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Biotransformation of Sinapic Acid Catalyzed by *Momordica charantia* Peroxidase

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Biotransformation of sinapic acid (**1**) with H_2O_2/*Momordica charantia* peroxidase, which exists in the widely used food *M. charantia*, at pH 5.0, 43 °C, in the presence of acetone resulted in six compounds, including four new compounds. Compound **2** was the first isolation of a new unique sinapic acid tetrameric derivate, which is formed by peroxidase catalysis in vitro. Besides **2**, three other new sinapic acid dimers, **3**−**5** have also been isolated. Their structures were established on the basis of spectroscopic data. Compound **5** showed a stronger antioxidative activity than the parent sinapic acid (**1**). Compounds **4** and **5** significantly inhibited the growth of HL-60 cell at the concentration of 10^{-5} mol/L.

KEYWORDS: Biotransformation; *Momordica charantia* peroxidase; sinapic acid; cinnamic acid; tetramer

INTRODUCTION

The fruit of *Momordica charantia* is one of the most widely used vegetables in China. Previously, we reported the physical and chemical characterization of *M. charantia* peroxidase (MCP), a novel plant peroxidase with high acidic amino acid purified from the fruits of *M. charantia* (*1*). We found that although MCP shared spectroscopic and kinetic features with other peroxidases, the enzyme had several unique characteristics, including enzyme pH stability (pH 3.8∼8.0) and thermostability (20∼45 °C) wider than those of other peroxidases such as horseradish peroxidase (*2*). We also found that the transformation efficiency could be higher at 43 °C compared with the room temperature. Therefore MCP can be expected to oxidize a wider range of substrates, especially cinnamic acid derivatives, considering its potential applications for useful biotransformation. Recently, we have applied purified MCP to transform ferulic acid, which is an extremely abundant and widespread cinnamic acid derivative, into some oxidation products. Among them, two novel dehydrotrimers have been obtained and a dimer has shown more powerful anti-inflammation activity than the parent ferulic acid (*1*, *3*).

Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid, **1**, **Figure 1**), which is also a widespread cinnamic acid derivative (*4*) with anti-inflammation and antioxidation capabilities (*5*, *6*), exists in many foods and fruits, such as the fruit of *Citrus limon*, *Vitis vinifera*, and the root of *Allium cepa* (*7*−*9*). Sinapic acid is a good substrate for many oxidoreductases such as polyphenol oxidase from the white-rot fungus *Trametes versicolor* (*10*−*12*), cell wall-bound peroxidases (*13*), and horseradish peroxidase (*14*). Some dehydrodimers obtained both in vitro and in vivo by oxidoreductases have already been characterizied (*15*−*19*). They redsulted from oxidative coupling of sinapate ester and represented the products of 8−8 radical coupling. Some peroxidases such as CWPO-C were able to oxidize syringyl moieties which were hard to be oxidized by others (*20*−*22*). However, only a few dehydrodimers and higher oligomers of sinapic acid have been obtained. With the aim of studying the biotransformation of sinapic acid by purified MCP and screening the bioactive properties of higher oligomers, the biotransformation was carried out in this research.

MATERIALS AND METHODS

General Procedures. Positive-ion HR-FABMS was recorded with a JEOL HX-110 spectrometer using *m*-nitrobenzyl alcohol as a matrix. Positive-ion HR-ESIMS was recorded with an Agilent TOF MSD 1946D spectrometer. UV and IR spectra were recorded with Shimadzu UV-2501PC and Nicolet Impact 410 spectrometers, respectively. ^{1}H and ^{13}C NMR, HSQC, HMBC, and NOESY spectra were taken with Bruker ACF-500 (500 and 125 MHz, respectively) and Bruker ACF-600 spectrometers (600 and 150 MHz, respectively) in acetone-d_6, DMSO-d_6, or $CDCl_3$; chemical shifts are reported in ppm as δ relative to Me_4Si (internal standard). Column chromatography was carried out with Sephadex LH-20 (20−100 μm, Pharmacia) and ODS-C_{18} (100−200 μm, Waters). L-Cysteine hydrochloride (high pure grade) was purchased from AMRESCO. Sinapic acid, DMSO, *cis*-diamminedichloroplatinum (*cis*-DDP), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma. The malondialdehyde (MDA) kit was provided by Nanjing Jiancheng Bioengineering Institute. Other reagents and solvents used were of analytical grade and were purchased from reliable commercial sources.

Plant Material. Fruits of *M. charantia* were collected at a suburb of Nanjing, China, and identified by Dr. Mingjian Qin, China Pharmaceutical University. A voucher specimen (No. 000804) was deposited in the Department of Natural Medicinal Chemistry, China Pharmaceutical University.

Purification of MCP. MCP from fruits of *M. charantia* was purified to electrophoretic homogeneity by consecutive treatment of ammonium

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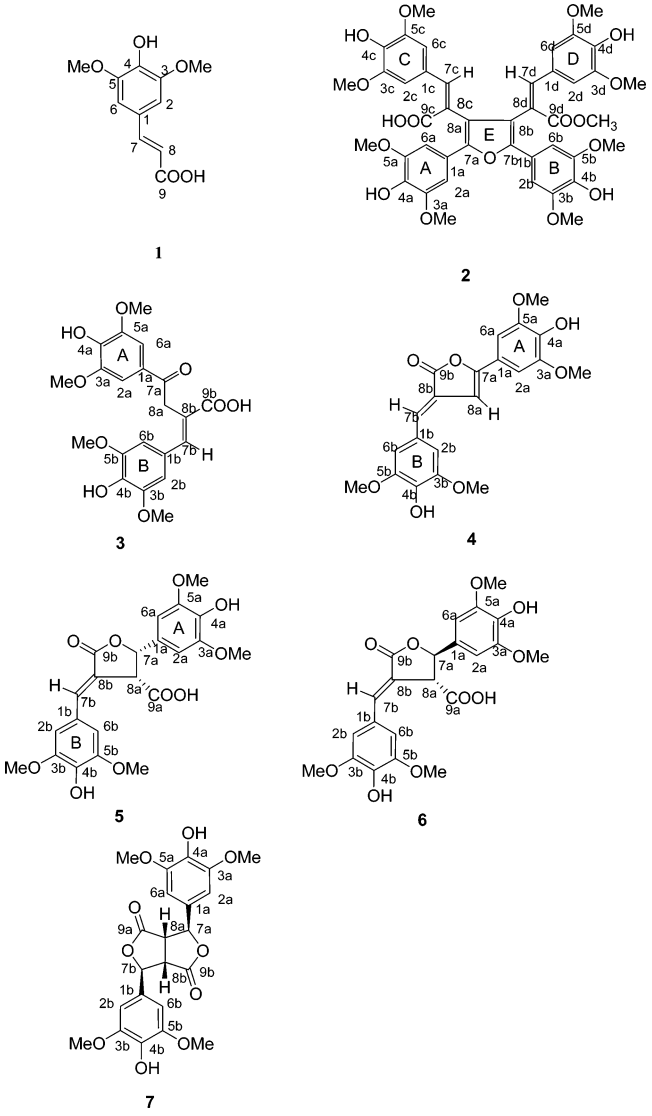

Figure 1. Chemical structures of **1**–**7**.

sulfate fractionation, ion exchange chromatography on DEAE-Sepharose FF, affinity chromatography on Con A Sepharose, and gel filtration on Sephadex G-150 as described in our previous report (*1*). The purified MCP exhibited a specific activity of 7757 E.U. of peroxidase per mg of protein, which was 46-fold higher than that of the crude extract.

Biotransformation of Sinapic Acid in Aqueous Acetone. A solution of sinapic acid (500 mg) in acetone (25 mL) and a solution of MCP (4×10^3 U) in buffer (100 mM NaOAC-HOAC, pH 5.0, 100 mL) were mixed and treated with hydrogen peroxide (3%, 5 mL) at 43 °C and stirred for 4 h, during which time the mixture gradually turned a brownish red color.

Isolation and Identification. The reaction mixture was evaporated to dryness at 40 °C under reduced pressure. The extractum was dissolved with water. Then the aqueous mixture was extracted with ethyl acetate and *n*-butanol, successively. The extract was washed with water, dried with anhydrous Na_2SO_4, and evaporated to dryness at 40 °C under reduced pressure. The combined ethyl acetate layer was concentrated to afford a brown powder (388 mg), which was subjected to RP-C18 column chromatography (Φ3.0 × 50, 80 g) eluted with MeOH/H_2O (30:70 and 40:60, v/v) to afford fraction 1 (233 mg) and fraction 2 (118 mg). Fraction 1 was subjected to Sephadex LH-20 column chromatography (Φ1.5 × 100, 70 g, 8 mL each) eluted with MeOH. The elution of 15 to 20 fractions (197 mg) was submitted to RP-C18 column chromatography (Φ1.5 × 30, 40 g) eluted with MeOH/H_2O (40:60, v/v) to give **5** (166 mg). Fraction 2 was subjected to Sephadex LH-20 column chromatography (Φ1.5 × 100, 70 g, 8 mL each) eluted with MeOH/$CHCl_3$. (1:1, v/v). The elution of 13 to 15 fractions (42 mg) was submitted to RP-C18 column chromatography (Φ1.5 × 30, 40 g) eluted with MeOH/H_2O (50:50, v/v) to give **6** (24 mg), and the elution of 18 to 23 fractions (60 mg) was submitted to RP-C18 column chromatography (Φ1.5 × 30, 40 g) eluted with MeOH/H_2O (45:55, v/v) to give **7** (30 mg). The combined *n*-butanol layer was concentrated to afford a strong brown powder (64 mg) and subjected to RP-C18 column chromatography (Φ3.0 × 50, 80 g) eluted with MeOH/H_2O (40:60, 50:50, and 60:40, v/v) to afford fraction 3 (25 mg), fraction 4 (18 mg), and fraction 5 (15 mg), respectively. Fraction 3 was subjected to Sephadex LH-20 column chromatography (Φ1.5 × 100, 70 g, 8 mL each) eluted with MeOH. The elution of 8 to 12 fractions (20 mg) was submitted to RP-C18 column chromatography (Φ1.5 × 30, 40 g) eluted with MeOH/H_2O (40:60, v/v) to give **2** (16 mg). Fraction 4 was subjected to Sephadex LH-20 column chromatography (Φ1.5 × 100, 70 g, 8 mL each) eluted with MeOH. The elution of 16 to 19 fractions (14 mg) was submitted to RP-C18 column chromatography (Φ1.5 × 30, 40 g) eluted with MeOH/H_2O (45:55, v/v) to give **3** (10 mg). Fraction 5 was subjected to Sephadex LH-20 column chromatography (Φ1.5 × 100, 70 g, 8 mL each) eluted with MeOH/$CHCl_3$ (1:1, v/v). The elution of 14 to 16 fractions (12 mg) was submitted to RP-C18 column chromatography (Φ1.5 × 30, 40 g) eluted with MeOH/H_2O (55:45, v/v) to give **4** (9 mg).

Compound **2**: green powder. IR ν_{max}^{KBr} (cm^{-1}) = 3445, 3423, 2939, 2840, 1701, 1610, 1514, 1456, 1332, 1253, 1220, 1114. Positive HR-FABMS Found: *m*/*z* 831.2498, $C_{43}H_{43}O_{17}$. For ^1H and ^{13}C NMR data, see **Table 1**.

Compound **3**: white powder. IR ν_{max}^{KBr} (cm^{-1}) = 3445, 2933, 1992, 1622, 1514, 1458, 1423, 1340, 1280, 1222, 1164,1118. Positive HR-FABMS Found: *m*/*z* 419.1336, $C_{21}H_{23}O_9$. For ^1H and ^{13}C NMR data, see **Table 2**.

Compound **4**: red powder. IR ν_{max}^{KBr} (cm^{-1}) = 3552, 3439, 2941, 1745,1614, 1512, 1460, 1336, 1251, 1217, 1164, 1114. Negative HR-FABMS Found: *m*/*z* 399.1085, $C_{21}H_{19}O_8$. For ^1H and ^{13}C NMR data, see **Table 2**.

Compound **5**: white powder. IR ν_{max}^{KBr} (cm^{-1}) = 3443, 3425, 2939, 1734, 1639, 1610, 1520, 1461, 1431, 1352, 1323, 1218, 11161, 1116. Positive HR-ESIMS Found: *m*/*z* 469.1113 $[M + Na]^+$, $C_{22}H_{22}O_{10}Na$. Negative HR-ESIMS Found: *m*/ *z* 401.1252 $[M - CO_2 - H]^-$, $C_{21}H_{21}O_8$. For ^1H and ^{13}C NMR data, see **Table 2**.

Compound **6**: white powder. ESI-MS: *m*/*z* = 445 $[M - H]^-$. ^1H NMR (acetone-d_6, 500 MHz): δ = 6.70 (s, 2, 2a-CH and 6a-CH), 5.75 (d, 1, J = 2.6 Hz, 7a-CH), 4.36 (m, 1, 8a-CH), 7.08 (s, 2, 2b-CH and 6b-CH), 7.63 (d, 1, J = 2.6, 7b-CH), 3.83 (s, 6, 3a-OCH_3 and 5a-OCH_3), 3.88 (s, 6, 3b-OCH_3 and 5b-OCH_3), 7.39 (br s, 1, 4a-OH), 7.92-(br s, 1, 4b-OH). ^{13}C NMR (acetone-d_6, 125 MHz): δ = 125.7 (C-1a), 104.6 (C-2a), 149.4 (C-3a), 137.8 (C-4a), 149.4 (C-5a), 104.6 (C-6a), 81.9 (C-7a) 54.6 (C-8a), 172.5 (C-9a), 131.7 (C-1b), 109.8 (C-2b), 149.2 (C-3b), 141.4 (C-4b), 149.2 (C-5b), 109.8 (C-6b), 140.1 (C-7b), 125.7 (C-8b), 172.0 (C-9b), 57.0 (OMe-3a and OMe-5a), 57.1 (OMe-3b and OMe-5b).

Compound **7**: white powder. ESI-MS: *m*/*z* = 445 $[M - H]^-$. ^1H NMR (acetone-d_6, 500 MHz): δ = 6.75 (s, 4, 2a-CH, 5a-CH, 2b-CH, and 5b-CH), 5.77 (s, 2, 7a-CH and 7b-CH), 4.13 (s, 2, 8a-CH and 8b-CH), 3.85 (s, 12, 3a-OMe, 5a-OMe, 3b-OMe and 5b-OMe). ^{13}C NMR (acetone-d_6, 125 MHz): δ = 130.3 (C-1a and C-1b), 104.7 (C-2a, C-6a, C-2b and C-6b), 149.7 (C-3a, C-5a, C-3b and C-5b), 138.0 (C-4a and C-4b), 83.8 (C-7a and C-7b), 49.5 (C-8a and C-8b), 176.5 (C-9a and C-9b), 57.2 (OMe-3a, OMe-5a, OMe-3b and OMe-3a).

Measurement of Antioxidative Activity. Male Sprague−Dawley SD rats (8−9 weeks old) were used. They were purchased from the Experimental Animal House of China Pharmaceutical University (Nanjing, China). All rats were maintained with free access to pellet food and water in plastic cages at 20 ± 2 °C and kept on a 12 h light/dark cycle. The animal study protocol was in compliance with the guidelines of China for animal care, which was conformed to the internationally accepted principles in the care and use of experimental animals. Rat was sacrificed by bloodletting and was perfused in situ via the portal vein with phosphate buffered saline (PBS). Liver tissue was collected and cut into species. After being weighed, the tissue was homogenized with PBS (1:4) using a high-speed homogenizer for 10 s each time at 0 °C, and the operation was repeated for 4 to 5 times.

Table 1. NMR Data for Compound **2** in Acetone-d_6[a]

position	^{1}H	^{13}C	HMBC	position	^{1}H	^{13}C	HMBC
1a		123.3		1b		123.4	
2a	6.92 (1H, s)	104.7	1a, 4a, 6a, 7a	2b	7.03 (1H, s)	104.6	1b, 4b, 6b, 7b
3a		149.4		3b		149.5	
4a		137.7		4b		137.7	
5a		149.4		5b		149.5	
6a	6.92 (1H, s)	104.7	1a, 2a, 4a, 7a	6b	7.03 (1H, s)	104.6	1b, 2b, 4b, 7b
7a		149.7		7b		150.1	
8a		122.5		8b		122.3	
1c		126.7		1d		126.7	
2c	6.99 (1H, s)	110.5	1c, 4c, 6c, 7c	2d	6.96 (1H, s)	110.6	1d, 4d, 6d, 7d
3c		148.8		3d		148.7	
4c		139.6		4d		139.4	
5c		148.8		5d		148.7	
6c	6.99 (1H, s)	110.5	1c, 2c, 4c, 7c	6d	6.96 (1H, s)	110.6	1d, 2d, 4d, 7d
7c	7.84 (1H, s)	144.9	8a, 8c, 9c	7d	7.77 (1H, s)	145.4	8b, 8d, 9d
8c		120.2		8d		119.6	
9c		169.0		9d		169.1	
3a, 5a-OMe	3.72 (6H, s)	57.3	3a, 5a	3b, 5b-OMe	3.70 (6H, s)	57.3	3b, 5b
3c, 5c-OMe	3.67 (6H, s)	57.0	3c, 5c	3d, 5d-OMe	3.65 (6H, s)	57.0	3d, 5d
–COOMe	3.12 (3H, s)	52.3	9d				

[a] Chemical shifts are given in δ values and followed by proton numbers and multiplicities. The spectra of ^{1}H and ^{13}C signals were measured in acetone-d_6 (^{1}H, 500 MHz and ^{13}C, 125 MHz), and the spectra of HSQC and HMBC were measured in acetone-d_6 (^{1}H, 600 MHz and ^{13}C, 150 MHz). The ^{1}H and ^{13}C signals were assigned by HSQC and HMBC spectra.

Table 2. NMR Data for Compounds **3**, **4**, and **5**[a]

	3		**4**		**5**	
position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1a	128.8		118.2		127.5	
2a	107.4	7.37 (1H, s)	103.2	7.13 (1H, s)	105.4	6.81 (1H, s)
3a	149.2		148.2		149.4	
4a	142.8		138.2		137.5	
5a	149.2		148.2		149.4	
6a	107.4	7.37 (1H, s)	103.2	7.13 (1H, s)	105.4	6.81 (1H, s)
7a	198.8		155.1		80.7	5.80 (1H, d, 7.5)
8a	39.4	4.30 (2H, s)	99.1	7.34 (1H, s)	53.4	4.71 (1H, dd, 7.5, 1.8)
9a	–	–	–	–	172.1	
1b	127.3		125.3		126.0	
2b	108.0	6.60 (1H, s)	108.8	7.10 (1H, s)	109.6	7.03 (1H, s)
3b	149.1		148.2		149.0	
4b	138.0		138.9		140.0	
5b	149.1		148.2		149.0	
6b	108.0	6.60 (1H, s)	108.8	7.10 (1H, s)	109.6	7.03 (1H, s)
7b	143.6	7.90 (1H, s)	134.7	7.25 (1H, s)	139.7	7.56 (1H, d, 1.8)
8b	126.5		121.8		123.6	
9b	171.4		169.3		171.4	
3a, 5a-OMe	56.6	3.64 (6H, s)	56.3	3.85 (6H, s)	57.1	3.83 (6H, s)
3b, 5b-OMe	57.0	3.88 (6H, s)	56.4	3.89 (6H, s)	57.1	3.88 (6H, s)
4a-OH				9.09 (1H, s)		7.31 (1H, br s)
4b-OH				9.28 (1H, s)		7.96 (1H, br s)

[a] Spectra of compound **3** were measured in CD_3OD (^{1}H, 500 MHz and ^{13}C, 125 MHz), those of compound **4** were measured in DMSO-d_6 (^{1}H, 500 MHz and ^{13}C, 125 MHz), and those of compound **5** were measured in acetone-d_6 (^{1}H, 500 MHz and ^{13}C, 125 MHz).

The homogenates (25%) were then centrifuged at 10 000 rpm for 20 min. The supernatants were collected and again centrifuged at 100 000 rpm for 60 min. The supernatants were discarded, and the microsomal pellet was suspended in 10 volume PBS containing 30% glycerol (glycerol:PBS = 30:70 (v/v)). Microsomal suspensions were stored in small portions at −80 °C and thawed before lipid peroxidation assay. Microsomal protein was determined by the biuret method.

Lipid peroxidation was induced in rat liver microsomes by $FeSO_4$−cysteine. A mixture of the microsomal suspension (10%, 100 μL), $FeSO_4$ solution (1 mmol/L, 50 μL), L-cysteine hydrochloride (10 mmol/L, 20 μL), the sample solution (final concentration of 10^{-4} mol/L, 10 μL) or vehicle (DMSO, 10 μL), and PBS (0.01 M, pH 7.4, 820 μL) was incubated at 37 °C for 30 min. Each reaction mixture was stopped at 4 °C. The concentration of MDA, the second derivative of lipid peroxidation, in the reaction mixture above-mentioned was determined using the commercial kit according to the guidelines provided. The inhibition ratio was defined according to the following equation:

$$\text{inhibition ratio} = (\text{the absorbance of vehicle} - \text{the absorbance of tested compound})/\text{the absorbance of vehicle} \times 100\%$$

Measurement of Inhibition of HL-60. Human leukemia cell line (HL-60) cells were obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. HL-60 cells were cultured in a humidified incubator at 37 °C in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (GIBCO BRL) and antibiotics (100 U/mL penicillin and 100 g/mL streptomycin) in an atmosphere containing 5% CO_2. Cells were passaged twice weekly and routinely examined for mycoplasma contamination.

When HL-60 cells began growth exponential, HL-60 cells were seeded onto 96-well microplates (Costar) at a density of 2×10^4 cells/well in RPMI-1640 medium (180 μL) and cultured in a humidified incubator for 24 h at 37 °C in 5% CO_2/air. After the supernatant was removed, 20 μL of tested compound (with final concentration of 10^{-5}, 10^{-6}, or 10^{-7} mol/L**)** was added and cultured for 48 h. Then cell growth was evaluated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) assay. A total of 20 μL of 5 mg/mL MTT in RPMI-1640 medium was added to each well for a further 4 h incubation. After the supernatant was removed, 150 μL of DMSO (dimethyl sulfoxide) was added to dissolve the formazan crystals. The plate was shaken for 5 min and then scanned with an ELISA reader (Multiskan Spectrum MSS 1500−320, Thermo Electron Corporation) at 570 nm. The inhibition ratio was calculated according to the following equation:

inhibition ratio = (the absorbance of negative control − the absorbance of tested compound)/ the absorbance of negative control × 100%

RESULTS AND DISCUSSION

Compound **2** was obtained as a green powder. Its molecular formula was determined as $C_{43}H_{42}O_{17}$ by positive HR-FABMS spectrometric analysis (data given above), indicating the formation of a sinapic acid tetramer. The IR spectrum exhibited absorption bands of hydroxyl (3445, 3423 cm^{-1}), ester carbonyl (1701 cm^{-1}), and phenyl (1610, 1514 cm^{-1}) groups. In the ^1H and ^{13}C NMR spectra (**Table 1**), four 1,3,4,5-tetra-substituted aromatic fragments (A, B, C, and D) were observed, and no *trans*-coupled disubstituted double bonds were detected, suggesting that the sinapic radical, the reaction intermediate, did not react with its aromatic ring but was tetramerized at the side chain. The side chains of two sinapic radicals involved decarboxylation and further formed a furan fragment (E) as shown by only two carbonyl groups (δ_C 169.1 and 169.0) and two oxygen-substituted aromatic carbons (δ_C 149.7 and 150.1) observed in the furan ring, with 12 carbons linked with oxygen atoms located in the four aromatic rings. The carboxylic acid group of one sinapic was present as a methyl ester, as indicated by one methoxy group linked to the carbonyl group (δ_H 3.12, δ_C 52.3), with eight methoxy groups located in the four aromatic rings. In addition, two trisubstituted double bonds (δ_H 7.84, 7.77; δ_C 144.9, 120.2 and 145.4, 119.6) were also characterized.

All the signals of protons and carbons of **2** were assigned with detailed analysis of the ^1H and ^{13}C NMR data associated with HSQC and HMBC spectra (**Table 1**). An HSQC experiment established all direct $^1H-^{13}C$ connectivities. The HMBC spectrum showed many informative $^1H-^{13}C$ long-range correlations which allowed us to connect partial fragments. The correlations of H-2a (6a) to C-7a and H-2b (6b) to C-7b suggested that aromatic fragments A and B were connected to the 7a and 7b positions of the furan fragment E through 1a and 1b carbon atoms, respectively. For the trisubstituted CH═C fragment (7c, 8c), the correlations of H-2c (6c) to C-7c and H-7c to C-2c (6c, 9c, 8a) supported connection of the aromatic fragment C to the CH═ group with δ 7.84 (7c) through the 1c carbon atom, the furan fragment E was connected to the ═C group (8c) through the 8a carbon atom, and a carboxylic acid group was also connected to the ═C group (8c). For the other trisubstituted CH═C fragment (7d, 8d), the correlations of H-2d (6d) to C-7d, H-7d to C-2d (6d, 9d, 8b), and OMe-9d to C-9d indicated that the aromatic fragment D was connected to the CH═ group with δ 7.77 (7d) through the 1d carbon atom. The furan fragment E was connected to the ═C group (8d) through the 8b carbon atom, and a methyl ester group was also connected to the ═C group (8d) (**Figure 2**). The stereochemistry of two olefinic bonds (7c, 8c and 7d, 8d) was determined to be *Z*-configuration on the basis of the obvious NOE correlation between H-7c and H-7d. Combining the above analysis, the structure of **2** was elucidated as 3-(4-hydroxy-3,5-dimethoxy)-2-[2,5-bis(4-hydroxy- 3,5-dimethoxy)-4-[3-(4-hydroxy-3,5-dimethoxy)-[(*E*)-2-carboxyethenyl]-2-yl]furan-3-yl]-(*E*)-2-propenoic acid methyl ester. This is the first successful directed conversion of the sinapic acid into a tetramer derivate possessing two full conjugation systems in the molecule, which would be rather difficult to obtain through organic synthesis.

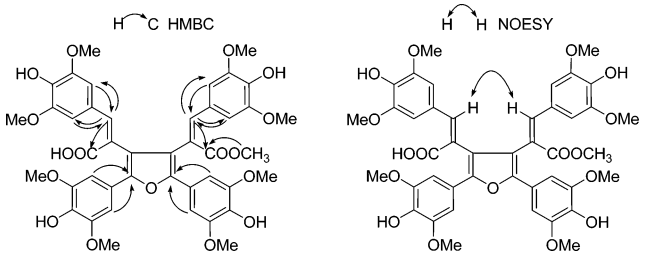

Figure 2. Key HMBC and key NOESY for compound **2**.

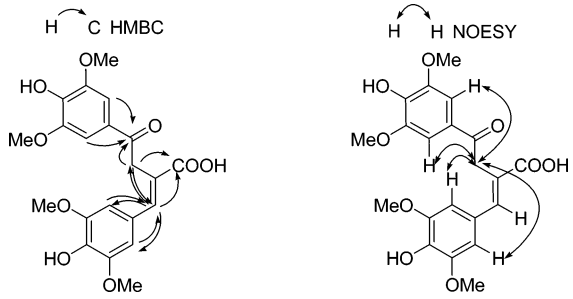

Figure 3. Key HMBC and key NOESY for compound **3**.

Compound **3** was obtained as a white powder. Its molecular formula was determined as $C_{21}H_{22}O_9$ by positive HR-ESIMS spectrometric analysis, indicating a sinapic acid dehydrodimer. The IR spectrum exhibited absorption bands of hydroxyl (3445 cm^{-1}), ketone carbonyl (1992 cm^{-1}), and phenyl (1622, 1514 cm^{-1}) groups. In the ^1H and ^{13}C NMR spectra (**Table 2**), two 1,3,4,5-tetra-substituted aromatic fragments (A and B), one trisubstituted double bond, one $-CH_2CO-$ fragment, one carboxylic acid, and four methoxy groups were observed, suggesting that the sinapic radical was dimerized at the side chain.

Detailed analysis of the ^1H and ^{13}C NMR data associated with HSQC and HMBC spectra allowed assignment of all the H and C signals (**Table 2**). An HSQC experiment established all direct $^1H-^{13}C$ connectivities. The HMBC spectrum showed many informative $^1H-^{13}C$ long-range correlations. The correlations of H-2a (6a) to C-7a suggested that aromatic fragment A was connected to the carbonyl group of the $-CH_2CO-$ fragment through the 1a atom. For the trisubstituted CH═C fragment (7b, 8b), the correlations of H-2b (6b) to C-7b, H-7b to C-2b (6b, 9b, 8a), and H-8a to C-7b (9b) supported that the aromatic fragment B was connected to the CH═ group with δ 7.90 (7b) through the 1b atom, the $-CH_2CO-$ fragment was connected to the ═C group (8b) through the 8a atom, and the carboxylic acid group was also connected to the ═C group (8b) (**Figure 3**). The stereochemistry of olefin carbon (C-7b) was determined to be *E*-configuration, because NOE correlation peaks between H-8a and H-2a (6a, 2b, 6b) were observed in the NOESY spectrum. According to the above analysis, the structure of compound **3** was elucidated as 3-(4-hydroxy-3,5-

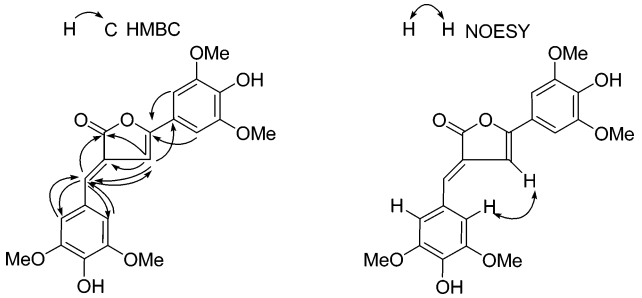

Figure 4. Key HMBC and key NOESY for compound **4**.

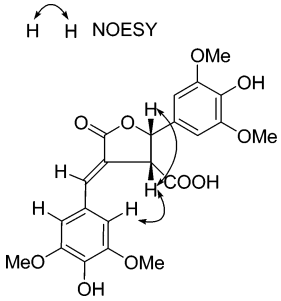

Figure 5. Key NOESY for compound **5**.

dimethoxyphenyl)-2-[1-(4-hydroxy-3,5-dimethoxyphenyl)-acetyl]-(*E*)-2-propenoic acid. Compound **3** was a new compound.

Compound **4** was obtained as a red powder. Its molecular formula was determined as $C_{21}H_{20}O_8$ by negative HR-ESIMS spectrometric analysis, indicating a sinapic acid dehydrodimer. The IR spectrum exhibited absorption bands of hydroxyl (3522, 3439 cm^{-1}), lactone carbonyl (1745 cm^{-1}), and phenyl (1614, 1512 cm^{-1}) groups. In the ^1H and ^{13}C NMR spectra (**Table 2**), two 1,3,4,5-tetra-substituted aromatic fragments (A, B), two trisubstituted double bonds, one lactone carbonyl group, and four methoxy groups were observed, suggesting that the sinapic radical was dimerized at the side chain.

By detailed analysis of the ^1H and ^{13}C NMR data associated with HSQC and HMBC spectra, all the H and C signals could be assigned (**Table 2**). An HSQC experiment established all direct $^1H-^{13}C$ connectivities. The HMBC spectrum showed many informative $^1H-^{13}C$ long-range correlations. The correlations of H-7b to C-9b (8a) and H-8a to C-1a (7b, 8b, 9b) supported the presence of the 3-methylene-2(3*H*)-furanone ring system. The correlations of H-2a (6a) to C-7a and H-8a to C-1a as well as the correlations of H-2b (6b) to C-7b and H-7b to C-2b (6b) suggested that the aromatic fragments A and B were connected to the 7a and 7b positions of the 3-methylene-2(3*H*)-furanone moiety through the 1a and 1b atoms, respectively (**Figure 4**). The stereochemistry of olefin carbon (C-7b) was determined to be *E*-configuration, because NOE correlation peaks between H-8a and H-2b were observed in the NOESY spectrum. According to the above analysis, the structure of **4** was elucidated as (*E*)-5-(4-hydroxy-3,5-dimethoxyphenyl)-3-[(4-hydroxy-3, 5-dimethoxy-phenyl)-methylene]-2(3*H*)-furanone. Compound **4** was a new compound.

Compound **5** was obtained as a white powder. Its molecular formula was determined as $C_{22}H_{22}O_{10}$ by positive HR-ESIMS spectrometric analysis and negative HR-ESIMS spectrometric analysis, indicating a sinapic acid dehydrodimer. The IR spectrum exhibited absorption bands of hydroxyl (3443, 3425 cm^{-1}), lactone carbonyl (1734 cm^{-1}), olefin (1639 cm^{-1}), and phenyl (1610, 1520 cm^{-1}) groups. In the ^1H and ^{13}C NMR spectra (**Table 2**), two 1,3,4,5-tetra-substituted aromatic fragments, one trisubstituted double bond, one saturated CH−CH fragment, two carbonyl groups, and four methoxy groups were observed, suggesting that the sinapic radical was dimerized at the side chain. It further possessed a mono-lactone ring system. The stereochemistry of olefin carbon (C-7b) was determined to be *Z*-configuration, because NOE correlation peaks between H-8a and H-2b were observed in the NOESY spectrum and the chemical shift of 7b (δ 7.56) affected by lactone carbonyl group (9b) in the ^1H NMR spectrum was located relatively downfield when compared with that in the *E*-configuration (**Figure 5**). The relative configuration of H-7a/H-8a was deduced to be a *cis* relationship, because the NOE correlation peak between H-7a and H-8a was detected and the coupling constant of H-7a/H-8a was $J = 2.6$ Hz compared with that ($J = 7.6$ Hz) in *trans* relationship in the mono-lactone reported in the literature (**6**). According to the above analysis, the structure of **5** was elucidated as *cis*-(*4*)*E*-2-(4-hydroxy-3,5-dimethoxyphenyl)-4-[(4-hydroxy-3,5-dimethoxyphenyl)-methylene]tetrahydro-5-oxo-3-furancarboxylic acid. Compound **5** was a new compound.

The known compounds, *trans*-(*4*)*E*-2-(4-hydroxy-3,5-dimethoxyphenyl)-4-[(4-hydroxyl-3,5-dimethoxyphenyl)-methylene]tetrahydro-5-oxo-3-furancarboxylic acid (**6**) (*6*), tetra-hydro-3,6-bis(4-hydroxy-3,5-dimethoxyphenyl)-1*H*,4*H*-furo[3,4-*c*]furan-

Table 3. Effect of Compounds on Lipid Peroxidation of Rat Liver Microsomes[a]

compd	concn (mol/L)	inhibition ratio of MDA formation
4	1×10^{-4}	65.6×2.5
5	1×10^{-4}	43.1×4.0
6	1×10^{-4}	45.7×4.5
7	1×10^{-4}	46.7×3.3
sinapic acid	1×10^{-4}	49.8×1.8

[a] Lipid peroxidation was induced in rat liver microsomes by $FeSO_4$–cysteine. The concentration of MDA, the second derivative of lipid peroxidation, was determined. The inhibition rate was calculated. Data are expressed as the mean ± SD of three experiments, and each experiment included triplicate sets.

Table 4. Effects of Compounds on the Growth of HL-60 Cells[a]

compd	concn (mol/L)	inhibition ratio for HL-60 growth (%)
4	1×10^{-5}	98.4
	1×10^{-6}	34.3
	1×10^{-7}	0.0
5	1×10^{-5}	92.3
	1×10^{-6}	0.0
	1×10^{-7}	0.00
6	1×10^{-5}	88.3
	1×10^{-6}	0.0
	1×10^{-7}	0.0
7	1×10^{-5}	98.2
	1×10^{-6}	0.0
	1×10^{-7}	0.0
cis-DDP	1×10^{-5}	93.9
	1×10^{-6}	71.3
	1×10^{-7}	51.3

[a] HL-60 cells (2×10^4 in 180 μL of RPMI-1640 medium) were pre-cultured in a 96-well microplate for 24 h. After the supernatant was removed, 20 μL of tested compound (with final concentration of 10^{-4}, 10^{-5}, or 10^{-6} mol/L) was added and cultured for 48 h. Then cell growth was evaluated with MTT assay. The inhibition ratio was calculated. Each data represented the mean of three experiments, and each experiment included triplicate sets.

1,4-dione (**7**) (*19*, *23*), were identified by comparing their physical and spectroscopic data with those reported in the literature.

The fruit of *M. charantia* is one of the most widely used foods in China for thousands of years. As a vegetable, it showed obvious hypoglycemic activity (*24*). A novel peroxidase (*M. charantia* peroxidase, MCP) was purified from the fruit of *M. charantia*. We applied the purified MCP to catalyze the transformation of sinapic acid, which exists in many foods, and showed many activities such as anti-inflammation and antioxidation capabilities (*5*, *6*). A new tetramer and three new dimers of sinapic acid were successfully obtained. In addition, the known compound **7** which was obtained exists in *Gmelina arborea* (*19*).

In order to investigate the effects of H_2O_2 or the polymerization of sinapic acid, a negative contrast experiment was conducted. The experimental result has shown that H_2O_2 cannot catalyze the polymerization of sinapic acid, when MCP did not exist in the reaction system. During our research, a new tetramer derivate (**2**) of cinnamic acid derivatives has been obtained with peroxidase in vitro. In the pharmacological research, compound **5** has shown stronger antioxidative activity than the parent sinapic acid (**1**), see **Table 3**. Its antioxidant activity appears to be related to the existence of a fully conjugated system in the molecule. Compounds **4** and **5** significantly inhibited the growth of HL-60 cell at the concentration of 10^{-5} mol/L, see **Table 4**.

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Supporting Information Available: ^{1}H, ^{13}C, HSQC, HMBC, and NOESY spectra for **2** and **4**; ^{1}H, ^{13}C, HSQC, and HMBC spectra for **3**; ^{1}H, ^{13}C, and NOESY spectra for **5**; and ^{1}H and ^{13}C spectra for **6** and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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