New Crinine-Type Alkaloids with Inhibitory Effect on Induction of Inducible Nitric Oxide Synthase from *Crinum yemense*

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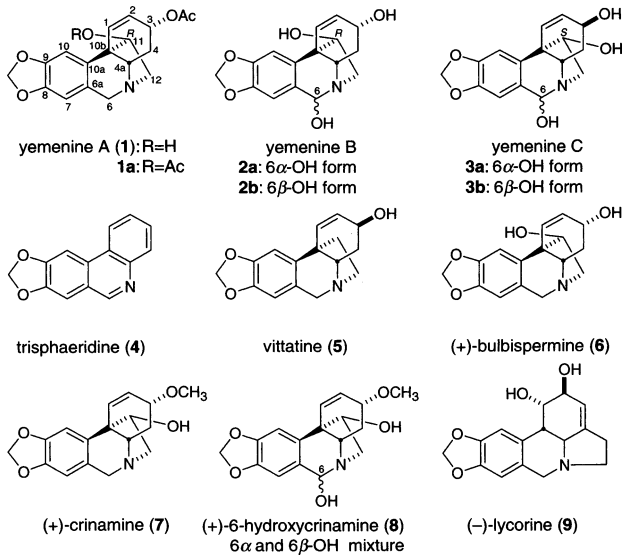
The 80% aqueous methanolic extract from the bulbs of *Crinum yemense* showed a potent inhibitory effect on nitric oxide production in lipopolysaccharide-activated macrophages. Three new crinine-type alkaloids, yemenines A (**1**), B (**2**), and C (**3**), were isolated from the herbal extract together with six known alkaloids. The absolute configurations of **1**−**3** were determined on the basis of chemical and physicochemical evidence. The effects of the isolated alkaloids on nitric oxide production in lipopolysaccharide-activated macrophages were examined, and several alkaloids, e.g. **1**, (+)-bulbispermine (**6**), (+)-crinamine (**7**), (+)-6-hydroxycrinamine (**8**), and (−)-lycorine (**9**), showed inhibitory effects on nitric oxide production and induction of inducible nitric oxide synthase.

The Amaryllidaceae plant *Crinum yemense* Deflers ex Schweinf. [syn. *C. album* (Forssk.) Herb.] is distributed in Yemen and the neighboring countries.[1−3] Chemical and biological studies on several *Crinum* plants have been reported.[4−6] However, the pharmacological activity and biological constituents of *C. yemense* are left uncharacterized.

In the course of our characterization studies on alkaloid constituents of natural medicines,[7−12] we found that the aqueous methanolic extract from the bulbs of *C. yemense* showed potent inhibitory activities on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages. From the methanolic extract, we have isolated three new crinine-type alkaloids, yemenines A (**1**), B (**2**), and C (**3**), together with six known alkaloids. This paper deals with the isolation and structure elucidation of three new alkaloids (**1**−**3**) as well as the inhibitory effects of the alkaloid constituents on nitric oxide production and induction of inducible nitric oxide synthase (iNOS).

Results and Discussion

The 80% aqueous methanolic extract from the bulbs of *C. yemense* collected in Ibb Province, Yemen (IC_{50} for NO production = 28 μg/mL), was partitioned into an aqueous hydrochloric acid (HCl)−$CHCl_3$ mixture. The acidic aqueous phase was extracted with $CHCl_3$, and then the acidic aqueous phase was made basic with 28% aqueous ammonium hydroxide (NH_4OH) followed by extraction with $CHCl_3$ to furnish the acidic $CHCl_3$ and basic $CHCl_3$-soluble fractions and aqueous layer. The aqueous layer was further extracted with *n*-BuOH to give the *n*-BuOH- and H_2O-soluble fractions. The basic $CHCl_3$-soluble fraction (active fraction, IC_{50} = 1.8 μg/mL) was crystallized in $CHCl_3$ to furnish (−)-lycorine[13] (**9**, 0.036%) and a mother liquid fraction. The mother liquid fraction was subjected to normal-phase and reversed-phase silica gel column chromatography and repeated HPLC to give yemenine A (**1**, 0.0011% from the natural medicine) together with trisphaeridine[14,15] (**4**, 0.0006%), vittatine[16] (**5**, 0.0006%), (+)-bulbispermine[17] (**6**, 0.012%), (+)-crinamine[13,18] (**7**, 0.0086%), and (+)-6-hydroxycrinamine[19] (**8**, 0.016%). The *n*-BuOH-soluble fraction was subjected to normal-phase and reversed-phase silica gel column chromatography and repeated HPLC to give yemenines B (**2**, 0.0002%) and C (**3**, 0.0006%).



Yemenine A (**1**) was isolated as a white powder with a positive optical rotation ($[\alpha]_D^{25}$ +61.7°) and was deduced to possess a nitrogen function based on TLC examination using the Dragendorff's reagent. The EIMS of **1** showed a molecular ion peak at *m*/*z* 329 [M^+] in addition to fragment ion peaks at *m*/*z* 300, 269 [base peak], and 181. The molecular formula $C_{18}H_{19}NO_5$ of **1** was determined from the molecular ion peak observed in the EIMS and by HREIMS measurement. The IR spectrum of **1** showed absorption bands at 3340, 1730, and 930 cm^{-1} ascribable to hydroxyl, ester carbonyl, and methylenedioxy groups, respectively. In the UV spectrum of **1**, absorption maxima were observed at 243 nm (log ϵ 3.56) and 292 nm (3.80), characteristic of a methylenedioxy-substituted benzene ring.[16] The ^1H ($CDCl_3$) and ^{13}C NMR (Table 1) spectra[20] of **1** showed signals assignable to an acetyl methyl, a methylene (H_2-4), two methylenes (H_2-6, 12), and three methines (H-3, 4a, 11) bearing a heteroatom, a methylenedioxy, and

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Table 1. ^{13}C NMR Data for Yemenines A−C (**1**, **2a**, **2b**, **3a**, **3b**)

	1[a]	**2a**[b]	**2b**[b]	**3a**[b]	**3b**[b]
C-1	124.7	124.7	124.9	127.6	127.8
C-2	133.9	137.4	137.2	132.9	132.8
C-3	70.0	68.6	68.2	64.8	64.5
C-4	29.7	34.2	34.3	32.8	33.0
C-4a	63.8	61.5	61.6	57.5	57.5
C-6	61.0	89.0	89.9	89.1	87.0
C-6a	126.2	129.3	127.8	129.2	130.8
C-7	106.8	110.2	109.0	110.1	108.9
C-8	146.1	149.3	149.3	149.3	148.8
C-9	146.4	147.7	147.7	147.4	147.5
C-10	103.0	103.6	103.6	103.6	103.6
C-10a	134.6	138.5	139.0	138.2	137.4
C-10b	50.0	51.6	51.6	51.3	51.9
C-11	79.9	79.6	79.5	79.5	80.3
C-12	63.3	59.0	59.2	59.1	59.1
$-OCH_2O-$	100.8	102.3	102.1	102.3	102.3
$-OCOCH_3$	170.4				
$-OCOCH_3$	21.3				

[a] Measured in $CDCl_3$ at 125 MHz. [b]Measured in CD_3OD at 125 MHz.

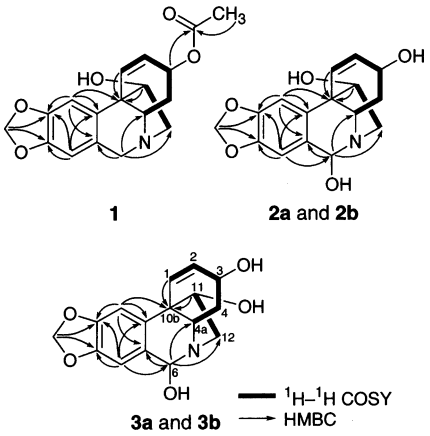

Figure 1. $^1H-^1H$ COSY and HMBC correlations of yemenines A−C (**1**−**3**).

two olefinic (H-1, 2) and two aromatic protons (H-7, 10). The structure of **1** was clarified by $^1H-^1H$ correlation spectroscopy ($^1H-^1H$ COSY) and heteronuclear multiple bond connectivity (HMBC) experiments. As shown in Figure 1, the $^1H-^1H$ COSY experiment on **1** indicated the presence of two partial structures drawn with bold lines (C-1−C-4a, C-11−C-12). In the HMBC experiment, long-range correlations were observed between the following proton and carbon pairs (H-1 and C-10b; H-3 and −OCOCH3; H-6 and C-4a, 7, 12; H-7 and C-8, 9, 10a; H-10 and C-6a, 9, 10a, 10b; H-11 and C-10b; $-OCH_2O-$ and C-8, 9), thus defining the connectivities of the quaternary carbons in **1**. The relative configuration of **1** was elucidated using a NOESY experiment, which showed correlations between the following proton pairs: H-3 and Hβ-4, H-4a; H-4a and Hβ-6 (Figure 2). To elucidate the absolute configuration, **1** was chemically related to (+)-bulbispermine (**6**), whose absolute configuration has been reported.[16] Thus, acetylation of **1** with acetic anhydride (Ac2O) in pyridine furnished diacetate (**1a**), which was also derived by acetylation of **6**. Compound **6** was obtained by alkaline hydrolysis of **1** with 5% aqueous KOH. Thus, compound **1** is a 3-*O*-acetyl derivative of (+)-bulbispermine.

Yemenine B (**2**) was obtained as white powder with a positive optical rotation ($[\alpha]_D^{25}$ +92.0°) and exhibited a positive Dragendorff's test. The EIMS of **2** showed a molecular ion at *m*/*z* 303 [M^+]. The molecular formula $C_{16}H_{17}NO_5$ of **2** was determined from the molecular ion peak observed in the EIMS and by HREIMS measurement. The IR spectrum of **2** showed absorption bands at 3350 and 930 cm^{-1}, suggesting the presence of hydroxyl and methylenedioxy functions. In the UV spectrum of **2**, absorption maxima were observed at 243 nm (log ϵ 3.56) and 292 nm (3.68) ascribable to a methylenedioxy-substituted benzene ring.[16] The proton and carbon signals of the ^1H (CD_3OD) and ^{13}C NMR (Table 1) spectra of **2** were superimposable on those of **6** possessing the 3α and 11(*R*)-hydroxyl groups, except for the signals due to the 6-position. NMR data also indicated that **2** exists as a mixture (ca. 2:1) of two C-6 epimers (**2a** and **2b**). Thus, the ^1H and ^{13}C NMR spectra[20] of the major (**2a**) and minor (**2b**) isomers showed signals assignable to a methylene (H_2-4), a methylene (H_2-12), and four methines (H-3, 4a, 6, 11) bearing a heteroatom, a methylenedioxy group, two olefinic protons (H-1, 2), and two aromatic protons (H-7, 10). The relative configuration of **2a** and **2b** was characterized by the NOESY experiment shown in Figure 2, in which NOE correlations were observed between the following proton pairs, respectively [**2a** (H-3 and Hβ-4, H-4a; Hα-4 and Ha-12; H-4a and H-6; H-11 and Ha-12), **2b** (H-3 and Hβ-4, H-4a; Hα-4 and Ha-12; H-6 and Hb-12; H-11 and Ha-12]. On the basis of this evidence, the relative configuration of **2** was elucidated as a mixture (ca. 2:1) of two C-6 epimers [6α-OH form (**2a**) and 6β-OH form (**2b**)]. A similar epimeric C-6 relationship was also observed for (+)-6-hydroxycrinamine (**8**).[19] In addition, the circular dichroic (CD) spectrum of **2** showed characteristic Cotton effects [244 nm ($\Delta\epsilon$ −1.90), 291 (+1.43) in MeOH] of crinane-type alkaloids possessing a 5α,10b-ethanophenanthridine system.[21,22] Consequently, the absolute configuration of **2** was determined as shown.

Yemenine C (**3**) was obtained as colorless needles (mp 152.0−154.0 °C from MeOH) with a negative optical rotation ($[\alpha]_D^{25}$ −14.0°) and exhibited a positive Dragendorff's test. The molecular formula $C_{16}H_{17}NO_5$, which was the same as that of **2**, was determined from the EIMS and by HREIMS analysis. The IR and UV spectra of **3** were very similar to those of **2**. That is, the IR spectrum of **3** showed absorption bands at 3350 and 930 cm^{-1}, while in the UV spectrum, absorption maxima were observed at 241 nm (log ϵ 3.58) and 293 nm (3.70). The proton and carbon signals of the ^1H and ^{13}C NMR (Table 1) spectra[20] of **3** were very similar to those of **8**, except for the signals assignable to the 3-hydroxyl group. The NMR data of **3** indicated that **3** was a mixture (ca. 2:1) of two epimers (**3a** and **3b**). The ^1H NMR (CD_3OD) and ^{13}C NMR (Table 1) spectra[20] showed the presence of the following functions: a methylene (H_2-4), a methylene (H_2-12), and four methines (H-3, 4a, 6, 11) bearing a heteroatom, a methylenedioxy group, two olefinic protons (H-1, 2), and two aromatic protons (H-7, 10). In the NOESY experiments on **3a** and **3b**, correlations were observed between the following proton pairs, respectively [**3a** (H-3 and Hα-4; Hα-4 and Ha-12; Hβ-4 and H-4a; H-4a and H-6; H-10 and H-11; H-11 and Hb-12), **3b** (H-3 and Hα-4; Hα-4 and Ha-12; Hβ-4 and H-4a; H-6 and Hb-12; H-10 and H-11; H-11 and Hb-12)]. Consequently, the relative configuration of **3** was elucidated as a mixture (ca. 2:1) of two epimers [6α-OH form (**3a**) and 6β-OH form (**3b**)] at C-6.[19] Finally, the absolute configuration of **3** was determined on the basis of the CD spectrum [244 nm ($\Delta\epsilon$ −2.82), 290 (+2.31) in MeOH].[21,22]

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

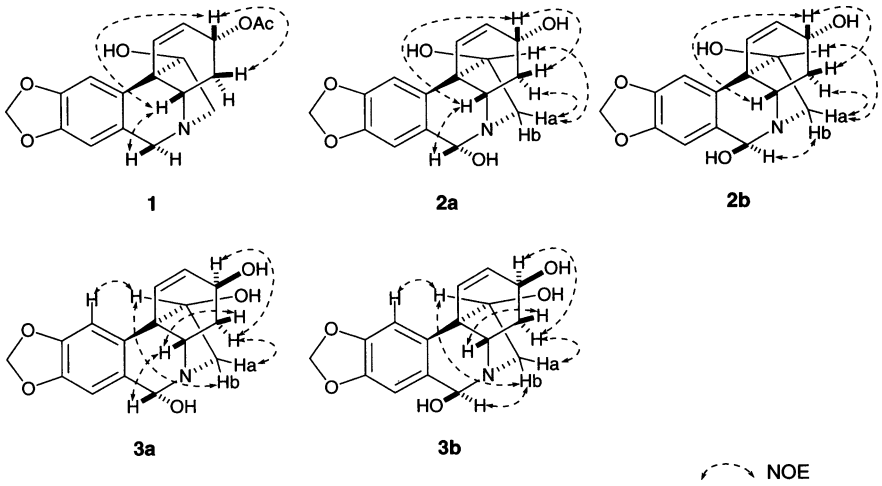

Figure 2. NOE correlations of yemenines A−C (**1**−**3**).

Table 2. Inhibitory Effects of Constituents from *C. yemmense* on NO Production in LPS-Activated Mouse Peritoneal Macrophages

	inhibition (%)[a]						
	0 μM	1 μM	3 μM	10 μM	30 μM	10 μM	IC_{50} (μM)
yemenine A (**1**)	0.0 ± 2.3 (100.0%)	10.4 ± 6.3 (110.5%)	38.1 ± 2.0[c] (100.6%)	69.0 ± 1.8[c] (80.1%)	93.0 ± 0.9[c,d] (66.6%)	98.5 ± 0.4[c,d] (51.0%)	4.9
yemenine C (**3**)	0.0 ± 2.4 (100.0%)	9.7 ± 3.3 (97.3%)	1.6 ± 3.7 (103.2%)	0.5 ± 0.6 (102.7%)	18.3 ± 5.5[c] (112.8%)	31.3 ± 1.4[c] (116.6%)	
trisphaeridine (**4**)	0.0 ± 4.5 (100.0%)	−7.1 ± 4.8 (92.8%)	−6.9 ± 5.3 (87.4%)	−3.4 ± 4.2 (86.3%)	−7.0 ± 2.7 (96.1%)	0.3 ± 2.3 (84.3%)	
vittatine (**5**)	0.0 ± 2.7 (100.0%)	−0.2 ± 6.2 (108.0%)	−12.5 ± 5.1 (106.7%)	7.1 ± 4.6 (108.7%)	13.1 ± 5.7 (102.7%)	42.4 ± 3.7[c] (105.7%)	
(+)-bulbispermine (**6**)	0.0 ± 8.4 (100.0%)	3.1 ± 1.6 (100.3%)	7.4 ± 2.0 (101.2%)	28.6 ± 4.1[c] (102.4%)	55.6 ± 1.4[c] (97.6%)	82.0 ± 0.9[c] (96.7%)	24
L-NMMA	0.0 ± 4.0 (100.0%)	5.9 ± 0.9 (81.6%)	10.3 ± 3.7 (90.5%)	15.0 ± 1.6[c] (96.7%)	034.1 ± 3.2[c] (105.7%)	63.1 ± 1.2[c] (131.9%)	57
CAPE	0.0 ± 0.7 (100.0%)	3.8 ± 0.1 (103.8%)	1.4 ± 0.1 (121.1%)	68.2 ± 0.0[c] (101.3%)	93.7 ± 0.2[c] (138.4%)	99.6 ± 0.0[c,d] (22.1%)	15
GED	0.0 ± 0.0 (100.0%)	6.2 ± 0.1 (85.1%)	24.4 ± 0.1[c] (92.0%)	57.9 ± 0.1[c] (116.0%)	89.7 ± 0.2[c] (130.0%)	97.9 ± 0.0[c] (115.3%)	7.4

	inhibition (%)[a]						
	0 μM	1 μM	3 μM	10 μM	30 μM	10 μM	IC_{50} (μM)
(+)-crinamine (**7**)	0.0 ± 1.1 (100.0%)	8.1 ± 7.5 (100.1%)	7.8 ± 5.4 (100.6%)	30.2 ± 2.5[c] (100.1%)	65.2 ± 2.9[c] (100.5%)	86.1 ± 1.1[c] (84.5%)	1.8
(+)-6-hydroxycrinamine (**8**)	0.0 ± 6.7 (100.0%)	−11.2 ± 11.0 (110.1%)	−23.1 ± 5.0 (120.0%)	6.3 ± 6.8 (117.3%)	30.3 ± 6.2[b] (115.3%)	069.5 ± 0.7[c] (108.3%)	5.4
(−)-lycorine (**9**)	0.0 ± 3.0 (100.0%)	0.0 ± 8.0 (98.2%)	2.8 ± 3.8 (101.4%)	20.6 ± 3.4[b] (105.4%)	54.4 ± 3.4[b] (98.3%)	91.4 ± 1.0[c,d] (76.1%)	2.5
herbimycin A	0.0 ± 6.7 (100.0%)	48.0 ± 2.0[c] (102.7%)	77.0 ± 2.4[c] (101.6%)				0.094

[a] Each value represents the means ± SEM ($N = 4$). Significantly different from the control. [b] $p < 0.05$. [c] $p < 0.01$. [d] Cytotoxic effect was observed. Viability of macrophages in parentheses (%, $N = 4$).

the family of NOS, iNOS (inducible nitric oxide synthase) is particularly involved in pathological aspects with overproduction of NO and can be expressed in response to proinflammatory 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 several NO production inhibitors, i.e., higher unsaturated fatty acids,[23] polyacetylenes,[24,25] coumarins,[24] flavonoids,[25,26] stilbenes,[27,28] lignans,[29,30] sesquiterpenes,[31−37] diterpenes,[38,39] triterpenes,[40−42] diarylheptanoids,[43,44] cyclic peptides,[42] and alkaloids.[12]

In our continuing study of the antiinflammatory principles, effects of the alkaloid constituents from the bulbs of *C. yemense* on NO production in LPS-activated macrophages were examined, and the results are summarized in Table 2. Yemenine A (**1**, $IC_{50} = 4.8$ μM), (+)-crinamine (**7**, 1.8 μM), (+)-6-hydroxycrinamine (**8**, 5.4 μM), and (−)-lycorine (**9**, 2.5 μM) exhibited inhibitory activity without cytotoxic effects in the MTT assay. Their inhibitory activities were more potent than that of guanidinoethyl disulfide (GED), a selective inducible nitric oxide synthase (iNOS) inhibitor ($IC_{50} = 7.4$ μM).

Next, the effects of the four alkaloids (**1**, **7**−**9**) on iNOS enzyme activity and iNOS induction were examined. A reference compound, N^G-monomethyl-L-arginine (L-NMMA, a nonselective inhibitor of NOS), inhibited iNOS enzyme activity with an IC_{50} of 13 μM, but **1** and **7**−**9** showed weak inhibition for iNOS activity (data not shown).[29] iNOS was detected at 130 kDa after a 20 h incubation with LPS by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE)−Western blotting analysis as shown in Figure 3.[26,30,42] iNOS induction of LPS-activated macrophages was suppressed by four alkaloid constituents (**1**, **7**−**9**) closely related to their inhibitions of NO. These results

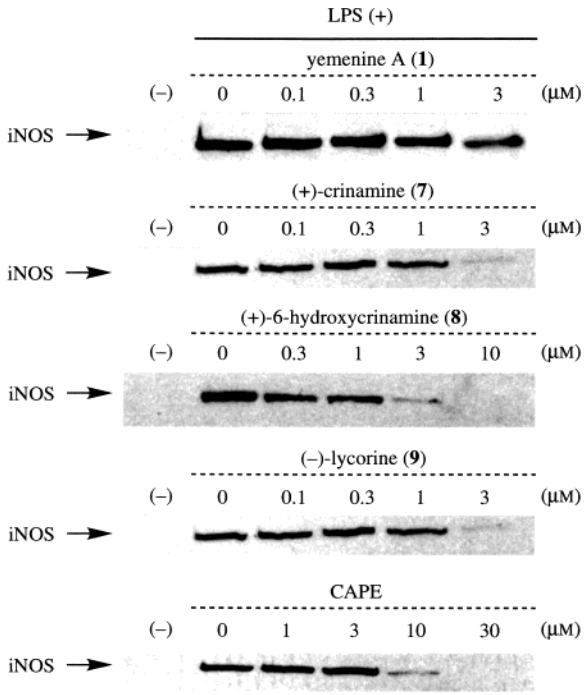

Figure 3. Effects of **1**, **7**−**9**, and CAPE on iNOS induction in LPS-activated mouse macrophages.

suggested that the four alkaloids (**1**, **7**−**9**) inhibited NO production mainly due to their inhibitory activities against iNOS induction in LPS-activated macrophages.

Experimental Section

General Experimental Procedures. The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l = 5 cm); CD spectra, JASCO J-720WI spectrometer; UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^{1}H NMR spectra, JEOL JNM LA-500 (500 MHz) spectrometer; ^{13}C NMR spectra, JEOL JNM LA-500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; EIMS and HREIMS, JEOL JMS-GCMATE mass spectrometer; 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, 150−350 mesh); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, 100−200 mesh); Diaion HP-20 column chromatography (Nippon Rensui); HPLC column, YMC-Pack ODS-A (YMC, 250 × 20 mm i.d.); TLC, precoated TLC plates with silica gel 60F_{254} (Merck, 0.25 mm) (normal-phase) and silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed-phase); reversed-phase HPTLC, precoated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm); detection was achieved by spraying with Dragendorff's reagent or 1% Ce$(SO_4)_2$−10% aqueous H_2SO_4 followed by heating.

Plant Material. The bulbs of *Crinum yemense* were collected in Ibb Province, Yemen, in September 2001 and identified by one of the authors (O.B.A.-H.). A voucher of the plant is on file in our laboratory.

Extraction and Isolation.[45,46] The fresh bulbs of *C. yemense* (3.1 kg) were cut and extracted three times with 80% aqueous MeOH under reflux for 3 h. Evaporation of the solvent under reduced pressure provided the aqueous MeOH extract (310 g, 10.0%). The aqueous MeOH extract (250 g) was dissolved in H_2O (ca. 250 mL) and acidified with dilute HCl until pH 2 and then extracted with $CHCl_3$. Removal of the solvent in vacuo from the $CHCl_3$-soluble portion yielded the acidic $CHCl_3$-soluble fraction (12.0 g, 0.48%). The pH of the acidic aqueous solution was adjusted to 9 with 28% NH_4OH and then extracted with $CHCl_3$. Removal of the solvent in vacuo yielded the basic $CHCl_3$-soluble fraction (7.5 g, 0.30%). The aqueous layer was extracted with *n*-BuOH, and removal of the solvent in vacuo yielded 21.0 g (0.84%) and 209.5 g (8.38%) of residue, respectively.

The basic $CHCl_3$-soluble fraction (7.0 g) was crystallized from $CHCl_3$ to furnish **9** (850 mg, 0.036%) and mother liquid fraction (6.15 g). The mother liquid fraction (6.15 g) was subjected to normal-phase silica gel column chromatography {250 g, MeOH−[$CHCl_3$−28% NH_4OH (500:1, v/v)] (0:100−10:90, v/v, gradient)} to give 11 fractions [fractions 1 (432 mg), 2 (870 mg), 3 (71 mg), 4 (627 mg), 5 (62 mg), 6 (301 mg), 7 (450 mg), 8 (102 mg), 9 (75 mg), 10 (350 mg), 11 (2.81 g)]. Fraction 3 (71 mg) was separated by reversed-phase silica gel column chromatography [20 g, MeOH−H_2O (50:50, v/v)−MeOH] and HPLC [YMC-Pack ODS-A, MeOH−H_2O (70:30, v/v)] to give **4** (15 mg, 0.0006%). Fraction 5 (62 mg) was separated by reversed-phase silica gel column chromatography [15 g, MeOH−H_2O (30:70, v/v)−MeOH] and HPLC [MeOH−H_2O (60:40, v/v)] to give **1** (25 mg, 0.0011%). Fraction 6 (301 mg) was separated by reversed-phase silica gel column chromatography [30 g, MeOH−H_2O (40:60, v/v)−MeOH] and HPLC [MeOH−H_2O (55:45, v/v)] to give **7** (201 mg, 0.0086%). Fraction 7 (450 mg) was separated by reversed-phase silica gel column chromatography [35 g, MeOH−H_2O (30:70, v/v)−MeOH] and HPLC [MeOH−H_2O (48:52, v/v)] to give **8** (372 mg, 0.016%). Fraction 9 (75 mg) was separated by reversed-phase silica gel column chromatography [20 g, MeOH−H_2O (30:70, v/v)−MeOH] and HPLC [MeOH−H_2O (50:50, v/v)] to give **5** (15 mg, 0.0006%). Fraction 10 (350 mg) was separated by reversed-phase silica gel column chromatography [30 g, MeOH−H_2O (40:60, v/v)−MeOH] and HPLC [MeOH−H_2O (70:30, v/v)] to give **6** (280 mg, 0.012%).

The *n*-BuOH-soluble fraction (15.0 g) was subjected to Diaion HP-20 column chromatography [450 g, MeOH−H_2O (0:100−30:70−50:50−80:20, v/v)−MeOH] to give five fractions [fractions 1 (6.5 g), 2 (2.3 g), 3 (1.2 g), 4 (3.1 g), 5 (1.9 g)]. Fraction 3 (1.2 g) was separated by reversed-phase silica gel column chromatography [40 g, MeOH−H_2O (30:70, v/v)−MeOH] and HPLC [MeOH−H_2O (30:70, v/v)] to give **2** (4 mg, 0.0002%) and **3** (10 mg, 0.0006%).

The known compounds were identified by comparison of their physical data ($[\alpha]_D$, IR, ^{1}H NMR, ^{13}C NMR, MS) with reported values.[13−19]

Yemenine A (1): white powder; Dragendorff's reagent positive; $[\alpha]_D^{25}$ +61.7° (c 0.50, $CHCl_3$); CD (MeOH) λ_{max} ($\Delta\epsilon$) 244 (−1.27), 293 (+0.98) nm; UV (MeOH) λ_{max} (log ϵ) 243 (3.56), 292 (3.80) nm; IR (KBr) ν_{max} 3340, 1730, 930 cm^{-1}; ^{1}H NMR ($CHCl_3$, 500 MHz) δ 2.09 (3H, s, −OAc), 2.13 (1H, ddd, J = 3.6, 5.1, 13.4 Hz, Hβ-4), 2.24 (1H, ddd, J = 10.0, 13.0, 13.4 Hz, Hα-4), 3.28 (1H, dd, J = 3.6, 13.0 Hz, H-4a), 3.37 (2H, m, H_2−12), 3.72, 4.32 (1H each, both d, J = 17.5 Hz, H_2−6), 4.01 (1H, m, H-11), 5.46 (1H, m, H-3), 5.90 (2H, br s, −OCH_2O−), 6.10 (1H, dd, J = 1.1, 10.2 Hz, H-2), 6.33 (1H, dd, J = 2.1, 10.2 Hz, H-1), 6.48 (1H, br s, H-7), 6.79 (1H, br s, H-10); ^{13}C NMR data, see Table 1; EIMS m/z 329 [M$^+$] (10), 269 (100), 181 (80); HREIMS m/z 329.1271 (calcd for $C_{18}H_{19}NO_5$ [M$^+$], 329.1263).

Yemenine B (2): white powder; Dragendorff's reagent positive; $[\alpha]_D^{25}$ +92.0° (c 0.20, MeOH); CD (MeOH) λ_{max} ($\Delta\epsilon$) 244 (−1.90), 291 (+1.43) nm; UV (MeOH) λ_{max} (log ϵ) 243 (3.56), 292 (3.68) nm; IR (KBr) ν_{max} 3350, 930 cm^{-1}; ^{1}H NMR (CD_3-OD, 500 MHz) **2a**: δ 1.90 (1H, ddd, J = 3.1, 5.5, 13.2 Hz, Hβ-4), 2.13 (1H, ddd, J = 8.8, 13.0, 13.2 Hz, Hα-4), 3.12 (1H, dd, J = 3.3, 14.1 Hz, Hb-12), 3.38 (1H, dd, J = 7.1, 14.1 Hz, Ha-12), 3.52 (1H, dd, J = 5.5, 13.0 Hz, H-4a), 3.85 (1H, dd, J = 3.3, 7.1 Hz, H-11), 4.33 (1H, m, H-3), 4.91 (1H, br s, H-6), 5.90 (2H, br s, −OCH_2O−), 6.04 (1H, dd, J = 1.0, 10.2 Hz, H-2), 6.23 (1H, dd, J = 2.0, 10.2 Hz, H-1), 6.76 (1H, br s, H-7), 6.87 (1H, br s, H-10); **2b**: δ 1.97 (1H, ddd, J = 3.1, 5.0, 13.2 Hz, Hβ-4), 2.17 (1H, ddd, J = 8.8, 13.0, 13.2 Hz, Hα-4), 2.82 (1H, dd, J = 3.1, 14.0 Hz, Hb-12), 3.31 (1H, dd, J = 7.0, 14.0 Hz, Ha-12), 3.52 (1H, dd, J = 5.0, 13.0 Hz, H-4a), 3.80 (1H, dd, J = 3.1, 7.0 Hz, H-11), 4.05 (1H, m, H-3), 4.89 (1H, br s, H-6), 5.89 (2H, d, J = 1.2 Hz, −OCH_2O−), 6.02 (1H, dd, J = 1.1, 10.2 Hz, H-2), 6.18 (1H, dd, J = 1.9, 10.2 Hz, H-1), 6.76 (1H, br s, H-7), 6.83 (1H, br s, H-10); ^{13}C NMR data, see Table 1;

EIMS *m/z* 303 [M^+] (22), 285 (100), 268 (85), 256 (18), 226 (50), 210 (40), 181 (20), 57 (30); HREIMS *m/z* 303.1103 (calcd for $C_{16}H_{17}NO_5$ [M^+], 303.1106).

Yemenine C (3): colorless fine needles; mp 152.0−154.0 °C (from MeOH); Dragendorff's reagent positive; $[\alpha]_D^{25}$ −14.0° (*c* 0.20, MeOH); CD (MeOH) λ_{max} ($\Delta\epsilon$) 244 (−2.82), 290 (+2.31) nm; UV (MeOH) λ_{max} (log ϵ) 241 (3.58), 293 (3.70) nm; IR (KBr) ν_{max} 3350, 930 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz) **3a**: δ 1.76 (1H, ddd, J = 2.2, 4.2, 13.6 Hz, Hβ-4), 2.22 (1H, ddd, J = 4.5, 13.6, 13.6 Hz, Hα-4), 3.08 (1H, dd, J = 3.3, 14.2 Hz, Ha-12), 3.36 (1H, dd, J = 7.0, 14.2 Hz, Hb-12), 3.71 (1H, dd, J = 4.2, 13.6 Hz, H-4a), 3.88 (1H, dd, J = 3.3, 7.0 Hz, H-11), 4.28 (1H, m, H-3), 4.92 (1H, br s, H-6), 5.90 (2H, d, J = 1.1 Hz, $-OCH_2O-$), 6.18 (1H, dd, J = 5.2, 10.2 Hz, H-2), 6.39 (1H, d, J = 10.2 Hz, H-1), 6.76 (1H, br s, H-7), 6.90 (1H, br s, H-10); **3b**: δ 1.78 (1H, ddd, J = 1.8, 3.8, 13.2 Hz, Hβ-4), 2.37 (1H, ddd, J = 4.1, 13.2, 13.2 Hz, Hα-4), 2.81 (1H, dd, J = 3.2, 14.2 Hz, Ha-12), 3.32 (1H, dd, J = 7.1, 14.2 Hz, Hb-12), 3.40 (1H, dd, J = 3.8, 13.2 Hz, H-4a), 3.96 (1H, dd, J = 3.2, 7.1 Hz, H-11), 4.26 (1H, m, H-3), 4.86 (1H, br s, H-6), 5.89 (2H, d, J = 1.1 Hz, $-OCH_2O-$), 6.15 (1H, dd, J = 5.0, 10.2 Hz, H-2), 6.37 (1H, d, J = 10.2 Hz, H-1), 6.76 (1H, br s, H-7), 6.87 (1H, br s, H-10); ^{13}C NMR data, see Table 1; EIMS *m/z* 303 [M^+] (40), 285 (35), 256 (25), 226 (100), 210 (32), 181 (19), 57 (28); HREIMS *m/z* 303.1101 (calcd for $C_{16}H_{17}NO_5$ [M^+], 303.1106).

Acetylation of Yemenine A (1) and (+)-Bulbispermine (6). A solution of **1** (10.0 mg) in pyridine (3.0 mL) was treated with acetic anhydride (Ac_2O, 2.0 mL), and the mixture was stirred at room temperature for 3 h. The reaction mixture was poured into ice−water and extracted with EtOAc. The EtOAc extract was successively washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ powder and filtered. Removal of the solvent under reduced pressure furnished a residue, which was purified by silica gel column chromatography {1.0 g, MeOH−[$CHCl_3$−28% NH_4OH (500:1, v/v)] (3:97, v/v)} to give **1a** (4.5 mg, 40%).

Through a similar procedure, **1a** (5.0 mg, 39%) was obtained from **6** (10.0 mg) using Ac_2O (2.0 mL) in pyridine (3.0 mL).

Compound 1a: colorless fine crystals; mp 146.0−148.0 °C (from *n*-hexane−EtOAc); Dragendorff's reagent positive; $[\alpha]_D^{25}$ +23.5° (*c* 0.40, $CHCl_3$); ^1H NMR ($CHCl_3$, 500 MHz) δ 2.04, 2.09 (3H each, both s, −OAc), 2.12−2.17 (2H, m, H_2-4), 3.28 (1H, dd, J = 4.1, 13.0 Hz, H-4a), 3.41 (2H, d, J = 5.3 Hz, H_2-12), 3.72, 4.34 (1H each, both d, J = 17.1 Hz, Hα, Hβ-6), 4.97 (1H, m, H-11), 5.45 (1H, m, H-3), 5.86 (1H, dd, J = 1.1, 10.3 Hz, H-2), 5.91 (2H, br s, $-OCH_2O-$), 6.23 (1H, dd, J = 2.1, 10.3 Hz, H-1), 6.47 (1H, br s, H-7), 6.86 (1H, br s, H-10); EIMS *m/z* 371 [M^+] (12), 311 (21), 269 (100), 211 (33), 149 (30).

Deacetylation of Yemenine A (1). A solution of **1** (5.0 mg) in 1,4-dioxane (2.0 mL) was treated with 5.0% aqueous KOH (2.0 mL), and the mixture was stirred at room temperature for 3 h. The reaction mixture was poured into ice−water and extracted with EtOAc. The EtOAc extract was successively washed with 5% aqueous HCl and brine, then dried over $MgSO_4$ powder and filtered. Removal of the solvent under reduced pressure furnished a residue, which was purified by silica gel column chromatography {1.0 g, MeOH−[$CHCl_3$−28% NH_4OH (500:1, v/v)] (3:97, v/v)} to give **6** (2.0 mg, 45%).[16]

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) 4 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 2-propanol 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). When the OD of the sample-treated group was reduced below 80% of the OD in the vehicle-treated group, the test compound was considered to exhibit cytotoxic effect. N^G-Monomethyl-L-arginine (L-NMMA), caffeic acid phenethylester (CAPE), guanidinoethyl disulfide (GED), and herbimycin A 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%). 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 (−)].

Detection of iNOS. In this experiment, TGC-induced peritoneal exudate cells (7.5×10^6 cells/3 mL/dish) from male ddY mice were precultured in culture dishes (6 cm i.d.) for 1 h, and the adherent cells were obtained as described previously.[26,30,42] After washing, the culture medium was exchanged for 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).

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

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