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A new ursane-type triterpenoid glycoside from *Centella asiatica* leaves modulates the production of nitric oxide and secretion of TNF-α in activated RAW 264.7 cells

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ABSTRACT

One new ursane-type triterpenoid glycoside, asiaticoside G (**1**), five triterpenoids, asiaticoside (**2**), asiaticoside F (**3**), asiatic acid (**4**), quadranoside IV (**5**), and 2α,3β,6β-trihydroxyolean-12-en-28-oic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**6**), and four flavonoids, kaempferol (**7**), quercetin (**8**), astragalin (**9**), and isoquercetin (**10**) were isolated from the leaves of *Centella asiatica*. Their chemical structures were elucidated by mass, 1D- and 2D-nuclear magnetic resonance (NMR) spectroscopy. The structure of new compound **1** was determined to be 2α,3β,23,30-tetrahydroxyurs-12-en-28-oic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester. The anti-inflammatory activities of the isolated compounds were investigated on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Asiaticoside G (**1**) potently inhibited the production of nitric oxide and tumor necrosis factor-α with inhibition rates of 77.3% and 69.0%, respectively, at the concentration of 100 μM.

Nitric oxide (NO) and tumor necrosis factor (TNF) are essential mediators of several biological processes, including the immune response. TNF stimulates NO production via the expression of inducible NO synthase (iNOS), with L-arginine being the only substrate.[1] The production of NO confers cytostatic or cytotoxic activity on macrophages against microbes and tumor cells.[2] TNF-α is one of the most important pro-inflammatory cytokines and is produced by activated monocytes and macrophages. NO and TNF-α generally act as defense factors against invading bacterial pathogens and are essential elements of antimicrobial immunity during inflammation.[3] They are secreted during the early phase of acute and chronic inflammatory diseases such as asthma, rheumatoid arthritis, septic shock and other allergic diseases, as well as during the activation of T cells.[4]

Centella asiatica (L.) Urban (Umbelliferae) is widely cultivated as a vegetable or spice in Asia, Africa, and Oceania. It is also used to treat skin diseases, rheumatism, inflammation, syphilis, mental illness, epilepsy, diarrhea, and wounds.[5] Previous phytochemical and biological investigations of *C. asiatica* have yielded a number of triterpenes,[6–8] polyacetylenes,[9] and flavonoids.[10] Studies have cited the anti-inflammatory activity of the ursane triterpenoids and a number of derivatives from this plant with the ursane-type skeleton been found to possess anti-inflammatory activity and the inhibitory production of NO and the secretion of TNF-α in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells; these compounds include madecassic acid,[11] madecassoside,[12] asiatic acid,[13,14] and asiaticoside.[14,15] Interestingly, our extensive phytochemical study led to the isolation of one new ursane-type triterpenoid glycoside as well as nine known compounds. In this study, we examined the effect of the new ursane-type triterpenoid glycoside and the nine known compounds on the production of NO and the secretion of TNF-α in LPS-stimulated RAW 264.7 cells.

In a preliminary study, the methanol extract of the leaves of *C. asiatica* inhibited the production of NO and secretion of TNF-α in LPS-stimulated RAW 264.7 cells. To clarify the active compounds from the leaves of *C. asiatica* caused this inhibition, one new ursane-type triterpenoid glycoside (**1**), five triterpenoids, asiaticoside (**2**),[10] asiaticoside F (**3**),[6] asiatic acid (**4**),[16] quadranoside IV (**5**),[17] and 2α,3β,6β-trihydroxyolean-12-en-28-oic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (**6**),[7] and four flavonoids, kaempferol (**7**),[10]

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quercetin (**8**),[10] astragalin (**9**),[18] and isoquercetin (**10**)[18] (see Fig. 1) isolated from the leaves of *C. asiatica*,[19] were tested their inhibitory effects on NO and TNF-α production in LPS-stimulated RAW 264.7 cells.

By using combined chromatographic separations, one new ursane-type triterpenoid glycoside together with nine known compounds were isolated from the methanol extract of *C. asiatica* leaves.[20] Compound **1** was obtained as a white amorphous powder. Its basic ion peak at *m/z* 975.5 $[M+H]^+$ was observed on positive-ion electrospray ionization mass spectrometry (ESI-MS), and high-resolution electronspray ionization mass spectroscopy (HR-ESI-MS) analysis revealed the molecular formula to be $C_{48}H_{78}O_{20}$, with a cluster ion peak at *m/z* 975.5160 $[M+H]^+$ (calcd for $C_{48}H_{79}O_{20}$: 975.5165). The ^1H NMR spectrum of **1** (in methanol-d_4) showed the following signals: four tertiary methyl groups at δ_H 0.67, 0.81, 1.03, and 1.10 (each 3H, s); two secondary methyl groups at δ_H 0.90 (d, 6.0 Hz) and 1.24 (d, 6.6 Hz); and three anomeric protons were at δ_H 4.35, 4.82, and 5.26, suggesting the presence of three sugar units (see Table 1). The ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) spectra revealed 48 carbon signals, of which, 30 were assigned to a triterpenoid sapogenol moiety and 18 belonged to three monosaccharide moieties. The aglycone of **1** was recognized to be an ursane-type triterpenoid on ^1H and ^{13}C NMR analysis (see Table 1), with the typical olefinic carbons at δ_C 126.9 and 139.4, four quaternary methyl carbons at δ_C 13.9, 17.8, 18.1, and 24.0, one secondary methyl carbon at δ_C 17.4, two oxymethine carbons at δ_C 69.6 and 77.9, two oxymethylene carbons at δ_C 65.8 and 66.3, and one carboxyl signal at δ_C 177.9. The ^1H and ^{13}C NMR data of **1** were similar to those of asiaticoside (**2**),[10] except for a difference of methyl group at C-20. In the heteronuclear multiple bond correlation (HMBC) spectrum (see Fig. 2), the methyl group H-24 (δ_H 0.67) correlated with carbons C-3 (δ_C 77.9), C-4 (δ_C 44.1), and C-23 (δ_C 66.3). The large coupling constant J_{23} = 10.8 Hz of the two protons at C-2 and C-3 and the chemical shifts of C-2 (δ_C 69.6) and C-3 (δ_C 77.9) in the aglycone confirmed the *equatorial* position of the 2- and 3-hydroxyl groups in comparison with the corresponding data for 2β,3α,23-trihydroxyurs-12-en-28-oic acid[21] [coupling constant between H-2 and H-3, J_{23} = 3.0 Hz, and δ_C values for C-2 (66.6) and C-3 (78.6)] and methyl 2α,3α,24-trihydroxyurs-12-en-28-oate[22] [coupling constant between H-2 and H-3, J_{23} = 3.0 Hz, and δ_C values for C-2 (66.2) and C-3 (73.3)]. This was further confirmed by correlation between proton signal at δ_H 3.67 (H-2) and proton signal at δ_H 1.03 (H-25). Moreover, the chemical shifts of C-3 (δ_C 77.9) and C-23 (δ_C 66.3) were quite difference from those reported for 2α,3β,24-trihydroxyurs-12-en-28-oic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (scheffursoside F)[23] [δ_C values for C-3 (85.7) and C-24 (65.7)]. These results suggested that there was a hydroxyl group at C-23, which was confirmed by a cross peak between H-24 (δ_H 0.67) and H-25 (δ_H 1.03) in the rotating overhouser effect spectroscopy (ROESY) spectrum. Furthermore, in the HMBC spectrum, the

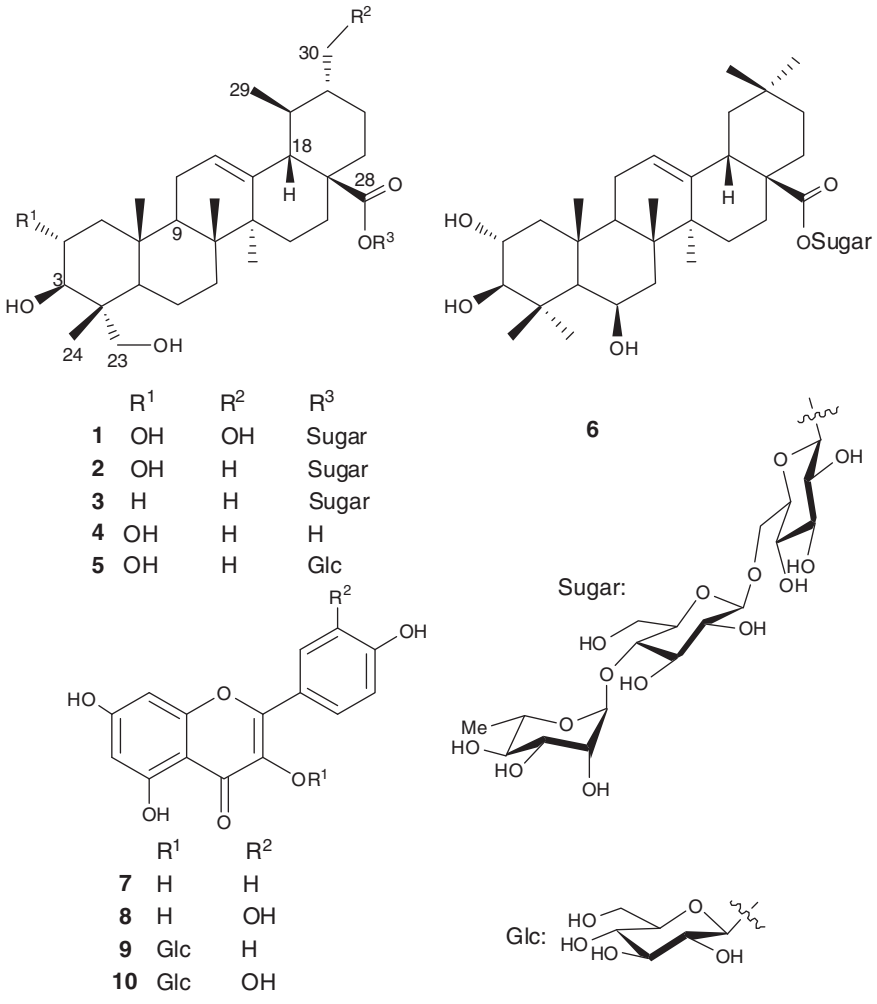

Figure 1. Structure of compounds (**1**–**10**) isolated from leaves of *C. asiatica*.

Table 1
The NMR spectral data of compound **1**

Position	1	
	δ_C[a,b]	δ_H[a,c] (mult., Hz)
Aglycone		
1	48.1	1.25 (m)
		1.94 (m)
2	69.6	3.67 (dt, 3.6, 10.8)
3	77.9	3.49 (d, 10.8)
4	44.1	—
5	48.3	0.87*
6	19.1	1.42 (m)
7	33.6	1.27 (m)
		1.60*
8	41.0	—
9	48.9	1.61*
10	39.0	
11	24.5	1.95 (m)
12	126.9	5.22 (t, 3.0)
13	139.4	—
14	43.4	
15	29.3	1.08*, 1.89*
16	25.2	1.73 (m)
17	49.4	—
18	54.1	2.21 (d, 11.5)
19	34.3	1.61 (m)
20	47.7	1.01 (m)
21	25.4	1.50*
22	37.6	1.58*
		1.72*
23	66.3	3.23 (d, 12.6)
		3.47 (d, 12.6)
24	13.9	0.67 (s)
25	17.8	1.03 (s)
26	18.1	0.81 (s)
27	24.0	1.10 (s)
28	177.9	—
29	17.4	0.90 (d, 6.0)
30	65.8	3.52*
		3.62 (dd, 3.0, 10.8)
C-28 O-glc		
1	95.8	5.26 (d, 7.8)
2	73.8	3.38 (m)
3	78.3	3.32 (m)
4	71.0	3.37 (m)
5	78.2	3.38 (m)
6	69.7	3.76 (dd, 4.2, 12.0)
		4.05 (d, 12.0)
glc′(1′→6)glc		
1′	104.5	4.35 (d, 7.8)
2′	75.3	3.21 (t, 7.8)
3′	76.8	3.27*
4′	79.6	3.51 (t, 7.8)
5′	76.7	3.42 (m)
6′	61.9	3.62 (dd, 4.2, 11.4)
		3.79 (d, 11.4)
rha(1″→4′)glc′		
1″	102.8	4.82 (d, 1.8)
2″	72.4	3.81 (m)
3″	72.2	3.60 (dd, 2.7, 9.0)
4″	73.7	3.30 (m)
5″	70.7	3.93 (dd, 6.6, 9.6)
6″	17.9	1.24 (d, 6.6)

[a] Measured in CD_3OD.
[b] 150 MHz.
[c] 600 MHz.
* Overlapped signals, assignments were done by HMQC, HMBC, COSY and ROESY experiments; glc, β-D-glucopyranosyl; rha, L-rhamnopyranosyl.

methyl group H-29 (δ_H 0.90) correlated with carbons C-18 (δ_C 54.1), C-19 (δ_C 34.3), and C-20 (δ_C 47.7), and the hydroxymethyl group H-30 (δ_H 3.52 and 3.62) correlated with carbons C-19 (δ_C 34.3), C-20 (δ_C 47.7), and C-21 (δ_C 25.4). In the ROESY spectrum, the cross peak between H-18 (δ_H 2.21) and H-29 (δ_H 0.90) and

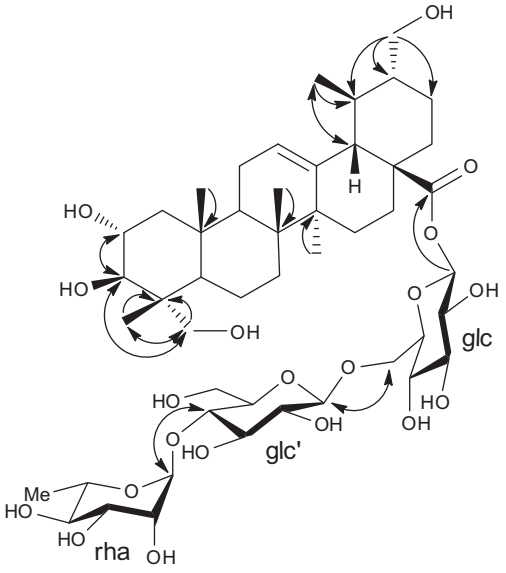

Figure 2. Selected HMBC spectrum of compound **1**.

the absence of a correlation between H-30 (δ_H 3.52 and 3.62) and H-18 (δ_H 2.21) indicated that the methyl and hydroxyl methyl groups at C-19 and C-20 had the β and α configurations, respectively. Acid hydrolysis of **1** provided the monosaccharide components of L-rhamnose and D-glucose (identified as TMS derivatives by gas chromatography (GC) method).[24] Moreover, HMBC correlations between the inner glc H-1 (δ_H 5.26) and C-28 of the aglycone (δ_C 177.9), between the outer glc H-1′ (δ_H 4.35) and the inner glc C-6 (δ_C 69.7), and between the rha H-1″ (δ_H 4.82) and the glc C-4′ (δ_C 79.6) were observed. These evidences suggested the sequence of the sugar linkages of **1**. The carbon signals of the sugar moieties were superimposable on those of characteristic triterpenoid glycosides isolated from *Centella* species.[6–8] Consequently, the structure of **1** was determined to be 2α,3β,23,30-tetrahydroxyurs-12-en-28-oic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester, named asiaticoside G.[25]

To evaluate the inhibitory effects of the isolates on the production of NO in the LPS-stimulated RAW 264.7 cells, cells were seeded in 96-well plates at a density of 1×10^5 cells/mL. After 3 h, the cells were treated with various concentrations of the compounds (1, 10, and 100 μM) and stimulated for 24 h with or without 1 μg/mL of LPS (Sigma Chemical Co., St. Louis, MO, USA). The nitrite concentration was measured in the supernatant of RAW 264.7 cells by the Griess reation.[26] In absence of both test the compounds and LPS, NO was produced at a very low concentration (0.87 ± 0.11 μM). LPS markedly induced the NO production by concentration of 6.09 ± 0.50 μM. In contrast, the dexamethasone (10 μM), positive control, resulted in a level of 1.54 ± 0.07 μM and compounds **1**, **2**, **4**, and **7**–**10** (1, 10, and 100 μM) significantly decreased the NO production to levels between 1.38 ± 0.26 to 5.16 ± 0.52 μM (see Fig. 3). One oleane-type triterpenoid glycoside (**6**) and two ursane-type triterpenoid glycosides (**3**, **5**) showed no activity at the concentration of 100 μM (IC_{50} >100 μM). Since isolates from *C. asiatica* significantly inhibited production of NO, we investigated the effects of these compounds on the secretion of TNF-α in LPS-stimulated RAW 264.7 cells.[27] LPS markedly induced the TNF-α production, to a concentration of 80.07 ± 1.00 ng/mL. In contrast, the dexamethasone (10 μM), positive control, resulted in a level of 27.01 ± 0.73 ng/mL and compounds **1**, **2**, **4**, **5**, and **7**–**10** (1, 10,

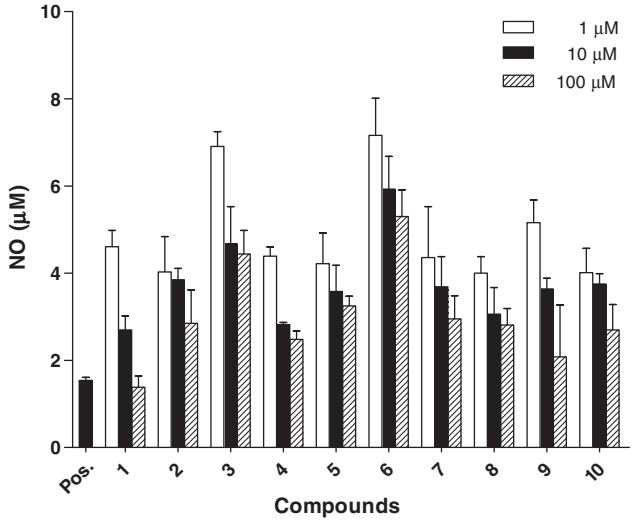

Figure 3. Effect of isolated compounds on NO production in RAW 264.7 cells. The cells were seeded in 96 well plates at a density of 1×10^5 cells/well. After 3 h, the cells were treated with various concentrations of compounds (1, 10, and 100 μM), and then LPS (1 μg/mL) was added and the cells were incubated for 24 h. Dexamethasone was used as a positive control. The values represent the means ± S.E.M. from three independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.001 versus LPS-treated group.

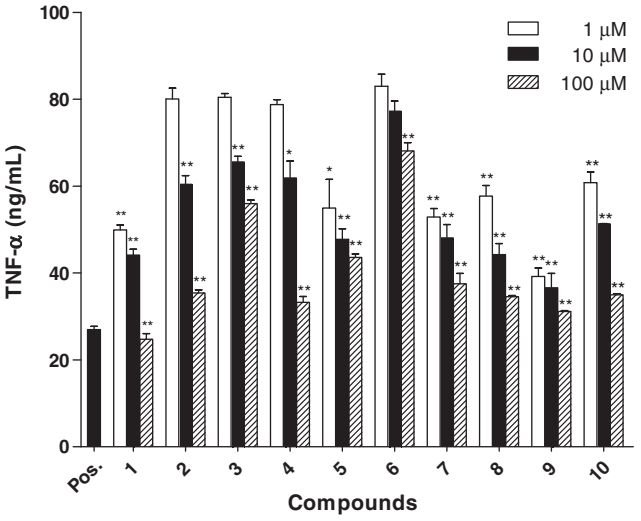

Figure 4. Effect of isolated compounds on TNF-α production in RAW 264.7 cells. The cells were seeded in 96 well plates at a density of 1×10^4 cells/well. After 1 h, the cells were treated with various concentrations of compounds (1, 10, and 100 μM), and then LPS (1 μg/mL) was added and the cells were incubated for 18 h. Dexamethasone was used as a positive control. The values represent the means ± S.E.M. from three independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.001 versus LPS-treated group.

and 100 μM) significantly decreased TNF-α secretion to levels from 78.77 ± 1.14 to 24.78 ± 1.28 ng/mL (see Fig. 4).

In the structure–activity relationship of ursane triterpenoids (**1**–**5**), the presence of the hydroxyl group at C-30 affected NO production and increase TNF-α activity. In contrast, the presence of sugar moieties at C-28 decreased activity; as previous reported, the aglycones were more active than their glycosides.[11,14] Specifically, the absence a hydroxyl group at C-2 (compound **3**) caused inactivity. When compared activity of flavonoids (**7**–**10**), the presence of the hydroxyl group at C-3′ or glucose moiety at C-3 affected on NO and TNF-α production decrease activity. We also examined the cytotoxic effects of isolated compounds at concentration of 100 μM in the presence or absence of LPS using MTT assays. It was found that neither compound affected the viability of RAW 264.7 cells after 24 h (data not shown), indicating that their inhibitory effects were not due to any cytotoxic effects. Our results showed that ursane-type triterpenoids (**1**–**4**) from *C. asiatica*, except compound **3** with absence hydroxyl group at C-2, suppressed the production of NO and secretion of TNF-α in LPS-stimulated RAW 267.4 cells. Thus, the isolated ursane-type triterpenoid glycosides might be important anti-inflammatory constituents of this plant. Among the tested compounds, asiaticoside G (**1**), with a hydroxyl group at C-30, had the most potent the anti-inflammatory effect, with IC_{50} of 8.4 and 35.3 μM for the production of NO and secretion of TNF-α, respectively (see Table 2). The in vivo anti-inflammatory effects of asiaticoside G (**1**) need to be further studied.

Table 2
IC_{50} values of the compounds for NO and TNF-α production in LPS-stimulated RAW 264.7 cells

Compounds	IC_{50} (μM)	
	NO	TNF-α
1	8.4 ± 1.1	35.3 ± 2.1
2	67.9 ± 18.4	85.7 ± 1.3
3	>100	>100
4	8.6 ± 1.8	74.8 ± 2.7
5	70.2 ± 10.3	86.4 ± 4.9
6	>100	>100
7	60.2 ± 36.3	80.3 ± 8.5
8	63.0 ± 27.9	66.6 ± 3.0
9	64.0 ± 19.4	67.1 ± 8.9
10	61.2 ± 17.2	76.3 ± 2.6
Dexsamethasone*	0.8 ± 0.07	0.01 ± 0.0001

* Dexsamethasone was used as positive control. Data presented is the mean ± S.D. of samples run in triplicate.

Acknowledgements

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19. The sample of *Centella asiatica* (L.) was collected at Tam Dao National Park, Vinh Phuc, Vietnam, during June 2009 and identified by Dr. Ninh Khac Ban (Institute of Ecology and Biological Resources, VAST). A voucher specimen (IMBC CA-0609) was deposited at the Herbarium of Institute of Marine Biochemistry, VAST.
20. The dried leaves of *C. asiatica* (3.0 kg) were extracted with MeOH three times under reflux for 15 h to yield 250 g of a dark solid extract. This extract was suspended in water and partitioned with ethyl acetate to yield ethyl acetate extract (CA1, 100 g) and water extract (CA2, 150 g). The ethyl acetate extract (CA1) was then subjected to chromatography on a silica gel column eluting with a gradient of $CHCl_3$/MeOH (from 50:1 to 5:1 v/v) yielded five fractions CA1A–CA1E. Fraction CA1A was chromatographed on an LH-20 column eluting with $CHCl_3$/MeOH (1:1 v/v) to yield **7** (30.5 mg) and **8** (84.7 mg). Fraction CA1C was chromatographed on a silica gel column using $CHCl_3$/MeOH (10:1, v/v) as an eluent to give fractions CA1C1–CA1C4. The CA1C3 fraction was chromatographed on an YMC RP-18 column eluting with MeOH/H_2O (5:1 v/v) to give **4** (60.0 mg). Fraction CA1C4 was chromatographed on an YMC RP-18 column eluting with MeOH/H_2O (4:1 v/v) to yield **5** (10.5 mg). The H_2O soluble fraction (CA2) was chromatographed on a Diaion HP-20P column (Mitshubishi Chemical Co., Japan) eluted with a step gradient of MeOH in water (0%, 25%, 50%, 75%, and 100% MeOH) yielding the five fractions, CA2A–CA2E. The fraction CA2B was chromatographed on a silica gel column eluting with $CHCl_3$/MeOH/H_2O (75:20:3, v/v/v) yielding the four fractions, CA2B1–CA2B4. Fraction CA2B2 was chromatographed on a silica gel column eluting with $CHCl_3$/MeOH/H_2O (75:20:3, v/v/v) yielded compound **6** (120.9 mg). Fraction CA2C was chromatographed on an YMC RP-18 column eluting with acetone/H_2O (2:1, v/v) to yield four sub-fractions, CA2C1–CA2C4. Fraction CA2C1 was chromatographed on a silica gel column (50 g, 2 × 50 cm) eluting with $CHCl_3$/MeOH/H_2O (30:10:1, v/v/v), yielding compound **1** (7.1 mg). Fraction CA2C2 was further chromatographed on a silica gel column (50 g, 2 × 50 cm) eluting with CH_2Cl_2/MeOH/H_2O (35:10:1, v/v/v), yielding compounds **2** (10.2 mg) and **3** (8.0 mg). Fraction CA2E was chromatographed on an YMC RP-18 column eluting with acetone/H_2O (1:1, v:v) to yield three fractions, CA2E1–CA2E3. Fraction CA2E1 was chromatographed on a silica gel column eluting with $CHCl_3$/MeOH/H_2O (55:10:1, v/v/v) to yield compound **9** (5.0 mg). Fraction CA2E2 was chromatographed on a silica gel column eluting with $CHCl_3$/MeOH/H_2O (35:10:1, v/v/v), yielding compound **10** (7.0 mg).
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24. Each compound (2.0 mg) was dissolved in 1.0 N HCl (dioxane/H_2O, 1:1, v/v, 1.0 mL) and then heated to 80 °C in a water bath for 3 h. The acidic solution was neutralized with silver carbonate and the solvent thoroughly driven out under N_2 gas overnight. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2 gas. The residue was dissolved in 0.1 mL of dry pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solution. The reaction mixture was heated at 60 °C for 2 h, and 0.1 mL of trimethylsilylimidazole solution was added, followed by heating at 60 °C for 1.5 h. The dried product was partitioned with *n*-hexane and H_2O (0.1 mL, each), and the organic layer was analyzed by gas liquid chromatography (GC): Column: column SPB-1 (0.25 mm × 30 m); detector FID, column temp 210 °C, injector temp 270 °C, detector temp 300 °C, carrier gas He (2.0 mL/min). The retention times of persilylated glucose and rhamnose were founded to be 14.11 and 4.50 min, respectively, when compared with the standard solutions prepared by the same reaction from the standard monosaccharides. (The retention times of persilylated D-glucose, L-glucose, and L-rhamnose were 14.11, 14.26, and 4.50 min, respectively).
25. White amorphous powder; $[\alpha]_D^{25}$ −18° (*c* 0.1, MeOH); UV λ_{max} (log ε, MeOH) 211 (1.75) nm; IR (KBr) n_{max} 3407, 2954, 1728, 1231, and 1064 cm^{-1}; ^1H and ^{13}C NMR are given in Table 1; ESI-MS *m*/*z* 975 $[M+H]^+$, 997 $[M+Na]^+$; HR-ESI-MS *m*/*z* 975.5160 $[M+H]^+$ (calcd for $C_{48}H_{79}O_{20}$: 975.5165).
26. The nitrite, which accumulated in the culture medium, was measured as an indicator of NO production by means of the Griess reaction. Briefly, 100 mL of cell culture medium (without phenol red) was mixed with an equal volume of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl), incubated at room temperature for 10 min, and then the absorbance was measured at 550 nm using a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was obtained by means of the $NaNO_2$ serial dilution standard curve and the nitrite production was measured.
27. TNF-α production in the supernatant of RAW 264.7 cells was quantified using an OptEIA™ assay kits according to the manufacturer's instructions.