of **6**, $C_{30}H_{48}O_4$ (DEI, *m*/*z* 472.3552 $[M^+]$), is the 22,23-dihydro derivative of **5**. These structural differences between **5** and **6** were fully supported by an analysis similar to that described for **3** and **4**; however, an observed doubling of side-chain signals in the ^{13}C NMR spectra suggested that **5** and **6** were each an inseparable 1:1 epimeric mixture, most likely at C-24, from biosynthetic precedents (*cf.* sitosterol and clionasterol, *vide infra*). Consequently, phorbasterones C and D were each characterized as a 1:1 epimeric mixture at C-24 (epimers are indicated by suffices **a** and **b**).

Analysis of the ^1H NMR spectrum of **5** ($CDCl_3$) supported a disubstituted *E*-double bond at C22,23 (δ 5.14, ddd, J = 2.8, 8.4, 15.2 Hz, 1H; 5.01, ddd, J = 2.0, 8.4, 15.2 Hz, 1H). Inspection of the DQFCOSY spectrum showed four methyl groups (δ 1.02, d, J = 6.4 Hz, 3H; 0.84−0.76, overlapped, m, 9H), one of which was part of an ethyl group, as suggested by a cross-peak between an overlapped CH_3 signal and a CH_2 signal at δ 1.65 (m). However, the ^1H NMR signals were not sufficiently dispersed to allow accurate ^1H NMR chemical shift or coupling constant measurements. Instead, examination of the ^{13}C NMR spectra of **5**, including the DEPT spectra, revealed the expected additional CH_2 (δ 25.4, t, C28) and exceptionally high-field CH_3 signal (δ 12.2, q, C29) of the ethyl group and allowed complete assignment of the structures of **5** and **6**, including stereochemistry, as follows.

The stereochemical assignment of the C-24 configuration followed from careful pairwise comparison and least-difference analysis of ^{13}C NMR chemical shifts in **5a** and **5b** with those of the assigned side-chain signals of the known C-24 epimers, stigmasterol[10,12] and poriferasterol[10,13] (Table 1). Since *diastereomeric* differences observed in the side-chain ^{13}C NMR signals are most likely influenced by the nearest stereogenic center, C-20 (which is invariably 20*R* in natural sterols), the pairwise analysis of ^{13}C NMR chemical shifts allows assignment of *absolute* C-24 stereochemistry in **5a** and **5b** as 24*S* and 24*R*, respectively. Similarly, pairwise comparison of ^{13}C NMR signals of **6** with those of sitosterol[10,14] and its C-24 epimer clionasterol[10,15] allowed assignment of the 24*R* and 24*S* configurations to the epimers of phorbasterone D, **6a** and **6b**, respectively (note the change in CIP priorities at C24).

Phorbasterones A−D (**3**−**6**) showed moderate cytotoxicity (IC_{50} 1−3 μg/mL) toward cultured HCT-116 colon tumor cells. The solvent fraction from which compounds **1**−**6** were derived was not active as a fish feeding deterrent in assays with *Thallasoma bifasciatum*.

In summary, we have identified four new ring A-contracted steroids, phorbasterones A−D (**3**−**6**), from *P. amaranthus*. Work on the chemical nature of the feeding deterrent principles is ongoing and will be reported in due course.

Experimental Section

Experimental Procedures. General procedures are described elsewhere.[16] Mass spectrometric measurements were performed at University of California, Riverside Mass Spectrometry Facility. NMR measurements were carried out on a Varian Inova 400 MHz NMR spectrometer equipped with either a $^1H\{^{15}N-^{31}P\}$ pulsed-field gradient (PFG) indirect-detection probe or $^1H/^{13}C/^{15}N/^{31}P$ PFG auto-switchable probe. DQFCOSY experiments were carried out with gradient-enhanced pulse sequences.

Animal Material. *Phorbas amaranthus* (02-13-054) was collected by hand using scuba at −3 to −10 m at North Dry Rocks, Key Largo, FL (25°07.850′ N, 080°17.521′ W) and identified by one of the authors, J.R.P. The sponge was stored for 2 months at −20 °C before extraction. A voucher specimen stored at UNC Wilmington is available from J.R.P.

Collection and Extraction of *P. amaranthus*. The lyophilized tissue (339 g) was gently agitated in MeOH (800 mL) and H_2O (200 mL) using an overhead stirrer (5 °C for 24 h). After filtration, extraction of the tissue was repeated twice with fresh MeOH (900 mL) and H_2O (100 mL), and a third time with MeOH (100 mL) and $CHCl_3$ (900 mL). Removal of volatiles from the $CHCl_3$−MeOH extract gave a deep-red gum (9.3 g). The majority of the extract (9.0 g) was applied to a silica column and eluted with a gradient (EtOAc in $CHCl_3$, then MeOH in $CHCl_3$). The 20% EtOAc fraction contained the crude anthosterones **1** and **2** and phorbasterones **3**−**6**. This fraction was further separated on Sephadex LH-20 (1:3 hexane−CH_2Cl_2) to yield a purified fraction of **1**−**6** (90 mg). Final purification was achieved by reversed-phase HPLC (C_8 Microsorb 90:10 MeOH−H_2O, then C_{18}, Microsorb, CH_3CN-H_2O) to afford, in order of elution, anthosterone A (**1**, 1.5 mg, 0.00037% dry wt), anthosterone B (**2**, 2.4 mg, 0.00059% dry wt), phorbasterone A (**3**, 4.4 mg, 0.0011% dry wt), phorbas-

Table 2. Selected ^{1}H NMR Chemical Shifts of **2**−**6** (δ, $CDCl_3$)[a]

#	2	3	4	5[d]	6
1	2.10, d, J = 13.5 Hz 2.18, d, J = 13.5 Hz	2.08, d, J = 13.8 Hz 2.17, d, J = 13.8 Hz	2.09, d, J = 13.8 Hz 2.18, d, J = 13.8 Hz	2.08, d, J = 14 Hz 2.17, d, J = 14 Hz	2.08, d, J = 13.6 Hz 2.17, d, J = 13.6 Hz
6	6.73, t, J = 3.3 Hz	6.71, t, J = 3.6 Hz	6.73, t, J = 3.3 Hz	6.72, t, J = 3.6 Hz	6.72, t, J = 3.6 Hz
7	2.38, ddd, J = 4.0, 6.0, 21.0	2.38, ddd, J = 3.9, 6.0, 20.7	2.40, ddd, J = 3.9, 6.3, 20.7	2.37, ddd, J = 3.6, 5.7, 21.0 1.85, ddd, J = 3.6, 9.3, 21.0	2.38, ddd, J = 4.0, 6.0, 21.0 1.85, ddd, J = 4.0, 9.0, 21.0
18	0.73, s	0.72, s	0.72, s	0.72, s	0.71, s
19	1.20, s	1.20, s	1.20, s	1.20, s	1.18, s
21	0.96, d, J = 6.7 Hz	1.00, d, J = 6.6 Hz	0.93, d, J = 6.6 Hz	1.02, d, J = 6.4 Hz	0.91, d, J = 6.4 Hz
22	*c*	5.13−5.17, m	*c*	5.14, ddd, J = 2.8, 8.4, 15.2 Hz	*c*
23	*c*	5.13−5.17, m	*c*	5.01, ddd, J = 2.8, 8.4, 15.2 Hz	*c*
26[b]	1.04, d, J = 6.5 Hz	0.89, d, J = 6.9 Hz	0.87, d, J = 6.6 Hz	0.83, d, J = 6.6 Hz	0.82, d, J = 6.6 Hz
27[b]	1.04, d, J = 6.5 Hz	0.82, d, J = 6.9 Hz	0.86, d, J = 6.6 Hz	0.79, d, J = 6.6 Hz	0.79, d, J = 6.6 Hz
28		0.80, d, J = 6.6 Hz	-	*c*	*c*
OH	3.78, brd, J = 1.2 Hz	3.78, brd, J = 0.9 Hz	3.80, brs	3.78, brd, J = 0.9 Hz	3.78, brs
MeO	3.77, s	3.76, s	3.78	3.76	3.76

[a] Spectra of **2** and **6** were recorded at 400 MHz, while compounds **3**, **5**, and **6** were recorded at 300 MHz. [b] Chemical shifts for H26 and H27 are interchangeable. [c] Unresolved. [d] Assignments from DQFCOSY (600 MHz).

terone B (**4**, 6.7 mg, 0.0017% dry wt), phorbasterone C (**5**, 1.1 mg, 0.0003% dry wt), and phorbasterone D (**6**, 0.7 mg, 0.0002% dry wt).

(−)-Phorbasterone A (3): colorless solid, $[\alpha]_D$ −45.4° (*c* 0.19, $CHCl_3$); UV (CH_3CN) λ_{max} 250 nm (ϵ 9600); IR (film) ν_{max} 3453, 2956, 1745, 1720, 1651 cm^{-1}; ^{1}H NMR ($CDCl_3$, see Table 2); ^{13}C NMR (100 MHz, $CDCl_3$, see Table 1); HRMS (DEI) *m*/*z* 456.3247 [M^+] (calcd for $C_{29}H_{44}O_4$, 456.3239).

(−)-Phorbasterone B (4): colorless solid, $[\alpha]_D$ −54.6° (*c* 0.28, $CHCl_3$); UV (CH_3CN) λ_{max} 250 nm (ϵ 9300); IR (film) ν_{max} 3469, 2952, 1745, 1720, 1650 cm^{-1}; ^{1}H NMR ($CDCl_3$, see Table 2); ^{13}C NMR (100 MHz, $CDCl_3$, see Table 1); HRMS (DEI) *m*/*z* 444.3240 [M^+] (calcd for $C_{28}H_{44}O_4$, 444.3239).

Phorbasterone C (5a and 5b): colorless solid, UV (CH_3CN) λ_{max} 250 nm (ϵ 9100); IR (film) ν_{max} 3357, 2958, 1745, 1720, 1650, 1384 cm^{-1}; ^{1}H NMR ($CDCl_3$, see Table 2); ^{13}C NMR (100 MHz, $CDCl_3$, see Table 1); HRMS (DEI) *m*/*z* 470.3382 [M^+] (calcd for $C_{30}H_{46}O_4$, 470.3396).

Phorbasterone D (6a and 6b): colorless solid, UV (CH_3CN) λ_{max} 250 nm (ϵ 9000); IR (film) ν_{max} 3357, 2958, 1745, 1720, 1650 cm^{-1}; ^{1}H NMR ($CDCl_3$, see Table 2); ^{13}C NMR (100 MHz, $CDCl_3$, see Table 1); HRMS (DEI) *m*/*z* 472.3537 [M^+] (calcd for $C_{30}H_{48}O_4$, 472.3552).

Cytotoxicity Assays. Cytotoxicity was measured with HCT-116 cells using the MTS method.[17] Briefly, compounds were assayed with compounds in DMSO (final concentration, 1% v/v) and run against etoposide as positive control. HCT-116 cells were incubated in 96-well plates for 72 h before addition of MTS [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt]. The Promega CellTiter 96 Aqueous cell proliferation assay (Technical Bulletin No. 169) was used. Well absorbances (λ 490 nm) were corrected for background and expressed as a percentage of the negative control (DMSO, only).

Acknowledgment. We thank J. Cowart (University of North Carolina, Wilmington) for assistance with collection of *Phorbas* sp., and S. Kelly (Scripps Institution of Oceanography) and S. Lievens (UCD) for performing the HCT-116 bioassays. The Bruker Avance 600 MHz spectrometer was purchased with funds provided by NIH RR11973. The Varian Inova 400 MHz spectrometer was purchased with funds provided by NSF CHE-9808183. This work was supported, in part, by grants from NIH (CA 85602) to T.F.M, and the NSF Biological Oceanography Program (OCE-0095724) and NOAA/National Undersea Research Center at UNCW to J.R.P.

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NP034037J