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EFFECTS OF SESQUITERPENES AND TRITERPENES FROM THE RHIZOME OF *ALISMA ORIENTALE* ON NITRIC OXIDE PRODUCTION IN LIPOPOLYSACCHARIDE-ACTIVATED MACROPHAGES: ABSOLUTE STEREOSTRUCTURES OF ALISMAKETONES-B 23-ACETATE AND -C 23-ACETATE

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Abstract The methanolic extract from a Chinese herbal medicine, the rhizome of *Alisma orientale*, was found to exhibit inhibitory activity of nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages. Novel triterpenes, alismaketones-B 23-acetate and -C 23-acetate, were isolated from the active extract together with eight sesquiterpenes and eighteen protostane-type triterpenes. The absolute stereostructures of new triterpenes were characterized on the basis of chemical and physicochemical evidence, which included the chemical correlations with known triterpenes. The guaiane-type sesquiterpenes (alismol, orientalols A and C) and protostane- and *seco*-protostane-types triterpenes (alisols C monoacetate, E-23-acetate, F, H, I, L-23-acetate, and M-23-acetate, alismaketones-B 23-acetate and -C 23-acetate, alismalactone 23-acetate, and 3-methylalismalactone 23-acetate) inhibited LPS-induced NO production (IC_{50} = 8.4–68 μM). Other triterpenes (alisols A, A monoacetate, B, B monoacetate, E, G, K-23-acetate, and N-23-acetate and 11-deoxyalisol B) also showed the potent inhibitory activity, but they showed cytotoxic effects more than 30 μM (MTT assay). In addition, alismol and alisol F were found to suppress iNOS induction.

Nitric oxide (NO), an inorganic free radical, has been implicated in physiological and pathological processes, such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation. NO is produced by the oxidation of L-arginine by a NO synthase (NOS). In the family of NOS, especially inducible NOS (iNOS) is involved in pathological aspect with overproduction of NO, and can be expressed in response to pro-inflammatory agents such as interleukin-1β, tumor necrosis factor-α, and lipopolysaccharide (LPS) in various cells including macrophages, endothelial cells, and smooth muscle cells.[1] Inhibition of iNOS enzyme activity or the induction may have provide therapeutic benefits in various types of inflammation.[2]

We previously reported that the guaiane-type sesquiterpene, and protostane-type triterpene constituents from a Chinese herbal medicine, Alismatis Rhizoma (the rhizome of *Alisma orientale* JUZEP.), showed relaxant effect on the contraction of isolated aortic or bladder smooth muscles[3–6] and inhibitory activities for experimental models of type I–IV allergies.[7,8] As a continuing study on Alismatis Rhizoma, the methanolic extract was found to show the inhibition of nitrite (NO_2^-, a product of NO) accumulation in LPS-activated macrophages. This report deals with the effects of the guaiane-type sesquiterpene and *seco*-protostane- or protostane-type triterpene constituents from Alismatis Rhizoma on NO production and iNOS induction in LPS-stimulated macrophages. In addition, we describe the structure elucidation of novel triterpenes, alismaketones-B 23-acetate (**25**) and -C 23-acetate (**26**).

Materials and Methods

Isolation of Chemical Constituents from the Dried Rhizome of Alisma orientale The MeOH extract from Chinese Alismatis Rhizoma was partitioned into a mixture of AcOEt-water. Twenty-five less polar constituents [alismol (**1**),[5] alismoxide (**2**),[5] orientalols A (**3**)[5] and C (**4**),[5] germacrene D (**8**),[9] and alisols A (**9**),[10] A monoacetate (**10**),[10] B (**11**),[10] B monoacete (**12**),[10] C monoacetate (**14**),[10] E (**15**),[10] E-23-acetate (**16**),[10] F (**17**),[10] G (**18**),[10] H (**19**),[11] I (**20**),[11] K-23-acetate (**21**),[11] L-23-acetate (**22**),[11] M-23-acetate (**23**),[11] and N-23-acetate (**24**),[11] 11-deoxyalisol B (**13**),[9] alismaketones-B 23-acetate (**25**)[12] and -C 23-acetate (**26**),[12] alismalactone 23-acetate (**27**),[4] and 3-methylalismalactone 23-acetate (**28**)[4]] were isolated from the AcOEt-

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soluble portion, while three sesquiterpenes [sulfoorientalols b (**5**),[6] c (**6**),[6] and d (**7**)[6]] were isolated from the water-soluble portion. Their isolation procedure was reported in previous papers, except for new triterpenoids (**25**, **26**).[12]

Table 1. ^{13}C-NMR Data for **25** and **26** ($CDCl_3$, δc, 125 MHz)

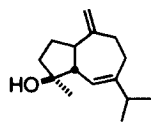
	25	26		25	26
1	30.8	32.3	17	135.4	213.9
2	33.6	33.2	18	23.7	15.2
3	219.8	219.1	19	25.5	24.9
4	47.0	47.1	20	29.0	42.1
5	48.3	45.0	21	19.9	18.2
6	20.0	19.6	22	38.5	34.7
7	34.1	31.5	23	73.1	72.3
8	40.7	44.3	24	77.8	64.8
9	49.5	47.4	25	72.0	58.8
10	37.0	36.1	26	26.4[a)]	19.3
11	70.0	128.8	27	27.0[a)]	24.8
12	34.5	146.3	28	29.6	29.3
13	142.7	205.9	29	20.1	19.1
14	55.0	53.2	30	23.6	22.0
15	39.9	23.3	OAc	170.3	170.6
16	84.0	38.1		21.5	21.1

[a)]Assignments may be interchangeable.

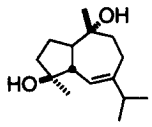
Absolute Stereostructures of Alismaketones-B 23-Acetate and C 23-Acetate: Alismaketone-B 23-acetate (**25**), a white powder, $[\alpha]_D^{25}$ +42.7°, $C_{32}H_{50}O_6$, IR (KBr): 3460, 1732, 1704 cm^{-1}, showed quasimolecular ion peaks at *m/z* 531 $(M+H)^+$ and *m/z* 553 $(M+Na)^+$ in its positive-ion FAB-MS. The proton and carbon signals in the ^{1}H-NMR[13] and ^{13}C-NMR (Table 1) spectra of **25** were very similar to those of alisol F (**17**)[10] and its 24-acetate, except for the signals due to the 23 and 24-positions. The 16, 24-seven membered oxide structure of **25** was characterized on the basis of detailed examination of its H-H COSY and HMBC experiment, which showed long-range correlations between the following protons and carbons: 16-H and 17, 24-C; 23-H and acetyl-C; 24-H and 23, 25-C. Finally, reduction of **25** with Li in ethylenediamine furnished dihydroalisol A (**29**)[10] and consequently, the absolute stereostructure of **25** having the seven membered oxide ring was determined.

Alismaketone-C 23-acetate (**26**), a white powder, $[\alpha]_D^{25}$ +22.3°, $C_{32}H_{48}O_6$, UV ($CHCl_3$): 240 nm (log ε 3.8), IR (KBr): 1738, 1705, 1701, 1674 cm^{-1}, showed quasimolecular ion peaks at *m/z* 527 $(M-H)^-$, *m/z* 529 $(M+H)^+$, and *m/z* 551 $(M+Na)^+$ in the negative and positive-ion FAB-MS. The ^{1}H-NMR[13] and ^{13}C-NMR spectra of **26** indicated the presence of seven *tert.* methyls, a *sec.* methyl, an epoxide, three ketones, and a disubstituted olefin. Detailed examination of the NMR experiments on **26** led us to presume the tricyclic structure of **26**. The absolute stereostructure of 13, 17-*seco*-protostane-type triterpene (**26**) was determined by the following chemical correlation of **26** with alisol B monoacetate (**12**). Namely, oxidative cleavage of the 13, 17-olefin in **12** yielded **30**, which was treated with $SOCl_2$ to give the enone **26**.

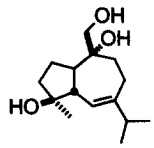
Reagents: Lipopolysaccharide (LPS, from *Salmonella enteritidis*) and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma; 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl tetrazolium bromide (MTT) was from Dojin, Japan; caffeic acid phenethyl ester (CAPE) and guanidinoethyldisulfide (GED) was from Calbiochem; RPMI 1640 was from Gibco; protease inhibitor cocktail (Complete Mini) was from Boehringer Mannheim; fetal calf serum (FCS) was from Bio Whittaker; nitrocellulose membrane (0.25 μm) was from Bio Rad; 96-well microplate and culture dish (6 cm) were from Nunc; anti-mouse iNOS antibody (monoclonal) was from Transduction Laboratories; anti-mouse IgG antibody conjugated to horseradish peroxidase and the enhanced chemiluminescense (ECL) kit were from Amersham; thioglycolate (TGC) medium from Nissui Seiyaku, and all other chemicals were from Wako.



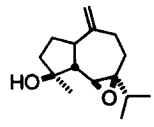
alismol (**1**)



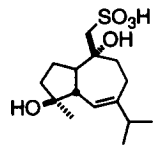
alismoxide (**2**)



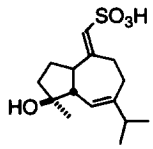
orientalol A (**3**)



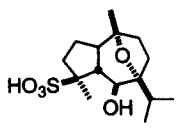
orientalol C (**4**)



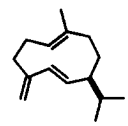
sulfoorientalol b (**5**)



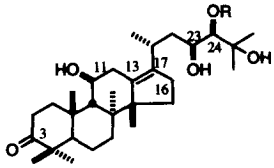
sulfoorientalol c (**6**)



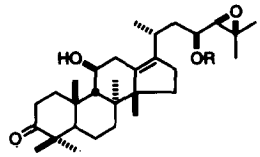
sulfoorientalol d (**7**)



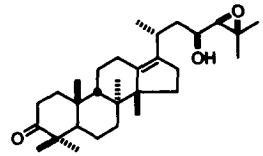
germacrene D (**8**)



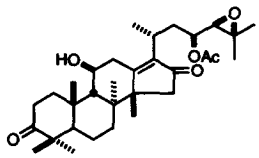
alisol A (**9**): R=H
alisol A monoacetate (**10**): R=Ac



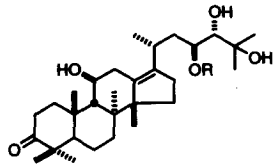
alisol B (**11**): R=H
alisol B monoacetate (**12**): R=Ac



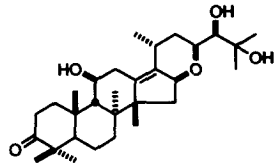
11-deoxyalisol B (**13**)



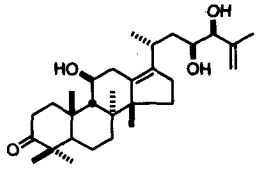
alisol C monoacetate (**14**)



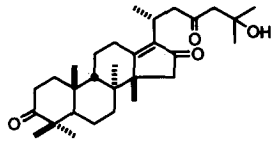
alisol E (**15**): R=H
alisol E 23-acetate (**16**): R=Ac



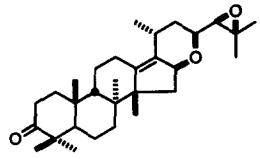
alisol F (**17**)



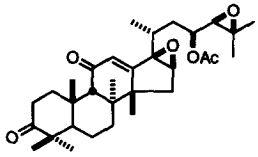
alisol G (**18**)



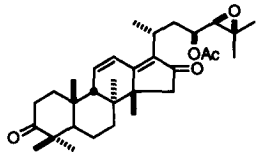
alisol H (**19**)



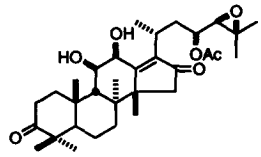
alisol I (**20**)



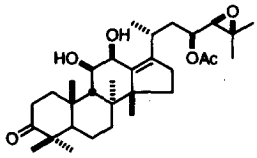
alisol K-23-acetate (**21**)



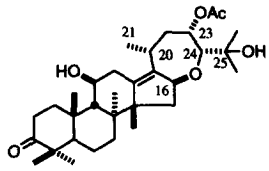
alisol L-23-acetate (**22**)



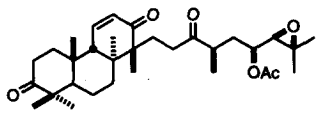
alisol M-23-acetate (**23**)



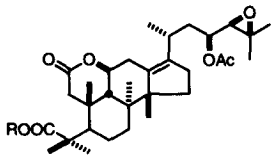
alisol N-23-acetate (**24**)



alismaketone-B 23-acetate (**25**)

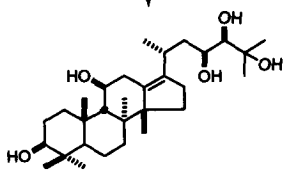


alismaketone-C 23-acetate (**26**)



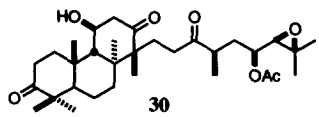
alismalactone 23-acetate (**27**): R=H
3-methylalismalactone
23-acetate (**28**): R=CH_3

Li | $NH_2CH_2CH_2NH_2$



dihydroalisol A (**29**)

$SOCl_2$ | pyridine



30

O_3

alisol B monoacetate (**12**)

Bioassay Methods

Screening for NO Production: Peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice by washing with 6–7 ml of ice-cold PBS, and cells (5 x 10^5 cells/well) were suspended in 200 μl of RPMI 1640 supplemented with 5% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml), and pre-cultured in 96-well microplates at 37°C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing the cells with PBS, and the adherent cells (more than 95% macrophages as determined by Giemza staining) were cultured in the fresh medium containing 10 μg/ml LPS and test compound (1, 3, 10, 30 and 100 μM) for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent.[14] Cytotoxicity was determined by MTT colorimetric assay. Briefly, after 20-h incubation with test compounds, MTT (10 μl, 5 mg/ml in PBS) solution was added to the wells. After 4 h culture, the medium was removed, and isopropanol containing 0.04N HCl was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm (reference: 655 nm). CAPE, L-NMMA, and GED were used as reference compounds. Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration was 0.5%)

Detection of iNOS: In this experiment, peritoneal exudate cells were obtained from the peritoneal cavities of male ddY mice that had been peritoneally injected with 4% TGC medium 4 days previously to get large numbers of cells. Cells (7.5 x 10^6 cells/3 ml/dish) were pre-cultured in culture dishes (6 cm i.d.) for 1 h, and the adherent cells (more than 95% macrophages) were obtained as described above. After washing, culture medium was exchanged for fresh medium containing 5% FCS, 20 μg/ml LPS and test compound for 12 h. Cells were collected in lysis buffer [100 mM NaCl, 10 mM Tris, Complete Mini (1 tab/10 ml), 0.1% Triton X-100, 2 mM EGTA] and sonicated. After determination of protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension were boiled in Laemmli buffer[15]. For SDS-PAGE, aliquots of 50 μg protein of each sample were subjected to electrophoresis in 10% polyacrylamide gel. Following electrophoresis, the proteins were electrically transferred onto nitrocellulose membrane. The membrane were incubated with 5% nonfat dried milk in Tris buffered saline (TBS, 100 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) and probed with a mouse monoclonal IgG (dilution of 1:1000) against iNOS. The blots were washed in TBS and probed with secondary antibody, anti-mouse IgG antibody conjugated to horseradish peroxidase (dilution of 1:5000). Detection was performed using ECL kit and X-ray film (Hyper Film, Amersham).

Results and Discussion

Nitrite, a NO product, was accumulated in the medium after 20-h incubation with LPS. Nitrite concentration in the medium without inhibitors (control group) was 33.6±6.7 μM and that in without LPS (unstimulated group) was 3.0±1.8 μM (mean±S.D. of 31 experiments). Reference compounds, CAPE (an inhibitor of nuclear factor κB activation),[16] L-NMMA (a non-selective inhibitor of NOS),[17] and GED (an inhibitor of iNOS)[18] potently inhibited the nitrite accumulation in the medium.

First, we examined the effects of eight sesquiterpenes (**1–8**) and twenty triterpenes (**9–28**) isolated from the methanolic extract on the nitrite concentration in LPS-activated macrophages. As shown in Table 2, three guaiane-type sesquiterpenes [alismol (**1**) and orientalols A (**3**) and C (**4**)] and a germacrane-type sesquiterpene [germacrene D (**8**)] inhibited the LPS-induced NO production (IC_{50} = 23–68 μM). Whereas, sulfoorientalols b (**5**), c (**6**), and d (**7**) with a sulfonic acid function showed little activity (IC_{50} >100 μM). These results indicated that the polar function of guaiane-type sesquiterpenes reduces the activity, though detail structure-activity relationships are still unclarified.[19]

All *seco*-protostane- and protostane-types triterpenes (3–30 μM) significantly inhibited nitrite accumulation in the medium. But, alisols A (**9**), A monoacetate (**10**), B (**11**), B monoacetate (**12**), E (**15**), G (**18**), K-23-acetate (**21**), and N-23-acetate (**24**) and 11-deoxyalisol B (**13**) showed cytotoxic effects at 30 μM by MTT assay. Therefore, IC_{50} values of them could not be calculated. Whereas, alisols C monoacetate (**14**), F (**17**), H (**19**), I (**20**), L-23-acetate (**22**), and M-23-acetate (**23**) and alismaketone-B 23-acetate (**25**), which possess an oxygen function (*e.g.* =O, -O-) at the 16 position, tended to show less cytotoxicity than others, except for alisol

Table 2. Inhibitory Effects of Constituents from the Rhizome of *A. orientale* on NO Production in LPS-activated Mouse Macrophages

	Inhibition (%)					
compounds	1	3	10	30	100	IC_{50} (μM)
alismol (**1**)	3.4±1.6	17.6±2.7**	27.0±2.9**	57.5±1.2**	97.4±1.4**	23
alismoxide (**2**)	-2.5±1.6	-1.8±1.6	0.4±4.2	12.0±2.6*	33.1±4.1**	>100
orientalol A (**3**)	-4.3±1.8	-3.1±0.8	4.8±3.2	21.2±3.9**	64.1±3.6**	68
orientalol C (**4**)	0.9±1.2	5.0±2.9	14.1±1.2**	29.2±2.1**	74.9±0.9**	53
sulfoorientalol b (**5**)	2.8±1.9	-2.2±2.5	4.9±2.4	16.3±2.8**	21.3±3.2**	>100
sulfoorientalol c (**6**)	8.4±3.9	14.3±2.5**	11.0±2.9*	21.5±1.5**	27.1±2.6**	>100
sulfoorientalol d (**7**)	3.3±2.0	-2.1±3.0	4.3±1.3	12.6±1.2**	24.7±2.4**	>100
germacrene D (**8**)	7.5±1.8*	1.6±1.9	16.7±1.6**	29.0±1.7**	68.5±1.5**	58
alisol A (**9**)	0.2±1.8	21.4±2.2**	26.2±1.7**	84.2±0.3**▼	113.5±0.2**▼	>10
alisol A monoacetate (**10**)	-2.9±1.7	6.4±0.7*	13.4±0.6**	71.0±1.3**▼	111.5±0.8**▼	>10
alisol B (**11**)	13.2±1.4*	21.3±6.3**	36.3±2.5**	108.9±0.2**▼	108.2±0.1**▼	>10
alisol B monoacetate (**12**)	0.0±4.2	13.4±3.8	29.7±3.6**	105.8±0.4**▼	105.0±0.5**▼	>10
11-deoxyalisol B (**13**)	3.9±1.7	0.7±2.8	38.7±1.2**	107.1±0.0**▼	106.5±0.1**▼	>10
alisol C monoacetate (**14**)	2.9±2.0	13.3±1.4**	21.1±2.5**	43.8±2.8**	67.3±1.8**	42
alisol E (**15**)	5.8±2.6*	21.4±1.6**	30.0±0.9**	112.7±0.2**▼	111.2±0.2**▼	>10
alisol E-23-acetate (**16**)	9.2±9.7	10.0±7.2	32.1±5.1**	81.0±0.7**	103.7±0.3**▼	16
alisol F (**17**)	7.5±1.6	14.8±4.0**	40.3±1.5**	69.6±1.4**	99.5±0.6**	15
alisol G (**18**)	8.8±2.2*	21.4±3.0**	29.7±1.7**	104.2±0.6**▼	106.4±0.5**▼	>10
alisol H (**19**)	-5.1±10.7	17.2±1.5	26.7±3.9**	71.3±0.5**	97.2±1.4**	17
alisol I (**20**)	-5.1±3.8	21.7±1.2**	31.2±1.7**	84.2±1.1**	100.4±1.4**	12
alisol K-23-acetate (**21**)	9.9±2.2**	11.9±1.4**	31.1±2.9**	83.6±0.7**▼	113.6±0.2**▼	>10
alisol L-23-acetate (**22**)	17.8±3.9**	27.9±5.4**	54.8±2.1**	86.5±1.6**	104.0±0.3**▼	8.4
alisol M-23-acetate (**23**)	19.0±2.8*	29.7±7.4**	50.8±3.1**	86.3±1.4**	104.1±0.2**▼	9.3
alisol N-23-acetate (**24**)	5.9±4.6	4.0±2.2	32.3±3.1**	116.9±0.1**▼	115.7±0.3**▼	>10
alismaketone-B 23-acetate (**25**)	-4.5±5.9	7.5±4.7	13.5±3.6	42.9±2.8**	90.8±2.3**	36
alismaketone-C 23-acetate (**26**)	2.9±2.5	10.2±0.7	22.7±2.5**	61.0±5.7**	113.8±0.3**▼	23
alismalactone 23-acetate (**27**)	10.3±2.0**	23.2±1.7**	38.2±1.6**	65.6±1.1**	90.7±0.6**	16
3-methylalismalactone 23-acetate (**28**)	-2.6±3.6	17.2±5.5	49.2±6.5**	84.2±3.5**	110.3±0.6**▼	10
CAPE	-1.9±3.3	25.5±3.9**	97.5±1.4**	104.1±0.7**	103.5±1.0**▼	4.0
L-NMMA	4.4±2.0	2.0±1.6	17.7±2.8**	52.3±1.5**	79.2±0.9**	28
GED	39.1±3.1**	76.3±1.5**	102.3±0.4**	105.1±0.3**	105.3±0.3**▼	1.4

$$\text{Inhibition (\%)} = \frac{A-B}{A-C} \times 100$$

A–C: NO_2^- concentration [A: LPS (+), Sample (-); B: LPS (+), Sample (+); C: LPS (-), Sample (-)]

Values represent means±S.E.M.(N=4) and IC_{50} was determined graphically. Asterisks denote significant differences from each control at $*p<0.05$, $**p<0.01$. ▼: Cytotoxic effect was observed.

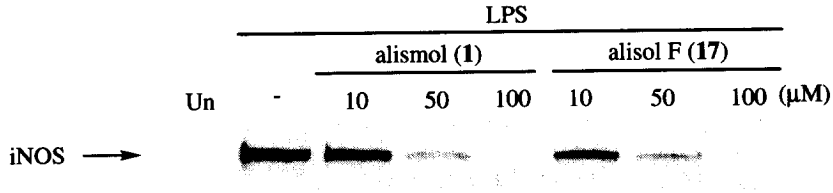


Fig. 1. Effects of Alismol (**1**) and Alisol F (**17**) on iNOS Induction in LPS-activated Mouse Macrophages
Un: unstimulation

K-23-acetate (**21**) with 16,17-epoxide moiety. These results indicated that the 16-oxygen function of alisols might reduce the cytotoxic effects on the macrophages.

Next, we examined the effects of two active constituents (**1** and **17**) on iNOS induction. As shown in Fig. 1, iNOS was detected at 130 kDa after 12-h incubation with LPS by SDS-PAGE-Western blotting. Compounds **1** and **17** at 50 and 100 μM apparently inhibited iNOS induction. These results suggested that the active constituents, at least in part, inhibit the up-stream signaling pathway of iNOS induction thereby preventing NO production.

References and Notes

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12. Two new triterpenes, **25** (0.0019% from the natural medcine) and **26** (0.0020%), were isolated together with **27** and **28** from Alismatis Rhizoma originating in Fukien province, China.
13. **25**: High-resolution positive ion FAB-MS: Calcd for $C_{32}H_{51}O_6$ $(M+H)^+$: 531.3685, Found : 531.3701. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.90 (3H, s, 30-H_3), 1.05 (3H, s, 29-H_3), 1.07 (3H, s, 19-H_3), 1.07 (3H, s, 28-H_3), 1.17 (3H, d, *J*=7.0 Hz, 21-H_3), 1.19 (3H, s, 26-H_3), 1.19 (3H, s, 27-H_3), 1.23 (3H, s, 18-H_3), 1.69 (1H, ddd, *J*=4.6, 4.6, 14.0 Hz, 22β-H), 1.76 (1H, d, *J*=10.7 Hz, 9-H), 1.91 (1H, ddd, *J*=7.0, 7.0, 14.0, 22α-H), 2.06 (3H, s, 23-OAc), 2.78 (1H, m, 20-H), 3.44 (1H, d, *J*=1.5 Hz, 24-H), 3.83 (1H, ddd, *J*=5.5, 10.7, 10.7 Hz, 11-H), 5.13 (1H, ddd, *J*=1.2, 4.6, 9.5 Hz, 16-H), 5.20 (1H, ddd, *J*=1.5, 4.6, 7.0 Hz, 23-H).
26: High resolution positive ion FAB-MS: Calcd for $C_{32}H_{49}O_6$ $(M+H)^+$: 529.3529, Found : 529.3536. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.96 (3H, s, 19-H_3), 1.05 (3H, s, 30-H_3), 1.07 (3H, s, 29-H_3), 1.10 (3H, s, 28-H_3), 1.17 (3H, d, *J*=7.0 Hz, 21-H_3), 1.18 (3H, s, 30-H_3), 1.34 (3H, s, 27-H_3), 1.40 (3H, s, 26-H_3), 2.08(3H, s, 23-OAc), 2.35 (1H, m, 5-H), 2.53 (1H, dd-like, 9-H), 2.79 (1H, d, *J*=8.9 Hz, 24-H), 4.67 (1H, ddd, *J*=3.4, 8.9, 11.3 Hz, 23-H), 5.91 (1H, dd, *J*=3.4, 10.4 Hz, 11-H).
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