Structure Elucidation at the Nanomole Scale. 3. Phorbasides G−I from *Phorbas* sp.

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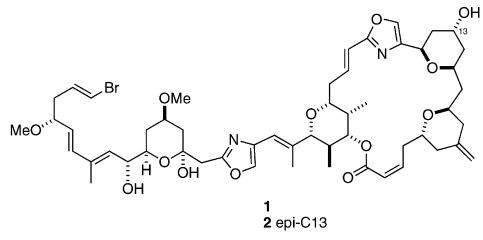
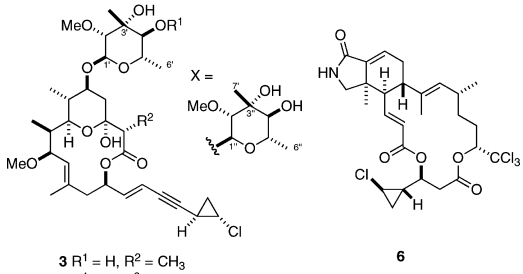
Three new phorbasides (G−I), chlorocyclopropyl ene-yne macrolide glycosides, were isolated from the sponge *Phorbas* sp. in yields of 7−9.5 μg and fully characterized by MS, CD, and microcryoprobe NMR. The structures of the new compounds differ only in the nature of the sugar residues. The absolute configurations of the new compounds were correlated by ROESY and CD with the parent compounds phorbasides A and B.

The use of microcryoprobe NMR spectroscopy, integrated with other "nanomole-scale" techniques (CD, FTIR, FTMS) for natural products discovery, has expanded our view to extremely minute amounts of compound.[1] Nanomole-scale methods exploit technical improvements in signal-to-noise of spectroscopic methods, but more importantly they open new vistas for discovery of new compounds from rare organisms and uncovering chemical complexity and diversity within single specimens.[2] The marine sponge *Phorbas* sp., collected off Muiron Island in Western Australia, has been shown to contain highly cytotoxic macrolides including phorboxazoles A (**1**) and B (**2**)[3] and phorbasides A (**3**) and B (**4**).[4] Recently, the structures of very minor natural products from this one specimen of *Phorbas* were elucidated to reveal appreciably broader chemical diversity within the sponge than encountered in earlier studies. The new compounds, which were fully characterized by MS, CD, and NMR (COSY, ROESY, HSQC, HMBC), include phorbaside F (**5**, 8 μg, 13 nmol),[5] hemiphorboxazole A (16.5 μg),[6] and muironolide A (**6**, 90 μg).[7] The latter represents the first member of an entirely new carbon skeleton based on a hexahydro-1*H*-isoindolin-1-one. We now report additional phorbasides, G−I (**7**−**9**), in total yields of 7−9.5 μg. The structures were solved by integrated application of microcryoprobe NMR, including quantitative analysis, MS, and CD analysis.



1
2 epi-C13

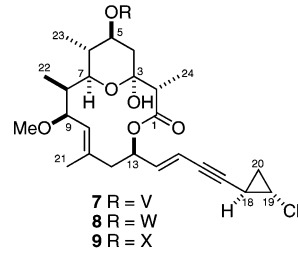


3 R^1 = H, R^2 = CH_3
4 R^1 = X, R^2 = CH_3
5 R^1 = H, R^2 = H

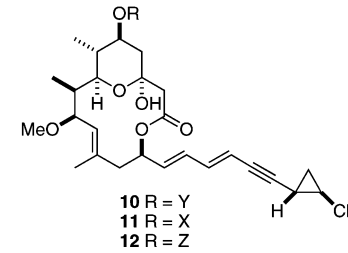
6

Results and Discussion

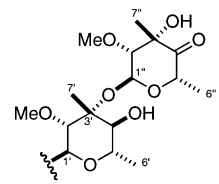
Reexamination of the CCl_4-soluble fraction of the sponge *Phorbas* sp., which previously afforded **3** and **4**,[4] revealed very minor components, which were further purified by reversed-phase HPLC to give *total yields* of pure compounds in amounts ranging from 7 to 9.5 μg (quantitation by comparative NMR "^{13}C satellite" integration):[5] phorbaside F[5] (**5**), 8 μg (3.8 × 10^{-6} %), phorbaside G (**7**), 9.5 μg (4.0 × 10^{-6} %), phorbaside H (**8**), 7.0 μg (2.9 × 10^{-6} %), and phorbaside I (**9**), 9.4 μg (3.9 × 10^{-6} % dry wt).



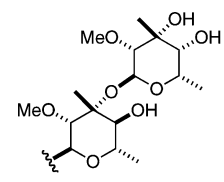
7 R = V
8 R = W
9 R = X



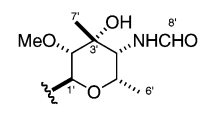
10 R = Y
11 R = X
12 R = Z



V



W



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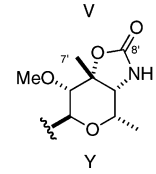
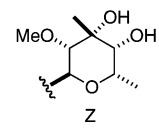


Y



Z

The molecular formula of phorbaside G (**7**), $C_{41}H_{61}ClO_{14}$, derived from HRESITOFMS (*m*/*z* $[M + Na]^+$, 835.2631), is two H atoms less than that of phorbaside B (**4**). The ^{1}H, COSY, NOESY, HSQC, and HMBC experiments revealed the same macrolide carbon framework present in **4**, but differing in the disaccharide group attached at C-5 (Tables 1 and 2). The second sugar unit of **7** is oxidized with respect to **4** and has a keto group at C-4″ (δ_C 208.0). In addition, the ^{13}C NMR chemical shifts of C-3′ (δ 80.5) and C-4′ (δ 70.4) in the first sugar unit appeared downfield of those in **4** by ~10 ppm. The configuration of the sugar unit was established by ROESY as a 6-deoxy-2,3-*C*,*O*-dimethyl-α-L-*manno*-pyranoside (*O*-methyl-L-evalose). Careful analysis of the HMBC spectrum showed that the anomeric proton signal H-1″ (δ 5.82, s) was correlated with the quaternary C-3′, thus indicating a 1″→3′ disaccharide linkage. The downfield ^{13}C NMR chemical shift of C-3′ at δ 80.5 is characteristic of a 1″→3′ linkage when the tertiary OH of

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Table 1. NMR Data of Phorbasides G−I, **7**−**9** (600 MHz, $CDCl_3$)

no.	**7** $^{\delta}$H, mult. (*J* in Hz)	**8** $^{\delta}$H, mult. (*J* in Hz)	**9**[a] $^{\delta}$H, mult. (*J* in Hz)
1			
2	2.52, q (7.3)	2.52, q (7.3)	2.51, m
3			
4	1.10, m 2.31, dd (12.3, 5.2)	1.10, m 2.29, dd (12.0, 4.7)	1.15, m 2.31, dd (12.0, 4.5)
5	3.63, td (10.6, 4.4)	3.61, td (10.5, 4.7)	3.64, td (10.6, 4.5)
6	1.46, m	1.46, m	1.45, m
7	3.66, dd (10.6, 2.6)	3.66, dd (10.5, 2.4)	3.67, m
8	2.18, m	2.19, m	2.18, m
9	3.77, dd (9.5, 2.6)	3.77, dd (9.5, 2.4)	3.78, dd (9.5, 2.2)
10	5.28, d (9.5)	5.28, d (9.5)	5.28, d (9.5)
11			
12	2.25, d (7.3)	2.25, d (7.3)	2.25, d (7.0)
13	5.72, dd (14.0, 6.5)	5.72, dd (16.0, 6.2)	5.72, dd (16.0, 6.2)
14	6.05, dd (15.8, 6.5)	6.05, dd (16.0, 6.2)	6.05, dd (16.0, 6.2)
15	5.66, d (15.8)	5.66, d (16.0)	5.66, d (16.0)
18	1.78, m	1.78, m	1.77, m
19	3.17, ddd (9.0, 6.6, 3.0)	3.17, ddd (9.0, 6.6, 3.0)	3.17, ddd (9.0, 6.6, 3.0)
20	1.29, m	1.27, m	1.27, m
21	1.73, s	1.73, s	1.73, s
22	0.98, d (6.7)	0.97, d (7.2)	0.98, d (7.0)
23	0.92, d (6.7)	0.91, d (6.4)	0.92, d (6.4)
24	1.13, d (7.0)	1.13, d (7.2)	1.13, d (7.2)
OMe	3.23, s	3.23, s	3.23, s
OH	4.79, s	4.78, s	
1′	4.88, s	4.85, s	4.96, s
2′	3.26, s	3.18, s	2.97, s
3′			
4′	3.94, d (5.4)	3.94, dd (9.4. 3.2)	3.97, d (10.5)
5′	3.76, m	3.75, dd (9.4, 6.5)	4.11, q
6′	1.30, d (6.6)	1.31, d (6.5)	1.11, d (6.4)
7′	1.45, s	1.38, s	1.43, s [1.45, s[b]]
8′			8.37, s [(7.93, d (12.0)[b]]
3′-OH			
2′-OMe	3.43, s	3.41, s	3.47, s [3.46, s[b]]
1″	5.82, s	5.76, s	
2″	3.44, s	3.00, s	
3″			
4″		3.14, d (12.0)	
5″	4.70, q (6.8)	4.22, q (6.5)	
6″	1.31, d (6.0)	1.27, d (6.2)	
7″	1.63, s	1.38, s	
2″-OMe	3.49, s	3.48, s	
-NH			6.32, d (10.6)[a] [6.14, t, (11.4)[b]]

[a] Mixture of rotamers (2:1), some signals doubled. [b] Minor rotamer.

C-methyl sugars is glycosylated and matches ^{13}C NMR chemical shifts of L-evalose residues with similar glycosylation patterns found in glycolipid antigens from *Mycobacterium avium*.[8]

Phorbaside H (**8**) has a molecular formula of $C_{41}H_{63}ClO_{14}$ as established by HRESITOFMS (*m/z* 837.3780 $[M + Na]^+$) and is isomeric with phorbaside B (**4**). Significant differences in NMR chemical shifts were again seen in the sugar units as revealed by the ^{1}H, COSY, ROESY, HSQC, and HMBC experiments (Tables 1 and 2). The configuration of the first sugar unit in **8** is 6-deoxy-2,3-*C*,*O*-dimethyl-α-L-*manno*-pyranoside, the same as in **4**, while the second sugar unit is a 6-deoxy-2,3-*C*,*O*-dimethyl-α-L-*talo*-pyranoside on the basis of interpretation of a ROESY experiment (600 MHz, $t_m = 400$ ms, Figure 1). Key ROESY correlations were observed from the anomeric signal H-1″ to H-2″, H-2″ to H-7″, and H-4″ to H-5. Furthermore, the ^{1}H NMR signal due to the methyl group at C-7″ (δ 1.38) showed ROESY correlations to H-2″ and H-5″. The C4″ configuration in **8**, therefore, is epimeric to C-4 in evalose residues that occur in **3** and **4** and is the first example of a *talo*-hexose in the callipeltoside-phorbaside family, although the amino sugars in callipeltosides A (**10**) and B (**11**)[9] are also *talo*. The linkage of the sugar units was established from cross-peak assignments of HMBC and HSQC data. As with **7**, a 1″→3′ disaccharide linkage was identified by characteristic changes in ^{13}C chemical shifts and heteronuclear correlation; the signal of the anomeric proton H-1″ (δ 5.76, s) in the second sugar of **8** shared an HMBC cross-peak with the quaternary C-3′ (δ 80.1) of the first sugar, which was also shifted downfield by about 10 ppm compared to **3**.

Phorbaside I (**9**) has a molecular formula of $C_{34}H_{50}ClNNaO_{10}$, as determined by HRESITOFMS (*m/z* $[M + Na]^+$, 690.3020). Analysis of ^{1}H, COSY, HSQC, and HMBC experiments indicated that **9** has the same aglycone as phorbaside A (**3**), and deductive reasoning, which accounted for the differences in the NMR signals and the presence of N in the formula, led to an assignment of the amino sugar glycone (Tables 1 and 2). The ^{1}H NMR spectrum of **9** showed several signals that were doubled in the ratio of 2:1, characteristic of interconverting rotamers of a formamide NH−CH═O group. The associated ^{1}H NMR signals were split into two sets: a formyl signal H-8′ (δ 8.37, s, major and 7.93, d, $J = 12$ Hz, minor) and an NH signal (6.32, d, $J = 10.6$ Hz, major and 6.14, t, $J = 11.4$ Hz, minor). Signal doubling was also observed for H-7′ (δ 1.43, s, major and 1.45, s, minor) and H-2′-OMe (δ 3.47, s, major and 3.46, s, minor). A *talo* configuration for the sugar was supported by ROESY (600 MHz, $t_m = 400$ ms) data cross-peaks between *syn*-facial hydrogens across the pyranose ring: H-1′ to H-2′, H2′ to H-7′, and H′-4 to H-6′ and H-7′. These lines of evidence pointed to an *N*-formyl 4-amino-4,6-dideoxy-2,3-*C,O*-dimethyl-α-L-*talo*-pyranoside residue, first seen in callipeltoside B (**11**), but assigned as β-L.[9] Thus the configuration at each anomeric carbon in **9** is α-L, the same as in **3** and **4** derived from similar evidence.[10] Note that the disaccharides **7** and **8** have 1″→3′ disaccharide linkages instead of the 1″→4′ assigned to phorbaside C and the 1″→2′ linkage for phorbaside E.[4]

The anomeric configuration of evalose residues and related sugars in the callipeltose-phorbaside family of glycosides has been a matter of some equivocation. The ^{1}H NMR signals for both H-1 and H-2 in evalose residues are singlets with zero vicinal couplings that would otherwise resolve axial or equatorial dispositions from simple Karplus relationships. D'Auria and co-workers assigned a β-L configuration to callipeltosides B and C;[9] however, we assigned α-L to the anomeric carbons C-1′ and C-1″ in **3**, **4**, and other phorbasides[4] on the basis of consistent observation of a weak

Table 2. ^{13}C NMR Data[a] of Phorbasides G−I, **7**−**9** (600 MHz, $CDCl_3$)

no.	**7** C, mult.	**8** C, mult.	**9** C mult.
1	176.1, C	176.2, C	176.6
2	47.0, CH	47.4, CH	47.0, CH
3	97.4, C	97.4, C	97.0, C
4	46.3, CH_2	46.9, CH_2	46.3, CH_2
5	79.3, CH	79.1, CH	80.0, CH
6	38.7, CH	38.8, CH	38.5, CH
7	74.9, CH	74.8, CH	74.7, CH
8	37.0, CH	37.1, CH	36.7, CH
9	79.5, CH	79.8, CH	79.5, CH
10	127.9, CH	127.8, CH	127.5, CH
11	133.0, C	132.4, C	132.6, C
12	46.0, CH_2	46.9, CH_2	46.4, CH_2
13	70.5, CH	70.8, CH	71.0, CH
14	139.7, CH	139.9, CH	140.0, CH
15	111.2, CH	111.3, CH	111.2, CH
16	[b]	90.6, C	90.5, C
17	[b]	[b]	[b]
18	[b]	[b]	[b]
19	[b]	35.0, CH	[b]
20	18.0, CH_2	19.6, CH_2	[b]
21	16.8, CH	16.7, CH	16.5, CH
22	6.5, CH_3	6.7, CH_3	6.2, CH_3
23	12.4, CH_3[c]	12.8, CH_3	12.4, CH_3
24	12.4, CH_3[c]	12.6, CH_3	12.6, CH_3
OMe	55.4	55.3	55.1
1′	99.0, CH[d]	99.5, CH[d]	98.6, CH
2′	86.4, CH	87.0, CH	83.0, CH
3′	80.5, C	80.1, C	67.8, C
4′	70.4, CH	69.8, CH	55.4, CH
5′	67.0, CH	67.0, CH	64.4, CH
6′	18.2, CH_3	18.3, CH_3	17.3, CH
7′	19.4, CH_3	19.5, CH_3	23.5 [38.5][c] CH_3
8′			149.1, C
2′-OMe	58.8	59.3	55.3 [59.2][c]
1″	92.4, CH[e]	92.9, CH[e]	
2″	89.3, CH	84.6, CH	
3″	76.3, C	68.0, C	
4″	208.0, C	76.5, CH	
5″	68.2, CH	65.8, CH	
6″	14.7, CH_3	17.3, CH_3	
7″	23.3, CH_3	22.4, CH_3	
2″-OMe	59.2	59.4	

[a] δ's measured by indirect detection, HSQC and HMBC ($^1J_{CH}$ = 8 Hz). [b] Not detected. [c] Minor rotamer. [d] $^1J_{CH}$ = 167 Hz. [e] $^1J_{CH}$ = 178 Hz.

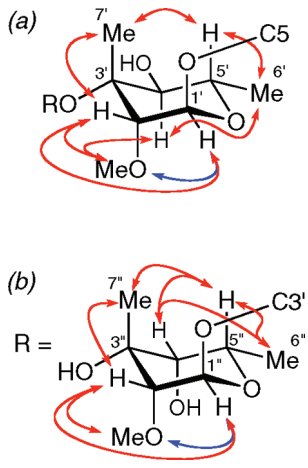

Figure 1. ROESY correlations in the sugar residues of **8**: (a) 6-deoxy-2,3-*C*,*O*-dimethyl-α-L-*manno*-pyranoside (first sugar unit); (b) 6-deoxy-2,3-*C*,*O*-dimethyl-α-L-*talo*-pyranoside (second sugar unit). Correlations marked with blue arrows are key to assignment of the anomeric configuration (see text).

ROESY cross-peak between each acetal proton (H-1′ or H-1″) and the adjacent *syn*-C-2′-OMe group. Similar ROEs were observed for phorbaside G (**7**) and phorbaside H (**8**, see Figure 1). Here, with the enhanced mass sensitivity of microcryoprobe NMR

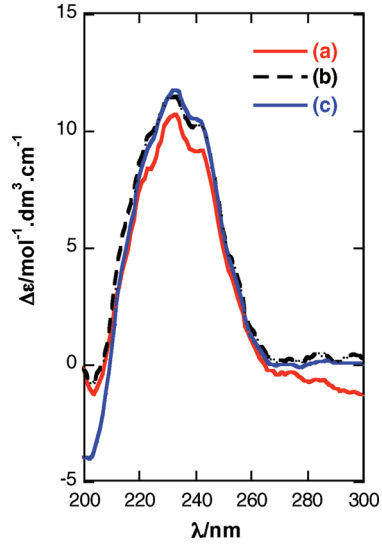

Figure 2. Circular dichroism (CD) spectra of phorbasides G−I (MeOH, 23 °C): (a) **7** ($c = 1.0 \times 10^{-4}$ M); (b) **8** ($c = 7.8 \times 10^{-5}$ M); (c) **9** ($c = 1.78 \times 10^{-4}$ M).

spectroscopy, we were able to provide independent evidence of the axial configuration of the C-1′ and C-1″ OR groups in **7** and in **8** the form of $^1J_{CH}$'s of the anomeric CH signals measured from ^{1}H coupled HSQC spectra (Table 2). The value of $^1J_{CH}$ of the anomeric CH in aldopyranoses is sensitive to the axial or equatorial disposition of the C-1 alkoxy substituent and falls in the ranges 169−171 and 158−162 Hz, respectively.[11] The measured values for C-1′ (J = 167 Hz) and C1″ (J = 178 Hz) for **7** and **8** are essentially the same and fall into the higher range, consistent with axial alkoxy substituents, O-C-5 and O-C-3′, respectively.

The absolute configurations of all the new compounds were readily assigned by comparisons with **3** and **4** after quantitation,[5] quantitative recovery from the 1.7 mm NMR tubes, and measurement of their CD spectra (Figure 2). The Cotton effects observed at λ 231 and 241 nm in **7**−**9** arise from $\pi-\pi^*$ transitions of the hyperconjugated cyclopropylenyne chromophore.[4] These Cotton effects are identical in sign and magnitude compared to those of **3** and **4** that we had earlier assigned[4] by correlation with synthetic models of known configuration, prepared from diastereomers of *O*-menthyl 2,2-dichlorocyclopropanecarboxylate.[12] Independently, the sugar configurations of **3** and **4** were assigned as L by methanolysis (HCl, MeOH), conversion of the resultant *O*-methylglycosides to bichromophoric derivatives, and CD comparison with synthetic standards prepared in several steps from L-rhamnose.[13] Since all phorbsides A−I were obtained from the same single specimen of *Phorbas* sp., it is highly likely that the sugars in **7**−**9** are also L.

Unlike phorbasides A−E, where differential cytotoxicities against the colon tumor cell line HCT116 were reported,[4b] the amounts of **6**−**9** available were insufficient to assess cytotoxic activity. Nanomole-scale natural products methods provide powerful tools for structure identification;[1,2] however fuller investigation of their properties is a challenge that is presently met only by synthesis and well-designed SAR studies. This was nicely illustrated recently by SAR studies of the antifungal hemiphorboxazole A,[6] a compound that was obtained in a yield of only 16.5 μg, but subsequently prepared in milligram quantities by directed synthesis.[14]

In conclusion, the structures of the new phorbasides G−I (**7**−**9**) were elucidated from vanishingly small samples (7−9.5 μg) using CD, MS, and NMR data obtained from a 600 MHz NMR spectrometer equipped with a 1.7 mm microcryoprobe. The outstanding quality of NMR data obtained from this instrument allowed full assignments and revealed differences in the glycone units. Phorbaside G (**7**) contained a novel C-4-oxidized pyranoside,

and **8** and **9** were variations on the *C*-methyl pyranoside sugars first reported by Zampella and co-workers in callipeltosides A (**10**), B (**11**), and C (**12**).

Experimental Section

General Experimental Procedures. UV−vis spectra were recorded on a JEOL V-630 dual-beam spectrometer in 0.2 or 1 cm quartz cells. CD spectra were recorded on a Jasco 810 spectropolarimeter in 0.2 cm quartz cells at 23 °C unless otherwise stated. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a 600 MHz spectrometer equipped with a Bruker 1.7 mm {^{13}C,^{15}N}^{1}H CPTCI microcryoprobe. NMR spectra were measured in $CDCl_3$ and referenced to residual solvent signals (^{1}H, δ 7.26 ppm; ^{13}C, δ 77.16 ppm). HRMS measurements were measured under ESI conditions with an Agilent 6000 series TOF mass spectrometer or a ThermoFinnigan Orbitrap mass spectrometer. Semi-preparative HPLC was carried out with a Varian high-capacity dual-pump system coupled to a Rainin UV-1 high-dynamic UV detector under specified conditions. All solvents for HPLC purification were redistilled from commercial HPLC grade solvent.

Extraction and Isolation. The sponge *Phorbas* sp. (sample ID: 93-054) was collected from Muiron Island, Western Australia, in 1993. A taxonomic description of the specimen has appeared elsewhere.[15] The sample was immediately frozen and stored at −20 °C until extraction (∼2 months). The CCl_4-soluble fraction (350 mg) of the MeOH extract of *Phorbas* sp.[3] was separated by flash chromatography (silica cartridge, Analogix RS-12, 12 g, 2 cm × 7.5 cm) using mixtures of *n*-hexane and EtOAc in increasing polarity (0−100%) to yield seven fractions. Fraction 5 (93-054-B1-5, 12.5 mg) was further purified twice by reversed-phase HPLC (phenylhexyl column, 250 × 10 mm, 90:10 MeOH/H_2O, 2 mL min^{-1}, then phenylhexyl column, 250 × 4.6 mm, 60:40 CH_3CN/H_2O, 1 mL min^{-1}) to yield pure phorbaside I (**9**), t_R = 9 min, 9.4 μg (3.9×10^{-6} % dry wt), phorbaside F[5] (**5**), t_R = 12 min, 8 μg (3.8×10^{-6} % dry wt), phorbaside G (**7**), t_R = 18 min, 9.5 μg (4.0×10^{-6} % dry wt), and phorbaside H (**8**) t_R = 19 min, 7.0 μg (2.9×10^{-6} % dry wt).

Phorbaside G (7): colorless film; UV (MeOH) λ_{max} (log ε) 233 nm (4.07), 241 (4.07); CD (MeOH) λ_{max} ($\Delta\varepsilon$), 231 nm (+11.07), 241 (+9.60); ^{1}H and ^{13}C NMR (see Tables 1 and 2); HRESITOFMS *m*/*z* 835.3631 $[M + Na]^+$ (calcd for $C_{41}H_{61}ClNaO_{14}$, 835.3642).

Phorbaside H (8): colorless film; UV (MeOH) λ_{max} (log ε) 233 nm (4.07), 241 (4.07); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 231 nm (+11.6), 241 (+10.3); ^{1}H and ^{13}C NMR (see Tables 1 and 2); HRESITOFMS *m*/*z* 837.3780 $[M + Na]^+$ (calcd for $C_{41}H_{63}ClNaO_{14}$, 837.3799).

Phorbaside I (9): colorless film; UV (MeOH) λ_{max} (log ε) 233 nm (4.07), 241 (4.07); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 231 nm (+11.4), 241 (+10.5); ^{1}H and ^{13}C NMR (see Table 1 and 2); HRESITOFMS *m*/*z* 690.3020 $[M + Na]^+$ (calcd for $C_{34}H_{50}ClNNaO_{10}$, 690.3021).

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Supporting Information Available: ^{1}H NMR and 2D NMR spectra of **7**, **8**, and **9**. These materials are available free of charge via the Internet at http://pubs.acs.org.

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