ORGANIC LETTERS

2009
Vol. 11, No. 9
1967−1970

Structure Elucidation at the Nanomole Scale. 2. Hemi-phorboxazole A from *Phorbas* sp.†

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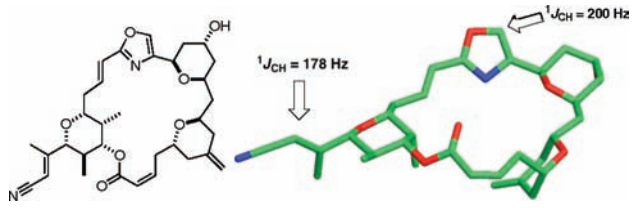
Received February 28, 2009

ABSTRACT



Hemi-phorboxazole A, a minor truncated analogue of phorboxazole A from the marine sponge *Phorbas* sp., was isolated in a total yield of 16.5 μg (28 nmol). The structure was elucidated by application of integrated nanomole-scale natural product techniques, including cryomicroprobe NMR, ^{1}H-coupled HSQC, and circular dichroism (CD).

Recent developments in capillary and microprobe NMR, particularly cryomicroprobes,[1] have increased the mass sensitivity of NMR methods for structure elucidation, including 2D NMR,[2] and expanded the window of discovery to natural products to the nanomole scale.[3] Here, we report a powerful application of this approach with the discovery of a new oxazole-containing macrolide, hemi-phorboxazole A (**1**, Figure 1) from the same sponge *Phorbas* sp. that provided phorboxazoles A (**2**) and B (**3**)[4] and, more recently phorbasides A (**4**), B−E[5] and F (2-demethylphorbaside A).[6]

Phorbasides A−E exhibit modest cytotoxicity toward colon tumor cells (HCT-116; IC_{50} 2−30 μM); however, **2** and **3** show exceptionally highly cytostatic activity and induce cell growth inhibition (GI) in a range of cancer cells (e.g., leukemia, CCRF-CEM, GI_{50} = 0.25 nM; HCT-116, GI_{50} = 0.44 nM, mean GI_{50} of ∼1 nM, NCI 60-cell panel). The complete characterization of the **1**—including their chiroptical properties, and stereochemical assignments—was carried out with a *total sample* size of 16.5 μg (28 nmol), an accomplishment that would have been almost impossible a few years ago with previous-generation spectroscopic instrumentation.

Hemi-phorboxazole A (**1**) is the first reported natural variation on the phorboxazole carbon skeleton since disclosure of **2** and **3** in 1995.[4] Unlike **2** and **3**, which were isolated in a combined yield of over 100 mg, the μg amounts available of **1** required adaptation to new operational

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10.1021/ol9004189 CCC: $40.75
Published on Web 04/02/2009

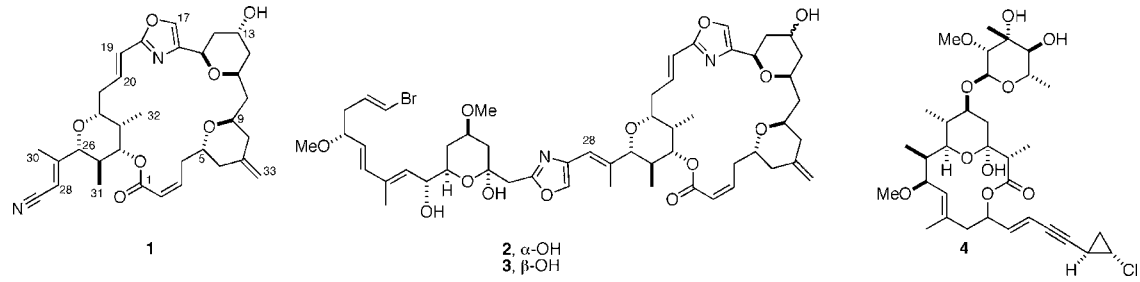

Figure 1

protocol. Purification, structure elucidation and quantitation of **1** exploited nanospray-LC HRMS (ESI TOF), cryomicroprobe 2D NMR (COSY, HSQC, HMBC, NOESY), including strategic interpretation of $^1J_{CH}$ coupling constants (*J*-coupled HSQC) and circular dichroism (CD).

The CCl_4-soluble fraction of the methanol extract of the *Phorbas* sp. was prepared as previously described and partially fractionated by silica flash chromatography (silica, MeOH−CH_2Cl_2 gradient), and reversed-phase HPLC (MeOH−H_2O). Final purification[7] was achieved using phenyl-bonded reversed phase HPLC (4.6 × 250 mm, CH_3CN-H_2O, 1 mL/min, λ 254 nm detection) to afford **1** (16.5 μg) as a colorless glass.[8] Quantitation of **1**, which was critical for determination of other molar spectroscopic quantities (ε, UV−vis; $\Delta\varepsilon$, CD) was made by a comparative integration of CH signals in **1** with the (constant) $^1H-^{13}C$ satellite peaks of residual $CDCl_3$ (99.8% D) after external calibration with standard solutions of cholesterol (1−50 μg/35 μL).[6]

Nanospray LC high-resolution time-of-flight mass spectrometric (HRTOFMS) measurement of **1** provided a tentative formula $C_{33}H_{43}N_2O_7$ from accurate mass measurement of pseudomolecular ions $[M + H]^+$ (*m/z* 579.3050, 579.3064, Δmmu = −1.4) and $[M + Na]^+$ (*m/z* 601.2872, Δmmu = −1.7), although one alternate formula ($C_{34}H_{44}NO_7$) was also plausible (see below). Preliminary inspection of the ^1H NMR spectrum revealed only about half the signals of **2**, including only one oxazole singlet (δ 7.42, s, $^1J_{CH}$ = 200.4 Hz). Full analysis of **1** by ^1H and ^{13}C NMR, COSY, HSQC, and HMBC spectra was carried out on the limited sample of **1** at 600 MHz using a 1.7 mm inverse-detection cryoprobe, designed for optimal ^1H and ^{13}C detection. We had previously utilized the unmatched mass-sensitivity of 1 and 1.7 mm cryoprobes for structure elucidation of complex macrolides;[9] however, some modifications of our usual approaches were adopted for **1**.

J-coupled HSQC acquisition was optimized by use of a shorter fixed delay of 2.5 ms in the evolution period (corresponding to $^1J_{CH}$ = 200 MHz, compared to the typical 3.57 ms, $^1J_{CH}$ = 140 Hz), and no broadband ^1H decoupling during FID acquisition allowed measurement of cross-peaks with full $^1J_{CH}$ couplings, including possible cyclopropyl ($^1J_{CH} \approx 175-200$ Hz) and terminal acetylenic carbons ($^1J_{CH} \approx 250$ MHz) that often escape detection due to very low efficiency of polarization transfer.[10] This lends confidence to assignments and removes equivocal interpretations, particularly of *C*-Me, *N*-Me, and *S*-Me signals,[11] identification of oxazole-ring H-5 ^1H signals ($^1J_{CH}$ > 200 Hz[12]), and sp versus sp^2 $^{13}C-^1H$ assignments.

The contiguous scalar-coupled $^1H-^1H$ spin networks C-1 to C-17 and C-19−C-26 of **1** were mapped using COSY and HMBC experiments (600 MHz, $CDCl_3$). Both the ^1H and ^{13}C chemical shifts of **1** were virtually identical with those of **2** including the α-configuration of the OH group at C-13 group (δ_H 4.40 m, 1H, δ_C 64.8, CH, $^1J_{CH}$ = 144.8 Hz; cf. **3**[4a] δ_H 4.36, m, δ_C 64.8, CH).

Noticeably lacking in **1** were ^1H NMR signals for the second oxazole ring of **2**, the fourth oxane ring C-33−C-37, and the extended side chain, including the terminal C-15−C-46 vinyl bromide (Table 1).

Using COSY and HMBC data, the largest ^1H and ^{13}C NMR differences in **1** and **2** were narrowed down to the sp^2 carbons directly attached to the third oxane ring (O−C-22−C-26). Placement of an "end-group" and the elemental balance of the formula (CN or C_2H) required that the oxazole-substituted side chain at C-28 in **2** be replaced by either a terminal alkyne (C≡CH) or a nitrile (C≡N). Indeed, the presence of an ^1H signal (δ 2.67, 1H) and quaternary carbon signal (δ 99.0, C, HMBC) in the spectra of **1** was not inconsistent with an acetylene, and interpretation of the pseudomolecular ion in the HRMSESI spectrum could accommodate either a C≡CH end-group ($M + H^+$, Δmmu = −1.0) or C≡N (M^+, Δmmu = −1.4).

(7) Procedures for efficient handling of nanomole samples include use of *re*-distilled HPLC-grade solvent, prewashing (1:1 $CHCl_3$/MeOH) of all glassware used in handling, post-HPLC column, and avoidance of high dilutions. NMR samples were delivered in fixed volumes of $CDCl_3$ (35 μL) from a gastight syringe.

(8) **1**: oil, $C_{33}H_{43}N_2O_7$; UV (MeOH), λ 220 sh, 261 ($\log_{10}$ 4.06); CD (MeOH), λ 211 (−18.9), 259 (−1.9); FTIR (ATR, neat) ν 2220 m, 1716 s cm^{-1}.

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(10) The cyclopropyl ^{13}C signals of callipeltoside A were not observed in the DEPT and HMQC spectra with a fixed delay set to J = 135 Hz. See ref 23.

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(12) Historically, $^1J_{CH}$'s have been underutilized for two reasons: neglected appreciation of the useful correlation of their magnitude with structure (e.g., electronic environment, ring-strain) and experimental limitations for their measurement which required direct-detected coupled ^{13}C NMR (tens of mg samples), or coupled HSQC or HMQC (>1 mg samples). Cryomicroprobe measurements of $^1J_{CH}$'s from ^1H-coupled HSQC, even of microgram samples, is now simplified.

Table 1. NMR Data for Hemi-phorboxazole A (**1**) ($CDCl_3$, 600 MHz, 1.7 mm Cryomicroprobe)

no.	^{1}H, multiplicity (J, Hz)	^{13}C NMR[a] ($^1J_{CH}$ Hz)[b]
1		165.4
2	5.88, m[c]	120.0 (145)
3	5.93, m[c]	145.0 (145)
4a	2.32, m	30.7 (122)
4b	3.46, m	30.7 (122)
5	4.17, m	73.6 (134)
6a	2.05, br d (13.1)	36.9 (134)
6b	2.40, m	36.9 (122)
7		141.8
8a	1.86, m	39.1 (124)
8b	2.69, br d (12.3)	
9	3.98, m	69.3 (145)
10a	1.41	41.2 (122)
10b	1.86, m	
11	4.06, m	68.8 (134)
12a	1.56, m	38.9 (122)
12b	1.68, bd (12.7)	
13	4.40, m	64.8 (145)
14a,b	1.95, m	35.0 (122)
15	4.73, dd (11.0, 2.6)	67.2 (134)
16		142.6
17	7.42, s	134.0 (200)
18		161.3
19	6.28, d (15.8)	119.7 (178)
20	6.64, ddd (16.0, 9.9, 6.4)	133.5 (145)
21a	2.40, m	34.2 (134)
21b	2.50, ddd (12.8, 10.2, 5.7)	34.2 (122)
22	3.51, ddd (11.4, 5.1, 1.3)	78.5 (134)
23	2.30, m	32.2 (122)
24	4.48, dd (11.2, 4.4)	78.7 (145)
25	1.95, m	35.0 (122)
26	3.56, d (10.2)	86.3 (134)
27		160.8
28	5.37, s	99.0 (178)
29		116.0
30	2.09, s	16.6 (134)
31	0.75, d (6.4)	12.9 (134)
32	0.94, d (6.9)	6.1 (122)
33a	4.60, br s	110.0 (156)
33b	4.97, br s	110.0 (156)

[a] Measured from indirectly detected experiments (HMBC). 2D correlations from HSQC (optimized, $^1J_{CH} = 200$ Hz) and HMBC ($^{2,3}J_{CH} = 8$ Hz). [b] $^1J_{CH} \pm 5$ Hz, from ^{1}H-coupled HSQC ($^1J_{CH} = 200$ Hz). [c] Second-order coupling.

Unfortunately, the identities of the remaining quaternary carbons as sp^2 or sp were equivocal and not satisfactorily resolved from their ^{13}C NMR chemical shifts (δ 160.8; 116.0; 99.0 ppm), alone.

Two pieces of evidence confirmed the latter formula and an α,β-unsaturated nitrile. HSQC ($^1J_{CH} = 200$ Hz) showed no correlations from the ^{1}H NMR signal at δ 2.67, thereby associating this signal, by default, with the C-13 OH group. The remaining quaternary signals could now be readily assigned to a highly polarized trisubstituted C-28−C-29 double bond (HSQC and HMBC). The ^{13}C NMR chemical shifts of the C-28 and C-27 double bond (δ 99.0, Cq; 160.8, Cq, respectively) were only consistent with bond polarization by an electron-withdrawing group (CN) at C-28. A nitrile carbon, in turn, was assigned as C-29 (δ 116.0, Cq). In addition, the larger $^1J_{CH}$ observed for H-28 (δ 5.37, s, 1H, $J = 178$ Hz) is entirely consistent with the NMR spectral data for (*E*)-3,4-dimethylpent-2-enenitrile.[13] Finally, the configuration of the C-28−C-29 double bond was assigned as *E* by NOESY from observation of cross peak between H-28 and H-26 (Figure 2) but not between the C-27 vinyl Me signal and H-28.[14]

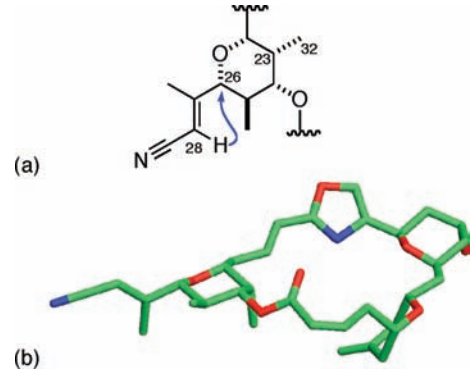

Figure 2. NOESY of **1** ($CDCl_3$, 600 MHz, mixing time, $t_m = 400$ mS). (a) Selected NOEs of **1** (b). Molecular model of **1** based on the X-ray coordinates of an intermediate in the synthesis of **2** (Forsyth et al.[27a]) and parsimonious group replacements.[15]

The possible origin of hemi-phorboxazole A (**1**) deserves some comment. With the exception of cyanogenic glycosides from plants,[16] nitrile groups are uncommon in nature. For example, nitrile groups are found in the structures of calyculin A[17] from the sponge *Calyx*, the microbial metabolites cyanosporaside A and B from "*Salinispora pacifica*",[18] and borrelidin from *Streptomyces parvulus*.[19] The C≡N group in the latter $\alpha,\beta,\delta,\gamma$-unsaturated nitrile macrolide is derived by cytochrome P450-mediated allylic oxidation of a vinyl methyl group precursor ("preborrelidin"), transamination to an oxime, and dehydration to the cyano group.[20] When applied to **1** (Figure 3a), this motif would be consistent with a putative precursor ***i*** that implies substitution of acetate for serine as the starter unit

(13) Prepared by Horner−Emmons−Wittig olefination of isopropyl methyl ketone [$(EtO)_2(P{=}O)CH_2CN$, NaH, THF, 45%, *E*/*Z* 3:1]. Selected assignments: *E*-isomer, δ_H 5.12, (s, 1H, $^1J_{CH} = 172$ Hz, H-3); δ_C170.8, s (C-3); 117.6 (Cq, CN); 93.6 (CH, C-2); *Z*-, δ 5.02 (s, H-3, $^1J_{CH} = 171$ Hz).

(14) The characteristic annular NOE of **2** (reference 4a) was also observed in **1** between H-32 (δ 0.95) and H-33a (δ 4.60). The CD spectra of **1** and **2** are similar (see the Supporting Information), and it is almost certain the absolute configurations of the two are identical. See ref 4b,c.

(15) Molecular mechanics calculations (MMFF94, Spartan' 04) of a simplified model of **1** reveal two stable rotamers, *anti* and *syn*, in which the π-system C-27−C-28−CN is coplanar with C-26−C-23−C-32. The depicted *anti* rotamer (Figure 2) is ~1 kcal mol^{-1} less stable than the *syn* rotamer, obtained by rotation of C-26−C-27 by ~180°.

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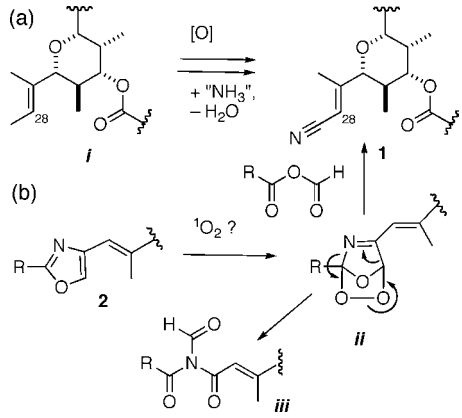

Figure 3. Possible origins of hemiphorboxazole A (**1**): (a) cf. borrelidin biosynthesis, ref 19; (b) $^1O_2$ [4 + 2] addition to **2** followed by rearrangement of ***ii***.

[C-28−C-29] of an abbreviated chain produced by a polyketide-nonribosomal peptide synthase (PKS-NRPS). Path a also maintains the correct alignment of C=O groups in subsequent ketide extender units (C-27−C-1).

Alternatively, **1** may arise from autoxidation of the oxazole ring in **2** (Figure 3, path b). Photosensitized [4 + 2] singlet oxygen addition to an oxazole ring gives a peroxide (cf. **ii**) that may further react to provide a labile *N*-formylimide (cf. **iii**) or nitrile by different rearrangement pathways.[21] Imides (C=O-NH-C=O), which have formally lost CO from *N*-formylimides, have been found as congeners of trisoxazole macrolides.[22] Conceivably, nitrile **1** may arise as an artifact of $^1O_2$ cycloaddition to **2** (Figure 3).

A summary of yields of compounds (Table 2) from a single specimen of *Phorbas* sp. and a literature report for callipeltoside from the sponge *Callipelta* sp[23a] puts the significance of **1** into sharp relief.

Phorboxazole A (**2**) is relatively abundant (400 ppm), while phorbasides A (**4**) and B−F are rare (∼0.1−0.01 × concentration of **2**). Hemi-phorboxazole A (**1**) is *∼10000 times less adundant* than **2** and **3** and among the rarest of the *Phorbas* macrolides described to date.

Table 2. Isolated Yields of Macrolides (ppm) from Sponges

macrolide	sponge	ppm, /dry wt	ref
hemi-phorboxazole A (**1**)	*Phorbas* sp.	0.07	*a*
phorboxazole A (**2**)	*Phorbas* sp.	400	*b*
phorboxazole B (**3**)	*Phorbas* sp.	170	*b*
phorbaside A (**4**)	*Phorbas* sp.	11.6	*c*
phorbaside B	*Phorbas* sp.	5.5	*c*
phorbaside C	*Phorbas* sp.	127	*c*
phorbaside D	*Phorbas* sp.	30	*c*
phorbaside E	*Phorbas* sp.	30	*c*
phorbaside F	*Phorbas* sp.	0.03	*d*
callipeltoside A	*Callipelta* sp.	1.4	*e*

[a] This work. [b] Reference 4. [c] Reference 5. [d] Reference 6. [e] Reference 23.

Presumably, **1** is a potent Michael acceptor; however, its limited quantity precludes anything but the most rudimentary bioassay. A full evaluation of the biological profile of this compound is currently incomplete. Like callipeltosides A−C,[23] further biological evaluation of **1** is not possible with current stocks of natural product. Compounds **2** and **3** have only been isolated twice—from the sponges *Phorbas* sp.[4] and *Raspailia* sp.,[24] each obtained under circumstances that militate against successful recollection of either sponge. Given the highly potent cytostatic activity of **2** and contemporary interest in its structure−activity relationships,[25] mechanism of action,[26] and total synthesis,[25b,27] the synthesis of **1** and its analogues presents itself as a significant and worthy goal.

Acknowledgment. We are very grateful to C. Forsyth (Ohio State University) for sharing X-ray coordinates to use for preparation of Figure 2 and A. Jansma (UCSD) for assistance with NMR experiments. HRMSESI TOF mass measurements were provided by the Scripps Research Institute mass spectrometry facility. This work was supported by grants from the NIH (RO1 CA122256, RO1 AI039987).

Supporting Information Available: Isolation procedures, ^{1}H, NMR (600 MHz), COSY, HSQC, ^{1}H-coupled HSQC, HMBC, NOESY, and CD spectra of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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