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New *ent*-kauranes from the fruits of *Annona glabra* and their inhibitory nitric oxide production in LPS-stimulated RAW264.7 macrophages

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ABSTRACT

Three new *ent*-kaurane diterpenoids, 7β,16α,17-trihydroxy-*ent*-kauran-19-oic acid (**1**), 7β,17-dihydroxy-16α-*ent*-kauran-19-oic acid 19-*O*-β-D-glucopyranoside ester (**2**), 7β,17-dihydroxy-*ent*-kaur-15-en-19-oic acid 19-*O*-β-D-glucopyranoside ester (**3**) along with five known compounds, paniculoside IV (**4**), 16α,17-dihydroxy-*ent*-kaurane (**5**), 16β,17-dihydroxy-*ent*-kaurane (**6**), 16β,17-dihydroxy-*ent*-kauran-19-al (**7**), and 16β,17-dihydroxy-*ent*-kauran-19-oic acid (**8**) were isolated from the fruits of *Annona glabra*. Their chemical structures were elucidated by physical and chemical methods. All compounds were evaluated for inhibitory activity against nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages. As the results, compound **3** showed potent inhibitory LPS-stimulated NO production in RAW 264.7 macrophages with the IC_{50} value of 0.01 ± 0.01 μM; compounds **1** and **7** showed significant inhibitory NO production with the IC_{50} values of 0.39 ± 0.12 μM and 0.32 ± 0.04 μM, respectively.

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Nitric oxide (NO) produced in large amounts by inducible nitric oxide synthase (iNOS) is known to be responsible for the vasodilation and hypotension observed during septic shock and inflammation.[1] The production of NO confers cytostatic or cytotoxic activity on macrophages against microbes and tumor cells.[2] NO mediates a variety of biological actions ranging from vasodilatation, neurotransmission, inhibition of platelet adherence and aggregation, as well as the macrophage and neutrophil-mediated killing of pathogens.[3] Although previous studies have demonstrated that cytokine-induced NO production can lead to eliminate parasites or tumor cells, NO has also been associated to damage of normal cells. Overproduction of NO causes excessive inflammatory response harmful to body in the inflammatory diseases.[4–6] They are secreted during the early phase of acute and chronic inflammatory diseases such as asthma, rheumatoid arthritis, septic shock, and other allergic diseases, and during the activation of T cells.[7]

Annona glabra L., belongs to Annonaceae family is a tropical tree growing wild in the America and Asia. It is used in traditional medicine to treat several diseases such as inflammation, and cancer. Phytochemical investigation led to the isolation of numerous acetogenins,[8] *ent*-kauranes,[9–12] peptides,[13] and alkaloids.[14,15] In addition, compounds exhibited anticancer,[12,16] anti HIV-reserve transcriptase,[11] and anti-malarial activities.[14] In the present study, three new along with five known *ent*-kaurane diterpenoids were isolated from the fruits of *A. glabra*. All compounds were evaluated for inhibitory activity against NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

The fruits of *A. glabra* were collected in Ho Chi Minh city, Vietnam, in May 2013, and authenticated by Dr. Bui Van Thanh, Institute of Ecology and Biological Resources, VAST, Vietnam. A voucher specimen (AG1605) was deposited at the Herbarium of Institute of Marine Biochemistry, VAST, Vietnam. The dried fruits of *A. glabra* (4.0 kg) were extracted with MeOH (3 × 5 L, 50 °C) under sonication for 1 h to yield 300.0 g extract after evaporation of the solvent. This extract was suspended in H_2O (2.0 L) and successively partitioned with *n*-hexane, $CHCl_3$, and ethyl acetate (EtOAc) to obtain the *n*-hexane (AG1, 51.0 g), $CHCl_3$ (AG2,

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190.5 g), EtOAc (AG3, 3.5 g), and H_2O (AG4, 54.0 g) extracts after removal of the solvents in vacuo. Using various chromatographic resin and isolation techniques, eight *ent*-kaurane-type diterpenoids were isolated.[17]

Compound **1**[18] was obtained as a white amorphous powder. The HR-ESI-MS analysis revealed the molecular formula to be $C_{20}H_{32}O_5$, with a pseudo ion peak at *m*/*z* 375.2159 $[M+Na]^+$ (Calcd for $C_{20}H_{32}O_5Na$: 375.2142). The ^1H NMR spectrum of **1** showed the signals for protons of two tertiary methyl groups at δ_H 1.00 (3H, s) and 1.18 (3H, s), one oxymethine at δ_H 3.63 (br s), and one oxymethylene group at δ_H 3.62 (d, *J* = 11.5 Hz) and 3.72 (d, *J* = 11.5 Hz). The ^{13}C NMR and DEPT spectra of **1** revealed signals for 20 carbons including one carboxylic, four quaternary (including an oxygenated carbon at δ_C 82.9), four methine, and nine methylene, and two methyl carbons (Table 1). A comparison of the ^{13}C NMR data of **1** to those of related *ent*-kaurane, 16α,17-dihydroxy-*ent*-kauran-19-oic acid[19] suggested that compound **1** possessed the same *ent*-kaurane skeleton. The HMBC correlations between H-18 (δ_H 1.18) and C-3 (δ_C 39.2)/C-4 (δ_C 44.2)/C-5 (δ_C 48.1)/C-19 (δ_C 182.0) confirmed the positions of both methyl and carboxylic groups at C-4 (Fig. 2). The configuration of the methyl group at C-4 was determined to be β by the observation of NOESY correlation between H-18 (δ_H 1.18) and H-5 (δ_H 1.77). The HMBC correlations from H-13 (δ_H 2.08)/H-15 (δ_H 1.56 and 1.74) to C-16 (δ_C 82.9)/C-17 (δ_C 66.7) confirmed two hydroxyl groups at C-16 and C-17. In addition, the α configuration of hydroxyl group at C-16 was confirmed by comparing the chemical shifts of C-13 (δ_C 46.1), C-16 (δ_C 82.9), and C-17 (δ_C 66.7) in **1** with those of the corresponding data of the 16β,17-dihydroxy-*ent*-kauran-19-oic acid[20] [δ_C 41.7 (C-13), 79.8 (C-16), and 70.5 (C-17)] and 16α,17-dihydroxy-*ent*-kauran-19-oic acid[20] [δ_C 45.9 (C-13), 81.7 (C-16), and 66.5 (C-17)]. The position of the hydroxyl group at C-7 was determined by the HMBC correlations from H-5 (δ_H 1.77)/H-6 (δ_H 1.98 and 2.11)/H-15 (δ_H 1.56 and 1.74) to C-7 (δ_C 78.1). Moreover, the NOESY correlations of H-7 (δ_H 3.63)/H-14 (δ_H 1.70) and broad singlet signal of H-7, proved the configuration of H-7 to be α (*equatorial* orientation). Consequently, compound **1** was elucidated to be 7β,16α,17-trihydroxy-*ent*-kauran-19-oic acid. The structure of **1** was previously assumed in the barley grain extract by Paul Gaskin *el al.*[21] using GC–MS analysis. However, the isolation of pure compound and clear elucidation its chemical structure have not been reported to date.

Compound **2**[22] was also obtained as a white amorphous powder. The ^1H NMR spectrum of **2** showed the following signals: two methyl groups at δ_H 0.99 (3H, s) and 1.22 (3H, s), assigned to the *ent*-kaurane aglycone; one anomeric proton at δ_H 5.42 (1H, d, *J* = 8.0 Hz), suggested the presence of a sugar moiety. The ^{13}C NMR and DEPT spectra revealed the presence of 26 carbons: one carboxyl, three quaternary, ten methine (including six oxymethine), ten methylene (including two oxymethylene), and two

Table 1
The ^1H and ^{13}C NMR data for compounds **1**–**3**

Compd	1		2		3	
	δ_C[a,b]	δ_H[a,c] (mult., Hz)	δ_C[a,b]	δ_H[a,c] (mult., Hz)	δ_C[a,b]	δ_H[a,c] (mult., Hz)
1	41.7	0.93 (m) 1.87 (m)	41.8	0.94 (m) 1.88 (m)	41.7	1.03 (dd, 3.5, 13.5) 1.87 (d, 13.5)
2	20.3	1.44 (m) 1.96 (m)	20.2	1.45 (m) 1.94 (m)	20.2	1.44 (dt, 5.0, 10.0) 1.96 (m)
3	39.2	1.08 (dd, 4.5, 13.5) 2.17 (d, 13.5)	39.1	1.13 (m) 2.22 (m)	39.1	1.12 (dd, 4.0, 13.5) 2.22 (m)
4	44.2	-	44.7	-	44.7	-
5	48.1	1.77 (d, 9.0)	49.5	1.78 (d, 13.0)	48.3	1.78 (m)
6	30.5	1.98 (m) 2.11 (m)	30.7	1.97 (m) 2.18 (dd, 13.0, 14.5)	29.3	1.96 (m) 2.23 (m)
7	78.1	3.63 (br s)	78.7	3.50 (br s)	75.6	3.59 (br s)
8	49.0	—	49.8	—	54.3	—
9	51.1	1.43 (d, 7.5)	50.6	1.43 (m)	43.6	1.39 (d, 7.5)
10	40.4	—	40.5	—	40.9	—
11	19.1	1.57 (m) 1.64 (m)	19.5	1.57 (m) 1.63 (m)	19.7	1.58 (m) 1.64 (m)
12	27.6	1.57 (m) 1.68 (m)	33.0	1.43 (m) 1.63 (m)	26.3	1.52 (m)
13	46.1	2.08 (m)	39.5	2.11 (m)	42.2	2.57 (m)
14	37.5	1.70 (dd, 4.0, 12.0) 1.83 (d, 12.0)	37.2	1.08 (m) 1.80 (d, 11.5)	43.5	1.42 (dd, 7.5, 10.5) 2.06 (d, 10.5)
15	50.1	1.56 (d, 13.5) 1.74 (d, 13.5)	42.6	1.12 (m) 1.71 (dd, 3.5, 10.0)	132.1	5.81 (s)
16	82.9	—	44.7	1.94 (m)	148.1	-
17	66.7	3.62 (d, 11.5) 3.72 (d, 11.5)	67.7	3.35 (m)	61.2	4.13 (d, 1.0)
18	29.3	1.18 (s)	28.8	1.22 (s)	28.8	1.22 (s)
19	182.0	—	178.7	—	178.6	—
20	16.1	1.00 (s)	16.3	0.99 (s)	16.1	1.02 (s)
19-O-Glc						
1′			95.7	5.42 (d, 8.0)	96.6	5.42 (d, 7.5)
2′			74.1	3.38 (m)	74.1	3.38 (m)
3′			78.7	3.45 (m)	78.7	3.42 (m)
4′			71.1	3.39 (m)	71.1	3.40 (m)
5′			78.6	3.39 (m)	78.7	3.39 (m)
6′			62.4	3.71(dd, 4.0, 11.5) 3.86 (d, 11.5)	62.4	3.71 (dd, 4.0, 12.0) 3.85 (dd, 2.0, 12.0)

Assignments were done by HMQC, HMBC, COSY, and NOESY experiments.
[a] Measured in CD_3OD.
[b] 125 MHz.
[c] 500 MHz.

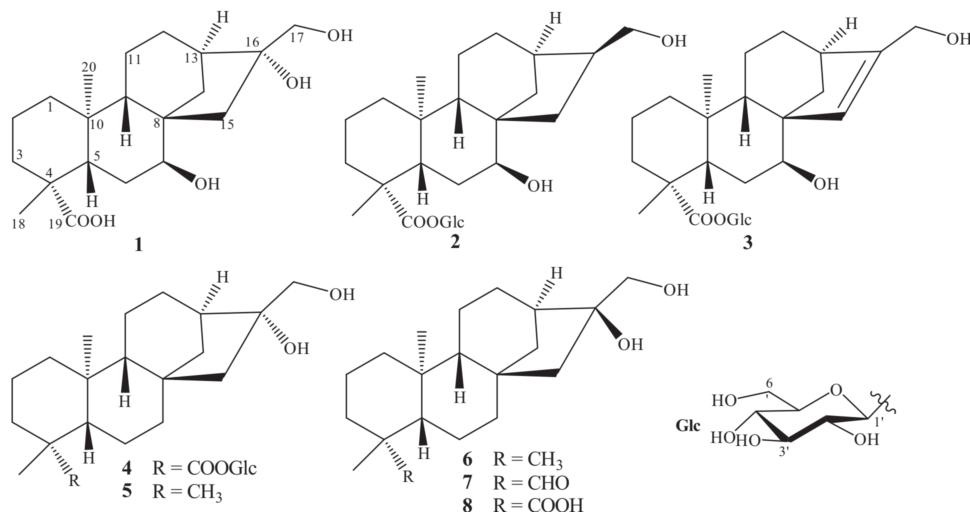

Figure 1. Structures of compounds **1**–**8** from the fruits of *Annona glabra*.

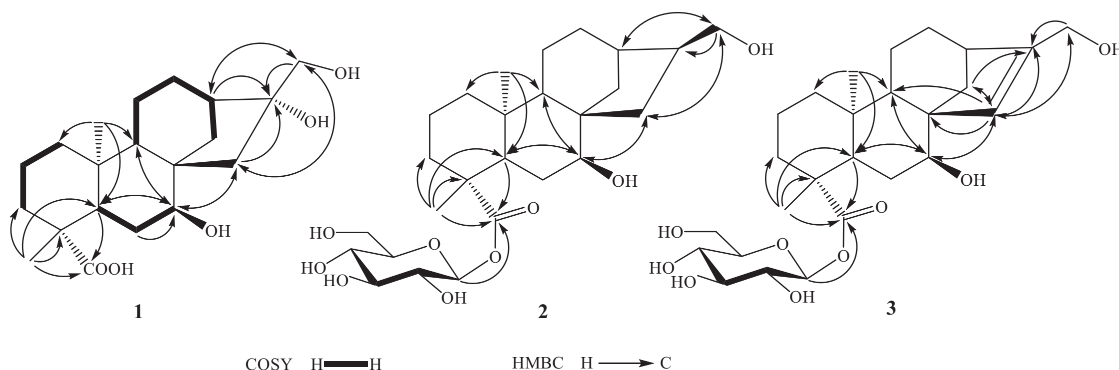

Figure 2. Selected HMBC and COSY correlations compounds **1**–**3**.

methyl carbons. The ^{1}H and ^{13}C NMR data of **2** were similar to those of helikauranoside A,[23] except for the additional hydroxyl group at C-7. The position of hydroxyl group at C-7 was confirmed by HMBC cross peaks from H-5 (δ_H 1.78)/H-9 (δ_H 1.43)/H-15 (δ_H 1.12 and 1.71) to C-7 (δ_C 78.7) as well as COSY correlations of H-5 (δ_H 1.78)/H-6 (δ_H 1.97 and 2.18)/H-7 (δ_H 3.50). Similarly to **1**, the configuration of H-7 in **2** was determined to be α-configuration by a broad singlet of H-7. The HMBC correlations between H-17 (δ_H 3.35) and C-13 (δ_C 39.5)/C-15 (δ_C 42.6)/C-16 (δ_C 44.7) suggested the hydroxyl group at C-17. In addition, the α configuration of hydrogen atom at C-16 was determined by comparing the chemical shifts of C-12 (δ_C 33.0) C-13 (δ_C 39.5), C-16 (δ_C 44.7), and C-17 (δ_C 67.7) with the corresponding data of 16α-*ent*-kaurane: helikauranoside A [δ_C 32.5 (C-12), 39.5 (C-13), 44.5 (C-16), and 67.7 (C-17)][23] and 16β-*ent*-kaurane: 17-hydroxy-16β-*ent*-kauran-19-al [δ_C 25.8 (C-12), 36.9 (C-13), 43.1 (C-16), and 64.1 (C-17)].[20] The HMBC cross peaks from H-18 (δ_H 1.22) to C-3 (δ_C 39.1), C-4 (δ_C 44.7), C-5 (δ_C 49.5), and C-19 (δ_C 178.7) confirmed the carboxyl group at C-4. Acid hydrolysis of **2** provided the D-glucose (identified as TMS derivatives). In addition, the coupling constant of glc H-1′ and glc H-2′; J = 8.0 Hz indicated the configuration of hydroxyl group at anomeric carbon in sugar to be β. The position of this sugar at C-19 of aglycone was confirmed by HMBC correlation between glc H-1′ (δ_H 5.42) and C-19 (δ_C 178.7). Based on the above evidence, the structure of **2** was elucidated to be 7β,17-dihydroxy-16α-*ent*-kauran-19-oic acid 19-*O*-β-D-glucopyranoside ester.

Compound **3**[24] was obtained as a white amorphous powder. Its molecular formula was determined to be $C_{26}H_{40}O_9$ by the appearance of the pseudo-ion peak at m/z 519.2550 in the HR-ESI-MS (Calcd for $C_{26}H_{40}O_9Na$: 519.2565). The ^{1}H-NMR of **3** showed one olefinic proton at δ_H 5.81 (1H, s) and two methyl groups at δ_H 1.02 (3H, s) and 1.22 (3H, s), suggested the presence of a *ent*-kaurane moiety; one anomeric proton at δ_H 5.42 (1H, d, J = 7.5 Hz), assigned to a sugar moiety. The ^{13}C NMR and DEPT spectra revealed the presence of 26 carbons, including 20 carbons of *ent*-kaurane-type diterpenoid and 6 carbons of sugar moiety. The ^{1}H and ^{13}C NMR data of **3** were similar to those of **2**, except for the additional double bond at C-15/C-16. The HMBC correlations between H-14 (δ_H 1.42 and 2.06)/H-17 (δ_H 4.13) and C-15 (δ_C 132.1) /C-16 (δ_C 148.1); H-15 (δ_H 5.81) and C-7 (δ_C 75.6)/C-8 (δ_C 54.3)/C-9 (δ_C 43.6)/C-14 (δ_C 43.5)/C-16 (δ_C 148.1)/C-17 (δ_C 61.2) confirmed the double bond at C-15/C-16. The hydroxyl group at C-7 was determined as the same with those of **2**. Consequently, structure of **3** was elucidated to be 7β,17-dihydroxy-*ent*-kaur-15-en-19-oic acid 19-*O*-β-D-glucopyranoside ester.

The other compounds were characterized as paniculoside IV (**4**),[25] 16α,17-dihydroxy-*ent*-kaurane (**5**),[9] 16β,17-dihydroxy-*ent*-kaurane (**6**),[9] 16β,17-dihydroxy-*ent*-kauran-19-al (**7**),[20] and 16β,17-dihydroxy-*ent*-kauran-19-oic acid (**8**),[20] (see Fig. 1) on the basis of NMR spectroscopic data, which were in good agreement with those reported in the literature.

To evaluate the inhibitory effects of the isolates on production of NO in the LPS-stimulated RAW 264.7 macrophages, cells were

seeded in the 24-well plates at a density of 5×10^5 cells/mL. After 3 h, the cells were pretreated with various concentrations of compounds and stimulated for 24 h with or without 1 μg/mL of LPS. Nitrite concentration was measured in the supernatant of RAW 264.7 cells by the Griess reaction.[26] First, the compounds were examined their cytotoxicity on cells at concentration of 30 μM. After that, each compound was screened its effects on the LPS induced the production of NO in RAW 264.7 cells at concentrations of 3, 10, and 30 μM since none of significant cytotoxic effects on the cells was found. In absence of both test compounds and LPS, the NO was produced at very low concentration (3.16 ± 0.27 μM). LPS markedly induced the NO production by concentration of 14.48 ± 0.43 μM. At the concentration of 30 μM, compounds **1**, **3**, and **7** potently decreased NO production with the inhibitory percent ranging from 90.0 to 97.6%; compounds **5**, **6**, and **8** significantly decreased NO production with the inhibitory percent ranging from 70.4% to 77.4% (Fig. 3). In order to get the IC_{50} values of highly potent inhibitory compounds, dose dependent inhibitory activities were tested. Suitable concentrations of each compound were roughly estimated from screening results and subjected for dose-dependent examination. As the results, compound **3** showed potent inhibitory LPS-stimulated NO production in RAW 264.7 macrophages with the IC_{50} value of 0.01 ± 0.01 μM; compounds **1** and **7** showed significant inhibitory NO productions with the IC_{50} values of 0.39 ± 0.12 μM and 0.32 ± 0.04 μM, respectively. Dexamethasone, a well-known anti-inflammatory and autoimmune drug, was used as a positive control throughout experiments. It showed the inhibitory NO production with the IC_{50} value of 0.80 ± 0.04 μM.

In the structure–activity relationship of *ent*-kaurane (**1–8**), the presence of the double bond at C-15/C-16 affected on inhibitory NO production. However, the presence of sugar moiety at C-19 did not affect to the NO production. In previous report, the methanol extract of *A. glabra* leaves showed potential anti-inflammatory activity.[27] Moreover, several *ent*-kauranes have been reported to possess inhibitory LPS-induced NO production in RAW 264.7 macrophages.[28] In our experiment, compound **3** showed potent inhibitory LPS-induced NO production in RAW 264.7 macrophages with the IC_{50} value of 0.01 ± 0.01 μM (Table 2). Thus, it is possible to demonstrate that isolated *ent*-kauranes might be important anti-inflammatory constituent of this plant. The in vivo anti-inflammatory effect of compound **3** need to be further studied.

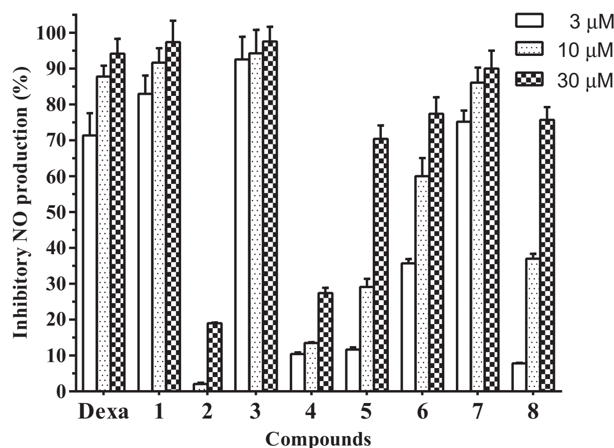

Figure 3. Effect of compounds **1**–**8** on NO production in RAW 264.7 cells. The cells were treated with various concentrations of compounds (3, 10, and 30 μM), and then LPS (1 μg/mL) was added and the cells were incubated for 24 h. Dexa, dexamethaxone. The values represent the means ± SD from three independent experiments.

Table 2
The inhibitory effects of **1**–**8** on NO production in LPS-stimulated RAW 264.7 macrophages

Compound	IC_{50} (μM)
1	0.39 ± 0.12
2	>30
3	0.01 ± 0.01
4	>30
5	17.06 ± 0.93
6	6.20 ± 0.47
7	0.32 ± 0.04
8	14.32 ± 1.06
Dexamethasone[a]	0.80 ± 0.04

[a] Dexamethasone was used as a positive control.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.11.059.

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17. The AG2 extract was chromatographed on a silica gel column and eluted with *n*-hexane/EtOAc gradient (100:1 to 1:1, v/v) to obtain four fractions AG2A–AG2D. The AG2B fraction was chromatographed on a silica gel column eluting with *n*-hexane/EtOAc (4:1, v/v) to obtain three fractions AG2B1–AG2B3. The AG2B1 fraction was further chromatographed on an YMC RP-18 column eluting with acetone/H_2O (3:1, v/v) to yield **5** (51.0 mg) and **6** (321.0 mg). The AG2D fraction was chromatographed on a silica gel column eluting with *n*-hexane/acetone (2:1, v/v) to obtain three fractions AG2D1–AG2D3. The AG2D1 was chromatographed on an YMC RP-18 column eluting with acetone–water (5:1, v/v) to yield **7** (7.0 mg). The AG2D3 fraction was chromatographed on a silica gel column eluting with CH_2Cl_2/EtOAc (10:1, v/v) to yield **8** (10.0 mg). The water soluble fraction AG4 was chromatographed on a Diaion HP-20P column (Mitsubishi Chem. Ind. Co., Tokyo, Japan) eluting with water containing increasing concentrations of MeOH (0%, 25%, 50%, 75%, and

100% MeOH) to give four fractions, AG4A−AG4D. The AG4C fraction was chromatographed on a silica gel column eluting with CH_2Cl_2/MeOH (6:1, v/v) to give three fractions AG4C1−AG4C3. The AG4C1 was chromatographed on an YMC RP-18 column eluting with MeOH/H_2O (1:1.5, v/v) to yield **1** (5.0 mg). The AG4C2 was chromatographed on a silica gel column eluting with CH_2Cl_2/EtOAc (10:1, v/v) to obtained two fractions AG4C2A and AG4C2B. The AG4C2A fraction was further separated on an YMC RP-18 column eluting with MeOH/H_2O (1:1, v/v) to yield **2** (4.0 mg), **3** (3.0 mg), and **4** (6.0 mg).

18. Compound (**1**): White amorphous powder; $[\alpha]_D$ −64.9 (*c* 0.1, MeOH); ^{1}H NMR and ^{13}C NMR (CD_3OD): see Table 1; HR-ESI-MS found *m*/*z* 375.2159 $[M+Na]^+$ (Calcd for $C_{20}H_{32}O_5Na$: 375.2142).

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22. Compound (**2**): White amorphous powder; $[\alpha]_D$ −40.7 (*c* 0.1, MeOH); ^{1}H NMR and ^{13}C NMR (CD_3OD): see Table 1; HR-ESI-MS found *m*/*z* 521.2732 $[M+Na]^+$ (Calcd for $C_{26}H_{42}O_9Na$: 521.2721).

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24. Compound (**3**): White amorphous powder; $[\alpha]_D$ −38.5 (*c* 0.1, MeOH); ^{1}H NMR and ^{13}C NMR (CD_3OD): see Table 1; HR-ESI-MS found *m*/*z* 519.2550 $[M+Na]^+$ (Calcd for $C_{26}H_{40}O_9Na$: 519.2565).

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