Structures of New Erythrinan Alkaloids and Nitric Oxide Production Inhibitors from *Erythrina crista-galli*

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Two new Erythrinan alkaloids, cristanines A and B (1, 2), were isolated from the bark of *Erythrina crista-galli* L. together with nine known Erythrinan alkaloids (3—5, 7—12) and an indole alkaloid (13). The structures of the compounds, cristanines A (1) and B (2), were elucidated by spectroscopic methods. Three of the twelve compounds isolated showed significant inhibitory activity on lipopolysaccharide induced nitric oxide (NO) production.

Key words *Erythrina crista-galli*; Leguminosae; alkaloid; nitric oxide production inhibitor

The *Erythrina* genus (Leguminosae) includes more than a hundred species and is distributed in South America and tropical and subtropical regions of South Asia. *Erythrina crista-galli* L. is known as cockspur coral tree. It is also commonly called "Corticeira" in Brazil, and its bark is used for rheumatism, hepatitis, sedation, and hypnogenesis.[1] Phytochemical studies on this plant showed the presence of Erythrinan alkaloids,[2—4] benzylisoquinoline alkaloids,[5] isoflavonoids,[6,7] and pterocarpans.[8,9] The study of biosynthesis of Erythrinan alkaloids in *Erythrina crista-galli* was reported.[10—12]

In a previous paper, we described the isolation and characterization of an indole derivative, hypaphorine (**13**), with a sleep-inducing effect on normal mice[13] and also reported an Erythrinan alkaloid, erysotrine (**6**), with TRAIL (tumor necrosis factor (TNF)-released apoptosis inducing ligand)-enhancing effect.[14] In this paper, we describe the isolation and structural characterization of new Erythrinan alkaloids, cristanines A (**1**) and B (**2**), together with ten known alkaloids (**3**—**5**, **7**—**13**) from the bark of *Erythrina crista-galli*, and these isolated compounds were evaluated for inhibitory activity of lipopolysaccharide (LPS)-induced nitric oxide (NO) production.[15] The overproduction of nitric oxide (NO) is associated with oxidative stress and with the pathophysiology of various diseases such as rheumatism, diabetes, and cardiovascular diseases with chronic inflammation.[16] In the search for various biologically active compounds from *Erythrina* plants, we focused on inhibitory activity against LPS-induced nitric oxide production for Erythrinan alkaloids in this study.

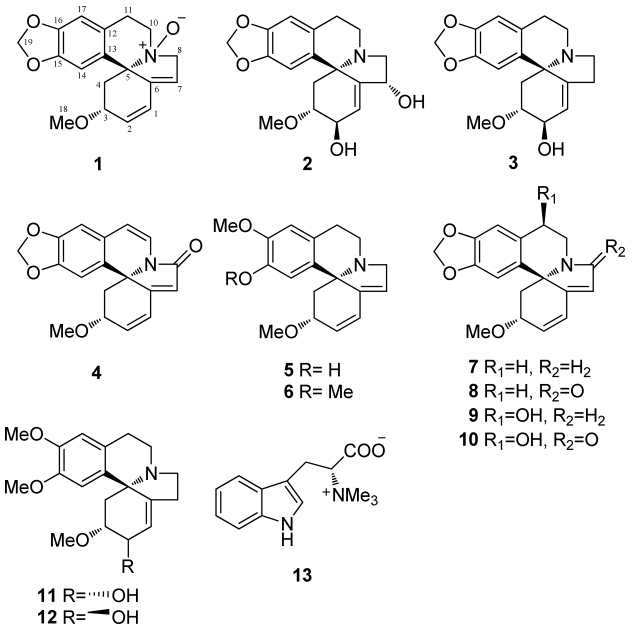

Fig. 1. Chemical Structure of Compounds **1**—**13**

Results and Discussion

Phytochemical study of alkaloidal $CHCl_3$ and *n*-BuOH extracts of *Erythrina crista-galli* led to the isolation of two new alkaloids, cristanines A (**1**) and B (**2**), together with ten known Erythrinan alkaloids, including erythratine (**3**),[17] crystamidine (**4**),[3,11] erysovine (**5**), erythraline (**7**), 8-oxo-erythraline (**8**),[18] erythrinine (**9**), 8-oxo-erythrinine (**10**),[19] erythratidine (**11**) and epi-erythratidine (**12**)[18] as well as one indole alkaloid, hypaphorine (**13**).[13] The structures of these known compounds were identified by comparison of their spectroscopic data with those reported in the literature (Fig. 1).

The molecular formula of cristanine A (**1**) was determined to be $C_{18}H_{19}NO_4$ by high resolution-electron ionization-mass spectra (HR-EI-MS) [*m*/*z* 313.1320 $(M)^+$, Calcd for 313.1314] and the IR spectrum implied the presence of an aromatic moiety and a conjugate olefin (1630, 1503, 1489 cm^{-1}). The ^1H- and ^{13}C-NMR and ^1H-detected heteronuclear single quantum coherence (HSQC) data for **1** indicated the presence of four methylenes, one sp^3 dioxy-methylene, one sp^3 oxy-methine, one sp^3 quaternary carbon, three sp^2 methines, one sp^2 quaternary carbon, two aromatic methines, two aromatic quaternary carbons, two aromatic oxy-quaternary carbons, and one methoxy group. With two double bonds and one aromatic ring accounting for six of ten un-

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Table 1. ^{1}H-NMR Data of Cristanines A (**1**) and B (**2**)

Position	**1** ($CDCl_3$)		**2** ($CDCl_3$)		**2**[a] (CD_3OD)	
	δ_H	mult (*J*, Hz)	δ_H	mult (*J*, Hz)	δ_H	mult (*J*, Hz)
1	6.63	dd (10.1, 2.2)	5.98	m	5.84	dd (3.0, 2.1)
2	6.14	br d (10.1)	4.34	m	4.20	ddd (7.3, 3.0, 2.1)
3	4.14	m	3.64	ddd (12.2, 6.0, 4.1)	3.59	m
4	2.10	dd (11.6, 5.7)	2.31	dd (12.2, 4.1)	2.29	dd (11.9, 4.2)
	3.24	t (11.6)	1.74	t (12.2)	1.68	t (11.9)
7	5.78	br s	4.33	m	4.29	dddd (8.2, 2.8, 2.1, 2.1)
8	4.17	dd (14.6, 3.3)	2.83	dd (10.7, 1.5)	2.75	dd (10.5, 2.8)
	4.42	dd (14.6, 1.0)	3.05	m	3.07	dd (10.5, 8.2)
10	3.79	ddd (13.6, 6.5, 4.3)	3.07	m	3.00	m
	3.85	ddd (13.6, 10.4, 4.3)	3.42	m	3.39	m
11	3.06	ddd (17.1, 10.4, 6.5)	2.58	dd (18.1, 6.5)	2.61	m
	3.19	dt (17.1, 4.3)	2.95	ddd (18.1, 9.2, 7.8)	3.00	m
14	6.67	s	6.70	s	6.75	s
17	6.62	s	6.57	s	6.51	s
18	3.35	s	3.33	s	3.33	s
19	5.94	d (1.3)	5.90	d (1.4)	5.89	d (1.2)
	5.95	d (1.3)	5.92	d (1.4)	5.88	d (1.2)

a) The overlapped signals of H-2 and H-7 could be separated using CD_3OD.

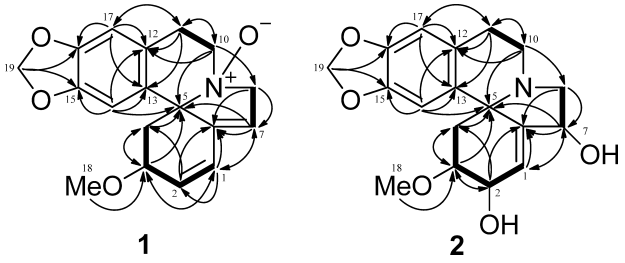

Fig. 2. ^{1}H–^{1}H COSY (—) and Key HMBC (→) Correlations of Cristanines A (**1**) and B (**2**)

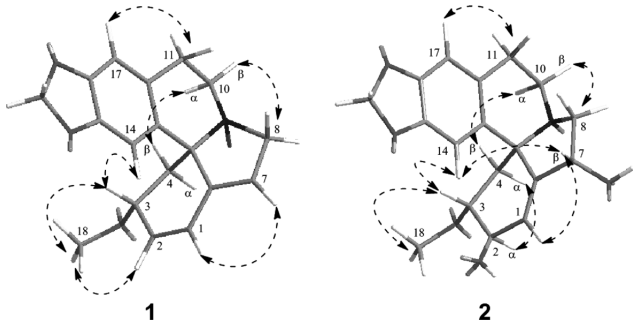

Fig. 3. Key NOESY Correlations of Cristanines A (**1**) and B (**2**)

saturations, it was concluded that **1** contains five rings including one aromatic ring. The ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum revealed connectivities of C-1 to C-4, C-7 to C-8, and C-10 to C-11 (Fig. 2). Heteronuclear multiple bond connectivity (HMBC) correlations (Fig. 2) were observed: H-17/C-11, C-12, C-13, C-16, H-14/C-5 (δ 83.3: a spiro-carbon), C-12, C-13, C-15, H-11/C-10, C-12, C-13, C-17, H-10/C-5, C-8, C-11, C-12, H-8/C-6, C-7, H-7/C-1, C-5, C-6, H-1/C-2, C-3, C-6, C-7, H-2/C-4, C-6, H-3/C-4, C-5, H-4/C-3, C-5, and H-19/C-15, C-16. These correlations showed that **1** was a typical Erythrinan alkaloid having a characteristic spiro-carbon positioned in the center of rings of A, B, and C with two olefins of $\Delta^{1,2}$ (δ_H 6.63 dd, δ_C 125.4, C-1; δ_H 6.14 br d, δ_C 133.8, C-2) and $\Delta^{6,7}$ (δ_C 138.7, C-6; δ_H 5.78 br s, δ_C 118.2, C-7) and a dioxy-methylene group (δ_H 5.94 d, 5.95 d, δ_C 101.3, C-19) attached to the C-15 and C-16 positions (δ_C 147.5, C-15; δ_C 147.0, C-16). Furthermore, one methoxy group was attached to C-3 as deduced from the HMBC correlations of methoxy protons H-18 to C-3. These data suggested that compound **1** has a gross structure similar to that of erythraline (**7**). Ultimately, the molecular formula of **1** indicated the presence of one extra oxygen atom. On ^{13}C-NMR, the spiro-carbon, C-5, and methylene carbons, C-8 and C-10, were markedly deshielded by 16.0, 14.6, and 16.5 ppm in comparison with those of erythraline (**7**), respectively. These facts suggested that the nitrogen atom was oxygenated. The direction of the oxygen atom of *N*-oxide might be α-facing due to the downfield shifts of H-4α (δ_H 3.24). The nuclear Overhauser effect spectroscopy (NOESY) correlations (Fig. 3) of H-14/H-3, H-4β/H-10α, and H-10β/H_2-8 suggested a methoxy group was located at C-3 in α-orientation and other NOESY correlations were satisfied with the relative stereochemistry of the new compound **1** depicted in Fig. 3. Therefore, the relative configuration of cristanine A (**1**) was assigned as erythraline *N*-oxide (Fig. 2). The positive optical rotation value ($[\alpha]_D^{25}$ +137.5°) of **1** suggested that **1** has an *S* configuration at C-5.[20,21]

The molecular formula $C_{18}H_{21}NO_5$ of cristanine B (**2**) was established by HR-EI-MS [*m*/*z* 331.1433 $(M)^+$, Calcd for 313.1420] and the IR spectrum implied the presence of an aromatic moiety and olefin (1622, 1503, 1484 cm^{-1}) and hydroxyl groups (3398 cm^{-1}). The ^{1}H- and ^{13}C-NMR and HMQC data for **2** indicated that compound **2** possessed an Erythrinan skeleton with one trisubstituted double bond (δ_H 5.84 dd, δ_C 122.8, C-1; δ_C 148.2, C-6), two secondary hydroxyl groups (δ_H 4.20 ddd, δ_C 72.7, C-2; δ_H 4.29 dddd, δ_C 69.1, C-7), one methoxy group (δ_H 3.59 m, δ_C 80.4, C-3; δ_H 3.33 s, δ_C 57.1, C-3-OMe: C-18), and one dioxy-methylene group (δ_H 5.88 d, 5.89 d, δ_C 100.9, C-19). The ^{1}H–^{1}H COSY connectivity from C-1 to C-4 comprised the sequence of a trisubstituted olefinic proton, an oxygenated proton, a methoxy-oxygenated proton, and methylene protons in this order. Furthermore, homo-allyl coupling was presented be-

Table 2. ^{13}C-NMR Data of Compounds **1**, **2**, **3** and **7** in $CDCl_3$

Position	**1** δ_C	**2** δ_C	**3** δ_C	**7** δ_C
1	125.4	122.8	122.4	125.2
2	133.8	72.7	72.3	131.5
3	75.4	80.4	80.3	76.1
4	31.0	39.9	39.4	41.8
5	83.3	65.1	64.6	67.3
6	138.7	148.2	143.1	142.3
7	118.2	69.1	21.6	122.9
8	72.2	57.2	39.6	57.6
10	61.0	40.0	46.0	44.5
11	27.5	22.1	26.1	25.2
12	124.2	126.0	126.3	127.9
13	129.8	129.1	128.6	132.5
14	105.8	108.0	107.4	106.1
15	147.5	146.8	146.9	146.0
16	147.0	145.7	145.7	145.8
17	107.9	109.0	108.6	108.6
18	55.6	57.1	56.2	55.9
19	101.3	100.9	100.7	100.6

tween H-2 and H-7 as deduced from the cross-peak in ^{1}H–^{1}H COSY and coupling patterns confirmed by homo-decoupling experiments (J=2.1 Hz). The HMBC correlations of H-1/C-6, C-7, H-2/C-3, C-4, C-6, H-3/C-2, C-4, C-5, and H_3-OMe/C-3 indicated the presence of $\Delta^{1,6}$-olefin, a hydroxyl group at C-2, and a methoxy group at C-3 similar to those of erythratine (**3**). However, one more oxygenated proton was observed instead of methylene signals at C-7, and the HMBC cross-peaks of H-8/C-6, C-7, H-7/C-5, C-6 supported the presence of a hydroxyl group at C-7 in the ring C. In the ^{13}C-NMR, the chemical shifts of C-6 and C-8 were deshielded by 5.1 and 17.6 ppm in comparison with those of erythratine (**3**), respectively (Table 2). The configurations of the hydroxyl group at C-2 and the methoxy group at C-3 were revealed to be in β-orientation and α-orientation as deduced from the NOESY correlations of H-2/H-4α and H-3/H-14, respectively. On the other hand, the configuration of the hydroxyl group at C-7 was oriented in α-configuration as deduced from the NOESY correlation of H-7/H-14. Other NOESY correlations were accountable for the relative stereochemistry of cristanine B (**2**) depicted in Fig. 3. Thus, cristanine B (**2**) was elucidated as 7α-hydroxy-erythratine.

We examined the inhibitory activity on LPS-induced nitric oxide (NO) production and the cell viability in RAW264.7 macrophages of the compounds isolated in present study. Three of the compounds tested showed significant inhibitory activity in a dose-dependent manner. Two Erythrinan alkaloids, erythraline (**7**) and erythrinine (**9**), and an indole alkaloid, hypaphorine (**13**), showed inhibitory activity on LPS-induced nitric oxide production with IC_{50} values of 8.8, 3.4, and 11.2 μg/ml, respectively. Furthermore, these compounds exhibited no cytotoxicity for murine macrophages RAW264.7 used in this assay. The other compounds tested (**1**—**6**, **8**, **10**—**12**) were inactive (IC_{50}: >25 μg/ml). Erythraline (**7**) with significant activity occurred as a major component among alkaloidal compounds in this plant. Therefore, it might play some role in treating inflammatory diseases such as rheumatism and hepatitis in prescriptions containing this medicinal plant.

Experimental

General Experimental Procedure Optical rotation was determined using a JASCO DIP-370 digital polarimeter. UV spectra were measured using a JASCO V-560 UV/VIS spectrometer. IR spectra were obtained with a Perkin Elmer, Spectrum 100, FT-IR spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained with a Bruker AVANCE 500 spectrometer using tetramethylsilane as an internal standard. HR-EI-MS were obtained with a JEOL HX-100 spectrometer.

Plant Materials The bark of *Erythrina crista-galli* was purchased in September 2004 at São Paulo, and identified by Dr. Goro Hashimoto (Centro de Pesquisas de História, São Paulo, Brazil). Herbarium specimens were deposited at the Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo, Japan (Specimen number: B-160).

Extraction and Isolation The bark of *E. crista-galli* (dry weight, 985 g) was extracted with MeOH. The MeOH extracts (69.1 g) were successively partitioned between petroleum ether, EtOAc, and 3% aqueous tartaric acid. Water-soluble materials were adjusted to pH 10 with Na_2CO_3 and partitioned with $CHCl_3$, EtOAc, and *n*-BuOH, successively.

The alkaloidal $CHCl_3$-soluble materials (2.55 g) were subjected to amino-silica gel column chromatography (hexane/AcOEt, 80 : 20→0 : 100, and then $CHCl_3$/MeOH, 100 : 0→0 : 100, Chromatorex-NH, Fuji Silysia, Japan) to obtain twenty fractions (F1-1—20). The F1-2 (hexane/EtOAc, 70 : 30, 772.4 mg) was fractionated with a silica gel column (hexane/EtOAc, 70 : 30→50 : 50, and then $CHCl_3$/MeOH, 90 : 10→0 : 100, Wako-gel C-300, Japan) to give seven fractions (F2-1—7). F2-2 (hexane/EtOAc, 70 : 30, 330.3 mg) was purified with a silica gel column ($CHCl_3$/MeOH, 98 : 2→0 : 100) to isolate erythraline (**7**, 248.4 mg) and the other fractions were further purified by silica gel CC and silica gel HPLC (hexane/EtOAc, 50 : 50, YMC-pack SIL, ϕ10×250 mm, Japan) to isolate crystamidine (**4**, 3.1 mg). F2-4 (hexane/EtOAc, 60 : 40, 50.0 mg) was purified on silica gel ($CHCl_3$/MeOH, 98 : 2→0 : 100) to give 8-oxo-erythraline (**8**, 8.6 mg). F1-5 (hexane/EtOAc, 40 : 60, 95.3 mg) was separated on silica gel ($CHCl_3$/MeOH, 100 : 0→0 : 100) to isolate erysovine (**5**, 3.1 mg). F1-8 (hexane/EtOAc, 10 : 90, 60.5 mg) was fractionated by silica gel column chromatography (CC) (EtOAc/MeOH, 95 : 5→0 : 100) to give erythratine (**3**, 5.5 mg). F1-10 ($CHCl_3$, 6.7 mg) was pure erythrinine (**9**). F1-11 ($CHCl_3$/MeOH, 98 : 2, 51.2 mg) was separated on silica gel (EtOAc/MeOH, 98 : 2→0 : 100) to isolate epierythratidine (**12**, 12.1 mg). F1-12 ($CHCl_3$/MeOH, 95 : 5, 43.0 mg) was fractionated by silica gel CC ($CHCl_3$/MeOH, 90 : 10→100 : 0, and then AcOEt/MeOH, 90 : 0→100 : 0) to isolate 8-oxo-erythrinine (**10**, 2.7 mg), erythratidine (**11**, 17.9 mg), and cristanine A (**1**, 7.1 mg). F1-13 ($CHCl_3$/MeOH, 90 : 10, 149.0 mg) was subjected to LH-20 (MeOH, Sephadex) followed by silicagel CC (EtOAc/MeOH, 90 : 10→0 : 100, and then $CHCl_3$/MeOH, 80 : 20→0 : 100) to give cristanine B (**2**, 4.0 mg). On the other hand, the *n*-BuOH extracts (9.9 g) were separated by silica gel CC ($CHCl_3$–MeOH, 90 : 10→0 : 100) to give hypaphorine (**13**, 5.8 mg).

Cristanine A (**1**): Colorless amorphous. $[\alpha]_D^{25}$ +137.5° (c=0.032, MeOH). UV (MeOH) λ_{max} (log ε): 204 (4.61), 240 (4.19), 292 (3.66) nm. IR (KBr) ν_{max}: 1630, 1503, 1489 cm^{-1}. ^{1}H- and ^{13}C-NMR (Tables 1, 2).

Cristanine B (**2**): Colorless amorphous. $[\alpha]_D^{25}$ +210.2° (c=0.03, MeOH). UV (MeOH) λ_{max} (log ε): 204 (4.70), 238 (3.78), 292 (3.77) nm. IR (KBr) ν_{max}: 3398, 1622, 1503, 1488 cm^{-1}. ^{1}H- and ^{13}C-NMR (Tables 1, 2).

Measurement of Nitric Oxide Nitrite concentrations in the conditioned medium were determined using Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylenediamine dihydrochloride, and 2.5% phosphoric acid). RAW264.7 (1.0×10^5 cells/well) cells were pre-incubated at 37 °C for 3 h with or without various concentrations of samples (1.56, 6.25, 25 μg/ml). After pre-incubation, LPS (10 μg/ml) was added, and cells were incubated for 20 h. After 20 h incubation, 100 μl aliquots of medium were mixed with an equal volume of Griess reagent. Absorbance was measured at 540 nm with a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, U.S.A.) after incubating for 5 min.

Determination of Cell Viability Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW264.7 cells plated in 96-well plates (1×10^5 cells/well) were treated with various concentrations of samples for 20 h. Then MTT (5 mg/ml) was added and incubated for 4 h. The culture medium was removed, and cells were dissolved in dimethyl sulfoxide (DMSO). The optical densities (OD) at 540 nm and 620 nm were measured using a microplate reader.

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