Cytotoxic and Insecticidal Constituents of the Unripe Fruit of *Persea americana*

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Unripe avocado fruit (*Persea americana*) was subjected to a bioactivity-directed fractionation, as monitored via the brine shrimp lethality assay, to isolate three major bioactive constituents (**1**−**3**). Compounds **1**−**3** all have a 1,2,4-triol moiety and a long aliphatic chain that terminates as either an alkane, alkene, or alkyne, respectively; **1** is new, while **2** and **3** have been described previously but not as cytotoxic or pesticidal agents. All have activity against six human tumor cell lines in culture and show selectivity for human prostate adenocarcinoma (PC-3) cells with **3** being nearly as potent as adriamycin. Also, when tested against yellow fever mosquito larva, **3** was more effective than rotenone, a natural botanical insecticide and positive control.

Persea americana Miller (Lauraceae) is grown throughout the tropics of the world for the harvest of its edible fruit, the avocado. From the leaves, Ye et al.[1] isolated methyl-2-(1-hydroxy-2-oxo-propyl)-(*Z*)-2-ene hexadecanoate, which had moderate activity against three human tumor cell lines in vitro; and, from aqueous and methanolic extracts of the leaves, Wigg et al.[2] found promising anti-HIV-1 activity in vitro, although they did not report the responsible chemicals. In our continuing search for antineoplastic agents from higher plants, using the brine shrimp lethality assay (BST) to prescreen the extracts and direct the fractionations, we examined the unripe fruit of *P. americana* for its rumored anticancer properties. Herein, we report the isolation, structure elucidation, and biological activities of 1,2,4-trihydroxynonadecane (**1**), 1,2,4-trihydroxyheptadec-16-ene (**2**), and 1,2,4-trihydroxyheptadec-16-yne (**3**) (Figure 1); compound **1** is new, while **2** and **3** have been described previously as the bitter[3] antibacterial[4,5] and antifungal[6] constituents of *P. americana*. In the literature, however, Adikaram et al.[6] switched some of the key ^{1}H NMR assignments between **2** and **3**. This manuscript corrects these and, as well, describes the unanticipated discovery that **3** is quite selective against human prostate adenocarcinoma (PC-3) cells when examined against six human tumor cell lines in vitro; furthermore, **3** is more potent than the natural botanical insecticide, rotenone, when tested in the yellow fever mosquito larvae assay (YFM) against *Aedes aegypti*.

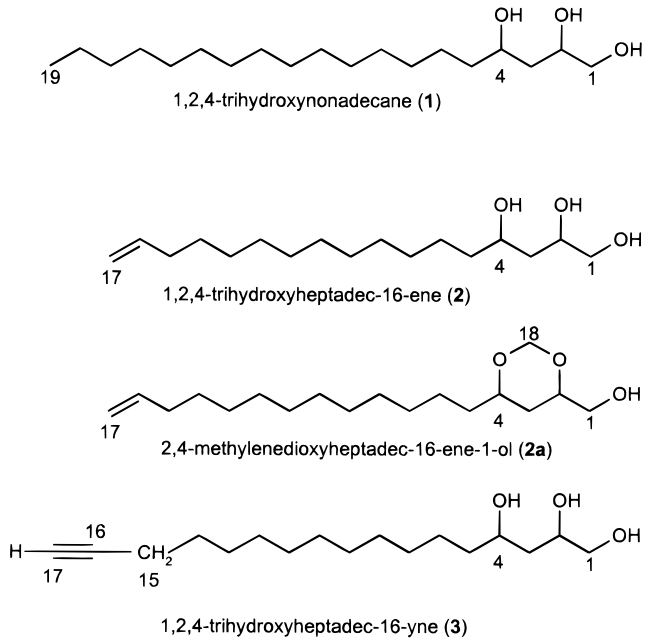

Figure 1. Structures of the compounds isolated from unripe fruit of *P. americana* and the formaldehyde-acetal derivative of **2**.

Results and Discussion

Activity-directed fractionation of the dried and pulverized unripe fruit of *P. americana* using BST and a combination of open column chromatography and HPLC (see Experimental) yielded compounds **1**−**3**. 1,2,4-Trihydroxynonadecane (**1**), isolated as a white powder, did not significantly absorb UV light with a λ_{max} of 200 nm. In the IR spectrum, four diagnostic peaks were found at about 3300 cm^{-1} for the OH stretching of an alcohol, 2918 and 2850 cm^{-1} for the respective asymmetric and symmetric stretching of methylene groups, and 1470 cm^{-1} for the scissoring vibrations of methylene groups. The protonated molecular ion of **1** (MH^+), as suggested by CIMS, was *m*/*z* 317 which, via HRCIMS, corresponded to an exact mass of 317.3062 and a formula of $C_{19}H_{40}O_3$+H (calcd 317.3056). Also prevalent in the CIMS were three subsequent losses of H_2O at *m*/*z* 299, 281, and 263, which, coupled with the molecular formula and the IR data, suggested the presence of three hydroxyl groups.

The ^{1}H NMR spectrum of **1** showed only three major regions: a methyl signal at δ 0.88, a large number of methylenes at δ 1.2 to 1.6, and several oxygenated moieties (hydroxyls) at δ 3.4 to 4.0. An integration of 40 total protons fitted well with the formula suggested

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Table 1. ^{1}H and ^{13}C NMR Data for 1,2,4-Trihydroxynonadecane (**1**)

position	^{1}H NMR (500 MHz) δ, multiplicity, (J in Hz)	^{13}C NMR (75 MHz) δ
1	3.49, dd, (11, 6.5)	66.78
1′	3.65, dd, (11, 3.6)	
2	3.98, m	72.62
3	1.59, m	38.96
4	3.91, m	72.50
5	1.49, m	38.31
6−17	1.44−1.24, m	31.91, 29.67, 29.58, 29.35, 25.30 28.95, 25.31
18	1.44−1.24, m	22.69
19	0.88, t, (6.5)	14.10

above. Due to its splitting (triplet) and upfield position, the methyl was assigned as being terminal to a methylene chain. In the oxygenated region, the two most downfield peaks did not show a distinct coupling pattern, and they integrated for one proton each. The two more upfield oxygenated peaks (δ 3.49 and 3.65), integrating for one proton each, had an interesting doublet of doublets pattern with a common *J* coupling of 11 Hz. The presence of one primary and two secondary alcohol moieties were suggested by the signals for three oxygenated carbons in the ^{13}C NMR spectrum at δ 66.78, 72.50, and 72.62.

COSY experiments were used to position the four hydroxylated proton signals. The primary alcohol gave two distinct signals (δ 3.49 and 3.65), due to the chirality at C-2, and each integrated for one proton (H-1 and H-1′). H-1 and H-1′ were coupled to each other and to the most downfield peak (δ 3.98, H-2), which integrated for one proton and, hence, suggested a vicinal diol. H-2 was also coupled to an upfield signal (δ 1.59, H-3); the other oxygenated proton (δ 3.91, H-4) was coupled to H-3 as well and suggested a diol relationship between H-2 and H-4 separated by one methylene unit. A 1,2,4-triol moiety was, thus, assigned to **1**. The complete ^{1}H and ^{13}C NMR assignments for **1** are shown in Table 1; HMQC was used to confirm the ^{13}C assignments.

The structural elucidations of **2** and **3** proceeded in a fashion similar to that of **1** except for the respective terminal alkene and alkyne moieties and a total of only 17 carbons for each molecule. In **2**, the most downfield ^{1}H NMR signal (δ 5.82, H-16) displayed an ABX coupling pattern (doublet of doublet of triplets, ddt) typical for a terminal double bond. *J* values of 17 and 10 Hz were observed for coupling to the respective trans and cis protons (H-17$_{trans}$ and H-17$_{cis}$) and 6.5 Hz for coupling to the allylic protons (H-15). H-17$_{trans}$ (δ 4.99) had a ddt pattern with *J* values of 17, 2, and 1.5 Hz for coupling to H-16, H-17$_{cis}$, and H-15; while H-17$_{cis}$ (δ 4.93), also with a ddt pattern, had *J* values of 10, 2, and 1.5 Hz for coupling to H-16, H-17$_{trans}$, and H-15. The signal for H-15 was then determined to be at δ 2.04 due its correlations in the COSY spectrum with H-16, H-17$_{trans}$, and H-17$_{cis}$. Alternatively, in **3**, an alkyne moiety was suggested by the distinct IR CH stretching band at 3281 cm^{-1}. In the ^{1}H and COSY NMR spectra of **3**, an alkyne was confirmed with a triplet (t) at δ 1.94 (H-17) coupled to the triplet of doublets (td) at δ 2.18 (H-15) with a *J* value of 2.5 Hz. The ^{13}C NMR data for the hydroxyl region of **3**, which had the typical 1,2,4-triol signals at δ 66.80, 72.66, and 72.46, respectively, had an additional signal at δ 68.03; HMQC data correlated the δ 68.03 (C-17) peak with δ 1.94 (H-17) via J_{CH} coupling of 245 Hz. In a similar fashion, C-16 (δ 84.82) had long-range coupling of 43 Hz to H-17 (δ 1.94), and C-15 (δ 18.39) correlated with H-15. The complete ^{1}H and ^{13}C NMR assignments for **2** and **3** are shown in Table 2. These data permitted the identification of **2** as 1,2,4-trihydroxyheptadec-16-ene and **3** as 1,2,4-trihydroxyheptadec-16-yne, both of which were previously described as the antifungal constituents from the immature fruit peel,[6] as the antibacterial constituents from the seeds,[4,5] and as the bitter components of the immature fruit.[3]

To clarify the relative configuration of positions C-2 and C-4 in **1**−**3**, compound **2** was derivatized to an intramolecular formaldehyde acetal (2,4-methylenedioxyheptadec-16-ene-1-ol, **2a**); the reaction was carried out in two stages and possibly proceeds through a Pummerer-type step. First described by Bal and Pinnick,[7] this procedure has been used successfully by Gu et al.,[8] Shi et al.,[9] and others to define the relative stereochemistries of the diol portions of several different Annonaceous acetogenins, and it has the advantage that the cyclic moiety of the product retains the original stereochemistries of the diols in the starting material. Gianni et al.[10] reported that the acetal protons in *cis*-4,7-dimethyl-1,3-dioxacycloheptane resonate as two well-separated doublets at δ 5.16 and 5.47; in the cis conformation, each proton is in a different magnetic environment. Alternatively, in *trans*-4,7-dimethyl-1,3-dioxacycloheptane, the acetal protons are a singlet at δ 5.30; in the trans conformation, each proton is in an almost identical magnetic environment. Hence, the relative configuration of the carbinol centers in a diol can be inferred from the ^{1}H NMR spectra of the acetal protons.

2,4-Methylenedioxyheptadec-16-ene-1-ol (**2a**) was synthesized from **2** as described in the Experimental Section. In the CIMS spectrum, **2a** had a protonated molecular ion (MH$^+$) at *m/z* 299, which, using HRCIMS, was determined to be *m/z* 299.2579 for $C_{18}H_{34}O_3$+H (calcd. 299.2586); when compared to the starting material, **2**, the reaction to form **2a** had added one carbon molecule (12 mass units) as anticipated.

The ^{1}H NMR spectrum for **2a** was similar to that of **2** except for the addition of two well-separated doublets at δ 4.74 and 5.12 (H-18 and H-18′). The COSY for **2a** illustrated that H-18 and H-18′ were coupled to each other with a geminal *J* of 6 Hz. In the ^{13}C NMR spectra, the acetal carbon resonated at δ 93.36 (C-18). The complete ^{1}H and ^{13}C NMR assignments for **2a** are given in Table 2. Most of the signals were very similar to those of **2**, with the exception of the positions associated with the acetal ring. C-2 and C-4 had shifted further downfield than C-1, relative to their respective positions in **2**; DEPT was used to assign C-1 as it has two protons attached as opposed to only one on either C-2 or C-4. The relative stereochemistry between positions C-2 and C-4 was, thus, assigned as cis in **2a** because H-18 and H-18′ resonate as two well-separated doublets.

Kashman et al.[11] studied the relative stereochemistry of positions C-2 and C-4 in an analogue of **2**, 1-acetoxy-2,4-dihydroxyheptadecane. By linking C-2 and C-4 with an acetonide and examining the resulting coupling

Table 2. ^{1}H (500 MHz) and ^{13}C NMR (75 MHz) Data for 1,2,4-Trihydroxyheptadec-16-ene (**2**), 2,4-Methylenedioxyheptadec-16-ene-1-ol (**2a**), and 1,2,4-Trihydroxyheptadec-16-yne (**3**)

position	^{1}H NMR for **2** δ, multiplicity, (J in Hz)	^{13}C NMR for **2** δ	^{1}H NMR for **2a** δ, multiplicity, (J in Hz)	^{13}C NMR for **2a** δ	^{1}H NMR for **3** δ, multiplicity, (J in Hz)	^{13}C NMR for **3** d
1	3.49, dd, (11, 6.5)	66.82	3.58, m	65.76	3.49, dd, (11, 6.5)	66.80
1′	3.65, dd, (11, 3)		3.65, dd, (11,3)		3.65, dd, (11, 3)	
2	3.98, m	72.62	3.74, m	76.70	3.98, m	72.66
3	1.58, m	38.99	1.46, m	33.05	1.57, m	38.94
4	3.91, m	72.55	3.58, m	76.22	3.91, m	72.46
5	1.48, m	38.34	1.59, m	35.98	1.42, m	38.28
6−14	1.44−1.26, m	29.59, 29.50, 29.16, 28.95, 25.31	1.22−1.42, m	29.54, 29.49, 29.14, 28.94, 24.89	1.26−1.43, m	29.55, 29.48, 29.09, 28.48, 25.31
15	2.04, m	33.83	2.04, m	33.81	2.18, td, (7, 2.5)	18.39
16	5.82, ddt, (17, 10, 6.5)	139.27	5.82, ddt, (17, 11, 7)	139.26		84.82
17_{cis}	4.93, ddt, (10, 2, 1.5)	114.09	4.93, ddt, (11, 3, 2)	114.07	1.94, t, (2.5)	68.03
17_{trans}	4.99, ddt, (17, 2, 1.5)		4.99, ddt, (17, 2, 1.5)			
18			4.74, d, (6)	93.36		
18′			5.12, d, (6)			

Table 3. Cytotoxic Activities of F005 (Unripe Fruit) and **1**−**3**

	A-549[a]	MCF-7[b]	HT-29[c]	A-498[d]	PC-3[e]	PaCa-2[f]
	(IC_{50} value in μg/mL)					
F005	>10	>10	>10	6.9	3.1	>10
1	3.0	3.2	3.0	2.7	1.2	>10
2	3.4	4.4	2.6	3.6	4.6×10^{-1}	5.3
3	4.8	6.5	8.9	4.1	6.1×10^{-2}	>10
adriamycin	2.2×10^{-3}	8.1×10^{-2}	5.3×10^{-3}	4.9×10^{-3}	1.1×10^{-2}	2.4×10^{-3}

[a] Human lung carcinoma (A-549).[20] [b] Human mammary adenocarcinoma (MCF-7).[21] [c] Human colon adenocarcinoma (HT-29).[22] [d] Human kidney carcinoma (A-498).[20] [e] Human pancreatic carcinoma (PaCa-2)[23]. [f] Human prostate adenocarcinoma (PC-3).[24]

constants, they also proposed a cis relationship. Sugiyama et al.[12] later synthesized all four possible stereoisomers of **2** and, by comparing the physical data with those of the natural compound, suggested the absolute stereochemistry as 2*R*, 4*R*. These data, then, confirm the utility of the formaldehyde acetal reaction to determine quickly the relative stereochemistry among diols. Either a singlet, integrating for two protons, or two well-separated doublets can be easily deduced from the ^{1}H NMR spectra. With acetogenins, formaldehyde acetals have been used to assign relative stereochemistries where the separation between the diols is 0−3 methylene units.[9]

The 95% ethanol extract of the unripe fruit of *P. americana* was quite potent in the BST (LC_{50} value of 31 μg/mL); and the BST was used at all phases of the purification process in order to prioritize the fractions. All the pure compounds isolated (**1**−**3**) had moderate to good activity against six human tumor cell lines in culture (Table 3); and they all showed some selectivity against the human prostate carcinoma (PC-3) cells. In the routine testing of F005 prior to large-scale column chromatography, some selectivity against the PC-3 cells was noted (Table 3) and served to drive this project. When the pure compounds were later examined in cell culture, more potent activity was observed, and the preferential cytotoxicity for the PC-3 cells was retained. The structure−activity relationships among **1**−**3** suggest that the terminal alkyne (**3**) is more potent than the terminal alkene (**2**), which is more potent than the longer terminal alkane (**1**). The experiment was repeated and yielded virtually identical results (data not shown). Also, in both the YFM and the BST, which are indicative of insecticidal activity, the same held structure−activity relationship with **3** being more potent than the natural botanical insecticide, rotenone (Table 4). The terminal acetylene group (alkyne) is known to impart fungicidal[13] and insecticidal[14,15] properties in several natural product compounds. As the fruit ripens, the activity of the extract decreases tenfold, presumably due to degradation of these active constituents[16,17] (Table 4).

Table 4. Activities of F005 and **1**−**3** in the YFM and the BST

	YFM	BST
	LC_{50} values in μg/mL (95% confidence intervals)	
F005 (unripe fruit)	not tested	11.1 (7/18)
F005 (ripe fruit)	not tested	111 (68/181)
1	1.8 (0.4/9)	2.8 (1/7)
2	2.1×10^{-1} (0.03/0.9)	1.3 (0.5/3)
3	7.7×10^{-2} (0.005/0.4)	6.4×10^{-1} (0.3/1)
rotenone	7.5×10^{-1} (0.2/3)	not tested

Experimental Section

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 300s and Varian VXR-500s spectrometers in $CDCl_3$ with TMS as an internal standard. LRCIMS and LREIMS were recorded on a Finnigan 4000 quadrupole mass spectrometer. HRCIMS were determined on a Kratos MS-50. IR spectra were recorded using KBr pellets with a Nicolet-Magna 550 spectrometer. Melting points were measured in capillaries on a Mel-temp apparatus and were uncorrected. Ranin instruments equipped with the Dynamax software system and a Ranin UV-1 detector set at 205 nm were used in the reversed phase with a C-18 column (250 × 21 mm, 8 μm, 60e).

Plant Material. Both ripe and unripe fruit of *P. americana* (Lulu variety) were collected by Mr. J. Crafton Clift on the avocado plantation of Dr. Eric Cohen, 6701 S. W. 125 Ave., Miami, FL 33183. The seeds were removed, and the fruits were cut into small pieces and dried in an oven at 100 °F for 7 days. The

dried fruit pieces were ground into a powder with a model 4 Wiley mill using a 2-mm screen; 2.7 kg of unripe and 2.1 kg of ripe fruit were stored separately in airtight glass jars.

Evaluation of Bioactivity. The brine shrimp (*Artemia salina*) lethality assay, as described in the literature,[18,19] was used at all steps of the fractionation process. Cytotoxicity tests against six human tumor cell lines in culture were performed by Vicki Croy of the Purdue Cell Culture Laboratory, Purdue Cancer Center. The assay used MTT to visualize the amount of cell growth inhibition against the cancer cells after a six-day exposure to the test compounds. The cells used were lung carcinoma (A-549),[20] mammary adenocarcinoma (MCF-7),[21] colon adenocarcinoma (HT-29),[22] kidney carcinoma (A-498),[20] pancreatic carcinoma (PaCa-2),[23] and prostate adenocarcinoma (PC-3).[24] Adriamycin was used as a positive control. Typically, IC_{50} values of less than 4 μg/mL are considered significantly active.

The YFM was used to determine the relative insecticidal activity of the pure compounds isolated from *P. americana*. Following the protocol of He et al.,[25] eggs of *A. aegypti*, provided by Dr. Steve Sackett (New Orleans Mosquito Control Board, New Orleans, LA 70126), were hatched in H_2O overnight. The larvae were then transferred to an aqueous solution of bovine liver powder (4 mg/mL, ICN Biochemicals) and allowed to grow for 4 days. Larvae were delivered to a 96-well microtiter plate containing 240 μL of 5 mM MES (2-{*N*-morpholino}ethanesulfonic acid). One larva and serial dilutions of test compound were added to each of eight wells per dose; rotenone was used as a positive control. The plates were incubated at room temperature in the dark for four more days; then, the numbers of live vs dead larvae were scored at each dose, and LC_{50} values were calculated as described above for the BST.

Extraction and Isolation. The dried and powdered unripe fruit of *P. americana* (945 g) was percolated with 95% EtOH until the eluent (F001) had only a pale-yellow color. The residue of F001 (240 g) was then partitioned between H_2O (F002) and CH_2Cl_2, and the residue of the CH_2Cl_2 extract (F003, 91 g) was fractionated between 10% aqueous MeOH (F005) and hexane (F006). Bioactivity was concentrated in fractions F001, F003, and F005, as monitored with the BST (Table 4). F005 (29.9 g) was loaded onto a Si gel column (160 g of 60−200 mesh silica) where a gradient of 100% hexane to 100% EtOAc to 25% MeOH was run. Fractions were combined based on similar TLC patterns and tested in the BST. In total, 55 pools were collected, which accounted for 95% of the weight loaded onto the column. Pools 25 to 31 were the most bioactive based on the BST; hence, they were combined (3.2 g) and loaded onto a second column (140 g of 60−200 mesh Si gel) that was developed with a gradient of 100% CH_2Cl_2 to 100% Me_2CO to 35% MeOH. Fractions were once again pooled based on TLC and tested with the BST to give 34 pools (A through HH), which recovered 88% of the weight loaded onto the column. Using HPLC in the reversed-phase with an MeCN−H_2O solvent system, compounds **1**−**3** from the bioactive fractions of column II were resolved. A portion (270 g) of dried and powdered ripe fruits were extracted and partitioned in a fashion similar to that of the unripe fruit to produce F005 (ripe fruit).

Intramolecular Conversion of 1,2,4-Trihydroxyheptadec-16-ene (2) to 2,4-Methylenedioxyheptadec-16-ene-1-ol (2a). All reagents were purchased from Mallinckrodt, except for chlorotrimethylsilane (TMS-Cl, Aldrich). First, a 1.2:1 molar ratio of dimethyl sulfoxide (DMSO)−TMS-Cl was mixed in 2 mL of benzene and placed in a capped 20-mL scintillation vial without stirring in a freezer (−15 °C) overnight. The benzene was decanted, and the white crystalline precipitate was washed with cold $CHCl_3$, and then approximately 5 mg was added to a solution of 8−12 mg of **2** in a minimum amount of $CHCl_3$ in a 20-mL scintillation vial. The vial was capped, and the reaction was stirred at room temperature and monitored by TLC. The reactant was driven to the final product (**2a**) by periodically (every 5−6 h) adding another 5 mg of the washed precipitate and was typically complete after 2 days with about a 30% yield.

1,2,4-Trihydroxynonadecane (**1**)**:** white powdery solid; 10.2 mg; yield 0.00108%; mp 79−81 °C; UV λ_{max} 200 nm; IR ν_{max} (KBr) cm^{-1}: 3312, 3300, 2918, 2850, 1470; CIMS (isobutane) *m/z* (%) $[MH]^+$ 317 (9), $[MH - H_2O]^+$ 299 (100), $[MH - 2H_2O]^+$ 281 (35), $[MH - 3H_2O]^+$ 263 (11); HRCIMS (isobutane) *m/z* 317.3062 for $C_{19}H_{41}O_3$ $[MH]^+$ (317.3056); see Table 1 for the ^1H NMR and ^{13}C NMR data.

1,2,4-Trihydroxyheptadec-16-ene (**2**)**:** white powdery solid; 58.5 mg; yield 0.00619%; mp 67−69 °C; UV λ_{max} 202 nm; IR ν_{max} (KBr) cm^{-1}: 3320, 3297, 2920, 2850; CIMS (isobutane) *m/z* (%) $[MH]^+$ 287 (25), $[MH - H_2O]^+$ 269 (100), $[MH - 2H_2O]^+$ 251 (65), $[MH - 3H_2O]^+$ 233 (50); HRCIMS (isobutane) *m/z* 287.2577 for $C_{17}H_{35}O_3$ $[MH]^+$ (287.2586); see Table 2 for the ^1H NMR and ^{13}C NMR data.

2,4-Methylenedioxyheptadec-16-ene-1-ol (**2a**)**:** white solid; CIMS (isobutane) *m/z* (%) $[MH]^+$ 299 (5), $[MH - H_2O]^+$ 281 (20), $[MH - 2H_2O]^+$ 263 (10), $[MH - 3H_2O]^+$ 245 (7), $[MH - CH_2O]^+$ 269 (35), $[MH - CH_2O - H_2O]^+$ 251 (20), $[MH - CH_2O - 2H_2O]^+$ 233 (51); HRCIMS (isobutane) *m/z* 299.2579 for $C_{18}H_{35}O_3$ $[MH]^+$ (299.2586), *m/z* 245.2274 for $C_{18}H_{29}$ $[MH - 3H_2O]^+$ (245.2269); see Table 2 for the ^1H NMR and ^{13}C NMR data.

1,2,4-Trihydroxyheptadec-16-yne (**3**)**:** white powdery solid; 37.9 mg; yield 0.00401%; mp 74−76 °C; UV λ_{max} 203 nm; IR ν_{max} (KBr) cm^{-1}: 3434, 3384, 3281, 2917, 2849, 1467; CIMS (isobutane) *m/z* (%) $[MH]^+$ 285 (18), $[MH - H_2O]^+$ 267 (55), $[MH - 2H_2O]^+$ 249 (73), $[MH - 3H_2O]^+$ 231 (40); HRCIMS (isobutane) *m/z* 285.2435 for $C_{17}H_{33}O_3$ $[MH]^+$ (285.2430); see Table 2 for the ^1H NMR and ^{13}C NMR data.

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