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Comparison of Antioxidative Properties of Carbazole Alkaloids from *Murraya koenigii* Leaves

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A new dimeric carbazole alkaloid, 8,10′-[3,3′,11,11′-tetrahydro-9,9′-dihydroxy-3,3′,5,8′-tetramethyl-3,3′-bis(4-methyl-3-pentenyl)]bipyrano[3,2-*a*]carbazole (**12**), was isolated from the CH_2Cl_2 extract of *Murraya koenigii* together with six known carbazole alkaloids, koenimbine (**6**), *O*-methylmurrayamine A (**7**), *O*-methylmahanine (**8**), isomahanine (**9**), bismahanine (**10**), and bispyrayafoline (**11**). Their structures were determined on the basis of ^{1}H and ^{13}C NMR spectroscopic and mass spectrometric (MS) data. The antioxidative properties of 12 carbazole alkaloids isolated from leaves of *M. koenigii* were evaluated on the basis of the oil stability index together with their radical scavenging ability against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. On the basis of the lag time to reach a steady state, the 12 carbazoles were classified into three groups. It is suggested that an aryl hydroxyl substituent on the carbazole rings plays a role in stabilizing the thermal oxidation and rate of reaction against DPPH radical.

KEYWORDS: Carbazole; *Murraya koenigii*; curry leaf; antioxidant; oil stability index (OSI); 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

INTRODUCTION

The plant *Murraya koenigii* (L.) Spreng., belonging to the family Rutaceae, is native to India and now distributed in most of southern Asia. The leaves of this plant, called curry leaves, are commonly used for flavoring southern Asian dishes. *Murraya* species have also been used in traditional medicine in eastern Asia (*1*). Previous studies on *Murraya* species include reports of coumarins (*2*), terpenoids (*3*), and many investigations on carbazole alkaloids (*4*−*10*). Recently, several biological activities have been reported for carbazole alkaloids. Bioactive coumarins, acridone alkaloids, and carbazole alkaloids from the family Rutaceae were reviewed by Ito (*7*). Wu et al. (*8*) have reported the evaluation for antiplatelet activity and vasorelaxing effects of carbazole alkaloids from *M. euchrestifolia*. Ramsewak et al. (*9*) reported antioxidant activity and other biological activities of mahanimbine, murrayanol, and mahanine isolated from *M. koenigii*. Activities of carbazoles from *M. koenigii* against Gram-positive and Gram-negative bacteria and fungi were reported by Chowdhuri et al. (*10*).

Previously we established the model oil system for the determination of antioxidative activity under high-temperature conditions by the oil stability index (OSI) method (*11*). We found that the CH_2Cl_2 extract of *M. koenigii* showed antioxidative activity, on the basis of OSI values, and reported the isolation of five carbazole alkaloids, euchrestine B (**1**), bismurrayafoline (**2**), mahanine (**3**), mahaninebicine (**4**), and mahaninebine (**5**), from the CH_2Cl_2 extract and the strong antioxidative activities of compounds **1**−**3** (*12*). This investigation was designed to isolate and identify additional carbazole alkaloids from the leaves of *M. koenigii* and to evaluate the antioxidant activity of 12 carbazoles by measuring OSI values and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.

MATERIALS AND METHODS

Materials. Methyl linoleate (95% grade) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Ascorbic acid, α-tocopherol, BHT, DPPH, acetone, benzene, chloroform, EtOAc, hexane, CH_2Cl_2, and methanol (MeOH) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Silicone oil (TSF451-100) was purchased from Toshiba Silicone Co. Ltd. (Tokyo, Japan).

General Experimental Procedures. The ^{1}H, ^{13}C, and 2D NMR spectra were obtained with a Varian Unity 500 (500 MHz) spectrometer using TMS as internal standard (Varian Inc., Palo Alto, CA). EI and HR EIMS were measured at 70 eV on a Hitachi M 2000 mass spectrometer (Hitachi Ltd., Tokyo, Japan). Specific rotations were measured on a Jasco (Tokyo, Japan) P1030 polarimeter. UV spectra were recorded on a UV-2500PC UV−vis spectrophotometer (Shimadzu, Kyoto, Japan). Preparative high-performance liquid chromatography

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(HPLC) was performed with a Hitachi L-6250 intelligent pump equipped with an L-4200 UV−vis detector. Preparative reversed phase HPLC was done with a 250 × 20 mm Nomura C-18 column. Silica gel 60, 70−230 mesh (Merck, Darmstadt, Germany), Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and Chromatorex DM1020T octadecyl silica gel (ODS) (Fuji Sylisia Chemical Ltd., Kasugai, Japan) were used for column chromatography, and silica gel 60 F_{254} plates (Merck) were used for TLC. An oxidative stability instrument (Omnion Inc., Rockland, MA) attached to an IBM-compatible computer was used for the OSI measurement. A Wallac 1420 multilabel counter (Amersham Pharmacia Biotech AB) was employed as a microplate reader for measuring DPPH radical scavenging activity.

Plant Material. Leaves of *M. koenigii* were collected in Selangor, Malaysia, in January 1998. A voucher specimen (no. 06367) was deposited at the herbarium of Universiti Putra Malaysia, Selangor, Malaysia.

Extraction and Isolation. Air-dried and ground leaves (400 g) of *M. koenigii* were successively extracted with CH_2Cl_2 (3 × 2 L) and 70% aqueous acetone (5 × 2 L) at room temperature. For each extraction, the plant material was soaked in the solvent and allowed to stand overnight. The combined CH_2Cl_2 solutions were evaporated in vacuo to give the crude CH_2Cl_2 extract (31.8 g). The 70% aqueous acetone layer was concentrated in vacuo, and the resulting aqueous solution was partitioned with EtOAc to give the EtOAc soluble fraction (9.3 g) and water soluble fraction (53.6 g). The CH_2Cl_2 extract (12.0 g) was chromatographed over silica gel (200 g) and eluted with a mixture solution of CH_2Cl_2 and acetone of increasing polarity to give 10 fractions. Fraction 3 (0.7 g) eluted with CH_2Cl_2 was rechromatographed on a silica gel column using *n*-hexane/CH_2Cl_2 (6:4) as an eluent to give 11 fractions. Compound **6** (1.4 mg), compound **7** (0.6 mg), and compound **8** (3.5 mg) were separated from the fifth fraction of fraction 3 by preparative HPLC with a reversed phase C-18 column, using a mixture of methanol and water (98:2) at a flow rate of 5 mL/min and monitored with a UV detector at 280 nm. Fraction 6 (0.5 g), eluted with CH_2Cl_2, was rechromatographed on Sephadex LH-20 (acetone) to give 10 fractions from which (fraction 6-10) compound **9** (101.0 mg) was obtained. Fraction 6-5 was chromatographed on a silica gel column eluted with hexane/Ac (75:25) to give compound **11** (13.8 mg). Fraction 7 (1.3 g) was rechromatographed on a Sephadex LH-20 column using acetone as an eluent to give eight fractions. Repeated column chromatography over silica gel and ODS gel gave compound **10** (10.5 mg). Fraction 8 (1.03 g) was subjected to Sephadex LH-20 column chromatography using acetone as an eluent to give seven fractions. Fraction 8-5 was rechromatographed on ODS columns to give 15 fractions. Fraction 8-5-10 was rechromatographed on a preparative HPLC with 95% MeOH as an eluent by a flow rate of 5 mL/min to afford compound **12** (10.5 mg).

The structures were identified as koenimbine (**6**), *O*-methylmurrayamine A (**7**), *O*-methylmahanine (**8**), isomahanine (**9**), bismahane (**10**), bispyrayafoline (**11**), and 8,10′-[3,3′,11,11′-tetrahydro-9,9′-dihydroxy-3,3′,5,8′-tetramethyl-3,3′-bis(4-methyl-3-pentenyl)]-bipyrano[3,2-*a*]carbazole (**12**) on the basis of their ^{1}H and ^{13}C NMR and mass spectrometric data. The structural formulas of compounds **1**−**12** are shown in **Figure 1**.

*Compound **6**:* UV λ_{max} (MeOH) nm (log ϵ) 230.4 (4.37), 236.8 (4.38), 296.2 (4.18), 335.6 (3.65), 359.6 (3.52); ^{13}C NMR δ 16.1 (5-CH_3), 27.6 (3-CH_3), 56.1 (OCH_3), 75.9 (C3), 102.6 (C7), 104.5 (C11b), 111.0 (C10), 113.1 (C9), 117.2 (C1), 117.2 (C6a), 118.4 (C5), 121.1 (C6), 124.5 (C7a), 129.3 (C2), 134.3 (C10a), 135.7 (C11a), 149.9 (C4a), 154.0 (C8); EIMS 70 eV, *m*/*z* (rel intensity) 293 $[M]^+$ (70), 278 (82); $C_{19}H_{19}NO_2$.

*Compound **7**:* UV λ_{max} (MeOH) nm (log ϵ) 221.2 (4.87), 240.4 (4.95), 282.6 (4.65 sh), 293.6 (4.82), 325.0 (4.25), 341.2 (4.22 sh), 356.6 (4.13 sh); ^{13}C NMR δ 16.1 (5-CH_3), 27.6 (3-CH_3), 55.7 (OCH_3), 75.7 (C3), 95.2 (C10), 104.2 (C11b), 107.7 (C8), 117.2 (C1), 117.8 (C6a), 117.8 (C7a), 118.4 (C5), 119.9 (C7), 120.4 (C6), 129.6 (C2), 134.7 (C11a), 140.6 (C10a), 148.9 (C4a), 158.0 (C9); EIMS 70 eV, *m*/*z* (rel intensity) 293 $[M]^+$ (54), 278 (100), 263 (12), 235 (9), 234 (6); $C_{19}H_{19}NO_2$.

*Compound **8**:* UV λ_{max} (MeOH) nm (log ϵ) 221.2 (4.23), 241.2 (4.31), 246.6 (4.29, sh), 284.6 (4.05 sh), 294.6 (4.20), 326.0 (3.57), 331.2 (3.58), 343.0 (3.59), 357.8 (3.50 sh); ^{1}H NMR δ 1.44 (3H, s, 3-CH_3), 1.58 (3H, br s, 4′-CH_3), 1.66 (3H, br s, 4′-CH_3), 1.72−1.78 (2H, m, H-1′), 2.11−2.20 (2H, m, H-2′), 2.32 (3H, br s, 5-CH_3), 3.88 (3H, s, 9-OCH_3), 5.11 (1H, m, H-3′), 5.65 (1H, d, J = 9.8 Hz, H-2), 6.62 (1H, d, J = 9.8 Hz, H-1), 6.80 (1H, dd, J = 2.2, 8.5 Hz, H-8), 6.89 (1H, d, J = 2.2 Hz, H-10), 7.56 (1H, br s, H-6), 7.76 (1H, d, J = 8.5 Hz, H-7), 7.73 (1H, br s, NH); ^{13}C NMR δ 16.0 (5-CH_3), 17.6 (4′-CH_3), 22.8 (C2′), 25.7 (4′-CH_3), 25.8 (3-CH_3), 40.7 (C1′), 55.7 (OCH_3), 78.0 (C3), 95.2 (C10), 104.3 (C11b), 107.6 (C8), 116.7 (C6a), 117.5 (C1), 117.9 (C7a), 118.3 (C5), 119.9 (C7), 120.5 (C6), 124.2 (C3′), 128.7 (C2), 131.7 (C4′), 134.7 (C11a), 140.7 (C10a), 148.9 (C4a), 157.9 (C9); EIMS 70 eV, *m*/*z* (rel intensity) 361 $[M]^+$ (44), 346 (8), 278 (100); $C_{24}H_{27}NO_2$; $[\alpha]_D^{25}$ +3.0 (*c* 0.10, $CHCl_3$).

*Compound **9**:* UV λ_{max} (MeOH) nm (log ϵ) 222.2 (4.39), 238.8 (4.43), 286.0 (4.13, sh), 296.2 (4.32), 321.6 (3.80), 351.2 (3.59); $[\alpha]_D^{25}$ −38.2 (*c* 0.22, $CHCl_3$).

*Compound **10**:* UV λ_{max} (MeOH) nm (log ϵ) 224.0 (4.75), 245.4 (4.78), 298.4 (4.69), 331.0 (4.20), 341.8 (4.18 sh), 359.2 (4.05 sh); ^{13}C NMR δ 16.1 (5-CH_3, 5′-CH_3), 17.6 (4″-CH_3, 4‴-CH_3), 22.7 (C2″, C2‴), 25.7 (4″-CH_3, 4‴-CH_3), 25.8 (3-CH_3, 3′-CH_3), 40.6 (C1‴), 40.7 (C1″), 78.0 (C3′), 78.2 (C3), 98.1 (C10), 104.5 (C11b, C11b′), 104.8 (C10′), 108.6 (C8′), 110.2 (C8), 116.2 (C6a), 117.1 (C6a′), 117.4 (C1), 117.6 (C1′), 118.0 (C7a′), 118.7 (C5′), 118.9 (C5), 119.1 (C7a), 120.5 (C6′, C7′), 120.6 (C6, C7), 124.1 (C3″, C3‴), 128.6 (C2′), 129.0 (C2), 131.7 (C4″, C4‴), 149.1 (C4a′), 149.6 (C4a), 151.4 (C9′), 151.8 (C9); EIMS 70 eV, *m*/*z* (rel intensity) 692 $[M]^+$ (100), 609 (48), 558 (30), 475 (23), 347 (55), 263 (53); $C_{46}H_{48}N_2O_4$; $[\alpha]_D^{25}$ +4.0 (*c* 0.65, $CHCl_3$).

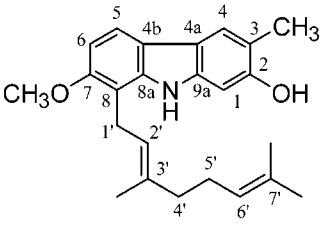
*Compound **11**:* UV λ_{max} (MeOH) nm (log ϵ) 224.2 (4.90), 238.4 (4.87), 297.2 (4.87), 332.4 (4.29); ^{13}C NMR δ 16.7 (8-CH_3, 8′-CH_3), 17.6 (4″-CH_3, 4‴-CH_3), 22.6 (C2″, C2‴), 25.7 (4″-CH_3, 4‴-CH_3), 25.9 (3-CH_3, 3′-CH_3), 40.8 (C1″, C1‴), 78.2 (C3, C3′), 99.0 (C10, C10′), 105.0 (C11b, C11b′), 109.9 (C5, C5′), 117.4 (C1, C1′), 117.7 (C6a, C6a′), 117.7 (C7a, C7a′), 117.9 (C8, C8′), 119.5 (C6, C6′), 122.3 (C7, C7′), 124.0 (C3″, C3‴), 128.8 (C2, C2′), 131.7 (C4″, C4‴), 136.0 (C11a, C11a′), 137.8 (C10a, C10a′), 150.3 (C9, C9′), 151.1 (C4a, C4a′); EIMS 70 eV, *m*/*z* (rel intensity) 692 $[M]^+$ (100); HR-EIMS 70 eV, found *m*/*z* 692.3611 $[M]^+$, $C_{48}H_{56}N_2O_4$ requires 692.3614; $C_{46}H_{48}N_2O_4$; $[\alpha]_D^{25}$ +22.2 (*c* 0.09, $CHCl_3$).

*Compound **12**:* UV λ_{max} (MeOH) nm (log ϵ) 224.0 (4.84), 243.4 (4.85), 298.4 (4.76), 327.4 (4.30); ^{13}C NMR δ 16.0 (5-CH_3), 16.7 (8′-CH_3), 17.6 (4″-CH_3, 4‴-CH_3), 22.7 (C2″, C2‴), 25.7 (4″-CH_3, 4‴-CH_3), 25.8 (3-CH_3), 25.9 (3′-CH_3), 40.7 (C1″, C1‴), 78.2 (C3, C3′), 98.0 (C10), 104.1 (C10′), 104.5 (C11b), 104.8 (C11b′), 109.6 (C5′), 110.2 (C8), 116.2 (C6a), 116.8 (C7a′), 117.9 (C6a′), 117.4 (C1), 117.5 (C1′), 117.5 (C8′), 118.9 (C5), 119.2 (C7a), 119.5 (C6′), 120.6 (C6), 121.4 (C7′), 121.7 (C7), 124.1 (C3″, C3‴), 128.7 (C2′), 129.1 (C2), 131.8 (C4″, C4‴), 135.0 (C11a), 136.0 (C11a′), 139.2 (C10a′), 141.6 (C10a), 149.6 (C4a), 150.9 (C4a′), 150.0 (C9′), 151.9 (C9); EIMS 70 eV, *m*/*z* (rel intensity) 724 $[M]^+$ (100), 479 (3), 362 (3); HR-EIMS 70 eV, found *m*/*z* 692.3623 $[M]^+$, $C_{48}H_{56}N_2O_4$ requires 692.3614; $[\alpha]_D^{25}$ +55.0 (*c* 0.12, $CHCl_3$).

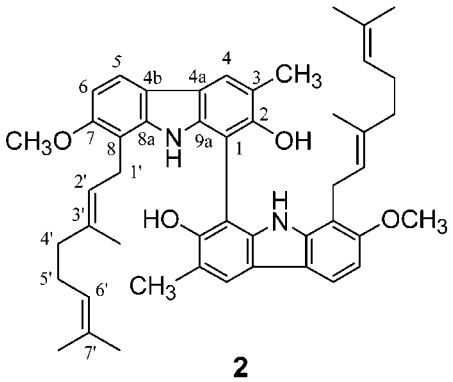
Evaluation of OSI. The OSI is defined as the point of maximum change of the rate of oxidation of fats and oils under high temperature (*11*, *13*). A stream of air was bubbled into 5 g of oil contained in a reaction tube placed in an electric heating chamber. The effluent air that contained volatile organic acids generated from the oil was collected in another tube containing distilled water (50 mL). The conductivity of the water as oxidation proceeded was measured automatically. The air flow rate was set at 2.5 mL/s for all determinations.

Methyl linoleate (10%) in silicone oil was used as a model substrate oil for measuring OSI values with and without samples. The test samples (5 μmol) were dissolved in 500 μL of methanol. A portion of these solutions (100 μL) was added to the 5 g of model substrate oil (final concentration = 0.2 μmol/g of model substrate oil).

The mixtures were shaken on a vortex mixer for 30 s under nitrogen. These were then preheated for 30 min without linking the conductivity measurement tubes. Then the reaction tubes were connected to the conductivity measurement tubes, and the OSI values were measured at 110 °C. Three replicates of OSI measurements were obtained on all samples (*11*). The OSI ratio was calculated according to the following equation:



1



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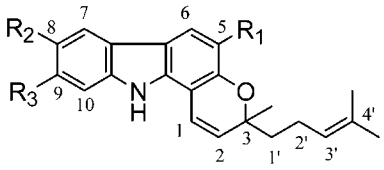
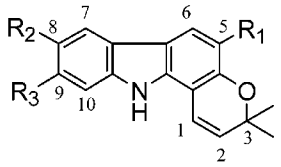
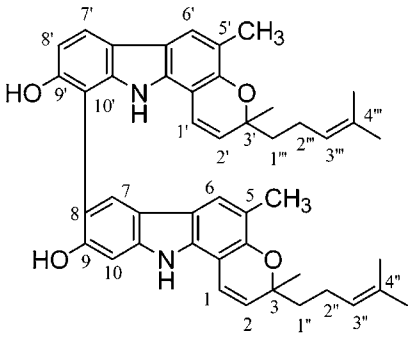


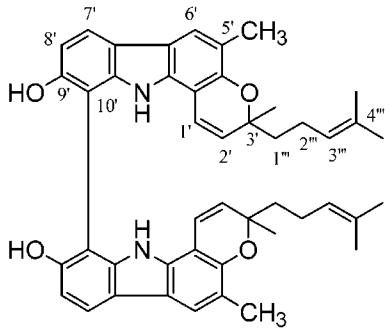
	R_1	R_2	R_3
3	CH_3	H	OH
4	H	CH_3	H
5	CH_3	H	H
8	CH_3	H	OCH_3
9	H	CH_3	OH



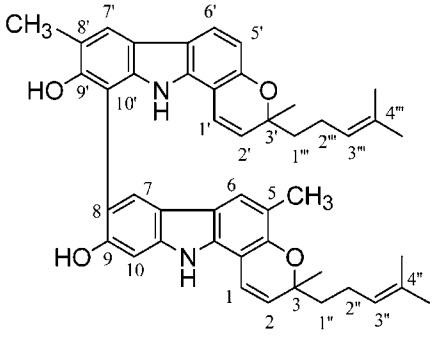
	R_1	R_2	R_3
6	CH_3	OCH_3	H
7	CH_3	H	OCH_3



10



11



12

Figure 1. Structures of carbazole alkaloids isolated from leaves of *M. koenigii*.

OSI ratio = OSI value of the model oil with sample ÷ OSI value of the model oil without sample

Evaluation of DPPH Radical Scavenging Activity. The scavenging activity of compounds isolated from the CH_2Cl_2 extract on DPPH radicals was measured according to the method of Brand-Williams et al. (*14*) with some modifications (*15*). To a well in a 96 well flat-bottom microplate was added 75 μL of an ethanolic solution of sample and 75 μL of a 200 μM ethanolic solution of DPPH (final concentration = 100 μM). Samples were prepared in triplicate for each concentration used (final concentrations were 0.1, 1, 3.2, 10, 31.5, and 100 μM). Ethanolic solution without samples (75 μL) was added as control. The absorbances of each reaction mixture were measured at 520 nm against a blank of ethanol without DPPH after 1 and 30 min and 1, 2, 3, 5, 7, 24, 72, and 99 h. All analyses were run in triplicate. The DPPH radical concentration in the reaction mixture was calculated according to the calibration curve determined by linear regression:

$$\text{absorbance at 520 nm} = 3.5 \times 10^{-3} \times [\text{concentration of DPPH radical } (\mu\text{M})] - (8 \times 10^{-4})$$

The percentage of the remaining DPPH radical at each reaction time was calculated as follows:

remaining DPPH radical (%) = [concentration of DPPH radical with samples at each reaction time ÷ concentration of DPPH radical without samples at each reaction time] × 100

Table 1. ^{1}H NMR Assignment for Compounds **10**–**12**

	10	11	12
H-1 (1′)	6.64 (1H, d, 9.8), 6.47 (1H, d, 9.8)	6.44 (2H, d, 9.7)	6.68 (1H, d, 9.8), 6.49 (1H, d, 9.8)
H-2 (2′)	5.69 (1H, d, 9.8), 5.54 (1H, d, 9.8)	5.55 (2H, d, 9.7)	5.71 (1H, d, 9.8), 5.56 (1H, d, 9.8)
H-5 (5′)		6.74 (2H, d, 8.3)	–, 6.71 (1H, d, 8.3)
H-6 (6′)	7.53 (1H, br s), 7.60 (1H, br s)	7.72 (2H, d, 8.3)	7.54 (1H, s) 7.70 (1H, d, 8.3)
H-7 (7′)	7.88 (1H, br s), 7.83 (1H, d, 8.3)	7.85 (2H, br s)	7.88 (1H, s), 7.76 (1H, s)
H-8 (8′)	–, 6.94 (1H, d, 8.3)		
H-10 (10′)	7.11 (1H, br s)		7.17 (1H, s), –
Ar-CH_3	2.31 (3H, br s), 2.33 (3H, br s)	2.50 (6H, br s)	2.31 (3H, br s), 2.47 (3H, br s)
Ar-OH	5.29 (1H, br s), 5.25 (1H, br s)	5.12 (2H, br s)	5.19 (1H, br s), 5.23 (1H, br s)
NH	7.95 (1H, br s), 7.61 (1H, br s)	7.50 (2H, br s)	7.98 (1H, br s), 7.55 (1H, br s)
3-CH_3	1.46 (3H, s), 1.40 (3H, s)	1.39 (6H, s)	1.47 (3H, s), 1.40 (3H, s)
3–4′-methyl-3′-pentenyl	1.74–1.80 (2H, m), 1.69–1.73 (2H, m)	1.65–1.76 (4H, m)	1.76–1.79 (2H, m), 1.70–1.75 (2H, m)
	2.09–2.22 (4H, m)	2.08–2.13 (4H, m)	2.16–2.20 (2H, m), 2.11–2.14 (2H, m)
	5.11 (1H, m), 5.08 (1H,m)	5.06 (2H, m)	5.12 (1H, m), 5.07 (1H, m)
	1.58 (3H, br s), 1.56 (3H, br s)	1.55 (6H, br s)	1.59 (3H, br s), 1.55 (3H, br s)
	1.66 (3H, br s), 1.64 (3H, br s)	1.63 (6H, br s)	1.67 (3H, br s), 1.64 (3H, br s)

DPPH radical scavenging activity (%) =
[concentation of DPPH radical without samples − concentration of DPPH radical containing samples (μM) ÷ concentration of DPPH radical without samples] × 100

The IC_{50} at each reaction time was defined as the concentration of compound that scavenged 50% concentration of DPPH radical against the concentration of DPPH radical of control at each reaction time. The IC_{50} at each reaction time was calculated graphically.

Statistical Analysis. We used a factorial analysis of variance (ANOVA) to test for overall differences among the compounds. Significances of individual differences were evaluated by using the Scheffé test, when ANOVA was significant. Significance was declared at $P < 0.05$. ANOVA and linear regression were performed by utilizing the Stat View program package (SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

Isolation and Structure Analysis of Compounds 6—12. The CH_2Cl_2 extract was subjected to repeated column chromatography using silica gel, Sephadex LH-20, and ODS to give a new dimeric carbazole alkaloid **12** and six known carbazole alkaloids **6**−**11**.

The UV spectra of isolated compounds **6**−**12** revealed a typical absorption of a carbazole skeleton (*4*).

Compound **6** had the molecular formula $C_{19}H_{19}NO_2$ on the basis of its EIMS and ^{13}C NMR spectra. The pyranocarbazole skeleton was deduced from its MS spectrum at a high-intensity peak of *m*/*z* 278, which was typical of the carbazolepyrilium ion (*4*). Compound **6** was identical with koenimbine isolated from the fruits (*16*) and leaves (*17*) of *M. koenigii* Spreng in 1968 and 1969, respectively, comparing the ^{1}H NMR spectra. Because ^{13}C NMR data of compound **6** have not been reported so far, the complete assignment of carbon signals of compound **6** was shown in the extraction and isolation section on the basis of ^{13}C NMR, HMQC, and HMBC measurements.

Compound **7** had the same molecular formula, $C_{19}H_{19}NO_2$, as compound **6** on the basis of its EIMS spectrum and showed ^{1}H and ^{13}C NMR spectra similar to those of compound **6**. The HMQC and HMBC measurements gave evidence for 5-methyl- and 9-methoxycarbazole skeletons. The assignment was also supported by nuclear Overhauser and exchange spectroscopy (NOESY) experiments. On the basis of the above data, compound **7** is identical with *O*-methylmurrayamine A, which was synthesized from murrayamine A by Wu (*18*). However, this is the first report of the isolation of this compound from a natural source. The complete assignment of carbon signals of compound **7** was shown in the extraction and isolation section; ^{13}C NMR data of *O*-methylmurrayamine A have not been reported.

Compound **8** had the molecular formula $C_{24}H_{27}NO_2$ as established by EIMS and ^{13}C NMR spectra. The pyranocarbazole skeleton was deduced on the basis of the presence of a high-intensity peak at *m*/*z* 278 in the EIMS spectrum. The ^{1}H and ^{13}C NMR spectra of compound **8** were similar to those of compound **7** except the signals at δ1.58 (3H, br s), 1.66 (3H, br s), 1.72−1.78 (2H, m), 2.11−2.20 (2H, m), and 5.11 (1H, m) due to the 4-methyl-3-pentenyl group. The HMQC, HMBC, and NOESY measurements allowed the complete assignments of proton and carbon signals as well as determination of the location of substituents on the pyranocarbazole skelton. On the basis of the above evidence, compound **8** was *O*-methylmahanine. Anwer et al. (*19*) reported the synthesis of DL-*O*-methylmahanine, but no spectroscopic data were reported. Then the data of compound **8** are shown in the extraction and isolation section. This is the first report of the isolation of *O*-methylmahanine from a natural source.

Compound **9** was identical with isomahanine previously isolated from fruits of *M. koenigii* (*20*) by comparison of its ^{1}H and ^{13}C NMR spectra.

The EIMS spectrum of compound **10** gave the molecular formula $C_{46}H_{48}N_2O_4$. On the basis of comparison of the ^{1}H spectra, compound **10** was identical with bismahanine isolated from stem bark of *Murraya koenigii* by Ito et al. (*21*). Although Ito et al. have not reported the ^{13}C NMR data of bismahanine, the assignment of carbon signals of compound **10** was shown in the extraction and isolation section.

The molecular formula of compound **11** was established as $C_{46}H_{48}N_2O_4$ by HRMS spectrometry. The ^{1}H and ^{13}C NMR spectra showed only half of the total signals for the protons and carbons available in its molecular formula, indicating that this compound had a completely symmetrical structure. The ^{1}H and ^{13}C NMR spectra were similar to those of **9** except for the lack of H-10 (**Table 1**), which suggested that the structure of **11** was indeed a symmetrical dimer of **9** with the linkage occurring at C-10 and C-10′. Compound **11** was therefore identified as bispyrayafoline, which was also previously isolated from the leaves of *M. koenigii* by Onoda et al. (unpublished results). The spectroscopic data of compound **11** are shown in the extraction and isolation section.

Compound **12** had the same molecular formula, $C_{46}H_{48}N_2O_4$, as compounds **10** and **11** on the basis of its HRMS and ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR spectra of compound **12** were similar to those of compound **10** (**Table 1**). The ^{1}H NMR spectrum indicated the presence of a mahanine (**3**) unit except

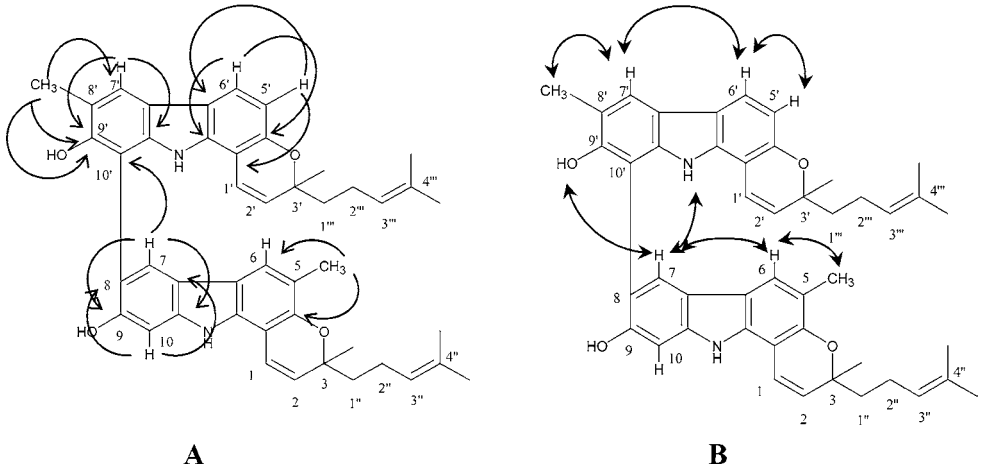


Figure 2. Correlation for compound **12** from 2D NMR spectrum: (**A**) selected HMBC correlation for compound **12**; (**B**) selected NOESY correlation for compound **12**.

for the lack of H-8 in the molecule. In the ^{1}H NMR spectrum of **12**, the ortho-coupled aromatic protons at δ 6.71 and 7.70 (J = 8.3 Hz) were observed. In the HMBC spectrum, cross-peaks occurred between the aromatic proton at δ 6.71 and 104.8 (C-11b′) and 117.9 (C-6a′), suggesting that the aromatic proton was located at C-5′. The HMBC correlation between another ortho-coupled aromatic proton at δ 7.70 and the aromatic carbon at δ 136.0 (C-11a′) and aromatic carbon atoms at 150.9 (C-4a′) indicated that the aromatic proton at δ 7.70 was located at position C-6′ (**Figure 2A**). Moreover, correlations between one of the aryl methyl protons (δ 2.47) and δ 121.4 (C-7′), 117.5 (C-8′), and 150.0 (C-9′) observed in its HMBC spectrum indicated that the aryl methyl substituent was located at C-8′ (**Figure 2A**). The assignment of the aromatic proton at δ 6.71 and the location of the benzylic methyl substituent could be further confirmed by the NOESY experiments as shown in **Figure 2B**. On the basis of the above evidence, another carbazole unit was isomahanine (**9**) except for the lack of H-10′. The HMBC spectrum showed that the aromatic proton of the mahanine unit at δ 7.88 (H-7) was correlated to C-10′ (δ 104.1) of the isomahanine unit (**Figure 2A**). Further information on the linkage of C-8 and C-10′ was obtained from NOESY correlations of H-7 to both NH and OH of the isomahanine unit (**Figure 2B**). From the above evidence, the structure of compound **12** was assigned as 8,10′-[3,3′,11,11′-tetrahydro-9,9′-dihydroxy-3,3′,5,8′-tetramethyl-3,3′-bis(4-methyl-3-pentenyl)]-bipyrano[3,2-*a*]carbazole.

Evaluation of OSI Ratio of Compounds 1—12. The 12 carbazole alkaloids isolated from the CH_2Cl_2 extract, together with α-tocopherol and BHT, were subjected to evaluation for OSI values. The ratios of OSI value against control measured at 110 °C are shown in **Figure 3**. Larger OSI ratios indicate a stronger antioxidant activity. The OSI value on the model oil without samples (control) showed a mean value of 1.48 h. The OSI ratios of model oil containing α-tocopherol and BHT were 6.01 and 3.82, respectively. The OSI ratios of compounds **1**, **3**, and **9**−**12** were significantly greater than that of BHT. In particular, the antioxidative activity of compound **3** was greater than that of α-tocopherol.

Among the monomeric carbazoles **1** and **3**−**9**, compounds **1** (OSI ratio of 9.01), **3** (10.40), and **9** (8.81) had stronger antioxidant activities than compounds **4**−**8** (0.90, 1.21, 3.27, 1.76, and 2.06, respectively). Iwatsuki et al. (*22*) reported that the antioxidant activity required the presence of a free hydroxyl group on the carbazole skeleton in the oxidations of methyl linoleate in a homogeneous solution and soybean phosphatidylcholine liposomes in aqueous dispersion, induced by free radicals at room temperature. Our result indicated that carbazole with free hydroxyl groups (compounds **1**, **3**, and **9**) also showed antioxidant activity under thermal oxidation. It seemed that hydroxy substituents on the carbazole ring extend induction time and the alkyl or methoxy substituents on carbazole rings had no influence on the activity. Among the dimeric carbazoles, the OSI ratios of compounds **10** (9.84), **11** (7.06), and **12** (6.72) were significantly greater than that of compound **2** (3.91). The activity discrepancy might be explained by the fact that the hydroxy group of compound **2** is hindered by the ortho-substituent, whereas each hydroxy group of compounds **10**−**12** is located at the outer part of the carbazole molecule.

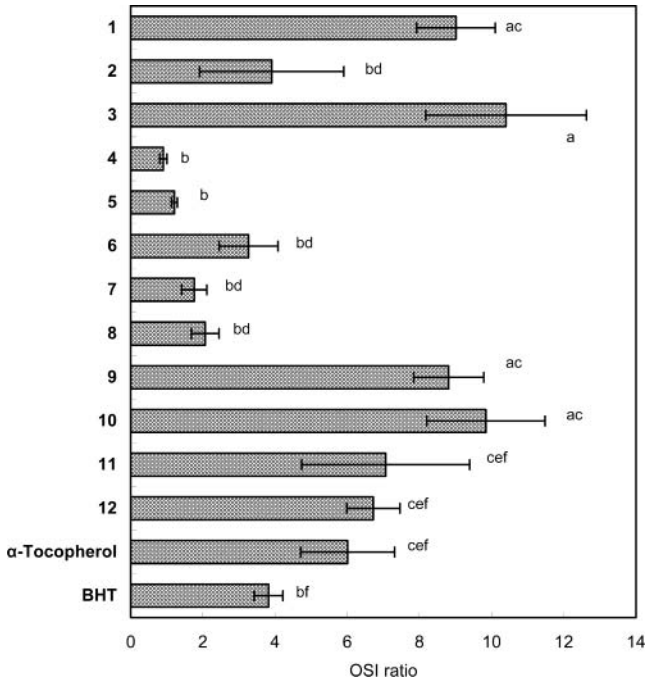


Figure 3. OSI ratios of carbazole alkaloids isolated from leaves of *M. koenigii* and α-tocopherol and BHT as standard antioxidants: OSI ratios are mean ± standard deviation of three replicates at 110 °C; bars with different letters are significantly (P < 0.05) different by analysis of variance with multiple comparisons. Samples were added at 0.2 μmol/g of model substrate oil.

Evaluation of DPPH Radical Scavenging Activity of Compounds 1—12. DPPH radical scavenging activity was

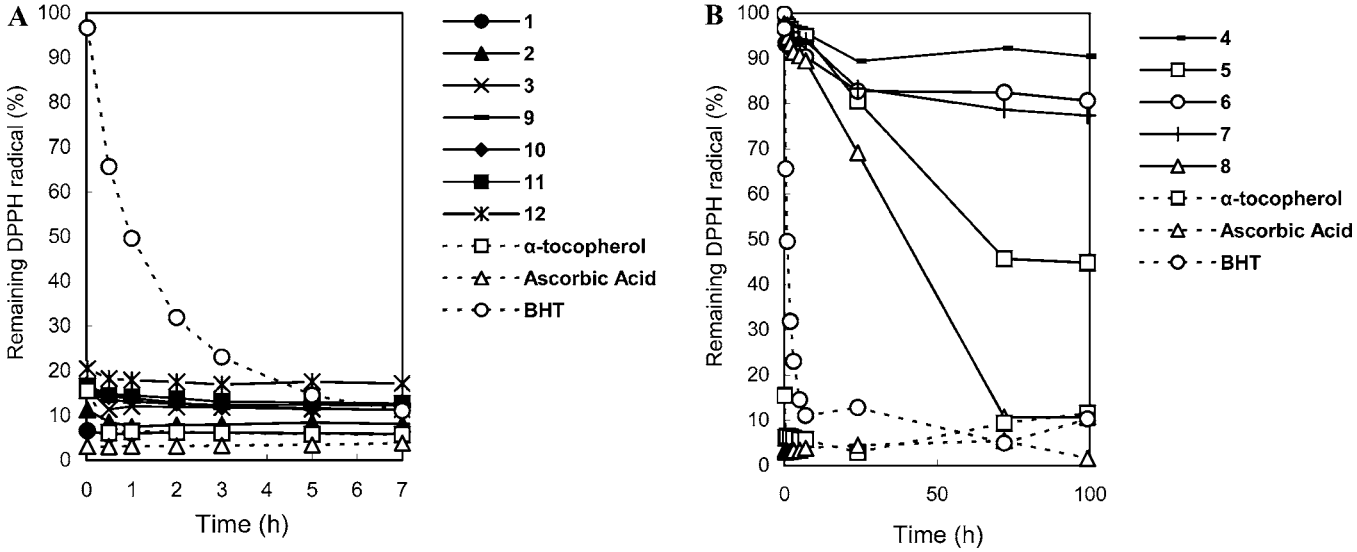


Figure 4. Kinetic behavior of compounds **1**–**12** and α-tocopherol, BHT, and ascorbic acid as standard antioxidants (concentration = 100 μM): (**A**) kinetic behavior of the fastest and faster reacting compounds; (**B**) kinetic behavior of the slow reacting compounds.

measured for compounds **1**−**12**, together with α-tocopherol, BHT, and ascorbic acid as standard antioxidants. Recently, Brand-Williams et al. (*14*) reported that 20 compounds were reacted with the DPPH radical and classified to three possible reaction kinetic types on the basis of the time to reach a steady state of the radical scavenging reaction (*23*). Sánchez-Moreno et al. (*24*) reported a new parameter, antiradical efficiency, that was estimated on the basis of EC_{50} and time to steady state. To our knowledge, there are no reports about kinetic behavior of carbazoles. Therefore, 12 carbazoles were classified on the basis of time to reach a steady state. The kinetic behaviors of the samples at 100 μM against reaction time are plotted in **Figure 4**. Ascorbic acid showed the fastest behavior and reached the plateau stage within 1 min. The faster behavior group included compounds **1**−**3** and **9**−**12** and α-tocopherol, showing the plateau stage reached after 30 min. BHT showed an intermediate behavior of 7 h. Compounds **4**, **6**, and **7** showed slow kinetic behaviors and reached steady state after 24 h. The kinetic behaviors of compounds **5** and **8** were very slow, requiring 72 h to reach the steady state. The remaining DPPH radicals were >80% at the steady state in the cases of compounds **4**, **6,** and **7**, but those of other samples were <20%. Therefore, the reaction rate of carbazole with free hydroxyl groups may be considered to be faster than that of carbazoles without free hydroxyl groups. The radical scavenging activity of compound **11** rose with increasing concentration (**Figure 5**). There were no differences of IC_{50} after 0.5, 1, 2, 5, 7, and 24 h. Compounds **1**−**3** and **9**−**12** and α-tocopherol, which are in the faster reaction group, showed similar behavior. **Table 2** shows the IC_{50} values after 30 min of scavenging activity of the compounds belonging to the faster group compared to that of control. The scavenging activity for these compounds was in the order **10**, **11**, **1**, **2** > **3** > **9**, **12**. There was no significant difference between α-tocopherol and these carbazoles. The dimeric carbazoles showed a tendency to raise DPPH radical scavenging activity in comparison with the monomeric carbazole alkaloids except for compound **12**. The hydroxyl-substituted carbazoles that belong to the faster group showed strong and rapid scavenging activities against the DPPH radical. Consequently, these hydroxylated carbazoles also consistently play an important role in expressing strong activity on the measurement of OSI. Meragelman et al. (*25*) reported that hydroxyl-substituted carbazole alkaloids from *Murraya siamensis* showed anti-HIV activity, whereas carbazole

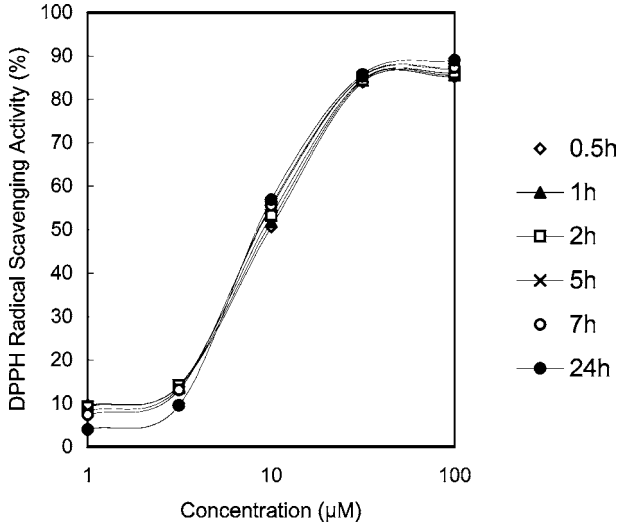


Figure 5. DPPH radical scavenging activity of compound **11** against 100 μM DPPH radical at several reaction times.

Table 2. DPPH Radical Scavenging Activities of Carbazoles and α-Tocopherol Belong to Faster Group

compound	IC_{50} of DPPH radical scavenging activities[a] (μM)
1	14.85 ± 0.77[a]
2	14.97 ± 0.76[a]
3	16.65 ± 3.05[ac]
9	24.00 ± 7.37[bc]
10	13.30 ± 0.96[a]
11	13.85 ± 5.45[a]
12	27.58 ± 10.69[b]
α-tocopherol	20.25 ± 1.94[abc]

[a] IC_{50} values are mean ± standard deviation of three replicates. Values with different letters are significantly (P < 0.05) different by analysis of variance with multiple comparisons.

without a hydroxyl substituent was inactive. Chowdhury et al. (*10*) reported antimicrobial activities of 6,7-dimethoxy-1-hydroxy-3-methylcarbazole and 1-formyl-3-methoxy-6-methylcarbazole. The hydroxyl-substituted carbazole was found to be highly active against both Gram-positive and Gram-negative bacteria and fungi, but carbazole without a hydroxyl substituent

showed a low activity against Gram-negative bacteria and fungi. The biological activity of hydroxyl-substituted carbazole is worthy of further study.

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