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Absolute stereostructures of polypodane- and octanordammarane-type triterpenes with nitric oxide production inhibitory activity from guggul-gum resins

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Abstract—The methanolic extract from guggul-gum resin, the resin of *Balsamodendron mukul*, was found to inhibit nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages ($IC_{50} = 13\ \mu g/mL$). From the methanolic extract, three new polypodane-type triterpenes, myrrhanol B and myrrhanones B and A acetate, and a new octanordammarane-type triterpene, epimansumbinol, were isolated together with 17 known compounds including progesterone and the related steroids. The absolute stereostructures of new triterpenes were elucidated on the basis of chemical and physicochemical evidence. The several constituents showed inhibitory effects on nitric oxide production and induction of inducible nitric oxide synthase.

1. Introduction

Balsamodendron (or *Commiphora*) *mukul* HOOK. (Burseraceae) is widely distributed in India, Sri Lanka, African northeast department, and Arabic southern part. The resin of *B. mukul* (common name: guggul-gum resin), have been extensively used as anti-obese, anti-inflammatory, anti-bacterial, anti-coagulant, and anti-atherosclerosis agents as Ayurvedic, Unani, and Siddha traditional medicines in India and Arabian countries. As chemical constituents of the resin of *B. mukul*, several sterols and terpenoids were isolated.[1–8] Previously, we reported that the 50% aqueous methanolic extract of the resin of *B. mukul* showed potent anti-inflammatory effect on adjuvant-induced air-pouch granuloma in mice and, from this extract, two new polypodane-type triterpenes named myrrhanol A (**5**) and myrrhanone A (**6**) were isolated as the active components.[9] As a continuation of the characterization study of the resin of *B. mukul*, the methanolic extract was found to exhibit inhibitory effect on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages. From the extract, we isolated three new polypodane-type triterpenes called myrrhanol B (**1**) and myrrhanones B (**2**) and A acetate (**3**), and a new octanordammarane-type triterpene termed epimansumbinol (**4**) together with 17 known constituents. This paper deals with the absolute structure elucidation of these new compounds (**1**–**4**). Furthermore, we describe the inhibitory effects of isolated compounds from the methanolic extract of the resin of *B. mukul* on NO production and/or induction of inducible nitric oxide synthase (iNOS) in LPS-activated mouse peritoneal macrophages.

2. Results and discussion

The gum resins of *B. mukul* (collected in Rajastan, India) were extracted with methanol at room temperature (42.5% from the natural medicine). The methanolic extract was found to exhibit inhibitory effects on NO production in LPS-activated mouse peritoneal macrophages ($IC_{50} = 13\ \mu g/mL$). The methanolic extract was subjected to normal- and reversed-phase silica gel column chromatography and finally HPLC to furnish three new polypodane-type triterpenes, myrrhanol B (**1**,

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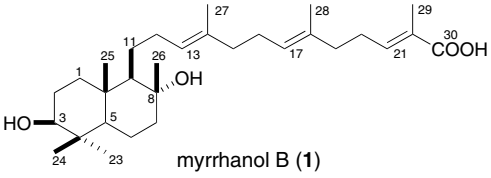
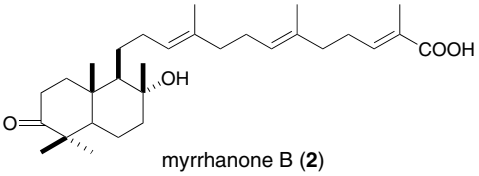
0.38%), myrrhanones B (**2**, 0.38%) and A acetate (**3**, 0.18%), and a new octanordammarane-type triterpene, epimansumbinol (**4**, 0.030%) together with four polypodane-type triterpenes, myrrhanol A[9] (**5**, 1.01%), myrrhanone A[9] (**6**, 0.080%), (8*R*)-3β,8-dihydroxy-polypoda-13*E*,17*E*,21-triene[10,11] (**7**, 0.41%), and (8*R*)-3-oxo-8-hydroxy-polypoda-13*E*,17*E*,21-triene[10] (**8**, 0.18%), nine pregnane-type sterols, progesterone[12] (**9**, 0.10%), 4-pregnene-3,16-dione[5] (**10**, 0.20%), 20*S*-acetyloxy-4-pregnene-3,16-dione[13] (**11**, 0.050%), 4,17(20)-(*cis*)-pregnadiene-3,16-dione[1] (**12**, 0.25%), 4,17(20)-(*trans*)-pregnediene-3,16-dione[1] (**13**, 0.38%), 16β-acetyloxy-pregn-4,17(20)-*trans*-dien-3-one[13] (**14**, 0.12%), 3α-acetyloxy-5α-pregnan-16-one[13] (**15**, 0.060%), 20*R*,22*R*-dihydroxycholest-4-en-3-one[14] (**16**, 0.16%), and guggulsterol-I[5,15] (**17**, 0.080%), three cembrane-type diterpenes, isocembrol[16] (**18** = thunbergol, 0.12%), 4-epiisocembrol[16] (**19**, 0.21%), and mukulol[2,4] (**20**, 0.97%), and a lignan, diayangambin[17] (**21**, 0.050%).

2.1. Stereostructures of myrrhanol B (1) and myrrhanones B (2) and A acetate (3)

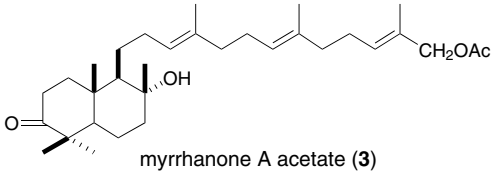
Myrrhanol B (**1**) was isolated as a colorless oil with positive optical rotation ($[\alpha]_D^{28} + 10.6$). The molecu-



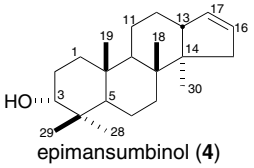
myrrhanol B (**1**)



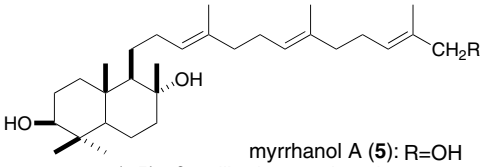
myrrhanone B (**2**)



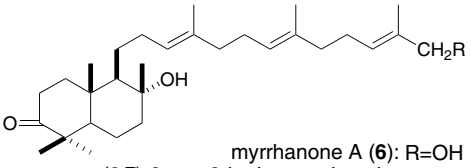
myrrhanone A acetate (**3**)



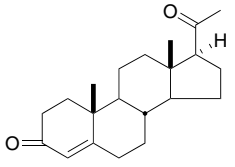
epimansumbinol (**4**)



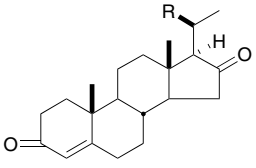
myrrhanol A (**5**): R=OH
(8*R*)-3β,8-dihydroxy-polypoda-13*E*,17*E*,21-triene (**7**): R=H



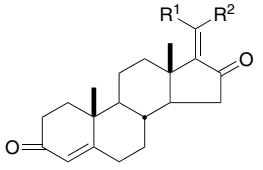
myrrhanone A (**6**): R=OH
(8*R*)-3-oxo-8-hydroxy-polypoda-13*E*,17*E*,21-triene (**8**): R=H



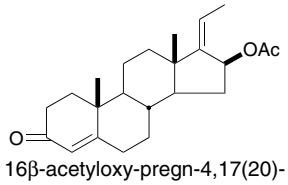
progesterone (**9**)



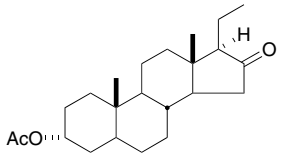
4-pregnene-3,16-dione (**10**): R=H
20*S*-acetyloxy-4-pregnene-3,16-dione (**11**): R=OAc



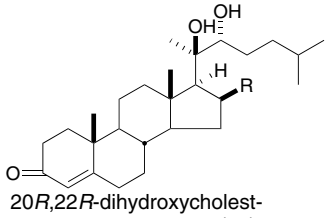
4,17(20)-(*cis*)-pregnadiene-3,16-dione (**12**): R^1=CH_3, R^2=H
4,17(20)-(*trans*)-pregnadiene-3,16-dione (**13**): R^1=H, R^2=CH_3



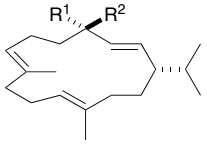
16β-acetyloxy-pregn-4,17(20)-*trans*-dien-3-one (**14**)



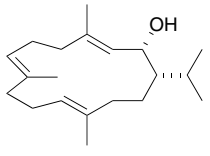
3α-acetyloxy-5α-pregnan-16-one (**15**)



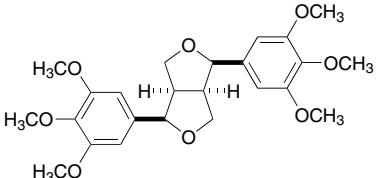
20*R*,22*R*-dihydroxycholest-4-en-3-one (**16**): R=H
guggulsterol-I (**17**): R=OH



isocembrol (**18** =thunbergol): R^1=CH_3, R^2=OH
4-epiisocembrol (**19**): R^1=OH, R^2=CH_3



mukulol (**20**)



diayagambin (**21**)

lar formula $C_{30}H_{50}O_4$ of **1** was determined from the quasimolecular ion peak at m/z 497 $(M+Na)^+$ on positive-ion fast atom bombardment (FAB)-MS and by high-resolution MS measurement. The IR spectrum of **1** showed absorption bands at 3432, 1720, and 1654 cm^{-1} ascribable to hydroxyl, carboxyl, and olefin functions. The ^1H NMR ($CDCl_3$) and ^{13}C NMR (Table 1) spectra of **1**, which were assigned by various NMR experiments,[18] showed signals assignable to seven tertiary methyls [δ 0.76, 0.80, 0.99, 1.15, 1.81 (3H each, all s, 24, 25, 23, 26, 29-H_3), 1.59 (6H, s, 27, 28-H_3)], a methine bearing a oxygen function [δ 3.23 (1H, dd, $J = 4.8$, 10.9 Hz, 3-H)], and three trisubstituted olefins [δ 5.10, 5.13 (1H each, both dd-like, 17, 13-H), 6.82 (1H, dd, $J = 5.8$, 7.0 Hz, 21-H)] together with a quaternary carbon bearing an oxygen function (δc 74.6, 8-C) and an carboxyl carbon (δc 171.7, 30-C). The planer structure of **1** was constructed on the basis of the ^1H–^1H correlation spectroscopy (^1H–^1H COSY) and heteronuclear multiple bond correlation (HMBC) experiments as shown in Figure 1. Thus, the ^1H–^1H COSY experiment indicated the presence of partial structures in bold lines. In the HMBC experiment of **1**, long-range correlations were observed between the following protons and carbons: 21-H and 30-C; 23-H_3 and 3, 4, 5, 24-C; 24-H_3 and 3, 4, 5, 23-C; 25-H_3 and 1, 5, 9, 10-C; 26-H_3 and 7, 8, 9-C; 27-H_3 and 13, 14, 15-C; 28-H_3 and 17, 18, 19-C; 29-H_3 and 21, 22, 30-C, so that the connectivities of the quaternary carbons in **1** was clarified. In addition, the relative stereostructure of **1** was characterized by a nuclear Overhauser enhancement spectrometry (NOESY), which showed the NOE correlations between the following proton pairs: 3-H and 5-H, 23-H_3; 5-H and 9-H, 23-H_3; 12-H_2 and 27-H_3; 16-H_2 and 28-H_3; 20-H_2 and 29-H_3; 24-H_3 and 25-H_3; 25-H_3 and 26-H_3 (Fig. 1).

Table 1. ^{13}C NMR data of myrrhanol B (**1**), myrrhanones B (**2**) and A acetate (**3**), and epimansumbinol (**4**)

	1	2	3	4
C-1	37.9	38.1	38.4	33.7
C-2	27.1	33.8	26.4	25.5
C-3	78.8	216.9	216.6	76.3
C-4	38.8	47.4	47.5	37.7
C-5	55.0	55.0	55.2	49.7
C-6	20.3	21.2	21.5	18.4
C-7	44.2	43.4	43.9	35.4
C-8	74.6	74.0	73.6	40.2
C-9	61.3	60.2	60.4	50.7
C-10	38.8	38.5	38.6	37.5
C-11	25.7	25.7	25.8	21.8
C-12	31.5	31.2	31.2	24.0
C-13	125.2	125.2	124.6	47.6
C-14	133.3	133.4	135.3	53.1
C-15	38.0	37.9	39.7	39.9
C-16	26.1	26.1	26.6	129.8
C-17	125.2	124.9	124.5	134.0
C-18	134.3	134.6	134.3	18.4
C-19	39.4	39.3	39.1	16.0
C-20	27.0	27.0	26.1	
C-21	143.8	144.0	129.8	
C-22	125.3	127.1	130.4	
C-23	28.2	26.2	26.4	
C-24	15.4	21.2	21.4	
C-25	15.6	14.7	14.9	
C-26	23.7	23.4	23.7	
C-27	16.2	16.0	16.3	
C-28	15.9	15.8	16.1	28.4
C-29	12.2	12.0	14.1	22.2
C-30	171.7	172.2	70.4	17.2
$CH_3\underline{C}O$–			170.9	
$\underline{C}H_3CO$–			21.1	

68 MHz, $CDCl_3$.

Finally, the absolute stereostructure of **1** was determined by the application of modified Mosher's method.[19] Namely, treatment of **1** with ethereal diazomethane ($CH_2N_2{\cdot}Et_2O$) gave the methyl ester derivative (**1a**),

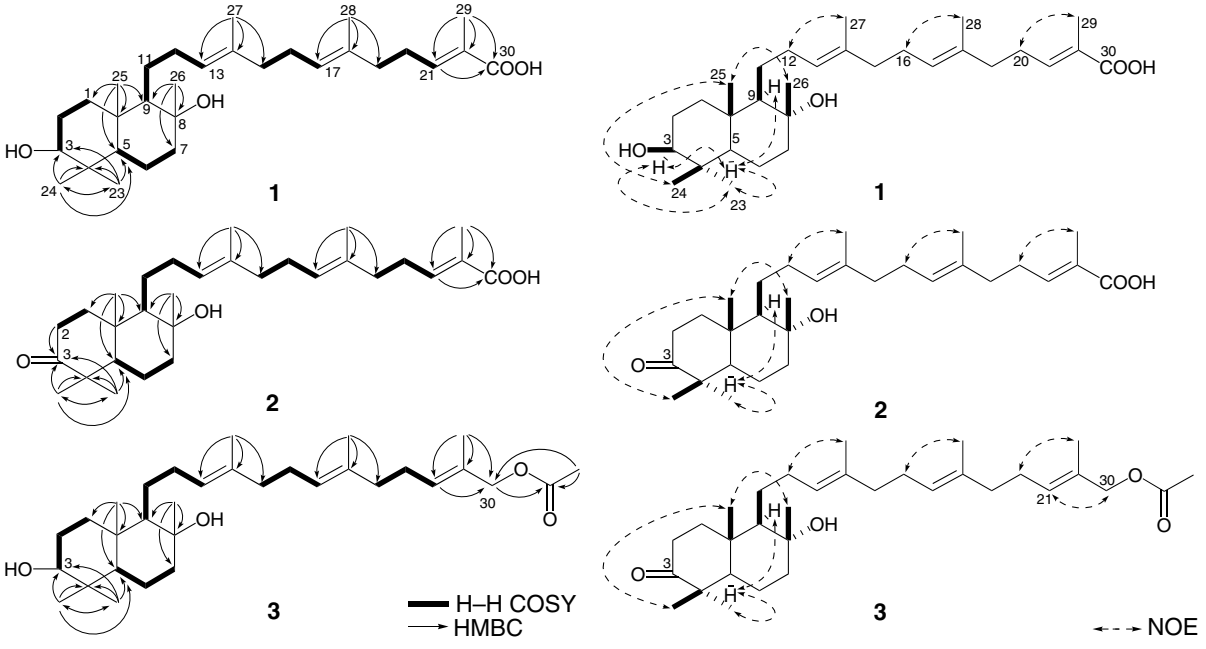


Figure 1. H–H COSY, HMBC, and NOESY experiments of **1**–**3**.

which was further treated with (*R*)- or (*S*)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and 4-dimethylaminopyridine (4-DMAP) to furnish the 3-mono-(*R*)-MTPA ester (**1b**) and 3-mono-(*S*)-MTPA ester (**1c**), respectively. As shown in Figure 2, the proton signals due to protons attached the 1, 2, and 25-positions in **1c** were observed at higher fields as compared to those of **1b** ($\Delta\delta$: negative), while the signal due to the 23 and 24-positions in **1c** were observed at lower fields as compared to those of **1b** ($\Delta\delta$: positive). Consequently, the absolute configuration of the 3-position was determined to be *S* and the absolute stereostructure of myrrhanol B was determined to be (3*S*,5*R*,8*R*,9*R*,10*S*)-3,8-dihydroxypolypoda-13*E*,17*E*,21*E*-trien-30-oic acid (**1**).

Myrrhanone B (**2**) was also isolated as a colorless oil with positive optical rotation ($[\alpha]_D^{28}$ + 13.5). A quasimolecular ion peak was observed at *m/z* 495 $(M + Na)^+$ in the positive-ion FAB-MS and the molecular formula of **2** was defined as $C_{30}H_{48}O_4$ from the high-resolution MS analysis. The IR spectrum of **2** showed absorption bands at 3432, 1720, 1709, and 1654 cm^{-1} due to hydroxyl, carboxyl, carbonyl, and olefin functions. The proton and carbon signals in the ^1H NMR ($CDCl_3$) and ^{13}C NMR (Table 1) spectra[18] of **2** showed signals due to seven tertiary methyls [δ 0.95, 1.02, 1.09, 1.21, 1.59, 1.61, 1.82 (s, 25, 24, 23, 26, 28, 27, 29-H_3)], three trisubstituted olefins [δ 5.12, 5.14 (dd-like, 17, 13-H), 6.84 (dd, J = 6.1, 7.2 Hz, 21-H)] together with a quaternary carbon bearing an oxygen function (δ*c* 74.0, 8-C), an carboxyl carbon (δ*c* 172.2, 30-C), and an carbonyl carbon (δ*c* 216.9, 3-C). The ^1H and ^{13}C NMR data of **2** resembled those of **1**, expect for the signals due to the 3-keto group. Finally, **2** was chemically related with **1**. Namely, diazomethane methylation of **2** followed by reduction with sodium borohydride ($NaBH_4$) yielded **1a**. Consequently, absolute stereostructure of myrrhanone B was determined to be (5*R*,8*R*,9*R*,10*S*)-3-oxo-8-hydroxypolypoda-13*E*,17*E*,21*E*-trien-30-oic acid (**2**).

Myrrhanone A acetate (**3**) was isolated as a colorless oil with positive optical rotation ($[\alpha]_D^{23}$ + 17.1). The molecular formula of **3**, $C_{32}H_{52}O_4$, was determined from the quasimolecular ion peak observed at *m/z* 523 $(M + Na)^+$ in the positive-ion FAB-MS and by high-resolution MS measurement. The IR spectrum of **3** showed absorption bands at 3496, 1738, 1705, and 1654 cm^{-1} ascribable to hydroxyl, ester carbonyl, carbonyl, and olefin functions. The proton and carbon signals in the ^1H NMR ($CDCl_3$) and ^{13}C NMR (Table 1) spectra[18] of **3** showed signals assignable to seven tertiary methyls [δ 0.95, 1.02, 1.10, 1.19, 1.65 (3H each, all s, 25, 24, 23, 26, 29-H_3), 1.60 (6H, s, 27, 28-H_3)], an acetyl group [δ 2.07 (s), δ*c* 170.9, 21.1 (-OAc)], a methylene bearing an oxygen function [δ 4.44 (2H, s, 30-H_2)], and three trisubstituted olefins [δ 5.11, 5.16, 5.44 (1H each, all dd-like, 17, 13, 21-H)] together with a quaternary carbon bearing a oxygen function (δ*c* 73.6, 8-C) and an carbonyl carbon (δ*c* 216.6, 3-C). The proton and carbon signals in ^1H and ^{13}C NMR spectra of **3** were superimposable on those of myrrhanone A (**6**),[9] except for the signal due to the acetyl group. In the HMBC experiment of **3**, a long-range correlation was observed between the acetyl methyl proton and 30-carbon as shown in Figure 1. Finally, **3** was obtained by acetylation of **6** with acetic anhydride (Ac_2O) in pyridine. Thus, the stereostructure of **3** was elucidated to be myrrhanone A 30-acetate.

2.2. Absolute stereostructure of epimansumbinol (4)

Epimansumbinol (**4**) was isolated as colorless oil with negative optical rotation ($[\alpha]_D^{24}$ − 28.1). The molecular formula $C_{22}H_{36}O$ of **4** was determined from the molecular ion peak at *m/z* 316 (M^+) in addition to the fragment ion peaks at *m/z* 298 $(M^+ - H_2O)$ and 190 (base peak) in the electron impact (EI)-MS and by high-resolution MS measurement. The IR spectrum of **4** showed absorption bands ascribable to hydroxyl and olefin functions at 3432 and 1690 cm^{-1}. The ^1H NMR ($CDCl_3$) and ^{13}C NMR (Table 1) spectra[18] of **4** showed signals

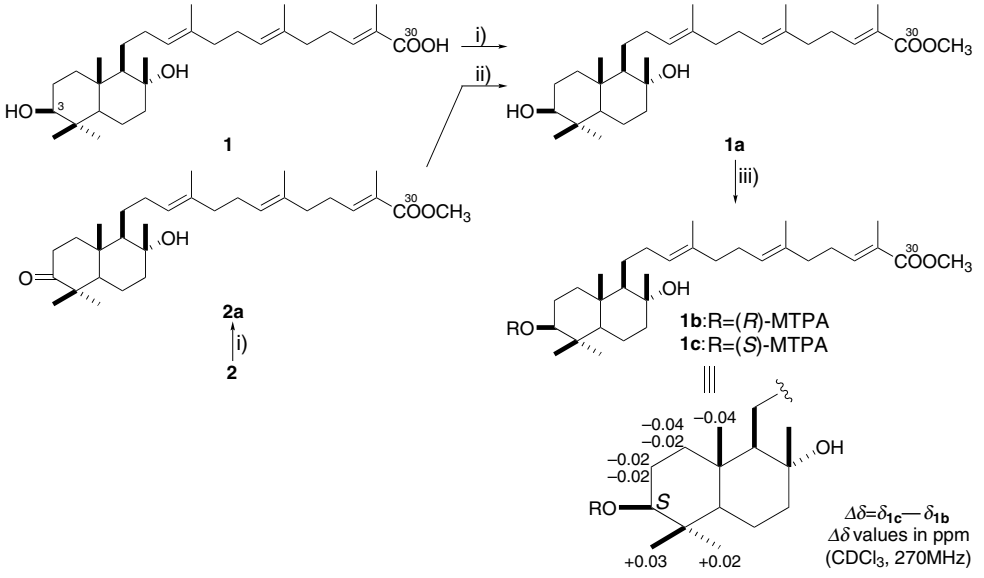


Figure 2. Reactions and conditions: (i) CH_2N_2·Et_2O/MeOH, rt; (ii) $NaBH_4$/MeOH, rt; (iii) (*R*)- [or (*S*)]-MTPA, EDC·HCl, 4-DMAP/CH_2Cl_2, rt.

assignable to five methyls [δ 0.84, 0.87, 0.94 (3H each, all s, 29, 19, 28-H_3), 1.01 (6H, s, 18, 30-H_3)], a methine bearing the oxygen function [δ 3.39 (1H, br s, 3-H)], and two olefins [δ 5.57, 5.64 (1H each, both m, 17, 16-H)] together with seven methylenes (1, 2, 6, 7, 11, 12, 15-C), three methines (5, 9, 13-C), and four quaternary carbons (4, 8, 10, 14-C). The proton and carbon signals in the ^1H and ^{13}C NMR spectra of **4** were similar to those of mansumbinol (**4′**),[20] except for the signals due to the 3-hydroxyl group. The planar structure of **4** was clarified by ^1H–^1H COSY and HMBC experiments as shown in Figure 3. In addition, the NOE correlations of **4** were observed between the following proton pairs: 3-H and 29-H_3; 5-H and 9-H, 28-H_3; 9-H and 30-H_3; 13-H and 18-H_3; 18-H_3 and 19-H_3; 19-H_3 and 29-H_3 (Fig. 3). Furthermore, oxidation of **4** with pyridinium chlorochromate (PCC) furnished mansumbinone (**4a**),[20] whose absolute stereostructure has been reported. On the basis of these observations, the absolute stereostructure of **4** was determined as a 3-epimer of mansumbinol (**4′**).

2.3. Inhibitory effects on NO production and iNOS induction in LPS-activated mouse peritoneal macrophages

The inorganic free radical NO 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 NO synthase (NOS). In the family of NOS, iNOS is particularly involved in pathological aspects with overproduction of NO, and can be expressed in response to pro-inflammatory agents such as interleukin-1β, tumor necrosis factor-α, and LPS in various cell types including macrophages, endothelial cells, and smooth muscle cells. As a part of our studies to characterize the bioactive components of natural medicines, we have reported various NO production inhibitors; that is higher unsaturated fatty acids,[21] polyacetylenes,[22,23] coumarins,[22] flavonoids,[23,24] stilbenes,[25,26] lignans,[27,28] sesquiterpenes,[29–35] diterpenes,[36,37] triterpenes,[38–40] diarylheptanoids,[41,42] cyclic peptides,[40] and alkaloids.[43,44] As a continuation of these studies, the effects of the constituents from the resin of *B. mukul* on NO production from LPS-activated macrophages were examined, and the results were summarized in Table 2. The isolated constituents significantly inhibited the accumulation of nitrite, a product of NO, in the medium. Among them, three polypodane-type triterpenes, myrrhanols B (**1**, $IC_{50} = 61\,\mu M$), A (**5**, 25 μM), and myrrhanone A (**6**, 35 μM), six pregnane-type sterols, progesterone (**9**, 11 μM), 4-pregnene-3,16-dione (**10**, 40 μM), 20*S*-acetyloxy-4-pregnene-3,16-dione (**11**, 56 μM), 4,17(20)-(*cis*)-pregnadiene-3,16-dione (**12**, 10 μM), 4,17(20)-(*trans*)-pregnadiene-3,16-dione (**13**, 8.2 μM), and 20*R*,22*R*-dihydroxycholest-4-en-3-one (**16**, 20 μM), and a cembrane-type diterpene, mukulol (**20**, 24 μM) showed stronger inhibitory effects without cytotoxic effects in the MTT assay.

Next, the effects of two active principle constituents (**5**, **20**) on iNOS induction were examined. iNOS was detected at 130 kDa after a 20 h incubation with LPS by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE)-Western blotting analysis (Fig. 4).[24,28,40] iNOS induction of LPS-activated macrophages was shown to be suppressed by these two active constituents (**5**, **20**) in a concentration-dependent manner. These results suggested that principal constituents from the resin of *B. mukul* (**5**, **20**) inhibited NO production due to their inhibitory activities against induction of iNOS in LPS-activated macrophages.

Previously, progesterone, a female sex steroid hormone, was reported to inhibit NO production and iNOS induction in murine macrophages.[45,46] In agreement with those reports, not only progesterone (**9**) but also other five pregnane-type sterols (**10**–**13**, **16**) showed inhibitory effects on NO production ($IC_{50} = 8.2$–$56\,\mu M$).

The inhibitory activities of these components against NO production in LPS-activated macrophages substantiated the traditional effects of this herbal medicine for the treatment of inflammation.

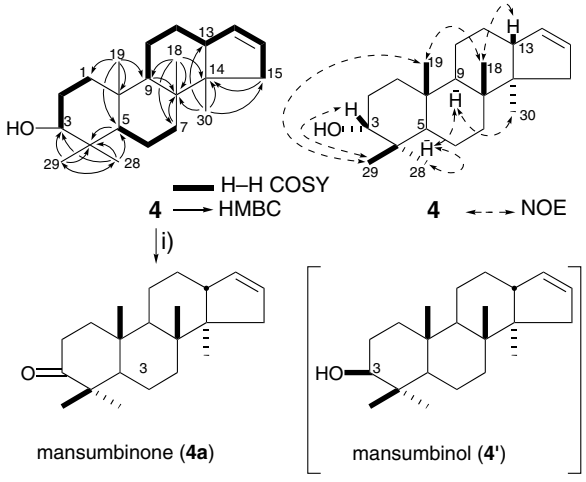


Figure 3. Reactions and conditions: (i) PCC/CH_2Cl_2, 0 °C.

3. Experimental

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter ($l = 5$ cm); IR spectra, Shimadzu FTIR-8100 spectrometer; EI-MS and high-resolution MS, JEOL JMS-GCMATE mass spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; ^1H NMR spectra, JEOL EX-270 (270 MHz) spectrometer; ^{13}C NMR spectra, JEOL EX-270 (68 MHz) spectrometer with tetramethylsilane as an internal standard; HPLC detector, Shimadzu RID-6A refractive index detector.

The following experimental conditions were used for chromatography: normal-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical,

Table 2. Inhibitory effects of MeOH extract and constituents from the resin of *B. mukul* on NO production in LPS-activated mouse peritoneal macrophages

	Inhibition (%)						IC_{50} (μg/mL)
	0 μg/mL	1 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	
MeOH extract	0.0 ± 2.8	10.5 ± 2.7[b]	18.9 ± 1.7[b]	39.1 ± 1.6[b]	93.8 ± 1.2[b]	99.1 ± 0.1[b,c]	13
	Inhibition (%)						IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	
Myrrhanol B (**1**)	0.0 ± 6.3	5.8 ± 5.9	10.9 ± 3.1	3.1 ± 4.2[a]	30.9 ± 2.6[b]	63.5 ± 0.5[b]	61
Myrrhanone B (**2**)	0.0 ± 1.3	3.3 ± 1.4	3.6 ± 1.0	4.5 ± 0.9	12.6 ± 0.6[b]	45.7 ± 0.8[b]	
Myrrhanone A acetate (**3**)	0.0 ± 6.4	−1.5 ± 1.7	−1.6 ± 7.4	−1.7 ± 7.4	21.5 ± 7.1[b]	96.3 ± 0.8[b,c]	
Epimansumbinol (**4**)	0.0 ± 0.6	3.3 ± 1.1	0.1 ± 0.9	6.9 ± 1.8	19.3 ± 1.6[b]	95.3 ± 2.7[b,c]	
Myrrhanol A (**5**)	0.0 ± 6.4	5.9 ± 1.9	4.2 ± 4.4	17.6 ± 1.4[b]	62.9 ± 2.3[b]	99.2 ± 0.2[b,c]	25
Myrrhanone A (**6**)	0.0 ± 1.0	−1.5 ± 1.7	0.0 ± 1.5	8.7 ± 0.7	43.2 ± 1.3[b]	99.6 ± 0.1[b]	35
7	0.0 ± 4.6	1.4 ± 2.5	2.6 ± 4.6	9.9 ± 3.3	16.7 ± 3.1[a]	33.7 ± 2.3[b]	
8	0.0 ± 1.9	−0.4 ± 2.0	1.4 ± 2.1	1.7 ± 0.7	23.5 ± 1.0[b]	90.5 ± 0.8[b,c]	
Progesterone (**9**)	0.0 ± 4.5	22.7 ± 2.7[b]	27.7 ± 2.8[b]	46.9 ± 2.2[b]	73.2 ± 1.6[b]	100.1 ± 0.2[b,c]	11
4-Pregnene-3,16-dione (**10**)	0.0 ± 1.8	1.2 ± 3.0	6.3 ± 2.7	6.7 ± 4.4	37.1 ± 4.0[b]	71.3 ± 1.9[b]	40
11	0.0 ± 2.1	0.0 ± 2.9	6.2 ± 1.6	6.6 ± 2.3	28.2 ± 3.7[b]	70.0 ± 1.8[b]	56
4,17(20)-(*cis*)-Pregnadiene-3,16-dione (**12**)	0.0 ± 2.1	14.9 ± 3.4	15.0 ± 5.3	48.9 ± 6.8[b]	90.2 ± 0.7[b]	98.9 ± 0.5[b]	10
4,17(20)-(*trans*)-Pregnadiene-3,16-dione (**13**)	0.0 ± 2.8	15.4 ± 3.5[a]	24.0 ± 4.6[b]	57.3 ± 4.8[b]	85.3 ± 2.9[b]	100.9 ± 0.2[b]	8.2
3α-Acetyloxy-5α-pregna-16-one (**15**)	0.0 ± 0.7	−3.2 ± 1.4	−3.0 ± 3.3	4.8 ± 1.3	26.8 ± 1.3[b]	100.4 ± 0.1[b,c]	
20*R*,22*R*-Dihydroxycholest-4-ene-3-one (**16**)	0.0 ± 1.4	3.9 ± 4.7	14.3 ± 1.7[b]	22.0 ± 1.1[b]	74.4 ± 4.0[b]	98.1 ± 0.2[b,c]	20
Guggulsterol-I (**17**)	0.0 ± 1.7	1.9 ± 1.9	3.7 ± 1.5	2.4 ± 2.0	3.5 ± 2.3	0.8 ± 0.2	
Isocembrol (**18**)	0.0 ± 0.6	1.3 ± 3.5	1.4 ± 1.8	5.8 ± 1.5	28.6 ± 2.6[b]	99.8 ± 0.1[b,c]	
4-Epiisocembrol (**19**)	0.0 ± 3.0	−4.4 ± 1.0	3.7 ± 2.2	14.1 ± 1.6[b]	31.5 ± 1.3[b]	98.6 ± 0.2[b,c]	
Mukulol (**20**)	0.0 ± 2.1	2.3 ± 2.4	6.6 ± 1.2	17.5 ± 1.5[b]	61.4 ± 6.5[b]	99.9 ± 0.2[b,c]	24
Diayagambin(**21**)	0.0 ± 0.9	0.0 ± 2.2	2.7 ± 1.8	4.9 ± 2.4	10.1 ± 3.6[a]	65.7 ± 1.4[b,c]	
L-NMMA	0.0 ± 4.0	5.9 ± 0.9	10.3 ± 3.7	15.0 ± 1.6[b]	34.1 ± 3.2[b]	63.1 ± 1.2[b]	57
GED	0.0 ± 0.0	6.2 ± 0.1	24.4 ± 0.1[b]	57.9 ± 0.1[b]	89.7 ± 0.2[b]	97.9 ± 0.0[b]	7.4
CAPE	0.0 ± 0.7	3.8 ± 0.1	1.4 ± 0.1	68.2 ± 0.0[b]	93.7 ± 0.2[b]	99.6 ± 0.0[b,c]	15

Each value represents the mean ± S.E.M. ($N = 4$).
Significantly different from the control, [a]$p < 0.05$, [b]$p < 0.01$.
[c] Cytotoxic effect was observed.

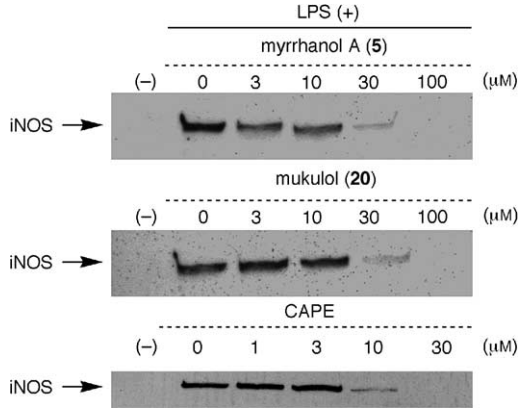


Figure 4. Effects of constituents (**5**, **20**) and CAPE on iNOS induction in LPS-activated mouse macrophages.

Ltd, 150–350 mesh); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100–200 mesh); TLC, pre-coated TLC plates with Silica gel $60F_{254}$ (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, pre-coated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

3.1. Extraction and isolation

The resin of *B. mukul* [800 g, collected in Rajastan, India and purchased from Sharangdhar Pharmaceuticals PTV. Ltd. (Pune, India)] was finely minced and extracted with methanol at room temperature (1 day × 3 times). Evaporation of the solvent under reduced pressure gave the MeOH extract (340 g, 42.5% from natural medicine).

The MeOH extract (70.0 g) was subjected to normal-phase silica gel column chromatography [2.0 kg, *n*-hexane–AcOEt (10: 1 → 5: 1 → 2: 1 → 1: 1) → $CHCl_3$–MeOH–H_2O (50:3:1, lower layer → 20:3:1, lower layer) → MeOH] to afford 11 fractions {Fr. 1 (1.8 g), Fr. 2 [1.6 g = mukulol (**20**, 0.97%)], Fr. 3 (1.1 g), Fr. 4 (1.6 g), Fr. 5 (6.3 g), Fr. 6 (3.3 g), Fr. 7 (20.5 g), Fr. 8 (8.0 g), Fr. 9 (1.7 g), Fr. 10 (14.5 g), Fr. 11 (9.6 g)}. Fraction 3 (1.1 g) was further separated by HPLC [YMC-pack ODS-A, 250 × 20 mm i.d., MeOH–H_2O (85:15)] to give epimansumbinol (**4**, 50 mg, 0.030%), isocembrol (**18**, 198 mg, 0.12%), and 4-epiisocembrol (**19**, 354 mg, 0.21%). Fraction 4 (1.6 g) was separated by HPLC [YMC-pack ODS-

A, MeOH–H_2O (85:15)] to give 3α-acetyloxy-5α-pregnan-16-one (**15**, 99 mg, 0.060%). Fraction 5 (1.0 g) was purified by HPLC [YMC-pack ODS-A, MeOH–H_2O (95:5)] to furnish (8*R*)-3β,8-dihydroxy-polypoda-13*E*,17*E*,21-triene (**7**, 114 mg, 0.41%) and (8*R*)-3-oxo-8-dihydroxy-polypoda-13*E*,17*E*,21-triene (**8**, 50 mg, 0.18%). Fraction 6 (0.5 g) was purified by HPLC [YMC-pack ODS-A, MeOH–H_2O (85:15)] to give myrrhanone A acetate (**3**, 50 mg, 0.18%), 16β-acetyloxy-pregn-4,17(20)-*trans*-dien-3-one (**14**, 33 mg, 0.12%). Fraction 7 (2.8 g) was purified by HPLC [Develosil C30-UG-5, 250×20 mm i.d., MeOH–H_2O (90:10)] to furnish nine fractions {Fr. 7-1 [85 mg = myrrhanone B (**2**, 0.38%)], Fr. 7-2 (120 mg), Fr. 7-3 (200 mg), Fr. 7-4 (300 mg), Fr. 7-5 (200 mg), Fr. 7-6 (30 mg), Fr. 7-7 [85 mg = myrrhanol B (**1**, 0.38%)], Fr. 7-8 (220 mg), Fr. 7-9 (35 mg = 20*R*,22*R*-dihydroxycholest-4-en-3-one (**16**, 0.16%), Fr. 7-10 (1525 mg)}. Fraction 7-3 (200 mg) was further purified by HPLC [YMC-pack ODS-A, MeOH–H_2O (80:20)] to give 4,17(20)-(*cis*)-pregnadiene-3,16-dione (**12**, 55 mg, 0.25%). Fraction 7-4 (300 mg) was subjected to HPLC [YMC-pack ODS-A, MeOH–H_2O (80:20) and YMC-pack Ph, 250×20 mm i.d., CH_3CN–H_2O (50:50)] to give progesterone (**9**, 23 mg, 0.10%) and 4,17(20)-(*trans*)-pregnadiene-3,16-dione (**13**, 84 mg, 0.38%). Fraction 7-5 (200 mg) was further purified by HPLC [YMC-pack ODS-A, MeOH–H_2O (85:15)] to give 4-pregnene-3,16-dione (**10**, 45 mg, 0.20%). Fraction 8 (8.0 g) was subjected to HPLC [YMC-pack ODS-A, MeOH–H_2O (85:15)] to furnish four fractions {Fr. 8-1 (2.50 g), Fr. 8-2 (1.50 g), Fr. 8-3 (0.64 g), Fr. 8-4 [1.67 g = myrrhanol A (**5**, 1.01%)], Fr. 8-5 (1.69 g)}. Fraction 8-1 (2.50 g) was further separated by HPLC [YMC-pack ODS-A, MeOH–H_2O (80:20)] to give 20*S*-acetyloxy-4-pregnene-3,16-dione (**11**, 80 mg, 0.050%). Fraction 8-3 (0.64 g) was recrystallized in AcOEt to furnish guggulsterol-I (**17**, 130 mg, 0.080%). Fraction 10 (10.0 g) was separated by normal-phase silica gel column chromatography [300 g, *n*-hexane–AcOEt (10:1 → 5:1 → 2:1 → 1:1) → MeOH] to afford five fractions {Fr. 10-1 (0.20 g), Fr. 10-2 [0.13 g = myrrhanone A (**6**, 0.080%), Fr. 10-3 (0.05 g), Fr. 10-4 (1.40 g), Fr. 10-5 (7.00 g), Fr. 10-6 (1.22 g)}. Fraction 10-4 (1.40 g) was subjected by HPLC [YMC-pack ODS-A, MeOH–H_2O (85:15)] to give diayangambin (**21**, 82 mg, 0.050%).

These constituents were identified by comparison of their physical data with reported values (**7**, **8**, **10**–**21**)[1,2,4–5,10–11,13–17] or with commercially obtained sample (**9**).[12]

Myrrhanol B (**1**): Colorless oil, $[\alpha]_D^{28}$ + 10.6 (*c* 1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{30}H_{50}O_4Na$ $(M+Na)^+$: 497.3607. Found: 497.3602. IR (KBr): 3432, 2938, 1720, 1654, 1457, 1387 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ: 0.76, 0.80, 0.99, 1.15, 1.81 (3H each, all s, 24, 25, 23, 26, 29-H_3), 0.90 (1H, d-like, 5-H), 1.02 (1H, dd-like, 9-H), 1.15, 1.70 (1H each, both m, 1-H_2), 1.26, 1.42 (1H each, both m, 11-H_2), 1.32 (1H, m, 6-H), 1.44, 1.90 (1H each, both m, 7-H_2), 1.59 (6H, s, 27, 28-H_3), 1.65–1.69 (3H, m, 2-H2, 6-H), 2.00 (2H, m, 19-H_2), 2.11 (6H, m, 12, 15, 16-H_2), 2.30 (2H, m, 20-H_2), 3.23 (1H, dd, *J* = 4.8, 10.9 Hz, 3-H), 5.10, 5.13 (1H each, both dd-like, 17, 13-H), 6.82 (1H, dd, *J* = 5.8, 7.0 Hz, 21-H). ^{13}C NMR (68 MHz, $CDCl_3$) δ*c*: given in Table 1. Positive-ion FAB-MS: *m*/*z* 497 $(M+Na)^+$.

Myrrhanone B (**2**): Colorless oil, $[\alpha]_D^{28}$ + 13.5 (*c* 1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{30}H_{48}O_4Na$ $(M+Na)^+$: 495.3450. Found: 495.3461. IR (KBr): 3432, 2930, 1720, 1709, 1654, 1456, 1385 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ: 0.95, 1.02, 1.09, 1.21, 1.59, 1.61, 1.82 (3H each, all s, 25, 24, 23, 26, 28, 27, 29-H_3), 1.16 (1H, dd, *J* = 4.0, 4.0 Hz, 9-H), 1.28, 1.50 (1H each, both m, 11-H_2), 1.28, 1.54 (1H each, both m, 6-H_2), 1.46 (1H, m, 5-H), 1.50, 1.90 (1H each, both m, 7-H_2), 1.54, 1.90 (1H each, both m, 1-H_2), 2.00 (2H, m, 19-H_2), 2.12 (6H, m, 12, 15, 16-H2), 2.30 (2H, m, 20-H_2), 2.40, 2.60 (1H each, both m, 2-H_2), 5.12, 5.14 (1H each, both dd-like, 17, 13-H), 6.84 (1H, dd, *J* = 6.1, 7.2 Hz, 21-H). ^{13}C NMR (68 MHz, $CDCl_3$) δ*c*: given in Table 1. Positive-ion FAB-MS: *m*/*z* 495 $(M+Na)^+$.

Myrrhanone A acetate (**3**): Colorless oil, $[\alpha]_D^{23}$ + 17.1 (*c* 1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{32}H_{52}O_4Na$ $(M+Na)^+$: 523.3757. Found: 523.3763. IR (KBr): 3496, 2938, 1738, 1705, 1654, 1456, 1385 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ: 0.95, 1.02, 1.10, 1.19, 1.65 (3H each, all s, 25, 24, 23, 26, 29-H_3), 1.12 (1H, dd, *J* = 3.9, 3.9 Hz, 9-H), 1.30, 1.32 (1H each, both m, 11-H2), 1.38, 1.60 (1H each, both m, 6-H_2), 1.45 (1H, m, 5-H), 1.45, 1.87 (1H each, both m, 7-H_2), 1.52, 1.94 (1H each, both m, 1-H_2), 1.60 (6H, s, 27, 28-H_3), 2.00 (4H, m, 15, 19-H_2), 2.07 (3H, s, -OAc), 2.10 (6H, m, 12, 16, 20-H_2), 2.40, 2.60 (1H each, both m, 2-H_2), 4.44 (2H, s, 30-H_2), 5.11, 5.16, 5.44 (1H each, all dd-like, 17, 13, 21-H). ^{13}C NMR (68 MHz, $CDCl_3$) δ*c*: given in Table 1. Positive-ion FAB-MS: *m*/*z* 523 $(M+Na)^+$.

Epimansumbinol (**4**): Colorless oil, $[\alpha]_D^{24}$ − 28.1 (*c* 1.00, MeOH). High-resolution EI-MS: Calcd for $C_{22}H_{36}O$ (M^+): 316.2766. Found: 316.2711. IR (film): 3432, 2943, 1690, 1451, 1387 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ: 0.84, 0.87, 0.94 (3H each, all s, 29, 19, 28-H_3), 1.01 (6H, s, 18, 30-H_3), 1.28 (1H, m, 5-H), 1.38–1.45 (6H, m, 1, 6, 7-H_2), 1.40, 1.70 (1H each, both m, 12-H_2), 1.50 (2H, m, 11-H_2), 1.58 (1H, m, 9-H), 1.58, 1.96 (1H each, both m, 2-H_2), 1.62 (1H, m, 15-H), 2.34 (1H br d, *J* = *ca*. 15 Hz, 15-H), 2.70 (1H, br d, *J* = *ca*. 13 Hz, 13-H), 3.39 (1H, br s, 3-H), 5.57, 5.64 (1H each, both m, 17, 16-H). ^{13}C NMR (68 MHz, $CDCl_3$) δ*c*: given in Table 1. EI-MS *m*/*z* (%): 316 (M^+, 14), 298 ($M^+ - H_2O$, 11), 190 (100).

3.2. Methylation of myrrhanol B (1) and myrrhanone B (2)

A solution of **1** (15.6 mg) in MeOH (2.0 mL) was treated with ethereal diazomethane (CH_2N_2·Et_2O, ca. 10.0 mL) until the yellow color persisted. The solution was stirred at room temperature (25 °C) for 30 min and then the solvent was removed under reduced pressure to furnish a residue. The residue was purified by normal-phase

silica gel column chromatography [1.0 g, *n*-hexane–AcOEt (4: 1)] to give **1a** (12.0 mg, 75%). Through a similar procedure, **2a** (12.0 mg, 58%) was prepared from **2** (20.0 mg).

1a: Colorless oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ: 0.76, 0.80, 0.99, 1.14, 1.83 (3H each, all s, 24, 25, 23, 26, 29-H_3), 0.90 (1H, d-like, 5-H), 1.04 (1H, m, 9-H), 1.19, 1.73 (1H each, both m, 1-H_2), 1.30, 1.45 (1H each, both m, 11-H_2), 1.35 (1H, m, 6-H), 1.45, 1.88 (1H each, both m, 7-H_2), 1.61 (6H, s, 27, 28-H_3), 1.62–1.70 (3H, m, 2-H_2, 6-H), 2.01 (2H, m, 19-H_2), 2.10 (6H, m, 12, 15, 16-H_2), 2.27 (2H, m, 20-H_2), 3.24 (1H, dd-like, 3-H), 3.73 (3H, s, –OCH_3), 5.16 (2H, dd-like, 13, 17-H), 6.74 (1H, dd-like, 21-H). Positive-ion FAB-MS: m/z 511 $(M + Na)^+$.

2a: Colorless oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ: 0.95, 1.02, 1.10, 1.20, 1.83 (3H each, all s, 24, 25, 23, 26, 29-H_3), 1.13 (1H, dd, $J = 4.0$, 4.0 Hz, 9-H), 1.32, 1.45 (1H each, both m, 11-H_2), 1.32, 1.54 (1H each, both m, 6-H_2), 1.45 (1H, m, 5-H), 1.45, 1.92 (1H each, both m, 7-H_2), 1.54, 1.92 (1H each, both m, 1-H_2), 1.61 (6H, s, 27, 28-H_3), 1.98 (2H, m, 19-H_2), 2.10 (6H, m, 12, 15, 16-H_2), 2.27 (2H, m, 20-H_2), 2.40, 2.60 (1H each, both m, 2-H_2), 3.73 (3H, s, –OCH_3), 5.14 (2H, m, 13, 17-H), 6.74 (1H, dd-like, 21-H). Positive-ion FAB-MS: m/z 509 $(M + Na)^+$.

3.3. Preparation of the (*R*)-MTPA ester (1b) and the (*S*)-MTPA ester (1c) from 1a

A solution of **1a** (1.0 mg) in CH_2Cl_2 (1.0 mL) was treated with (*R*)-MTPA (5.0 mg) in the presence of EDC·HCl (5.0 mg) and 4-DMAP (2.0 mg), and the mixture was heated under reflux for 2 h. The reaction mixture was poured into ice water and the whole was extracted with AcOEt. The AcOEt extract was successively washed with 5% aqueous HCl, aqueous saturated $NaHCO_3$, and brine, then dried over $MgSO_4$ and filtered. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was purified by a normal-phase silica gel column [1.0 g, *n*-hexane–AcOEt (3:1)] to give **1b** (0.3 mg, 41%) and **1a** (0.5 mg, recovered). Through a similar procedure, **1c** (0.1 mg, 12%) and **1a** (0.4 mg, recovered) were prepared from **1a** (2.0 mg) by the use of (*S*)-MTPA (5.0 mg), EDC·HCl (5.0 mg), and 4-DMAP (2.0 mg).

1b: Colorless oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ: 0.78, 0.83, 0.83, 1.13, 1.83 (3H each, all s, 24, 23, 25, 26, 29-H_3), 1.28, 1.78 (1H each, both m, 1-H_2), 1.61 (6H, s, 27, 28-H_3), 1.78, 1.88 (1H each, both m, 2-H_2), 3.56, 3.73 (3H each, both s, –OCH_3), 4.73 (1H, dd, $J = 4.9$, 10.8 Hz, 3-H), 5.15 (2H, dd-like, 13, 17-H), 6.73 (1H, dd-like, 21-H), 7.38–7.54 (5H, m, Ph-H).

1c: Colorless oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ: 0.79, 0.81, 0.85, 1.13, 1.83 (3H each, all s, 25, 24, 23, 26, 29-H_3), 1.24, 1.76 (1H each, both m, 1-H_2), 1.61 (6H, s, 27, 28-H_3), 1.76, 1.86 (1H each, both m, 2-H_2), 3.56, 3.79 (3H each, both s, –OCH_3), 4.73 (1H, dd-like, 3-H), 5.16 (2H, dd-like, 13, 17-H), 6.74 (1H, dd-like, 21-H), 7.39–7.52 (5H, m, Ph-H).

3.4. $NaBH_4$ reduction of 2a

A solution of **2a** (10.0 mg) in MeOH (2.0 mL) was treated with $NaBH_4$ (4.0 mg) and the whole mixture was stirred at room temperature for 30 min. The reaction mixture was quenched at acetone. Removal of the solvent under reduced pressure gave a product, which was purified by normal-phase silica-gel column chromatography [1.0 g, *n*-hexane–AcOEt (1:1)] to give **1a** (7.8 mg, 78%).

3.5. PCC oxidation of epimansumbinol (4)

A solution of **4** (10.0 mg) in CH_2Cl_2 (1.0 mL) was treated with pyridinium chlorochromate (PCC, 10.0 mg), and the whole mixture was stirred at 0 °C for 3 h under N_2 atmosphere. The reaction mixture was poured into ice water and the whole was extracted with AcOEt. The AcOEt extract was washed with brine and then dried over $MgSO_4$ powder and filtered. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was then purified by silica gel column chromatography [500 mg, *n*-hexane–AcOEt (10:1)] to give **4a**[20] (6.2 mg, 63%).

4. Bioassay

4.1. NO Production from macrophages stimulated by lipopolysaccharide

Peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice, which had been injected intraperitoneally with 4% thioglycolate medium (TGC) four days previously, by washing with 6–7 mL of ice-cold phosphate-buffered saline (PBS), and the cells (5×10^5 cells/well) were suspended in 200 μL of RPMI 1640 supplemented with 5% fetal calf serum, 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 Giemsa staining) were cultured in fresh medium containing 10 μg/mL lipopolysaccharide (LPS) and test compound (1–100 μM) for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent.

Cytotoxicity was determined using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (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 a 4 h culture, the medium was removed, and isopropanol containing 0.04 M 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). N^G-Monomethyl-L-arginine (L-NMMA, NOS inhibitor), guanidinoethyldisulfide (GED, iNOS inhibitor), and caffeic acid phenethylester (CAPE, inhibitor of NF-κB activation) were used as reference compounds.

Each test compound was dissolved in dimethyl sulfoxide (DMSO), and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated by the following formula and the IC_{50} was determined graphically ($N = 4$):

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

$A - C$: NO_2^- concentration (μM) [A: LPS (+), sample (−); B: LPS (+), sample (+); C: LPS (−), sample (−)].

4.2. Detection of iNOS

In this experiment, TGC-induced peritoneal exudate cells (7.5×10^6 cells/3 mL/dish) from male ddY mice were pre-cultured in culture dishes for 1 h, and the adherent cells were obtained as described previously.[26,30,42] After washing, the culture medium was exchanged with fresh medium containing 5% FCS, 20 μg/mL LPS and test compound for 20 h. Cells were collected in lysis buffer [100 mM NaCl, 10 mM Tris, protease inhibitor cocktail (1 tab/50 mL), 0.1% Triton X-100, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), pH 7.4] and sonicated. After determination of the protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension was boiled in Laemmli buffer. For SDS-PAGE, aliquots of 40 μg of protein from each sample were subjected to electrophoresis in 7.5% polyacrylamide gels. Following electrophoresis, the proteins were transferred electrophoretically onto nitrocellulose membranes. The membranes were incubated with 5% nonfat dried milk in Tris-buffered saline (T-TBS, 100 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) and probed with mouse monoclonal IgG (dilution of 1:1000) against iNOS. The blots were washed in T-TBS and probed with the secondary antibody, anti-mouse IgG antibody conjugated with horseradish peroxidase (dilution of 1:5000). Detection was performed using an ECL™ and X-ray film (Hyperfilm-ECL™, Amersham).

4.3. Statistics

Values were expressed as means ± S.E.M. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

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11. (a) Although the structure of **7** was reported,[10] the physical data of **7** were not reported; (b) **7**: Colorless oil, $[\alpha]_D^{28} + 14.6$ (*c* 1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{30}H_{52}O_2Na$ $(M + Na)^+$: 467.3865. Found: 467.3878. IR (KBr): 3453, 2940, 1658, 1456, 1387 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ: 0.76, 0.80, 0.99, 1.13, 1.61, 1.68 (3H each, all s, 24, 25, 23, 26, 29, 30-H_3), 0.90 (1H, d-like, 5-H), 1.02 (1H, dd, $J = 3.9$, 3.9 Hz, 9-H), 1.17, 1.74 (1H each, both m, 1-H_2), 1.27, 1.48 (1H each, both m, 11-H_2), 1.33 (1H, m, 6-H), 1.38, 1.87 (1H each, both m, 7-H_2), 1.60 (6H, s, 27, 28-H_3), 1.60–1.70 (3H, m, 2-H_2, 6-H), 2.00 (4H, m, 15, 19-H_2), 2.07 (6H, m, 12, 16, 20-H_2), 3.21 (1H, dd, $J = 5.0$, 10.9 Hz, 3-H), 5.11 (2H, dd-like, 13, 17-H), 5.17 (1H, dd-like, 21-H). ^{13}C NMR (68 MHz, $CDCl_3$) δc: 37.8 (t, 1-C), 27.1 (t, 2-C), 78.6 (d, 3-C), 38.7 (s, 4-C), 55.0 (d, 5-C), 20.2 (t, 6-C), 44.3 (t, 7-C), 73.7 (s, 8-C), 61.1 (d, 9-C), 38.7 (s, 10-C), 25.5 (t, 11-C), 31.3 (t, 12-C), 124.9 (d, 13-C), 135.1 (s, 14-C), 39.6 (t, 15-C), 26.6 (t, 16-C), 124.2 (d, 17-C), 134.8 (s, 18-C), 39.7 (t, 19-C), 26.7 (t, 20-C), 124.4 (d, 21-C), 131.0 (s, 22-C), 28.1 (q, 23-C), 15.3 (q, 24-C), 15.5 (q, 25-C), 23.7 (q, 26-C), 16.2 (q, 27-C), 16.0 (q, 28-C), 17.7 (q, 29-C), 25.7 (q, 30-C). Positive-ion FAB-MS: m/z 467 $(M + Na)^+$.
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