Tsaokoarylone, a Cytotoxic Diarylheptanoid from *Amomum tsao-ko* Fruits

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The crude methanol extract of the fruits of *Amomum tsao-ko* (Zingiberaceae) showed cytotoxic activity. Bioactivity-guided separation led to the isolation of a diarylheptanoid, tsaokoarylone [7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)-hepta-4*E*,6*E*-dien-3-one] (**2**). **2** showed cytotoxicity at 4.9 and 11.4 μg/mL (IC_{50}) against human nonsmall cell lung cancer A549 and human melanoma SK-Mel-2, respectively, determined by SRB colorimetric method. During purification 6-(4-hydroxyphenyl)-4-hydroxyhexan-2-one (**4**) together with three known diarylheptanoids was also isolated. Their structures were determined from interpretation of spectroscopic data (IR, UV, MS, and NMR) and synthesis confirmed the structure of **2**.

Key Words : *Amomum tsao-ko*, Tsaokoarylone, Cytotoxicity, Curcumin, Diarylheptanoid

Introduction

Many phenolic diarylheptanoids were isolated from the Zingiberaceae. Curcumin (**1**), the major pigment in tumeric (the rhizome of *Curcuma longa*), has shown to possess a broad range of biological activities such as antiviral (inhibition of HIV-1 and HIV-2 proteases),[1] Alzheimer's disease-related cell protection from beta-amyloid insult,[2] and cell cycle inhibition (inhibition of topoisomerase I and II).[3] Recently, curcumins have become synthetic targets for structural modifications to improve their anticancer efficacy and several synthetic analogues revealed cytotoxicity against multiple tumor cell lines.[4-6]

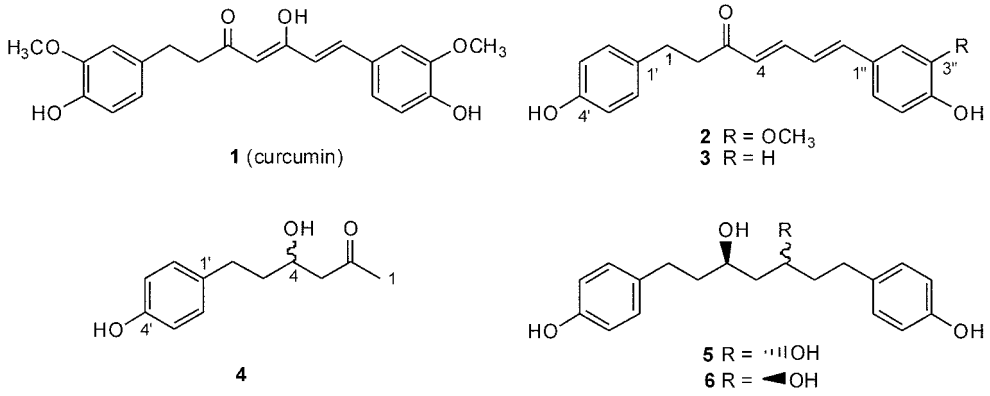
A methanol extract of the powdered fruits of *Amomum tsao-ko* (Zingiberaceae) which has been used in folk medicine for the treatment of stomach disorders and throat infections showed significant cytotoxic activity against human nonsmall cell lung cancer A549 and human melanoma SK-Mel-2 cell lines. In our previous work, the antifungal bicyclic nonanes, tsaokoin and isotsaokoin, were identified from the methanol extract of the fruits of the plant.[7] Further investigation of the fruits of this plant was undertaken to identify the cytotoxic principle. Bioactivity-guided purification led to the isolation of a new cytotoxic curcumin analogue, tsaokoarylone (**2**). In addition, one new 6-(4-hydroxyphenyl)-4-hydroxyhexan-2-one (**4**) and three known diarylheptanoids (**3**, **5**, **6**) were also isolated. Herein, we report isolation and structural determination of **2** and **4** from the fruits of *A. tsao-ko*.

Experimental Section

The melting points were measured on a Fisher melting point apparatus and are 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. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 400 NMR spectrometer at 400 and 100 MHz, respectively, in CD_3OD, and referenced to residual solvent signals (δ 3.31, ^{1}H; 49.15, ^{13}C for CD_3OD). 2D NMR spectra (gCOSY, TOCSY, gHSQC, gHMBC, ROESY) were recorded at 400 MHz using the manufacturer's software VNMR 6.1C. MPLC (medium-pressure liquid chromatography) was carried out on a FMI QSY lab pump system using silica gel 60 (230-400 mesh, 30 i.d. × 300 mm, Merck) at the flow rate of 10 mL/min. Preparative HPLC (High Performance Liquid Chromatography) was carried out on a Waters 600 model system with a Waters photodiode array detector 996 using a reversed phased C-18 silica gel column (Senshu Pak, 20 i.d. × 250 mm) with specified eluent at the flow of 7 mL/min at 225 nm. TLC was carried out on silica gel coated plastic sheets (Silica gel 60 F_{254}, Merck) and visualized under UV lamp (254 nm) or charred by heating after dipping in a solution of anisaldehyde-sulfuric acid in methanol. HRFAB Mass spectra were measured on a JEOL HX110A Tandem HR mass spectrophotometer at the Korea Basic Science Institute (Daejeon, Korea). Optical density for a 96-well microplate was measured on a Tecan Sunrise microplate reader at 520 nm.

Plant Material. The dried fruits of *A. tsao-ko* were purchased at the Kumsan herbal market, Chungnam, Korea, on December, 2003. It was 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 the Kongju National University (identification number: SM1099B).

Extraction and Isolation. The dried fruits (1.2 kg) of the plant *A. tsao-ko* were ground and extracted twice with 80% aqueous MeOH (5 L) in a incubating shaker (80 rpm) at 27 ^{o}C for 2 days. The methanol extracts combined were concentrated under vacuum and suspended in H_2O (500 mL) and extracted with EtOAc (200 mL × 4). The EtOAc layer was concentrated to yield brown oily syrup (20.1 g), which was chromatographed on a silica gel column (70-230 mesh, 100 id × 300 mm) with stepwise elution by mixtures of hexane and EtOAc of increasing polarity, to yield ten fractions by TLC monitoring. Fraction 7 (1.8 g, 4 : 6 hexane/



EtOAc eluate) was further fractionated using silica gel MPLC (7 : 3 to 6 : 4 hexane/EtOAc) into twelve subfractions. The 5th subfraction (179 mg) was subjected to C-18 HPLC (35 to 40% MeCN in H_2O for 120 min) to obtain **3** (0.29 mg) and **2** (2.78 mg) with retention time of 61.1 and 65.4 min, respectively. Fraction 8 (2.91 g, 2 : 8 hexane/EtOAc eluate) was applied onto a silica MPLC (6 : 4 to 0 : 10 hexane/EtOAc) to give seven portions. C-18 HPLC (10 to 40% MeCN in H_2O for 100 min) of the sixth portion (122 mg) provided **4** (3.5 mg) eluted at 45.9 min. The seventh portion (86 mg) was purified by C-18 HPLC with elution of 25 : 75 MeCN/H_2O to afford **5** (9.84 mg) and **6** (14.53 mg), which eluted at 38.7 and 42.3 min, respectively.

Tsaokoarylone [7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)-hepta-4*E*,6*E*-dien-3-one] (2). Yellow powder; mp 51-53 °C; UV (MeOH): λ_{max} (log ε) 261 (3.98), 269 (3.98), 364 (4.55) nm; IR: ν_{max} 3386, 1578, 1515, 1282, 1172, 829 cm^{-1}; ^1H and ^{13}C NMR: see Table 1; HRFABMS: *m/z* $[M + H]^+$ 325.1429 (calcd. for $C_{20}H_{20}O_4$+H: 325.1440); TLC (silica gel, 5 : 5 hexane/EtOAc): R_f 0.36.

6-(4-Hydroxyphenyl)-4-hydroxy-hexan-2-one (4). Colorless oil; $[\alpha]_D^{20}$ +8.3 (c = 0.12, MeOH); UV (MeOH): λ_{max} (log ε) 224 (3.99), 279 (3.41) nm; IR: ν_{max} 3355, 1705, 1614, 1515, 1365, 1242, 830 cm^{-1}; ^1H and ^{13}C NMR: see Table 1; HRFABMS: *m/z* $[M + H]^+$ 209.1184 (calcd. for $C_{12}H_{16}O_3$ + H: 209.1178); TLC (Silica gel, 2 : 8 hexane/EtOAc): R_f 0.50.

Synthesis of Tsaokoarylone (2). 4-Hydroxy-3-methoxycinnamaldehyde (124 mg, 0.696 mmol) and 4-(4-hydroxyphenyl)-2-butanone (93 mg, 0.566 mmol) were dissolved in EtOH (5 mL) and MeOH (2 mL), and sodium hydroxide (80 mg, 2.0 mmol) was added. The mixture was stirred to reflux overnight. The reaction mixture was acidified with cold HCl solution (30 mL, 1 M) and extracted with EtOAc (3 × 20 mL). The extracts combined were washed with saturated $NaHCO_3$ solution followed by brine and dried over anhydrous $MgSO_4$. Silica gel chromatography (6 : 4 hexane/EtOAc) afforded a yellow solid (103 mg) in 56% yield, which was identical with the natural product (**2**) in every aspect of spectral data.

***In vitro* Cytotoxicity Assay.** Human nonsmall cell lung cancer A549 and melanoma SK-Mel-2 cell lines were maintained in RPMI 1640 medium supplemented with L-glutamine (0.3 g/L), sodium bicarbonate (2.0 g/L), HEPES (4.75 g/L), fetal bovine serum (5% for A549 and 10% for SK-Mel-2), penicillin (55 units/mL), and streptomycin (55 mg/mL) in a 5% CO_2 humidified atmosphere at 37 °C. Cells were suspended into a 96-well microplate (5×10^4 for A549 and 1×10^5 cells/mL for SK-Mel-2; 100 mL/well) in RPMI medium and incubated in a CO_2 incubator at 37 °C for 24 h to allow cells to adhere. The test solutions (100 mL) at the two-fold serially diluted concentrations and control (100 mL) in RPMI medium containing DMSO (1%) were then added and incubated for 48 h. Cell growth was quantified by using protein-binding dye, sulforhodamine B (SRB).[8,9] In brief, adherent cells were fixed by cold 50% trichloroacetic acid at 4 °C for 1 h and stained with SRB solution. The bound SRB was solubilized with 10 mM tris base (pH 10.5) and the concentration was determined by optical density measurement at 520 nm on a microplate reader. The IC_{50} value, the compound concentration that inhibited cell line replication by 50 % relative to control was interpolated from dose-response graphs.

Results and Discussion

The methanol extract from the fruits of *A. tsao-ko* was suspended in water and extracted with EtOAc. The EtOAc layer was purified by repeated silica gel column chromatography followed by reversed phase HPLC to afford compounds **2-6**.

Compound **2** was obtained as yellow amorphous powder. The molecular formula was determined to be $C_{20}H_{20}O_4$ by HRFABMS (*m/z* $[M + H]^+$ 325.1429, calcd 325.1440). The IR broad absorption was observed at 3386 cm^{-1} suggesting the presence of hydroxyl group(s) and the strong absorptions were at 1578 and 1515 cm^{-1}. The ^1H NMR and ^1H-^1H COSY spectra indicated the presence of a conjugated diene group [δ 6.25 (d, *J* = 15.2 Hz), 6.85 (dd, *J* = 15.6, 10.4 Hz), 6.96 (d, *J* = 15.6 Hz), and 7.38 (dd, *J* = 15.2, 10.4 Hz)], a 1,4-disubstituted phenyl group [δ 6.68 (d, *J* = 8.8 Hz) and 7.03 (d, *J* = 8.8 Hz)], and a 1,2,4-trisubstituted phenyl group [δ 6.78 (d, *J* = 8.4 Hz), 7.00 (dd, *J* = 8.4, 2.0 Hz), and 7.13 (d, *J* = 2.0 Hz)]. In addition to those, the ^1H NMR spectrum also showed signals assignable to methyl protons [δ 3.88 (3H, s)] and two groups of methylene protons [δ 2.82 (2H, m) and 2.89 (2H, m)] coupled with each other. The ^{13}C

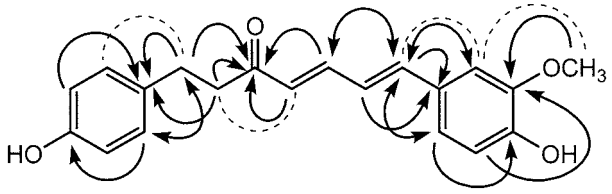


Figure 1. Important ^{1}H-^{13}C and ^{1}H-^{1}H correlations of **2** observed in HMBC (solid arrows, H to C) and ROESY (dashed lines) spectra, respectively.

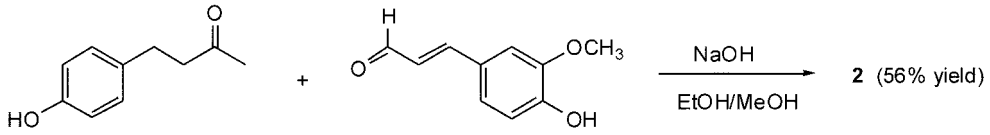
NMR and DEPT spectra exhibited eighteen signals corresponding to six quaternary carbons, nine methine carbons, two methylene carbons, and one methyl carbon. Among the methine carbons the peaks at δ 130.3 and 116.2 were almost two-fold higher in their intensity than other methine peaks, confirming of the presence of the symmetric 1,4-disubstituted phenyl group. The two double bonds in the diene unit were determined as both *trans* on the basis of the coupling constants of 15.6 and 15.2 Hz, respectively. In the HMBC spectrum the carbonyl peak at δ 202.7 correlated with the proton peaks at δ 2.82, 2.89, 6.25, and 7.38, to indicate that it was connected to both the diene unit and one of the methylene groups (Figure 1). This conjugated dienone unit may be contributable to the strong absorptions at 1578 and 1515 cm^{-1} in the IR spectrum. From interpretation of the ROESY spectrum the positions of the methoxy group at δ 3.88 and the methylene group (H-1) at δ 2.82 were determined to be placed to near to the aromatic methine proton (H-2'') at δ7.13 and the aromatic methine proton (H-2' or H-6') at δ7.03, respectively. Extensive analyses of the ^{1}H-^{1}H COSY, HSQC, HMBC, and ROESY spectra allowed the complete assignments of all protons and carbons of compound **2**, which was determined to be 7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)-hepta-4*E*,6*E*-dien-3-one, designated as tsaokoarylone (Table 1). For the structure-confirmation and material supply for bioassay a chemical synthesis was undertaken using the Aldol-type reaction of 4-hydroxy-3-methoxycinnamaldehyde with 4-(4-hydroxyphenyl)-2-butanone (Scheme 1). The reaction was carried out in the presence of sodium hydroxide under reflux to afford a yellow solid in 56% yield after chromatographic purification, whose spectral data were identical with those of the natural product confirming the structure of the natural product to be 7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)-hepta-4*E*,6*E*-dien-3-one.

Compound **4**, $[\alpha]_D^{20}$ +8.3° (*c* = 0.12, MeOH), was obtained as colorless liquid. The molecular formula was determined to be $C_{12}H_{16}O_3$ by HRFABMS (*m/z* $[M + H]^+$ 209.1184, calcd 209.1178). The IR absorptions at 3355, 1705, and 1614 cm^{-1} suggested the presence of hydroxyl, carbonyl, and aromatic groups, respectively. The ^{1}H NMR and ^{1}H-^{1}H COSY spectra indicated the presence of one 1,4-disubstituted phenyl group [δ 6.68 (2H, d, *J* = 8.4 Hz) and 7.01 (2H, d, *J* = 8.4 Hz)], one methyl group [δ 2.14 (3H, s)], three methylene groups [δ 2.58 (2H, d, *J* = 6.4 Hz), 2.55 (1H, m) and 2.64 (1H, m), and δ 1.69 (2H, m)], and one methine group [δ 4.01 (1H, quin, *J* = 6.4 Hz). The ^{13}C NMR and DEPT spectra showed ten carbons: three quaternary carbons, three methine carbons, three methylene carbons, and one methyl carbon. The carbon peaks at δ 129.1 and 114.9 were assigned to C-2' (C-6') and C-3' (C-5'), respectively. In the ^{1}H-^{1}H COSY spectrum the H-4 methine proton at δ 4.01 showed couplings with the H-3 protons at δ 2.58 and also coupled with the H-5 protons at δ 1.69, which in turn coupled with the H-6 protons at δ 2.55 and 2.64. In the

Table 1. NMR Spectral Data for Compounds **2** and **4** in CD_3OD (δ, ppm)[a]

Position	**2**		**4**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	31.1	2.82 (2H, m)	29.5	2.14 (3H, s)
2	43.4	2.89 (2H, m)	209.6	
3	202.7		50.7	2.58 (2H, d, 6.4)
4	128.9	6.25 (1H, d, 15.2)	67.1	4.01 (1H, quin, 6.4)
5	146.0	7.38 (1H, dd, 15.2, 10.4)	39.4	1.69 (2H, m)
6	125.2	6.85 (1H, dd, 15.6, 10.4)	30.8	2.55 (1H, m), 2.64 (1H, m)
7	143.8	6.96 (1H, d, 15.6)		
1'	133.2			132.9
2', 6'	130.3	7.03 (2H, d, 8.8)	129.1	7.01 (2H, d, 8.4)
3', 5'	116.2	6.68 (2H, d, 8.8)	114.9	6.68 (2H, d, 8.4)
4'	156.6			155.2
1"	129.7			
2"	111.1	7.13 (1H, d, 2.0)		
3"	149.2			
3"-OCH_3	56.5	3.88 (3H, s)		
4"	149.4			
5"	116.5	6.78 (1H, d, 8.4)		
6"	122.9	7.00 (1H, dd, 8.4, 2.0)		

[a] ^{1}H and ^{13}C NMR recorded at 400 MHz and 100 MHz, respectively.



Scheme 1

HMBC spectrum the C-2 carbonyl peak at δ 209.6 correlated with the proton peaks at δ 2.14 (H-1), 2.58 (H-3), and 4.01 (H-4). All protons and carbons were unambiguously assigned by interpretation of the ^{1}H-^{1}H COSY, HSQC, HMBC, and ROESY spectra (Table 1). Thus, the structure of **4** was determined to be 6-(4-hydroxyphenyl)-4-hydroxyhexan-2-one.

The known compounds, **3**, **5**, and **6**, were characterized as 1,7-bis(4-hydroxyphenyl)hepta-4*E*,6*E*-dien-3-one,[10] (+)-hannokinol,[11] and meso-hannokinol,[11] respectively, by comparing with their spectral data in the literatures.

Cytotoxicity of compounds **2**-**6** was evaluated against human cancer cell lines using the sulforhodamine B (SRB) colorimetric method.[8,9] **2** showed moderate cytotoxicity at the concentration (IC_{50}) of 4.9 and 11.4 μg/mL against lung cancer A549 and melanoma SK-Mel-2 cell lines, respectively, comparable to cisplatin (7.4 and 10.0 μg/mL, respectively) used as positive control. Compounds **3**-**6** were not active at the concentration lower than 100 μg/mL against those cell lines.

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