Isotsaokoin, an Antifungal Agent from *Amomum tsao-ko*

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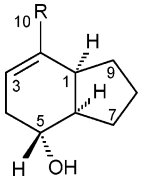
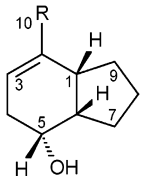
Bioassay-guided purification of a methanol extract from *Amomum tsao-ko* led to the isolation of the bicyclic nonane isotsaokoin (**1**) as the major active principle, an isomer of the previously reported tsaokoin (**2**). The stereochemical relationship of **1** and **2** was investigated by NOE experiments and Mosher ester derivatization. Compound **1** showed antifungal activity against *Trycophyton mentagrophytes*.

Amomum tsao-ko Crevost et Lemaire, which belongs to the Zingiberaceae, has been used in folk medicine for the treatment of stomach disorders and throat infections. The bicyclic nonane tsaokoin (**2**)[1] and antioxidants[2] including the hannokinol diarylheptanoids and the flavan catechin have been reported from this plant. The antibacterial spiroketal aculeatin,[3] an antimalarial diterpene peroxide,[4] and eicosenones[5] have been identified from other species of the genus *Amomum*. Although the plant *A. tsao-ko* has been used for a long time as spice and perfume in addition to medicinal usage, no antifungal constituents have been reported yet. As a result of our ongoing search for novel bioactive natural products from medicinal plants, the methanol extract of the powdered fruit of *A. tsaoko* was found to show significant antifungal activity against *Trycophyton mentagrophytes*. Bioassay-guided purification led to the isolation of a new bicyclic nonane, isotsaokoin (**1**), as the major active principle, an isomer of previously reported tsaokoin (**2**). Herein, we report the structural characterization of **1** from the fruits of *A. tsao-ko*.

The powdered fruits were extracted with methanol. The extract was partitioned between water and EtOAc. The EtOAc layer was fractionated on a silica gel flash column. The bioactive fractions were subjected to silica gel MPLC and preparative C_{18} HPLC to afford **1** and **2** in 0.00045% and 0.0065% yield, respectively, from the dried fruits. Compound structure determination was first focused on the major constituent, **2**.

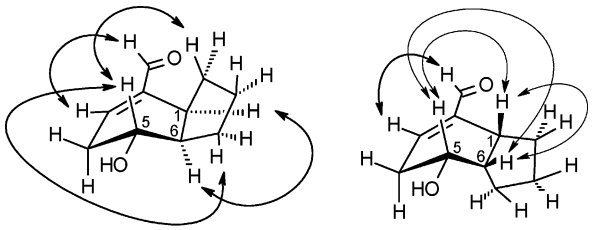


1 R = CHO
3 R = CH_2OH



2 R = CHO
4 R = CH_2OH

Compound **2** was obtained as a pale brown oil. The molecular formula was established as $C_{10}H_{14}O_2$ ($[M + H]^+$ *m*/*z* 167.1075, calcd 167.1072) by HRFABMS. The UV maxima observed at 231 and 228 nm in MeOH and the characteristic IR absorptions at ν_{max} 3396, 2728, 1683, and 1634 cm^{-1} indicated the presence of hydroxyl and conjugated aldehyde functional groups. Analysis of the ^{13}C NMR spectrum together with DEPT experiments (one quaternary, five methine, and four methylene carbons), the $^1H-^1H$ COSY spectrum (cross-peaks: H-3 or H-5 with H_β-4 and H_α-4; H-6 with H-5, H-1, and H-7), and significant HMBC correlations (H-1 or H-10 to C-2 and C-3; and H-9 to C-2) determined the gross structure of **2** as 5-hydroxybicyclo-[4.3.0]non-2-ene-2-carboxaldehyde. From a 1D NOESY experiment, critical NOEs were observed between H-1 and H-6; H-1 and H-5; and H-5 and H-6 to indicate that the two rings are *cis*-fused and those protons on the same side of the molecule (Figure 1). The H-5 proton was deduced to be pseudoaxial (β-oriented) on the basis of the observed coupling constants ($J_{H5-H4\alpha} = 9.2$ Hz and $J_{H5-H4\beta} = 5.0$ Hz), and the hydroxyl group at C-5 was therefore α-oriented. Also, the coupling constant (5.0 Hz) between H-5 and H-6 indicated that the H-6 proton occupies a pseudoequatorial position in the six-membered ring. Thus, compound **2** was identified as *rel*-(1*R*,5*R*,6*S*)-5-hydroxybicyclo[4.3.0]non-2-ene-2-carboxaldehyde, also known as tsaokoin.[1] Direct comparison of the NMR data of **2** with those of tsaokoin was not possible, because the NMR solvent used was not indicated in the literature. The ^1H NMR data of **2** (CD_3OD or $CDCl_3$) were in fairly good accord with those of tsaokoin, but the ^{13}C NMR data were slightly different. Compound **2** was reported to show an optical rotation of −0.11° (*c* 0.0221, $CHCl_3$).[1] However, we were not able to detect any optical rotation beyond instrumental error range even at the high concentration (*c* 8.64, MeOH). To determine its absolute stereochemistry by modified Mosher's ester analysis we decided to prepare the respective *R* and *S* Mosher esters of **2**.[6] Both the (*R*)-MTPA and (*S*)-MTPA esters of **2** showed an identical NMR spectrum, indicating that each ester so produced was a 1:1 diastereomeric mixture. Thus, compound **2** is racemic with no optical rotation.



1 **2**

Figure 1. Selected NOE correlations in compounds **1** and **2**.

Compound **1** was obtained as a pale brown oil. The molecular formula was determined to be $C_{10}H_{14}O_2$ by HRFABMS ($[M + H]^+$ *m*/*z* 167.1068, calcd 167.1072). The UV spectrum was the same as that of compound **2**. No optical rotation was detected in MeOH for **1**. The ^{13}C NMR spectrum together with the DEPT spectrum of **1** showed 10 carbons: a quaternary carbon, five methines, and four methylenes, with slight differences in chemical shift com-

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Table 1. NMR Spectral Data for Compound **1** in CD_3OD

position	δ_C (mult.)[a,b]	δ_H (int., mult., J in Hz)[c]	HMBC[d,e]
1	39.0 (d)	2.79 (1H, q, 8.4)	2, 3, 5, 6, 9
2	145.9 (s)		
3	149.6 (d)	6.79 (1H, dd, 5.0, 2.0)	1, 4, 5, 10
4	35.4 (t)	2.65 (1H$_\beta$, dt, 18.8, 5.0)	2, 3, 5, 6
		2.26 (1H$_\alpha$, ddt, 18.8, 9.2, 2.0)	2, 3, 5
5	68.5 (d)	3.58 (1H, td, 9.2, 5.0)	1, 3, 6, 7
6	45.7 (d)	2.02 (1H, m)	1, 4, 5, 8, 9
7	28.9 (t)	1.79 (2H, m)	1, 5, 6, 8, 9
8	24.6 (t)	1.70 (1H, m),	1, 6, 7, 9
		1.63 (1H, m)	1, 6, 7, 9
9	32.7 (t)	2.18 (1H$_\alpha$, dtd, 12.8, 8.4, 4.4)	1, 2, 6, 7, 8
		1.14 (1H$_\beta$, dtd, 12.8, 9.2, 8.4)	1, 2, 7, 8
10	195.8 (d)	9.39 (1H, s)	1, 2, 3

[a] Recorded at 100 MHz. [b] Multiplicity deduced by DEPT and HSQC spectra. [c] Recorded at 400 MHz. [d] Carbons showing long-range correlation with indicated proton. [e] Correlations observed for $^nJ_{CH} = 8$ Hz.

pared with **2** (Table 1). Interpretation of the $^1H-^1H$ COSY, HSQC, and HMBC spectra afforded the gross structure of **1** as being identical to that of **2**. However, a strong NOE was observed between the H-6 proton at δ 2.02 and the H-1 proton at δ 2.79, indicating a *cis* ring junction, but no NOE was seen between the H-5 proton at δ 3.58 and the H-6 or H-1 protons. Instead, the H-5 proton showed weak NOEs with the H$_\beta$-9 proton at δ 1.14 and the H-7 proton at δ 1.79 (Figure 1). The H-5 proton was determined to be pseudoaxial (β-oriented) on the basis of its coupling constants ($J_{H5-H\alpha4} = 9.2$ Hz and $J_{H5-H\beta4} = 5.0$ Hz) with the H-4 protons and to be *trans* to H-6 ($J_{H5-H6} = 9.2$ Hz), which was correspondingly pseudoaxial in the six-membered ring. The H-5 proton in **1** located in the shielding region above the plane of the adjacent five-membered ring was shifted upfield compared to that of **2** (δ 3.58 vs 3.95, respectively). Thus, the structure of **1** was deduced to be *rel*-(1*S*,5*R*,6*R*)-5-hydroxybicyclo[4.3.0]non-2-ene-2-carboxaldehyde, designated as isotsaokoin, which was also racemic, on the basis of an optical rotation measurement.

For structural confirmation and evaluation of biological activity, compounds **1** and **2** were converted to the allylic alcohols **3** and **4**, respectively, by $NaBH_4$ reduction. All the NMR assignments of the protons and carbons were consistent with the reduced forms. Reduction of the C-10 carboxaldehyde group resulted in upfield shift of the C-3 carbon signal about 31 ppm.

Compounds **1**−**4** were evaluated for growth inhibitory activity against *Trycophyton mentagrophytes*, one of the athlete's foot disease-causing fungi. The inhibition zone of **1** was 1.5 and 2.0 mm at 20 and 40 μg/disk, respectively, whereas that of amphotericin B as positive control was 2.5 and 3.0 mm at 20 and 40 μg/disk, respectively. Compounds **2** and **3** showed marginal activity and **4** no activity at a concentration of 60 μg/disk.

Experimental Section

General Experimental Procedures. The melting point was measured on a Fisher melting point apparatus and is uncorrected. Optical rotations were measured on a Perkin-Elmer polarimeter 341 LC model. UV spectra were recorded on a Shimadzu UV-2401PC spectrometer. The IR spectra were recorded on a Perkin-Elmer BX FT-IR spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian Mercury 400 NMR spectrometer at 400 and 100 MHz, respectively, in CD_3OD or $CDCl_3$ and referenced to residual solvent signals [δ 3.30, ^1H; 49.0, ^{13}C for CD_3OD; δ 7.26, ^1H; 77.24, ^{13}C for $CDCl_3$]. 2D NMR spectra (gCOSY, gTOCSY, gHSQC, gHMBC) were recorded using the manufacturer's software VNMR 6.1B with standard parameters. MPLC (medium-pressure liquid chromatography) was carried out on a FMI lab pump system using silica Daisogel (25−40 μm, 30 i.d. × 300 mm, Daiso Co., Ltd, Osaka, Japan) at a flow rate of 10 mL/min. Preparative HPLC was carried out on a Waters 600 model system with a photodiode array detector 996 using C_{18} Pegasil (Senshu Pak, 20 i.d. × 250 mm) with an eluent of 3:7 MeOH−H_2O at a flow rate of 7 mL/min at 225 nm. TLC was carried out on silica-coated plastic plates and visualized under a UV lamp (254 nm) or stained by dipping in a solution of anisaldehyde−sulfuric acid in methanol followed by heating. EIMS were recorded on a Shimadzu GCMS QP1000 mass spectrometer at an ionizing voltage of 70 eV at the Korea Research Institute of Chemical Technology and HRFABMS obtained on a JEOL HX110A Tandem HR mass spectrometer at the Korea Basic Science Institute (Daejeon, Korea).

Plant Material. The dried fruits of *Amomum tsao-ko* were purchased at the Kumsan herbal market, Chungnam, Korea, in March 2001. They were identified by Dr. Eunkyu Lim at the Busong Clinic of Medicinal Herbs (Iksan, Korea), and a voucher sample was deposited at the Natural Products Chemistry Laboratory of Kongju National University (identification number: SM1099).

Extraction and Isolation. The dried fruits (1082 g) of *A. tsao-ko* were powdered and percolated with MeOH (5 L) twice at room temperature for 7 days. The methanolic extracts combined were concentrated under vacuum to yield a brown oily syrup (34.8 g), which was suspended in H_2O (100 mL) and extracted with EtOAc (500 mL × 3). The EtOAc layer was concentrated (23.5 g) and chromatographed on a silica gel column (70−230 mesh, 70 i.d. × 400 mm) with elution by mixtures of hexane and EtOAc of increasing polarity, to yield 10 fractions (about 2 L each fraction). Fraction 8 (1.23 g, 3:7 hexane−EtOAc eluate) was further purified using silica MPLC (7:3 to 5:5 hexane−EtOAc) to give seven portions. The fourth portion (310 mg) was purified by C_{18} HPLC to yield **2** (70.3 mg) and **1** (4.9 mg), eluting at 52.7 and 59.2 min, respectively.

Isotsaokoin (1): colorless oil; UV (MeOH) λ_{max} (log ϵ) 231 (4.45), 228 (4.45) nm; IR (KBr, neat) ν_{max} 3383, 2950, 2871, 1682, 1640, 1450, 1168, 1059, 989, 838, 733 cm^{-1}; ^1H NMR and ^{13}C NMR data, Table 1; HRFABMS m/z $[M + H]^+$ 167.1068 (calcd for $C_{10}H_{14}O_2$+H, 167.1072); TLC (silica gel, 2:8 hexane−EtOAc) R_f 0.40.

Tsaokoin (2): pale brown oil; UV (MeOH) λ_{max} (log ϵ) 231 (4.44), 228 (4.45) nm; IR (KBr, neat) ν_{max} 3396, 2868, 2728, 1683, 1634, 1428, 1379, 1181, 1062, 1026, 969, 903, 740 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 9.35 (1H, s, H-10), 6.75 (1H, ddd, $J = 5.6$, 2.8, 1.2 Hz, H-3), 3.95 (1H, dt, $J = 9.2$, 5.0 Hz, H-5), 2.93 (1H, m, H-1), 2.48 (1H, dddt, $J = 18.8$, 5.6, 5.0, 1.4 Hz, H$_\beta$-4), 2.42 (1H, m, H-6), 2.38 (1H, ddt, $J = 18.8$, 9.2, 2.8 Hz, H$_\alpha$-4), 2.02 (1H, m, H$_\beta$-9), 1.73 (1H, m, H-7a), 1.54 (1H, m, H-7b), 1.50 (2H, m, H-8), 1.40 (1H, m, H$_\alpha$-9); ^{13}C NMR (CD_3OD, 100 MHz) δ 196.0 (CH, C-10), 149.6 (CH, C-3), 146.2 (C, C-2), 68.9 (CH, C-5), 44.3 (CH, C-6), 38.6 (CH, C-1), 33.5 (CH_2, C-9), 31.8 (CH_2, C-4), 26.1 (CH_2, C-7), 25.7 (CH_2, C-8); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 193.8 (CH, C-10), 146.6 (CH, C-3), 144.9 (C, C-2), 68.6 (CH, C-5), 43.1 (CH, C-6), 37.6 (CH, C-1), 32.6 (CH_2, C-9), 31.5 (CH_2, C-4), 25.3 (CH_2, C-7), 25.0 (CH_2, C-8); HRFABMS m/z $[M + H]^+$ 167.1075 (calcd for $C_{10}H_{14}O_2$+H, 167.1072); TLC (silica gel, 2:8 hexane−EtOAc) R_f 0.40.

$NaBH_4$ Reduction of Compounds 1 and 2. $NaBH_4$ (2.0 mg, 53 μmol) was added to a solution of **1** (2.3 mg, 14 μmol) in ethanol (1 mL), and the mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with water (0.5 mL) and extracted with EtOAc (7 mL) three times. The extract combined was washed with brine and dried over anhydrous $MgSO_4$. Concentration and purification by silica gel column chromatography (2:8 hexane−EtOAc) gave compound **3** (2.0 mg, 86%): colorless oil; $C_{10}H_{16}O_2$; UV (MeOH) λ_{max} <200 nm; ^1H NMR (CD_3OD, 400 MHz) δ 5.53 (1H, ddd, $J = 5.2$, 2.4, 1.6 Hz, H-3), 3.98 (1H, d, $J = 15.2$ Hz, H-10), 3.94 (1H, d, $J = 15.2$ Hz, H-10), 3.43 (1H, td, $J = 9.6$, 4.8 Hz, H-5), 2.54 (1H, q, $J = 8.8$ Hz, H-1), 2.28 (1H, ddd, $J = 16.8$, 5.2, 4.8 Hz, H$_\beta$-4), 2.03 (1H, ddd, $J = 16.8$, 9.6, 2.4 Hz, H$_\alpha$-4), 1.98 (1H, m, H-9a), 1.94 (1H, m, H-6), 1.82 (2H, m, H-7), 1.73 (1H, m, H-8a), 1.59

(1H, m, H-8b), 1.25 (1H, m, H-9b); ^{13}C NMR (CD_3OD, 100 MHz) δ 140.7 (C-2), 118.6 (C-3), 68.8 (C-5), 64.8 (C-10), 45.0 (C-6), 401.8 (C-1), 31.5 (C-9), 33.6 (C-4), 28.1 (C-7), 23.7 (C-8); EIMS *m*/*z* 168 $[M]^+$ (10), 150 (78), 135 (27), 123 (53), 119 (35), 107 (65), 97 (8), 93 (84), 83 (14), 79 (100), 67 (62), 56 (14), 53 (33); TLC (silica gel, 2:8 hexane−EtOAc) R_f 0.29. Compound **4** was obtained, by the same procedure as for **3**, from **2** (5 mg) in 79% yield: pale brown solid, mp 79−81 °C; UV (MeOH) λ_{max} <200 nm; ^{1}H NMR (CD_3OD, 400 MHz) δ 5.52 (1H, ddd, J = 6.0, 2.4, 1.2 Hz, H-3), 3.97 (1H, m, H-5), 3.93 (2H, m, H-10), 2.74 (1H, br s, H-1), 2.37 (1H, m, H-6), 2.15 (1H, dt, J = 16.4, 6.0 Hz, H_β-4), 2.09 (1H, m, H_α-4), 1.83 (1H, m, H-9a), 1.68 (1H, m, H-7a), 1.60−1.47 (4H, m, H-7b, H-8, H-9b); ^{13}C NMR (CD_3OD, 100 MHz) δ 140.5 (C-2), 118.8 (C-3), 68.4 (C-5), 64.1 (C-10), 43.8 (C-6), 40.4 (C-1), 31.1 (C-9), 29.0 (C-4), 24.3* (C-7), 24.2* (C-8) (*interchangeable assignments); EIMS *m*/*z* 168 $[M]^+$ (12), 150 (65), 135 (25), 123 (5), 119 (58), 107 (65), 97 (9), 93 (78), 83 (11), 79 (100), 72 (8), 67 (61), 55 (47); TLC (silica gel, 2:8 hexane−EtOAc) R_f 0.29.

(*R*)-MTPA Ester of Compound 2. A solution of compound **2** (5.3 mg) and DMAP (20 mg) in dry dichloromethane (0.5 mL) was added to a solution of (*R*)-MTPA (50 mg) and DCC (50 mg) in dry dichloromethane (1 mL) at room temperature, and the mixture was stirred for 12 h. The reaction mixture was diluted with EtOAc (50 mL), followed by washing with water (20 mL × 2), saturated $NaHCO_3$ (20 mL × 2), saturated NH_4Cl (20 mL × 2), and brine (20 mL × 2) and dried over anhydrous $MgSO_4$. Filtration and chromatography (silica gel, 70−230 mesh, 8:2 hexane−EtOAc) afforded the (*R*)-MTPA ester of compound **2**: colorless oil; ^{1}H NMR ($CDCl_3$, 400 MHz) of the ester showed sets of respective peaks with the same intensity such as at δ 9.42 and 9.36 (s, H-10) and δ 6.61 and 6.54 (td, J = 4.0, 0.8 Hz, H-3) to indicate the reaction product was a diastereomeric mixture (1:1 ratio); TLC (silica gel, 8:2 hexane−EtOAc) R_f 0.50.

(*S*)-MTPA Ester of Compound 2. The reaction was carried out using the same procedure as for the (*R*)-MTPA ester except that (*S*)-MTPA was used. The ^{1}H NMR spectrum of the product was the same as that of the (*R*)-MTPA ester.

Antifungal Activity Assay. Antifungal activity against *Trycophyton mentagrophytes* (KCTC No. 6085) was measured using a paper-disk agar diffusion method.[7] Dried paper disks (9 mm in diameter) containing test material (20, 40, or 60 μg) were placed on Sabouraud Dextrose Agar (SDA) plates seeded with fungi and followed by incubation at 27 °C for 4 days. The clear inhibition zone outside the paper disk was measured in millimeters.

Supporting Information Available: ^{1}H, ^{13}C, gCOSY, gHSQC, and gHMBC spectra for compounds **1** and **2** and ^{1}H NMR spectra of (*R*)- and (*S*)-MTPA esters of **2**. This information is available free of charge via the Internet at http://pubs.acs.org.

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