Oceanapiside, an Antifungal Bis-α,ω-amino Alcohol Glycoside from the Marine Sponge *Oceanapia phillipensis*

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The structure of oceanapiside, an antifungal α,ω-bis-aminohydroxylipid glycoside from the temperate marine sponge *Oceanapia* sp., was elucidated by a combination of 2D NMR, chemical degradation/correlation, and MALDI MS−MS spectrometry. Oceanapiside exhibits antifungal activity against *Candida glabrata* at 10 μg/mL (MIC).

The structures of sphingolipids from marine invertebrates share features of sphingolipids derived from plants and animals. In higher animals, the base is sphingosine (*erythro*-D-*E*-2-amino-4-octadecene-1,3-diol), while sphinganine (D-2-amino-octadecan-1,3-diol) is most common in plant sphingolipids.[1] A significant departure from this motif is found in the structures of dimeric amino alcohol bases from certain calcareous sponges. Leucettamols A (**1**) and B, from *Leucetta* sp.;[2] compound BRS1, from an unidentified sponge;[3] rhizochalin (**2**), from *Rhizochalina incrustata*,[4] and related compounds; coriacenins, from the Mediterranean *Clathrina coriacea*;[5] and rhapsamine, from the Antarctic *Leucetta leptorhapsis*,[6] are all aminolipids composed of symmetrical, or almost symmetrical, very long hydrocarbon chains (C_{28}−C_{30}) functionalized at both terminii as vicinal amino alcohols. We report here a new glycosidic amino alcohol lipid, oceanapiside (**3**) from a temperate-water, non-calcareous sponge, *Oceanapia phillipensis* Dendy, 1895 (Haplosclerida, Phloeodictyidae) collected in Southern Australia. Oceanapiside is a naturally occurring "bolaamphiphile"—a term coined[7] to describe amphiphilic lipids with polar functionality at both termini whose structures suggest a resemblance to the bola, a weapon fashioned from two leather balls tethered to the ends of a string. Oceanapiside exhibits significant antifungal activity against the pathogenic, Fluconazole-resistant yeast, *Candida glabrata*.

The *n*-BuOH-soluble portion of a MeOH extract of *Oceanapia phillipensis* was fractionated by reversed-phase (C_{18}) chromatography to afford a highly polar amino lipid, (−)-oceanapiside (**3**), $C_{34}H_{68}N_2O_9$ (HRFABMS (MH^+ 649.5003, Δ mmu = −1.3), which gave a positive ninhydrin test. Oceanapiside was insoluble in $CHCl_3$ and EtOAc, but soluble in MeOH and water. The UV spectrum of **3** showed no absorbance above λ 210 nm. Interestingly, the base peak in the ESIMS of **3** was the doubly charged ion at *m*/*z* 325 ($z = 2$) that corresponds to $[M + H_2]^{2+}$. Taken together with the positive ninhydrin reaction, this suggested a readily protonated diamine. The presence of a keto group was indicated by FT-IR (ν 1712 cm^{-1}, C=O stretch) and ^{13}C NMR spectra (δ 214.3, s). Although it was later confirmed that **3** contains two free primary amines, treatment of **3** with *t*-BOC carbonate (THF−MeOH−Et_3N) gave only a mono-*t*-BOC derivative **4** ($C_{39}H_{75}N_2O_{11}$, ESIMS, *m*/*z* 748, M^+), presumably because the second NH_2 group was hindered by the glycosyl group (see below). Oceanapiside gave no precipitate with $AgNO_3$ (absence of Cl^-), so we formulated **3** as the free base, but cannot exclude a salt with other anions.

Interpretation of the ^1H and ^{13}C NMR spectra of **3** in CD_3OD (Table 1) revealed one acetal group (δ 4.30, d, J = 7.6 Hz; δ_C 104.5 d), seven additional methines attached to oxygen (δ 80.1, 78.1, 77.7, 74.8, 73.1, 71.6), and a lipid chain (CH_2 envelope, δ 1.28, br s). Six of the ^{13}C signals were consistent with the presence of a sugar, which was identified as D-glucose as follows. Methanolysis of **3** (2M HCl in MeOH, 80 °C, 16 h) gave two fractions, separable by Si gel chromatography. The first fraction was a mixture of anomeric methyl glycosides ($C_7H_{14}O_6$, *m*/*z* 180, $MH^+ - Me$), identified as an α:β mixture of 1-*O*-methyl-D-glucopyranosides by high-performance TLC and comparison with an authentic sample (obtained by methylation of D-glucose, HCl, MeOH, 80 °C, 2 h). Glycoside **3** has the β-configuration at the anomeric carbon as shown by the coupling constant for the anomeric proton signal (δ 4.30, d, J = 7.6 Hz).[8] The second fraction eluted from the column with NH_3−MeOH−$CHCl_3$ and was identified as the novel polar aglycon **5** (FABMS; *m*/*z* 487.5, MH^+).

A downfield ^{13}C signal (δ 214.3, s) for a keto group and the glycopyranosyl ring accounted for all of the degrees of unsaturation required by the formula of **3**. The remainder of the formula was attributed to a linear aminolipid chain. Interpretation of COSY, gHMQC, and gHMBC data revealed the two terminal sequences of oceanapiside algycon as −CH(OH)CH(NH_2)CH_3 and −CH(O−)CH(NH_2)CH_2OH− and enabled assignment of the corresponding two sets of ^{13}C NMR signals: CH_2−O (δ 58.9, t, C-1), CH−N (57.2, d, C-2), CH−O (80.1, d, C-3) and CH_3 (16.0, q, C-28), CH−N (53.5, d, C-27), and CH−O groups (73.1, d, C-26). The two CH_2 groups (δ 2.43, t, J = 6.5 Hz, 4H; δ_C 43.4, t) α to the ketone carbonyl were clearly resolved and readily assigned by comparison with 3-heptanone (δ 2.37, t, J = 7 Hz; δ_C 44.9, t).[9] The methyl terminus (δ_H ~1.3, d, δ_C 16.0, q) was obscured in the ^1H NMR spectrum of **3** by the CH_2 envelope, but inferred from observation of the expected COSY and HMQC cross-peaks together with DEPT data, and conveniently revealed in the ^1H NMR spectrum ($CDCl_3$, Table 1) of the octaacetyl derivative **6** (δ 1.05, d, J = 6.7 Hz) or the *t*-BOC derivative **4** ($CDCl_3$, δ 1.14, d, J = 6.5 Hz).

Oceanapiside (**3**) gave an octaacetyl derivative, **6** (Ac_2O, pyridine, 16 h, 25 °C). The COSY spectrum (500 MHz) of **6** revealed downfield shifts (Δδ ~0.5−1 ppm) of carbinol ^1H signals due to acetylation of the OH groups, including both

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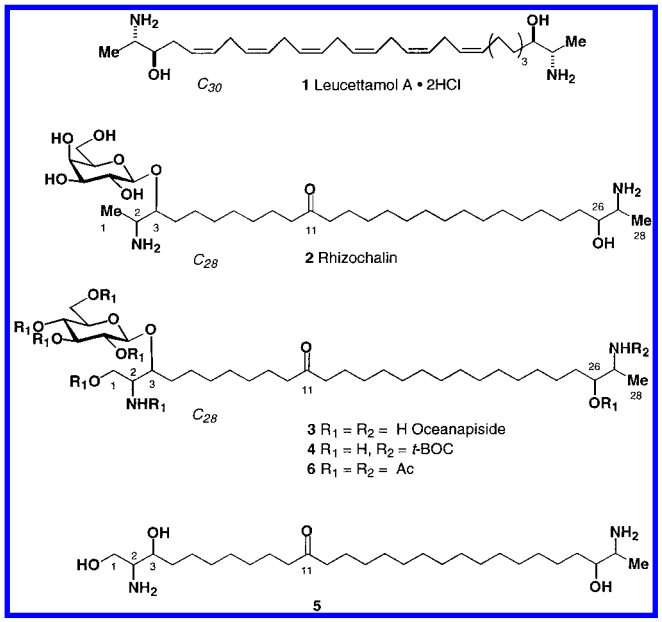
Table 1. ^{1}H, ^{13}C NMR Data for Oceanapiside (**3**) and the Octaacetyl Derivative **6**

	Oceanapiside **3** (CD_3OD, 500 MHz)				octaacetyl **6** ($CDCl_3$, 500 MHz)		
atom no.	δ_C	δ_H (mult, *J* Hz)	COSY	gHMBC[a]	δ_H (mult, *J* Hz)	COSY	ROESY
1a	58.9 (t)	3.86 (dd, 3.8, 11.8)		C2, C3	4.2 (m)		
1b		3.69 (dd, 9.2, 11.8)		C2, C3	4.08 (dd, 8, 11.5)	4.2	
2	57.2 (d)	3.35 (m)			4.2 (m)		
3	80.1 (d)	3.92 (m)	H4a, H4b	C2, C4, C5, C1′	3.65 (m)	1.58, 4.2	4.48, 6.43
4a	33.1 (t)	1.52 (m)	H3		1.58 (m)	3.65	
4b		1.70 (m)	H3	C3, C5, C6			
5	26.7 (t)	1.34 (m)					
6,7	30.5	~1.28 (br s)					
8	30.2 (t)	~1.28 (br s)					
9	24.8 (t)	1.51 (m)	H10	C10	1.55 (m)	2.36	
10	43.4 (t)	2.43 (t, 7)	H9	C9, C11	2.36 (m)	1.55	
11	214.3 (s)						
12	43.4 (t)	2.43 (t, 7)	H13	C13, C11	2.36 (m)	1.55	
13	24.8 (t)	1.51 (m)	H12	C12	1.55 (m)	2.36	
14	30.2 (t)	~1.28 (br s)					
15−23		~1.28 (br s)					
24	26.2	1.37 (m)					
25a	34.5 (t)	1.39 (m)	H26	C26, C27			
25b		1.56 (m)	H26	C26, C27			
26	73.1 (d)	3.45 (m)	H25a, H25b, H27	C24, C25, C27, C28	4.85 (m)	1.55, 4.20	1.05
27	53.5 (d)	3.10 (dq, 6.9, 6.9)	H28, H26	C25, C26, C28	4.2 (m)	4.85	
28	16.0 (q)	~1.25 (d)[b]	H27	C26, C27	1.05 (d, 6.7)	4.2	4.85
1′	104.5 (d)	4.30 (d, 7.6)	H2′	C3, C2′, C3′/C5′	4.48 (d, 8.0)	4.96	3.70, 5.20
2′	74.8 (d)	3.22 (dd, 3.6, 9.0)	H3′, H1′	C1′, C4′, C3′/C5′	4.96 (dd, 8.0, 9.5)	4.48, 5.20	
3′	77.7 (d)	3.35 (m)			5.20 (dd, 9.5, 9.5)		3.70, 4.48
4′	71.6 (d)	3.25 (m)		C2′, C3′/C5′, C6′	5.08 (dd, 9.5, 9.5)	3.70	
5′	78.1 (d)	3.35 (m)			3.70 (m)	4.2, 5.08	4.48, 5.20
6′a	62.6 (t)	3.63 (dd, 6.9, 11.6)	H5′, H6′	C4′, C3′/C5′	4.2		
6′b		3.95 (dd, 2.1, 11.6)	H5′, H6′	C4′, C3′/C5′	4.2		
2-NH					6.43 (d, 9.0)	4.2	3.65
27-NH					5.54 (d, 9.5)	4.2	
Ac					2.09, s; 2.07, s; 2.20, s; 2.01, s (× 2); 1.99, s; 1.97, s; 1.96, s		

[a] gHMBC recorded at 600 MHz. [b] Obscured by the CH_2 envelope.



primary OH groups (H_2-6′ and H_2-1), as well NH acetamide signals (δ 6.43, d, J = 9.0 Hz; 5.54, d, J = 9.5 Hz), which supported the presence of two primary NH_2 groups in **3**. COSY and RELAY experiments identified the two spin systems associated with NH acetamide proton signals at each chain terminus.

The location of the glucopyranosyl group in **3** was established by gHMBC. Severe overlap in the δ 3.0−4.0 region in the ^{1}H NMR spectrum of **3** (300 or 500 MHz) was relieved when spectra were recorded at 600 MHz (gHMQC, gHMBC). Clear assignments of the carbinol, CH−NH, and glycosidic ^{1}H signals were now made that allowed placement of the sugar group. The anomeric proton signal H-1′ (δ 4.30, d, J = 7.6 Hz) showed a correlation to δ 80.1 d, which, in turn, was assigned to C-3 from a combination of COSY and gHMBC data. In addition, the ROESY spectrum of **6** ($CDCl_3$) showed a strong dipolar correlation between the H-3 (δ 3.65, m) and H-1′ (δ 4.48, d, J = 8.0 Hz). Taken together, these data show the location of the D-glucopyranosyl group at C-3.

Initial attempts to assign the position of the C=O group in **3** by mass spectrometry were unsuccessful, as neither the ESIMS nor FABMS of **3** showed α or β cleavage fragment ions, while the EIMS fragmentation of **6** was dominated by multiple losses of ketene and CH_3CO. We recognized that the solution to this problem lay in soft-ionization, preferably with unambiguous charge-localization at the glucose group of the quasimolecular ion. Fortuitously, a sample of **3** left standing in CD_3OD (ca. 2 months, 4 °C) underwent adventitious deuterium exchange of the CH_2 hydrogens α to the carbonyl group. After reexchange of the labile OD and ND deuterons in the sample with CH_3OH, the ^{1}H NMR spectrum of **3**-d_4 showed a diminished ^{1}H signal for the α-CH_2 protons (δ 2.43, 2 × t)[10] and a single ^{2}H NMR (CH_3OH) signal (δ_D 2.41, br s) due the two α-CD_2 groups. The deuterium content of **3**-d_4 (74 atom % by MS − 49% d_4, and lesser amounts of other *d* isotopomers) was suitable for MS fragmentation studies. Matrix-assisted laser desorption ionization (MALDI) MS−MS of oligosaccharides in the presence of Li^+ salts has been shown to produce well-behaved fragmentation with defined charge-localization on sugar groups.[11] MALDI MS−MS of

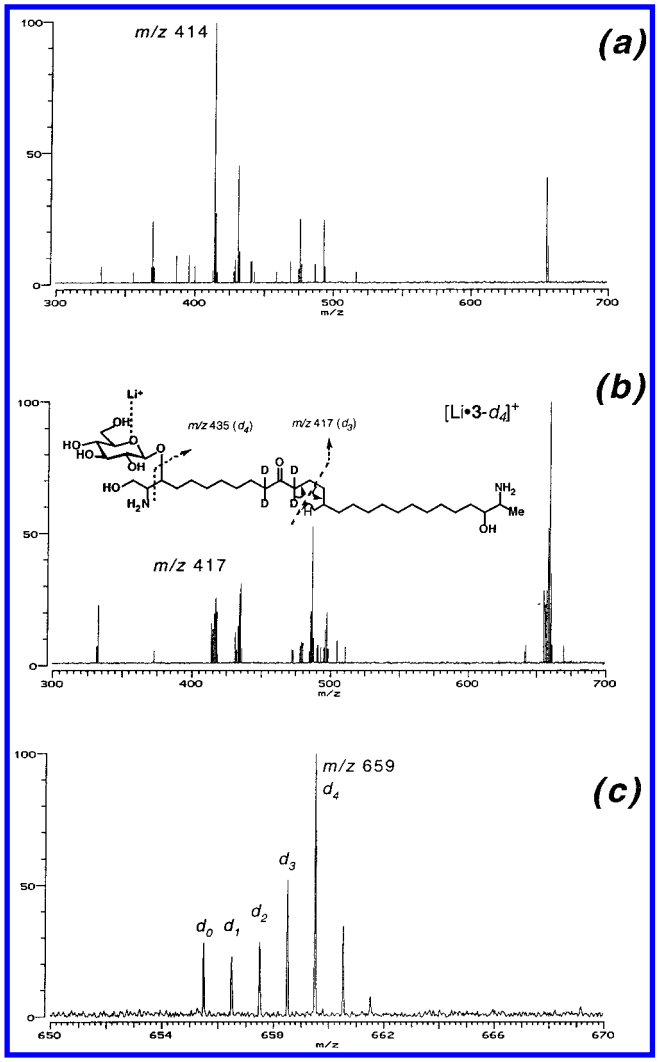


Figure 1. MALDI FT-MS spectra of **3**. (**a**) MALDI FT-MS spectra of **3** in the presence of Li^+. (b) MALDI FT-MS of **3**-d_4. (cd. 70 atom%) in the presence of Li^+. Laser pulse power was decreased with respect to (**a**) to retain parent ion [M + Li^+] intensity. (**c**) Expansion of M + Li^+ of **3**-d_4 parent ion showing d_0−d_4 isotopomer cluster.

3-d_4, (λ 347 nm laser irradiation) revealed the lithiated parent ion (*m*/*z* 659, [M + ^{7}Li]$^+$, d_4) and the fragment arising from charge-remote γ-cleavage two bonds from the C=O group (*m*/*z* 417, [M + ^{7}Li − $C_{15}H_{31}NO$]$^+$, d_3) only on the side of the carbonyl containing the glucopyranoside group.[11] Both peaks were clearly identified from observation of the expected deuterium and Li isotope patterns (Figure 1). Thus, the ketone carbonyl was located at C-11, closer to the more oxidized terminus, and the same relative position was found in **2**.

Oceanapiside (**3**) differs from rhizochalin (**2**) by the replacement of the 3-*O*-β-D-galactopyranosyl group in **2** with a 3-*O*-β-D-glucopyranoside. Presently, the stereochemistry of **3** is undefined due to the difficulty of independent assignment of the four stereogenic centers at dissimilar termini in this long-chain aminolipid.

Oceanapiside (**3**) showed antifungal activity against the Fluconazole-resistant yeast *Candida glabrata* with a minimum inhibitory concentration (MIC) of 10 μg/mL in broth dilution experiments. Under similar conditions, amphotericin B gave an MIC of ca. 1 μg/mL against *C. glabrata*, but **3** was essentially inactive against *C. krusei; C. albicans* ATCC 14503; and two Fluconazole-resistant strains, *C. albicans* 96-489 and *C. albicans* UCD-FR1. Interestingly, the aglycon **5** showed higher antifungal activity (MIC 3 μg/mL, *C. glabrata*) than the glycoside **3**, possibly due to better cell penetration.

Experimental Section

General Experimental Procedures. These procedures are described elsewhere.[12] FABMS measurements were performed at the University of California, Riverside Mass Spectrometry facility. MALDI FT-MS measurements were carried out on a custom-built instrument at the University of California, Davis.[13]

Animal Material. *Oceanapia phillipensis* Dendy, 1895 (90-12-095) was collected by hand using scuba at a depth of 30 m off Point Lonsdale in Port Phillip Bay, Victoria, Australia, in 1990, and stored at −20 °C until needed. The sponge sample has an encrusting flat base, mounted by fistula (40−70 mm in height), and terminating with apical oscula (ca. 10 mm diameter). The texture is very fibrous and spiculose. The ectoderm shows red pigmentation (3 mm penetration), while the interior is reddish brown. In life, the animal emits a pungent odor. The authoritative descriptive work on *Oceanapia* spp. from Port Phillip Bay is credited to Arthur Dendy.[14] Of the four *Oceanapia* species described by Dendy from the same location as our specimen, *O. mollis* and *O. imperfecta* are excluded by the absence of microsclera. The characteristic oxeas of 90-12-095 were slightly curved with a size range of 0.080−0.10 mm, entirely consistent with only one of the candidates, *Oceanapia phillipensis* (Dendy, 1895).[14,15] Type samples and spicule mounts are available from the corresponding author.

Extraction and Isolation. The lyophilized sponge (40.8 g) was repeatedly extracted with MeOH (× 3) and the combined MeOH extracts concentrated (~500 mL). The water content (% v/v) of the MeOH extract was adjusted before sequentially partitioning against *n*-hexane (10% H_2O), $CHCl_3$ (40%), and *n*-BuOH (100%). The *n*-BuOH extract was concentrated to give an orange solid (1.3 g), which was positive to ninhydrin. Further purification by reversed-phase flash chromatography on a solid-phase extraction cartridge (Varian, 20 g, C_{18} bonded-phase) with stepwise gradient elution using aqueous MeOH (40−100% MeOH: H_2O) afforded pure oceanapiside **3** (43.3 mg, 0.11% of dry wt). Early fractions from the column-cartridge gave a white-yellow precipitate upon addition of 1 drop of 0.1 M $AgNO_3$, (sea salt), but fractions containing pure **3** gave no precipitate. Oceanapiside (**3**) produced a distinct pink stain on TLC upon treatment with with ninhydrin reagent spray.

Oceanapiside (3): colorless solid; $C_{34}H_{68}N_2O_9$; $[\alpha]_D$ −5.5° (*c* 1.2, MeOH); no UV absorbance above 210 nm; IR (ZnSe, film), ν 3400−2900 br s, 1712, cm^{-1}; ^{1}H and ^{13}C NMR, Table 1; ESIMS *m*/*z* 649 (30%) [M + H]$^+$, 325 (100%) [M + H_2]$^{2+}$; HRFABMS *m*/*z* [M + H$^+$], 649.5016 (calcd for $C_{34}H_{69}N_2O_9$, 649.5003).

***N*-*t*-BOC Oceanapiside (4).** A solution of **3** in THF and MeOH (ca. 2 mL) was treated with freshly distilled Et_3N (50 μL) and *t*-BOC carbonate (ca. 50 mg) and allowed to stir for 2 h. Evaporation of the solvent and Si gel chromatography of the residue (0.7 × 6 cm, gradient, MeOH−$CHCl_3$) gave a mono-*t*-BOC derivative, **4**: ^{1}H NMR ($CDCl_3$) 1.14 (d, 3H, J = 6.5 Hz), 1.22 (br s), 1.41 (s, 9H), 2.35 (2 × t, 4H, J = 7 Hz), 3.3−4.3, (br m); $C_{39}H_{75}N_2O_{11}$, ESIMS *m*/*z* 748 [M]$^+$.

Methanolysis of Oceanapiside (3). A solution of **3** (11.7 mg) in 2M HCl in MeOH (1 mL) was heated in a sealed vial at 80 °C for 16 h. The mixture was cooled, concentrated, and subjected to chromatography on Si gel (0.7 × 6 cm) to provide two fractions.

For Fraction 1, elution of the column with 1:4 MeOH−$CHCl_3$ gave anomeric (+)-1-*O*-methyl glucopyranosides (2.7 mg); $[\alpha]_D$ +62.7° (*c* 0.15, MeOH), EIMS *m*/*z* 180 [M − MeOH]$^+$, CIMS *m*/*z* 212 (100%) [M + NH_4]$^+$, identical by ^{1}H NMR (300 MHz) and $[\alpha]_D$ with authentic (+)-1-*O*-methyl α/β-D-glucopyranosides prepared from D-glucose (see below). TLC: A 5 × 20 cm plate coated with silica GF, was sprayed with an aqueous solution of 0.1M NaH_2PO_4 and activated at 120 °C for 3 h prior to use. Adjacent lanes were spotted with either 1-*O*-methyl d-glu-

copyranosides (α:β mixture), or 1-*O*-methyl α-D-galactopyranoside (prepared from the D-glucose and D-galactose, respectively, 1M HCl in MeOH, 80 °C, 2 h) or each co-spotted together with the methanolysate of **3**. The plate was developed with 40:50:10 *n*-BuOH−Me_2CO−0.1 M NaH_2PO_4 aqueous and visualized with vanillin-H_2SO_4 (heat). The *O*-Me glycoside obtained from **3** coeluted with 1-*O*-methyl D-glucopyranoside (R_f, α-anomer, 0.55), ahead of 1-*O*-methyl D-galactopyranosides (R_f, α-anomer, 0.47).

For fraction 2, further elution of the column (12:18:1 MeOH−$CHCl_3$−NH_4OH aqueous) gave amino lipid (+)-**5**, $C_{28}H_{58}N_2O_4$, as a clear glass (4.8 mg): $[\alpha]_D$ +14.7°(*c* 0.3, MeOH); ^{1}H NMR (CD_3OD) 1.10 (d, 3H, J = 6.6 Hz), 1.28 (br s, CH_2 envelope), 1.53 (m, 8H), 2.43 (m, 4H), 2.74 (m, 1H), 2.80 (m, 1H), 3.48 (dd, 1H, J = 7.8, 10.5 Hz), 3.53 (m, 1H), 3.73 (dd, 1H, J = 4.2, 11.1 Hz) FABMS *m*/*z* 487.5 $[M + H]^+$.

Octaacetyl derivative, 6. A sample of **3** (11.7 mg) was dissolved in pyridine (0.5 mL) and acetic anhydride (0.5 mL) and allowed to stand at 25 °C for 18 h. Removal of the volatile material gave a residue (7.6 mg) that was purified by Si gel chromatography (0.7 × 5 cm, 2:98 MeOH−$CHCl_3$) to provide **6** (2.8 mg) as a clear glass; ^{1}H NMR ($CDCl_3$), see Table 1; EIMS *m*/*z* 925.5 (8%) $[M - OAc]^+$, 866.6 (43) $[M - 2OAc]^+$, 842.5 (28) $[M + H - 2CH_2{=}C{=}O]^+$, 782.5 (28), 578.5 (55), 518.4 (100).

Deuterium Content, ^{2}H NMR, and MALDI FT-MS of 3-*d*$_4$. A sample of **3** (ca. 25 mg) was stored in CD_3OD for ca. 2 months. The volatiles were removed under vacuum and the residue re-evaporated from CH_3OH (× 2) to obtain a sample of **3**-d_4. The presence of deuterium label in **3**-d_4 was detected by both ^{2}H NMR and MS. The ^{2}H NMR spectrum was recorded using a solution of **3**-d_4 in CH_3OH on a General Electric QE 300 using the lock channel in observe mode and referenced to the natural abundance of the ^{2}H solvent signal (CDH_2OH, δ_D 3.30), ^{2}H NMR (CH_3OH) δ 2.41 (br s). The isotopomer distribution of **3**-d_4 was measured from the MALDI FT-MS quasimolecular ion isotope-cluster in the presence of Na^+ $[M + Na]^+$ by Biemann's method[16] (74 atom %; *d*; 49% d_4, 25% d_3, 11% d_2, 4% d_1, 11% d_0). In separate experiments, samples of **3** or **3**-d_4 (ca 0.1 μg) were doped with LiCl (0.01 μmol) and dihydroxybenzoic acid (ca. 140 μg) and subjected to MALDI ionization on a custom-built FT-MS instrument.[13] The laser pulse energy was adjusted until appearance of daughter ions, *m*/*z* 414, $[M + {}^7Li\text{-}d_4]^+$ (100%), was observed. See Figure 1 for the fragmentation patterns.

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