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Studies on Dental Caries Prevention by Traditional Medicines. X.[1,2] Antibacterial Action of Phenolic Components from Mace against *Streptococcus mutans*

MASAO HATTORI,[a] SUMITRA HADA,[a] AKIKO WATAHIKI,[a] HITOMI IHARA,[a] YUE-ZHONG SHU,[a] NOBUKO KAKIUCHI,[a] TOKUO MIZUNO[b] and TSUNEO NAMBA*,[a]

Research Institute for Wakan-Yaku (Oriental Medicines), Toyama Medical and Pharmaceutical University,[a] 2630 Sugitani, Toyama, Japan and Scientific Instrument Division, JEOL Ltd.,[b] 1418 Nakagami, Akishima, Tokyo 196, Japan

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The methanolic extract of the aril of *Myristica fragrans* HOUTT. (mace) had anti-plaque action against a cariogenic bacterium, *Streptococcus mutans*. Through fractionation of the extract followed by microbial assay by using the tube dilution technique, dehydrodiisoeugenol (**1**) and 5′-methoxydehydrodiisoeugenol (**2**) were identified as the major antibacterial principles. Both compounds completely inhibited the bacterial growth at a concentration of 12.5 μg/ml. During this experiment, *threo*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol methyl ether (**6**) and guaiacin (**8**) were isolated for the first time from mace.

Keywords——antibacterial action; anti-plaque action; dehydrodiisoeugenol; guaiacin; LC-MS; mace; 5′-methoxydehydrodiisoeugenol; 2-(4-allyl-2,6-dimethoxyphenoxyl)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol methyl ether; *Myristica fragrans*; *Streptococcus mutans*

In the course of basic studies on dental caries prevention by traditional medicines,[1,3] we have previously screened various Ayurvedic medicines used in Sri Lanka for anti-plaque action and have found that the extracts of the roots of *Glycyrrhiza glabra* L., the seeds of *Nigera sativa* L., the aril of *Myristica fragrans* HOUTT., the seeds of *Peucedanum graveolens* BENTH., the roots of *Sida cordifolia* L. and the fruit of *Embelia ribes* BURM. potently inhibit the adherence of viable cells of *Streptococcus mutans* to smooth surfaces *in vitro*.[3g] The anti-plaque action of the extracts of *Glycyrrhiza glabra* and *Embelia ribes* was attributable to their antibacterial action against *S. mutans* as well as their inhibitory action against glucosyltransferase (GTase; EC 2.4.1.5) from *S. mutans*.[3g] In the present paper, we report the isolation and identification of antibacterial principles present in the aril of *Myristica fragrans* through *in vitro* bioassay.

Materials and Methods

Apparatus——All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Proton and carbon nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra were measured with JEOL GX-270 (^{1}H, 270 MHz), Varian XL-200 (^{1}H, 200 MHz) and JEOL FX-90Q (^{13}C, 22.5 MHz) spectrometers with tetramethylsilane as an internal standard. Mass spectra (MS) were measured with a JMS-DX 300 mass spectrometer (JEOL) at an ionization voltage of 70 eV. High-performance liquid chromatography (HPLC) was carried out on a Tri Rotar SR-1 (JASCO Ltd., Tokyo) equipped with a UVIDEC-100V detector (JASCO). Ultraviolet (UV) spectra and turbidity were measured with a Shimadzu UV-210 digital double beam spectrophotometer (Shimadzu Seisakujo, Kyoto). Circular dichroic (CD) spectra were recorded on a JASCO J-500 spectropolarimeter equipped with a JASCO DP-500 data processor. Infrared (IR) spectra were taken on a Hitachi 260-10 infrared spectrometer. Liquid chromatography-mass spectrometry (LC-MS) was carried out on a JMS-DX 300 (JEOL) equipped with a direct inlet LC-MS interface and a FAMILIC-300 HPLC system (JASCO).

Plant Material——The aril of *Myristica fragrans* (mace) used in this study was purchased from W. Wilbert and Co. (Colombo, Sri Lanka, 1983) and was ground before extraction.

Chemicals——Bacto brain heart infusion broth (BHI) was a product of Difco Laboratories (Detroit, U.S.A.). Dehydrodiisoeugenol (**1**), 5′-methoxydehydrodiisoeugenol (**2**), 2-(3,4-methylenedioxyphenyl)-2,3-dihydro-7-methoxy-3-methyl-5-(1(*E*)-propenyl)benzofuran (**3**), 2-(3-methoxy-4,5-methylenedioxyphenyl)-2,3-dihydro-7-methoxy-3-methyl-5-(1(*E*)-propenyl)benzofuran (**4**), *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)-propan-1-ol (**5**), 2-(4-allyl-2,6-dimethoxyphenoxy)-1-(3,4,5-trimethoxyphenyl)propane (**7**), elemicin, myristicin, eugenol methyl ether, isoeugenol methyl ether and safrol were isolated from mace according to the method of Isogai *et al.*[4a,b] These compounds were identified by comparison of the UV, ^{1}H-NMR, ^{13}C-NMR, IR and MS as well as melting point with those reported.[4] 6-*tert*-Butyl-*m*-cresol was purchased from Wako Pure Chemicals Inc. (Osaka).

Chromatography——Silica gel, Wako gel C-200, was used for column chromatography. Merck Kiselgel 60 F_{254} plates were used for thin layer chromatography (TLC) and Merck PSC-60 F_{254} plates for preparative TLC. Solvent systems used were as follows: A, $CHCl_3$–MeOH (9 : 1, v/v); B, benzene–EtOAc (9 : 1, v/v); C, benzene–acetone (9 : 1, v/v). Spots on the plate were detected under UV light or by exposure to iodine vapor.

Microorganisms——The following *Streptococcus mutans* strains were given by Professor S. Kotani of Osaka University: *S. mutans* E 49 (serotype a), *S. mutans* BHT (serotype b), *S. mutans* MT 5091 (serotype c), *S. mutans* OMZ 176 (serotype d), *S. mutans* MT 703R (serotype e), *S. mutans* MT 557 (serotype f) and *S. mutans* 6715 (serotype g). Each strain was cultured overnight at 37 °C in BHI broth (5 ml) and subcultured again for 6—8 h. This bacterial suspension (1.0 ml) was diluted with BHI broth (19 ml) and used for determination of minimal inhibitory concentrations (MICs) in the growth or adherence of the cells to glass surfaces.

Determination of MICs——A test compound (10 mg) was dissolved in MeOH (2 ml) and prepared for a series of 2-fold dilutions. The dilution (0.1 ml) and bacterial suspension (0.1 ml) were added to BHI broth (4.8 ml). The whole was mixed thoroughly and the tube was incubated for 48 h at 37 °C. The MIC was determined by judging visually the bacterial growth in the series of test tubes.

Inhibition of the Adherence of Viable Cells (Anti-plaque Action)——An assay mixture contained the following components: 0.25 ml of 50% sucrose–0.5 M phosphate buffer (pH 6.8), 2.1 ml of BHI broth, 0.05 ml of a diluted test solution and 0.1 ml of a bacterial suspension. The tube was incubated for 16—18 h at 37 °C at an angle of 30°. The cultured medium was removed by decantation and the tube was gently rinsed three times with water (3 ml each). The cells adhering to the tube surface were suspended in water (3 ml) and measured by turbidometry at 550 nm.

Extraction and Fractionation of the Components of Mace——Crude powder of mace (936 g) was extracted with MeOH (3.0 l×3) for 48 h at room temperature and the solution was evaporated *in vacuo* to give an oily residue (290 g). The residue was dissolved in 95% MeOH (2.0 l) and extracted with *n*-hexane (1.0 l × 3). The 95% MeOH and *n*-hexane phases were separately concentrated to give oily residues (95% MeOH soluble, 176.5 g; hexane-soluble, 113 g).

A portion of the 95% MeOH-soluble fraction (84.5 g) was dissolved in ether (1.0 l), and extracted with 5% HCl (150 ml × 3). The 5% HCl solution was neutralized to pH 7—8 and extracted with ether (100 ml × 3). The ether solution was evaporated to dryness *in vacuo* to yield a basic fraction (0.5 g). The 5% HCl-treated layer was neutralized and extracted with 5% $NaHCO_3$ (150 ml × 3). The 5% $NaHCO_3$ solution was acidified to pH 4 and extracted with ether (150 ml × 3). The ether solution was concentrated *in vacuo* to give an acidic fraction (1.0 g). The 5% $NaHCO_3$-treated organic layer was washed with 5% NaOH (150 ml × 3) and evaporated *in vacuo* to yield a neutral fraction (30 g). The washings were combined, acidified to pH 3 and extracted with ether (150 ml × 3). The ether solution was evaporated *in vacuo* to give a phenolic fraction (35 g). The MICs of the basic, acidic, neutral and phenolic fractions against *S. mutans* were >100, >200, >200 and 50 μg/ml, respectively. A portion of the phenolic fraction (31.5 g) was dissolved in benzene (10 ml) and applied to a column of silica gel (6 cm i.d. × 88 cm, 1.5 kg). The column was successively eluted with benzene containing increasing amounts of EtOAc. Each fraction was evaporated to dryness *in vacuo* and assayed for antibacterial action. By repeated column chromatography (LiChroprep Si 60, 30 cm × 2.5 cm, i.d.) of the active fractions, three known compounds, **1** (2.6 g), **2** (*ca.* 1 g) and **5** (300 mg), were isolated together with 6-*tert*-butyl-*m*-cresol (30 mg), guaiacin (**8**) (60 mg) and compound **6** (40 mg).

The hexane-soluble fraction (113 g) was dissolved in EtOH (2.0 l), kept overnight at 10 °C and filtered. The filtrate was evaporated *in vacuo* to give an oily residue, a portion (66 g) of which was similarly fractionated into basic, acidic, neutral (16 g) and phenolic fractions (14 g). The MICs of these fractions against *S. mutans* were 100 μg/ml, >200 μg/ml, >200 μg/ml and 25 μg/ml, respectively. Compound **1** (80 mg) was also isolated from this phenolic fraction and elemicin, myristicin, isoeugenol methyl ether, **3** (248 mg), **4** and **7** from the neutral fraction.

***threo*-2-(4-Allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol Methyl Ether (6)**——Prisms; $C_{22}H_{28}O_6$; mp 106—109 °C. MS *m/z*: 388 (M^+, 15%), 221 (25%), 194 (100%), 179 (8%), 167 (100%), 151 (20%). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.98 (3H, d, J=6.35 Hz, γ-H × 3), 3.22 (3H, s, α-OMe), 3.33 (2H, d, J=6.59 Hz, α′-H), 3.81 (6H, s, 2′-OMe and 6′-OMe), 3.89 (3H, s, 3-OMe), 4.30—4.41 (2H, m, α-H and β-H), 5.05—5.15 (2H, m, γ′-H_a and γ′-H_b), 5.57 (1H, s, –OH), 5.90—6.05 (1H, m, β′-H), 6.39 (2H, s, 3′-H and 5′-H), 6.87—6.93 (3H, ABX type, 2-H, 5-H and 6-H). ^{1}H-NMR (CD_3COOD, 270 MHz) δ: 0.97 (3H, d, J=6.22 Hz, γ-H × 3), 3.22 (3H, s, α-OMe), 3.33 (2H,

d, $J=6.78$ Hz, α′-H), 3.80 (6H, s, 2′-OMe and 6′-OMe), 3.85 (3H, s, 3-OMe), 4.39 (1H, d, $J=6.6$ Hz, α-H), 4.45 (1H, m, β-H), 5.04—5.14 (2H, m, γ′-H_a and γ′-H_b), 5.90—6.05 (1H, m, β′-H), 6.45 (2H, s, 3′-H and 5′-H), 6.855, 6.858, 7.01 (3H, 2-H, 5-H and 6-H). ^{13}C-NMR (see Table I).

Acetylation of 6 with Acetic Anhydride——On acetylation, **6** gave a monoacetate (**6c**). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.01 (3H, d, $J=6.1$ Hz, γ-H × 3), 2.31 (3H, s, 4-OAc), 3.26 (3H, s, α-OMe), 3.27 (2H, d, $J=6.84$ Hz, α′-H × 2), 3.80 (6H, s, 2′-OMe and 6′-OMe), 3.83 (3H, s, 3-OMe), 4.35 (1H, m, $J=6.1$ Hz, β-H), 4.42 (1H, d, $J=5.86$ Hz, α-H), 5.06—5.14 (2H, m, γ′-H_a and γ′-H_b), 5.89—6.04 (1H, m, β′-H), 6.39 (2H, s, 3′-H and 5′-H), 6.95—7.09 (3H, ABX type, 2-H, 5-H, 6-H). ^{13}C-NMR (see Table I).

Methylation of 6 with Dimethyl Sulfate——Compound **6** was not methylated with diazomethane, but was with dimethyl sulfate under alkaline conditions to give a mixture of fully methylated *threo*–*erythro* (3 : 2, determined by ^{1}H-NMR) isomers.

The two isomers (**6a** and **6b**) were then isolated by preparative TLC and column chromatography on silica gel. Compound **6a** (*threo* isomer): $C_{23}H_{30}O_6$. MS *m/z*: 402 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 0.98 (3H, d, $J=6.1$ Hz, γ-H × 3), 3.22 (3H, s, α-OMe), 3.34 (2H, d, $J=6.6$ Hz, α′-H × 2), 3.82 (6H, s, 2′-OMe and 6′-OMe), 3.88 (6H, s, 3-OMe and 4-OMe), 4.05—4.25 (1H, m, β-H), 4.37 (1H, dd, $J=2.69$ and 1.0 Hz, α-H), 5.06—5.13 (2H, m, γ′-H_a and γ′-H_b), 5.90—6.05 (1H, m, β′-H), 6.40 (2H, s, 3′-H and 5′-H), 6.83—6.95 (3H, ABX type, 2-H, 5-H and 6-H). ^{13}C-NMR (see Table I). Compound **6b** (*erythro* isomer): $C_{23}H_{30}O_6$. MS *m/z*: 402 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 1.26 (3H, d, $J=6.4$ Hz, α-H × 3), 3.33 (2H, d, $J=6.8$ Hz, α′-H), 3.38 (3H, s, α-OMe), 3.79 (6H, s, 2′-OMe and 6′-OMe), 3.86, 3.87 (each 3H, s, 3-OMe and 4-OMe), 4.05—4.15 (1H, m, β-H), 4.42 (1H, d, $J=3.66$ Hz, α-H), 5.05—5.15 (2H, m, γ-H_a and γ-H_b), 5.90—6.10 (1H, m, β′-H), 6.39 (2H, s, 3′-H and 5′-H), 6.75—6.95 (3H, ABX type, 2-H, 5-H and 6-H).

Methylation of 5 with Diazomethane——Compound **5** was reacted with diazomethane for 3 d at room temperature. The product was then purified by preparative TLC and identified as *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(3,4-dimethoxyphenyl)propan-1-ol (**5a**) on the basis of the following characteristics. $C_{22}H_{28}O_6$. MS *m/z*: 388 (M^+), 194 (base peak). ^{1}H-NMR ($CDCl_3$) δ: 1.12 (3H, d, $J=6.6$ Hz, γ-H × 3), 3.37 (2H, d, $J=6.6$ Hz, α′-H × 2), 3.86 (3H, s, –OMe), 3.87 (6H, s, 2 × –OMe), 3.88 (3H, s, –OMe), 4.12 (1H, d, $J=2.2$ Hz, α-H), 4.35 (1H, d q, $J=2.8$, 6.5 Hz, β-H), 4.81 (1H, br s, OH), 5.09—5.17 (2H, m, γ′-H_b and γ′-H_a), 5.91—6.06 (1H, m, β′-H), 6.46 (2H, s, 3′-H and 5′-H), 6.75—6.94 (3H, ABX type, 2-H, 5-H and 6-H).

Methylation of 5 with Dimethyl Sulfate——Compound **5** was reacted with dimethyl sulfate under alkaline conditions. The products were purified by preparative TLC and identified as a mixture of *threo*–*erythro* isomers (1 : 2) of 2-(4-allyl-2,6-dimethoxyphenoxy)-1-(3,4-dimethoxyphenyl)propane-1-ol methyl ether, which were identical with **6a** and **6b**. Under the same alkaline conditions except for addition of dimethyl sulfate, partial conversion of **5** (*erythro*) to the corresponding *threo*-isomer was not observed.

6-*tert*-Butyl-*m*-cresol——An oily substance was identified as 6-*tert*-butyl-*m*-cresol by comparing the spectral data with those of an authentic sample.[5]

Guaiacin (8)——Colorless prisms; $C_{20}H_{24}O_4$; mp 198—200 °C. This compound was identified by comparing the UV, CD, ^{1}H-NMR and mass spectra with those reported.[6-8]

LC-MS Measurement——The sample was injected into a column (150 mm × 4.6 mm, i.d.) of Zorbax ODS attached to the HPLC system. The column was eluted with 70% CH_3CN containing 0.5% AcOH. The effluent (200 μl/min) was introduced into a mass spectrometer through a direct inlet LC-MS interface (JEOL, heater temperature at 250 °C, split ratio of 5—10%). Mass detection was carried out by repetitive scanning (3 s/scan, in the chemical ionization (CI) mode from *m/z* 50 to *m/z* 500 under the following conditions: ionization voltage of 70 eV; chamber temperature at 200 °C. The pressure of the ion source was maintained at *ca.* 1×10^{-4} Torr during the operation.

Determination of the Contents of 1 and 2——The contents of **1** and **2** were determined by HPLC. A test solution (5 μl) was injected into a Develosil ODS-7 column (25 cm × 4.6 mm, i.d.) attached to a Shimadzu LC-4A HPLC system and analyzed under the following conditions: flow rate, 0.8 ml/min; UV trace, 270 nm; pressure, 10 kg/cm^2; mobile phase, CH_3CN–H_2O–AcOH (60 : 40 : 0.5). The contents of **1** and **2** were then calculated from standard curves which were prepared with authentic samples using diphenyl as an internal standard.

Results

Fractionation of the MeOH Extract and Isolation of the Components

The MeOH extract of mace was partitioned between 95% MeOH and hexane. The 95% MeOH-soluble fraction showed stronger antibacterial action with an MIC of 50 μg/ml against *S. mutans* than the hexane-soluble fraction, and was further fractionated into basic, neutral, acidic and phenolic fractions. The phenolic fraction showed antibacterial action with an MIC of 25 μg/ml, while the other fractions showed no action at concentrations below 100 or 200 μg/ml.

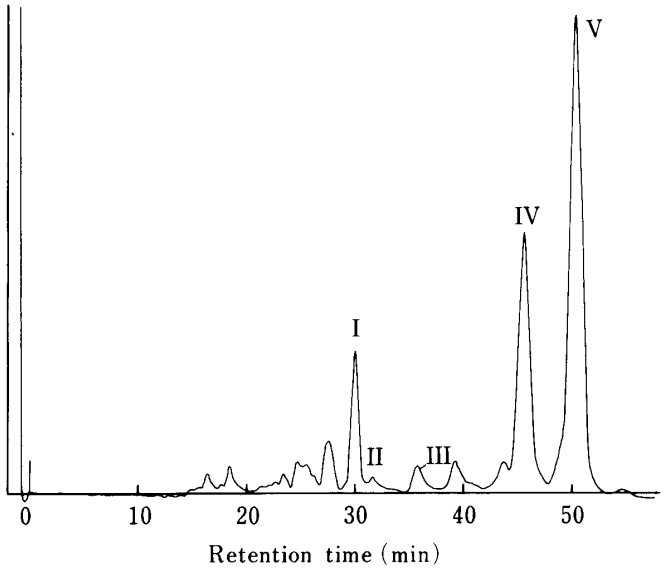


Fig. 1. HPLC Elution Profile of a Phenolic Fraction of Mace

HPLC was carried out by using a column of Zorbax ODS (150 mm × 4.6 mm, i.d.) under the following conditions: mobile phase, CH_3CN–H_2O–AcOH (70 : 30 : 0.5); flow rate, 100 μl/min.

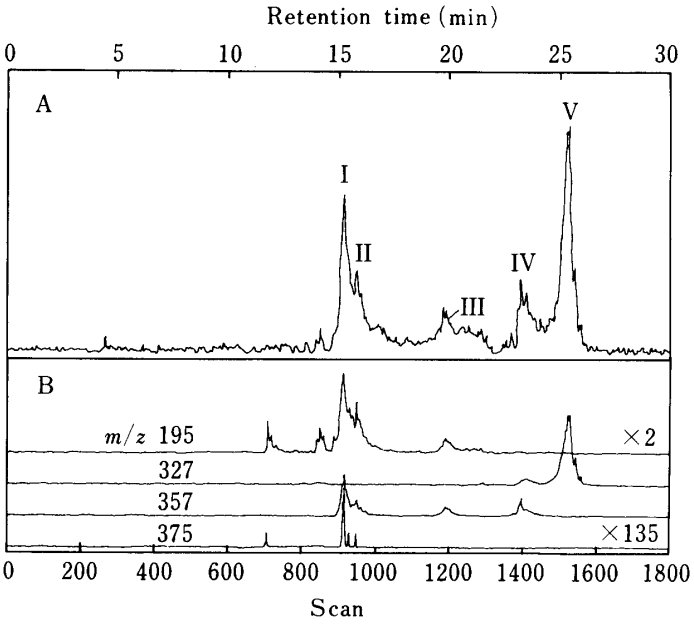


Fig. 2. Reconstructed Ion Current Chromatogram and Mass Chromatograms of the Phenolic Fraction

A, a reconstructed ion current chromatogram; B, mass chromatograms. HPLC was carried out under the same conditions as described in the legend to Fig. 1 except for flow rate (200 μl/min). The effluent was introduced on line into a mass spectrometer through a direct inlet LC-MS interface, operated in the positive CI mode. Characteristic mass fragments of each peak were as follows: peak I, *m/z* 357 (37%, $[M-OH]^+$), 195 (40%, $[C_{11}H_{13}O_3+H]^+$), 193 (14%), 181 (13%), 165 (13%), 143 (100%), 102 (81%); peak III, *m/z* 357 (98%, $[M-OCH_3]^+$), 195 (64%, $[C_{11}H_{13}O_3+H]^+$), 193 (28%), 181 (5%), 165 (10%), 141 (7%), 102 (100%); peak IV, *m/z* 357 (20%, $[M+1]^+$), 327 (7%), 101 (100%); peak V, *m/z* 327 (90%, $[M+1]^+$), 143 (23%), 101 (100%).

By using LC and LC-MS techniques, the phenolic fraction was analyzed. The LC profile (Fig. 1) and the total ion chromatogram (Fig. 2A) were essentially similar and three major peaks (I, IV, and V) were observed. These peaks were ascribed to the presence of compounds **5**, **2** and **1**, respectively, on the basis of the mass chromatogram (Fig. 2B), the MS (see the legend to Fig. 2) measured in the CI mode, and the retention times. Peak III showed mass fragment ions at *m/z* 357 and 195 similar to those of **5**, suggesting that the compound is an acyclic dilignol derivative.

The phenolic fraction was then chromatographed on silica gel to isolate these components. The eluate with benzene showed stronger antibacterial action against *S. mutans* than the others, and **1** was isolated along with 6-*tert*-butyl-*m*-cresol and guaiacin (**8**). The eluates with 0.3% and 2% EtOAc in benzene had moderate antibacterial action, and **2** and **5** were isolated from these fractions, respectively. In addition, a new compound **6** was isolated from the 10% EtOAc–benzene eluate, though it had no antibacterial action at concentrations less than 100 μg/ml.

Structure of Compound 6

Compound **6**, corresponding to peak III on the LC and LC-MS profiles (Fig. 1 and 2), was isolated as colorless prisms, $C_{22}H_{28}O_6$, mp 106—109 °C. The ^{1}H-NMR spectrum showed the presence of one *sec*-methyl (δ 0.98, 3H, d, J=6.35 Hz), one aliphatic methoxy (δ 3.22, 3H, s),[4h] three aromatic methoxyls (δ 3.81 and 3.89, 6H and 3H, respectively, each s) and one allyl group (δ 3.33 (d, J=6.59 Hz), 5.05—5.12 (m) and 5.90—6.05 (m); 2H, 2H and 1H, respectively). The spectral pattern was quite similar to that of **5**, except for the aliphatic methoxyl signal.[4h] The molecular ion peak in the EI-MS was higher than that of **5** by 14 mass units, attributable to a methyl residue, and characteristic mass fragment peaks at m/z 194, 221 and 167 were observed due to fragments AH, C and D, respectively as shown in Fig. 3.[4e] This evidence suggests tht **6** is a monomethyl ether of **5**. The signal of the α-methine proton was overlapped with that of the β-methine proton when measured in $CDCl_3$, but they were well separated in CD_3COOD. The relative large coupling constants of this methine proton in **6** (δ 4.39, d, J=6.6 Hz) and its monoacetate (**6c**) (δ 4.42, d, J=5.86 Hz) indicate that the relative configuration of the α- and β- oxygenated functional groups is *threo*.[9,10] This accounts for the difference in ^{1}H-NMR spectrum between **6** and its *erythro*-isomer (δ 1.24, 3H, d, J=6.5 Hz; 4.36, 1H, d, J=4 Hz), which has been obtained by anodic oxidation of a mixture

TABLE I. ^{13}C-NMR Spectral Data (in $CDCl_3$)

Carbon No.	Compounds				
	5 *erythro*	**6** *threo*	**5a** *erythro*	**6a** *threo*	**6b** *erythro*
1	133.1	134.9	133.1	134.9	134.6
2	108.6	109.9	109.4	110.6	110.6
3	146.4[a]	146.4[b]	148.8[c]	148.8[d]	148.8[e]
4	144.4[a]	145.1[b]	147.8[c]	148.5[d]	148.1[e]
5	113.8	113.7	110.9	110.6	110.3
6	118.7	121.1	118.1	120.3	119.7
α	82.2	87.4	82.2	87.3	85.1
β	72.7	81.0	72.8	81.0	82.3
γ	12.6	16.5	12.7	16.5	13.8
1′	136.0	135.2	135.9	135.3	135.2
2′	153.4	153.3	153.4	153.3	153.4
3′	105.5	105.7	105.5	105.6	105.7
4′	131.9	131.2	132.7	131.8	132.2
5′	105.5	105.7	105.5	105.6	105.7
6′	153.4	153.3	153.4	153.3	153.4
α′	40.4	40.4	40.4	40.4	40.3
β′	136.9	137.3	136.9	137.3	137.2
γ′	115.9	115.7	116.0	115.7	115.7
–OMe	55.8	55.9	55.8	55.8	55.8
	56.0	56.8	56.0	55.9	55.9
				56.8	57.3

a—*e*) Assignments may be interchanged in each column.

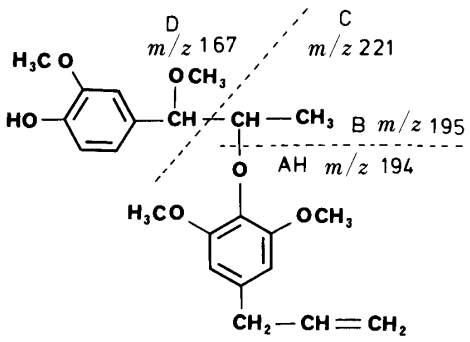
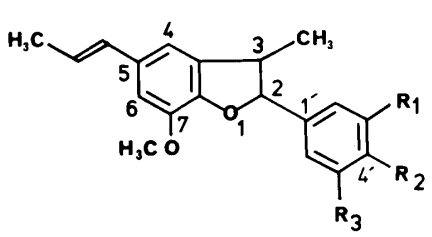
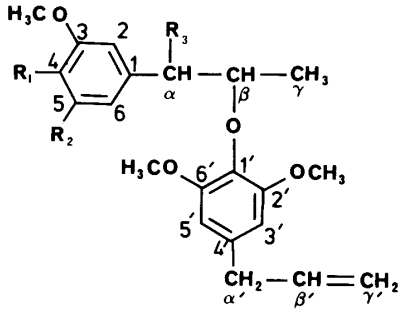
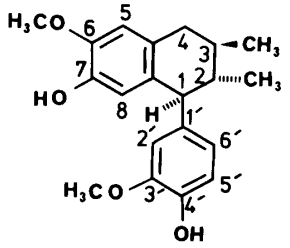


Fig. 3. Possible Mass Fragmentation Pattern of Compound **6**







	R_1	R_2	R_3
1:	OCH_3	OH	H
2:	OCH_3	OH	OCH_3
3:	O–CH_2–O		H
4	O–CH_2–O		OCH_3

		R_1	R_2	R_3
5:	*erythro*	OH	H	OH
5a:	*erythro*	OCH_3	H	OH
6:	*threo*	OH	H	OCH_3
6a:	*threo*	OCH_3	H	OCH_3
6b:	*erythro*	OCH_3	H	OCH_3
6c:	*threo*	OAc	H	OCH_3
7:		OCH_3	OCH_3	H

8
guaiacin

Fig. 4. Structures of Benzofurans, Diaryl Ethers and Guaiacin Isolated from Mace

of *Z*-isoeugenol and 4-allyl-2,6-dimethoxyphenol by Nishiyama *et al.*[9] Compound **6** was treated with dimethyl sulfate under alkaline conditions to give *threo* (**6a**, 60%) and *erythro* (**6b**, 40%) isomers, which were identical with the corresponding isomers (*threo*, 33%; *erythro*, 66%) obtained from **5** under the same conditions. The structure of **6** was finally established as *threo*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol methyl ether.

Antibacterial Action of Various Components Isolated from Mace

Table II shows the MICs of the components of mace against serotype c of *S. mutans*, which is most frequently detected in decayed teeth in Japan. The major phenolic components, **1** and **2**, had potent antibacterial action with an MIC of 12.5 μg/ml, determined by the tube dilution technique, while the others had no significant action. Aliquots of the culture which had been incubated with **1** or **2** at concentrations above the MIC were transferred to new BHI broth, but no appreciable bacterial growth was observed even after a 24—48 h incubation, indicating that both compounds are bactericidal against *S. mutans* at these concentrations.

Anti-plaque Action of 1 for Serotypes a—g of *S. mutans*

Table III shows the anti-plaque action of **1** against various serotypes of *S. mutans*. These strains were cultured in 5% sucrose-broth containing **1**, and the amounts of adhered cells were measured by turbidometry. Compound **1** almost completely inhibited the adherence of the cells to glass surfaces at concentrations of 6.25—12.5 μg/ml for serotypes c and g, 25 μg/ml for serotypes d, e and f, and 50 μg/ml for serotype a. Since the serotype b strain used in our experiment lacked the ability to adhere to smooth surfaces, anti-plaque activity could not be determined.

TABLE II. MICs of the Components Isolated from Mace against *S. mutans*

Compounds	MIC (μg/ml)	Compounds	MIC (μg/ml)
1[a]	12.5	Elemicin[b]	>200
2[a]	12.5	Myristicin[b]	>200
3[b]	>200	Eugenol methyl ether[b]	>200
4[b]	>200	Isoeugenol methyl ether[b]	>200
5[a]	>100	Safrol[b]	>200
6[a]	>100	Trimyristin	>200
7[b]	>200		
8[a]	>100		

The experiments were done in triplicate. Under the same conditions, magnolol, berberin hydrochloride, chlorhexidine gluconate and erythromycin lactobionate inhibited the growth with MICs of 6.25, 6.25, 50, 0.78 and 0.098 μg/ml, respectively. *a*) From the 95% MeOH-soluble, phenolic fraction. *b*) From the hexane-soluble, neutral fraction.

TABLE III. Effect of **1** on Plaque Formation of Various Serotypes of *S. mutans*

Sample concentration (μg/ml)	% inhibition of plaque formation					
	Serotype of *Streptococcus mutans*					
	a	c	d	e	f	g
6.25	1.3± 1.6	35.8±9.9	6.5±1.4	7.1± 0.0	41.5±2.5	94.7±1.7
12.5	9.4± 5.4	99.9±0.0	46.5±6.2	45.7±14.1	66.6±8.7	100.0±0.0
25	29.7±28.1	100.0±0.0	99.9±0.0	97.7± 2.5	98.6±1.5	100.0±0.0
50	96.4± 0.1	99.0±0.1	98.2±1.4	94.0± 2.4	96.3±1.4	99.4±0.3
100	86.0± 0.0	96.6±0.3	97.0±1.9	88.3± 4.7	94.1±4.4	96.7±0.0

The experiments were done in triplicate and the results are represented as percent inhibition relative to the control (mean±S.E.).

TABLE IV. Contents of Antibacterial Principles (**1** and **2**) in the Hexane-Soluble, 95% MeOH-Soluble and Phenolic Fractions

Fractions	Contents (%)	
	1	**2**
Hexane-soluble	1.53	0.73
95% MeOH-soluble	3.54	3.01
Phenolic	10.00	7.68

The contents were determined by HPLC using a Develosil ODS-7 column under the following conditions: column size, 4.6 mm i.d. × 250 mm; flow rate, 0.8 ml/min; mobile phase, CH_3CN–H_2O–AcOH (60:40:0.5); UV trace at 270 nm; internal standard, diphenyl.

Contents of Antibacterial Principles

Table IV shows the contents of **1** and **2** in the hexane-soluble, the 95% MeOH-soluble and the phenolic fractions, as determined by HPLC with diphenyl as an internal standard. The contents of both compounds were the highest in the phenolic fractions, next in the 95% MeOH-soluble fraction and the lowest in the hexane-soluble fraction; this order was the same as that of antibacterial potency represented by MIC values against *S. mutans*, suggesting that these two cyclic dilignols are mostly responsible for the antibacterial action of the mace extract.

Discussion

Plaque formation on the tooth surfaces is generally regarded as an initial stage of dental caries. Oral bacteria, especially *S. mutans*, are mainly responsible for this plaque formation. For prevention of dental caries, the most promising approach is therefore to suppress the plaque formation (plaque control). One of the possible ways of plaque control seems to be inhibition of cariogenic bacterial growth or inhibition of the adherence of the cells to tooth surfaces.

In this context, we have reported that diphenols such as magnolol and honokiol from the barks of *Magnolia obovata* or *M. officinalis*[3a,b,e] and coumarins such as glycyrol, glycyrin, isoglycyrol and glycycoumarin from *Glycyrrhiza uralensis*[1] have potent antibacterial action against *S. mutans*, and that flavonols from the spikes of *Artemisia capillaris*,[3d] embelin from the *Embelia* fruit,[3g] and galloyl glucoses from Chinese galls[3h] have potent inhibitory action against GTase, which mediates the adherence of the cells to smooth surfaces.

In the present work, we have found that two cyclic dilignols, **1**, and **2**, from mace inhibit the growth of *S. mutans* or the *in vitro* adherence of the viable cells to glass surfaces, while acyclic dilignols, **5**, **6** and **7**, do not. The mode of action of **1** and **2** was bactericidal at concentrations above the MICs, being similar to that of various phenols.[3a] Further, during the course of this experiment, we have isolated a new acyclic dilignol derivative (**6**) and guaiacin (**8**). The former is the first *threo* isomer among various acyclic dilignols isolated from Myristicious plants. This is the first report of the isolation of **8** from mace, though it has been isolated from *Guaiacum officinale* L. (Zygophyllaceae),[8a] *Machilus edulis* KING (Lauraceae),[8b] *Osteophloem platyspermum* (Myristicaceae),[8e] *Saururus cernuus* L. (Saururaceae),[8c] and *Virola carinata* (BENTH.) WARB. (Myristicaceae).[8d]

Zemek *et al.*[11] have studied the antimicrobial properties of lignin components with guaiacyl and syringyl structures. They have reported that isoeugenol exhibits the most pronounced inhibitory effect on the growth of bacteria, yeast, yeast-like organisms and molds, and is roughly tiwce as effective as **1**, which consists of two isoeugenol units. Contrary to their observation, we have found that **1** shows stronger antibacterial action (MIC, 12.5 μg/ml) against *S. mutans* than isoeugenol (MIC, >200 μg/ml).[3b,e] This is in good agreement with the previous finding that dimeric phenylpropanoids such as magnolol and honokiol have more potent antibacterial action against *S. mutans* than the corresponding monomeric units.[3b,e]

Mace has been widely used as a spice and an ingredient of many preparations and decoctions in Ayurvedic medicine, being considered to be hot, dry, carminative, digestive and expectorant.[12] It is also blended in dentifrice, a mixture of several plants which are said to prevent tooth decay in Sri Lanka and India.[13] Our present study suggests that mace as an ingredient of the dentifrice may play an important role in controlling plaque formation on the tooth surfaces as well as in adding flavoring.

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References and Notes

1) Part IX: M. Hattori, K. Miyachi, Y.-Z. Shu, N. Kakiuchi and T. Namba, *Shoyakugaku Zasshi*, in press (1986).
2) A part of this work was presented at the Annual Meeting of the Japanese Society of Pharmacognosy, Okayama, 1985, Abstract of Papers, p. 1.
3) *a*) T. Namba, M. Tsunezuka, K. Bae and M. Hattori, *Shoyakugaku Zasshi*, **35**, 295 (1981); *b*) T. Namba, M. Tsunezuka and M. Hattori, *Planta Medica*, **44**, 100 (1982); *c*) T. Namba, M. Hattori, M. Tsunezuka, T. Yamagishi and K. Konishi, *Shoyakugaku Zasshi*, **36**, 222 (1982); *d*) T. Namba, M. Tsunezuka, Y. Takehana, S.

Nunome, K. Takeda, Y. Shu and N. Kakiuchi, S. Takagi and M. Hattori, *ibid.*, **38**, 253 (1984); *e*) M. Hattori, M. Tsunezuka, S. Kadota, T. Kikuchi and T. Namba, *ibid.*, **39**, 76 (1985); *f*) T. Sakai, K. Kobashi, M. Tsunezuka, M. Hattori and T. Namba, *ibid.*, **39**, 165 (1985); *g*) T. Namba, M. Tsunezuka, D. M. R. B. Dissanayake, U. Pilapitiya, K. Saito, N. Kakiuchi and M. Hattori, *ibid.*, **39**, 146 (1985); *h*) N. Kakiuchi, M. Hattori, M. Nishizawa, T. Yamagishi, T. Okuda and T. Namba, *Chem. Pharm. Bull.*, **34**, 720 (1986).

4) *a*) A. Isogai, S. Murakoshi, A. Suzuki and S. Tamura, *Agric. Biol. Chem.*, **37**, 889 (1973); *b*) *Idem, ibid.*, **37**, 1479 (1973); *c*) J. E. Forrest, R. A. Heacock and T. P. Forrest, *Experimentia*, **15**, 139 (1973); *d*) T. P. Forrest, J. E. Forrest and R. A. Heacock, *Naturwiss.*, **60**, 257 (1973); *e*) J. E. Forrest, R. A. Heacock and T. P. Forrest, *J. Chem. Soc., Perkin Trans. 1*, **1974**, 205; *f*) E. Wenkert, *Phytochemistry*, **15**, 1547 (1976); *g*) K. K. Purushothaman and A. Sarada, *Indian J. Chem., Sect. B*, **19**, 236 (1980); *h*) Y. Kuo, Y. Lin and Y. Lin, *J. Chinese Chem. Soc.*, **30**, 63 (1983).

5) C. J. Pouchert, "The Aldrich Library of NMR Spectra," Edition II, Vol. 1, Aldrich Chem. Co., Inc., Milwaukee, 1983, p. 882.

6) J. Sakakibara, I. Ina and M. Yasue, *Yakugaku Zasshi*, **94**, 1377 (1974).

7) P. B. Hulbert, W. Klyne and P. M. Scopes, *J. Chem. Res.*, **1981**, 27.

8) *a*) F. E. King and J. G. Wilson, *J. Chem. Soc.*, **1964**, 4011; *b*) P. L. Majumder, A. Chatterjee and G. C. Sengupta, *Phytochem.*, **11**, 811 (1972); *c*) O. R. Gottlieb, J. G. S. Maia and M. N. De S. Ribeiro, *ibid.*, **15**, 773 (1976); *d*) K. V. Rao and F. M. Alvare, *J. Nat. Products*, **45**, 393 (1982); *e*) R. B. Fo, M. G. deCarralho and O. R. Gottlieb, *Planta Medica*, **1984**, 53.

9) A. Nishiyama, H. Eto, Y. Terada, M. Iguchi and S. Yamamura, *Chem. Pharm. Bull.*, **31**, 2834 (1983).

10) The above authors reported that α-proton *J* values in the *erythro* isomers of acyclic dilignols were in the range of 3 to 6 Hz, while those of the *threo* isomers were slightly larger than 6 Hz. In addition, methyl proton signals (γ-H) in the former seemed to appear at lower field than those in the latter.

11) J. Zemek, B. Kosikova and J. Augustin, *Folia Microbiol.*, **24**, 483 (1979).

12) J. Attygalle, "Sinhalese Materia Medica," M. D. Gunasena & Co., Ltd., Colombo, 1952, p. 139.

13) Personal communication by Dr. Upali Pilapitiya (Bandaranayake Memorial Ayurvedic Research Institute Nawinna, Maharagama, Sri Lanka).