Biotransformation of Ferulic Acid into Two New Dihydrotrimers by *Momordica charantia* Peroxidase

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As a result of a continuing investigation of the biotransformation of ferulic acid with H_2O_2/*Momordica charantia* peroxidase at pH 5.0 in the presence of acetone, triFA1 (1) and triFA2 (2), two new FA dehydrotrimers, have been isolated. The structures were elucidated from the spectroscopic data including 2D-NMR such as HSQC, HMBC and NOESY data. TriFA1 (1) is a unique FA dehydrotrimer possessing a 3a,9b-dihydro-1*H*-furo[3,4-*c*][1]benzopyran-3(4*H*)-one ring system. The possible mechanism for the formation of triFA1 was also discussed.

Key words *Momordica charantia* peroxidase; ferulic acid; FA dehydrotrimer

Previously, we reported the physical and chemical characterization of *Momordica charantia* peroxidase (MCP), a novel plant peroxidase with high acidic amino acid purified from the fruits of *Momordica charantia* and applied the purified MCP to transform ferulic acid (FA) into FA-2, a FA dehydrodimer, which had more powerful anti-inflammation than FA.[1)] We found that although MCP shared spectral 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)] So MCP can be expected to oxidize a wider range of substrates, especially cinnamic acid derivatives, when considering the potential applications of MCP for useful biotransformation.

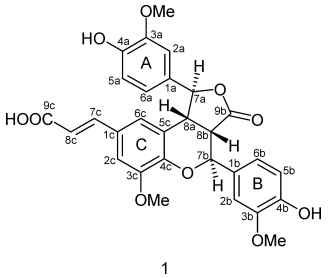
FA, which is an extremely abundant and widespread cinnamic acid derivative, was a good substrate for most plant peroxidases such as lignin peroxidase, wheat germ peroxidase and horseradish peroxidase.[3,4)] FA dehydrodimers produced both *in vitro* and *in vivo* by plant peroxidases have been extensively characterizied.[5—11)] They result from oxidative coupling of ferulate esters and represent mainly products of 8-5, 8-O-4, 4-O-5 and 5-5 radical couplings. Recently, it was reported that in incubation with lignin peroxidase and ectomycorrhizal fungi, dihydrotrimers of FA were formed.[12,13)] However, only two structures of FA dehydrotrimers were elucidated by NMR spectrum, although higher oligomers of FA on a template of a tyrosine-containing tripeptide have recently been yielded enzymically *in vitro*.[14)]

In order to further study the biotransformation of FA by purified MCP and analyze FA dehydrotrimers, we have investigated the oxidative coupling of FA with H_2O_2/MCP at pH 5.0 in the presence of acetone. Two new FA dehydrotrimers, triFA1 (**1**) and triFA2 (**2**), have been identified. TriFA1 (**1**) is a unique FA dehydrotrimer possessing a 3a,9b-dihydro-1*H*-furo[3,4-*c*][1]benzopyran-3(4*H*)-one ring system.

In the present paper, we report on the biotransformation of FA by MCP and the isolation and the structural characterization of the two new FA dehydrotrimers, as well as the possible formation mechanism for triFA1 (**1**).

Results and Discussion

TriFA1 (**1**): Amorphous powder, $[\alpha]_D^{20}$ +3.8° (c=0.12, MeOH), the high-resolution (HR) positive-ion ESI-MS give the quasi-molecular ion peak at m/z 557.1418 $[M+Na]^+$, indicating the molecular formula to be $C_{29}H_{26}O_{10}$, which suggested the structure of FA dehydrotrimer. By ^{1}H-NMR spectroscopy (Table 1), two nonequivalent tri-substituted aromatic fragments (A and B) were revealed. Fragment A was shown by an ABX system, for which the ^{1}H signals at δ 7.01 (dd) was *o*-coupled to that at δ 6.94 (d) and *m*-coupled to that at δ 7.25 (d), while fragment B was indicated by an AMX system, for which the ^{1}H signals at δ 6.96 (dd) was *o*-coupled to that at δ 6.82 (d) and *m*-coupled to that at δ 7.16 (d). One tetra-substituted aromatic fragment C was also revealed by an AX system of doublets at δ 7.26 and 6.72. *trans*-Coupling of signals at δ 7.45 (d, J=15.9 Hz) and 6.29 (d, J=15.9 Hz) identified an *E*-substituted vinyl group. Vicinal protons at δ 5.54 (d, J=7.0 Hz) and 3.98 (dd, J=7.0, 8.0 Hz) and those at δ 5.41 (d, J=8.1 Hz) and 3.61 (dd, J=8.1, 8.0 Hz) identified two sets of nonequivalent CH fragments in the saturated part of the molecule. Three methoxy groups at δ 3.83 and 3.90 (×2) were also characterized. ^{13}C-



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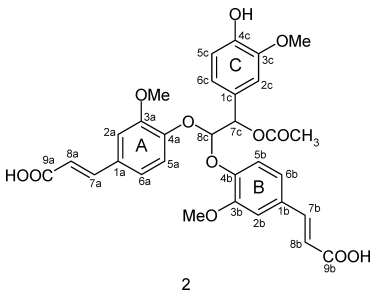

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Table 1. NMR Date of TriFA1 (**1**) (500 MHz, in Acetone-d_6) and TriFA2 (**2**) (500 MHz, in CD_3OD)

Position	triFA1 (**1**)		triFA2 (**2**)	
	^1H	^{13}C	^1H	^{13}C
1a		130.2		131.4
2a	7.25 (1H, d, 1.9)	111.3	7.28 (1H, d, 2.0)	112.5
3a		148.9		152.0
4a		148.3		148.2
5a	6.94 (1H, d, 8.1)	115.8	6.95 (1H, d, 8.4)	120.3
6a	7.01 (1H, dd, 1.9, 8.1)	121.1	7.09 (1H, dd, 2.0, 8.4)	122.5
7a	5.54 (1H, d, 7.0)	87.2	7.56 (1H, d, 16.0)	145.1
8a	3.98 (1H, dd, 7.0, 8.0)	43.7	6.41 (1H, d, 16.0)	118.2
9a	—	—		167.9
1b		130.2		131.3
2b	7.16 (1H, d, 2.0)	120.0	7.33 (1H, d, 2.0)	112.5
3b		148.3		151.9
4b		147.8		148.9
5b	6.82 (1H, d, 8.1)	115.6	7.05 (1H, d, 8.4)	119.9
6b	6.96 (1H, dd, 2.0, 8.1)	121.2	7.13 (1H, dd, 2.0, 8.4)	122.6
7b	5.41 (1H, d, 8.1)	75.1	7.58 (1H, d, 16.0)	145.1
8b	3.61 (1H, dd, 8.1, 8.0)	44.8	6.43 (1H, d, 16.0)	118.1
9b		174.3		167.9
1c		128.6		128.1
2c	7.26 (1H, d, 1.8)	110.1	7.24 (1H, d, 2.0)	113.1
3c		150.4		148.0
4c		147.0		147.8
5c		122.3	6.83 (1H, d, 8.2)	115.3
6c	6.72 (1H, d, 1.8)	123.1	7.04 (1H, dd, 2.0, 8.2)	122.4
7c	7.45 (1H, d, 15.9)	145.3	6.15 (1H, d, 5.4)	75.9
8c	6.29 (1H, d, 15.9)	117.4	6.29 (1H, d, 5.4)	103.3
9c		167.8	—	—
3a-OMe	3.90 (3H, s)	56.3	3.79 (3H, s)	56.4
3b-OMe	3.83 (3H, s)	56.5	3.84 (3H, s)	56.4
3c-OMe	3.90 (3H, s)	56.3	3.87 (3H, s)	56.4
7c-$COCH_3$	—	—	2.06 (3H, s)	169.9, 21.0

Chemical shifts were given in δ values and followed by proton numbers, multiplicities and J values (in Hz). The ^1H and ^{13}C signals were assigned by HSQC and HMBC spectra.

NMR signals (Table 1) at δ 174.3 (9b) confirmed the presence of lactone group. Signals at δ 167.8 (9c), 145.3 (7c) and 117.4 (8c) identified the presence of anacrylic acid side chain. Among 18 aromatic carbons, six were oxygen-substituted, four were carbon-substituted, and the remaining aromatic carbons were methines. Two nonaromatic oxygenated carbons at δ 87.2 (7a) and 75.1 (7b), two methylene carbons at δ 43.7 (8a) and 44.8 (8b) and three methoxy carbons at δ 56.3 (×2) and 56.5 were also detected.

Key heteronuclear multiple-bond connectivity (HMBC) (Fig. 1) led us to further conclude that: The methoxy group with δ_H 3.83, 3.90 (×2) were ascribed to aromatic fragments B, A and C, respectively, because the correlations of δ_H 3.83 to C-3b, δ_H 3.90 to C-3a and δ_H 3.90 to C-3c were shown. The di-substituted double bond with the chemical shift of the vinylic protons δ_H 7.45 (7c) and δ_H 6.29 (8c) was connected to aromatic fragment C, because the correlations of H-7c to C-2c (6c), H-8c to C-1c and H-2c (6c) to C-7c were observed. To one set of CH groups (7a, 8a) of the saturated part: The correlations of H-7a to C-2a (6a), H-2a (6a) to C-7a and H-8a to C-1a exhibited that the aromatic fragment A was connected to the CH group with δ 5.54 (7a) through 1a atom. The correlations of H-7a (8a) to C-9b and the chemical shift of the 7a carbon atom (δ_C 87.2) indicated that the oxygen atom of the lactone group was also connected to the CH group (7a); The correlations of H-8a to C-4c (6c), H-6c to C-8a and H-7a to C-5c clarified that CH group with δ 3.98 (8a)

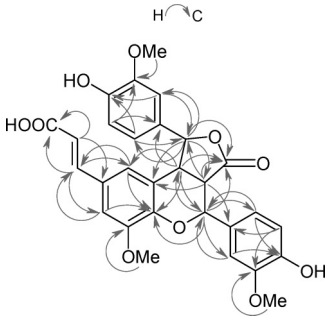

Fig. 1. The Main Correlations in the HMBC Spectrum of TriFA1 (**1**)

was connected to the position 5c of the tetra-substituted aromatic fragment C. To the other set of CH groups (7b, 8b) of the saturated part: The correlations of H-7b to C-2b (6b), H-2b (6b) to C-7b and H-8b to C-1b supported that the aromatic fragment B was connected to the CH group with δ 5.41 (7b) through 1b atom. The correlation of H-7b to C-4c and the chemical shift of the corresponding carbon atom (δ_C 75.1) indicated that the aromatic fragment C was also connected to the CH group (7b) through the phenolic oxygen atom; The correlations of H-8b (7b) to C-9b revealed that CH group with δ 3.61 (8b) was connected to the lactone carbonyl group. The two sets of CH groups of the saturated part were connected each other though CH group (8a) and CH group (8b), because the correlations of H-8b to C-7a, H-8a to

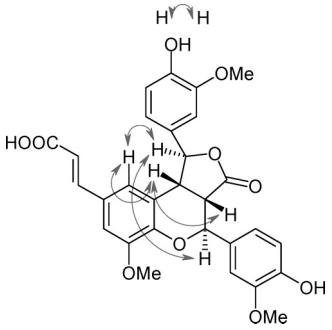

Fig. 2. The Main Correlations in the NOESY Spectrum of TriFA1 (**1**)

C-7b, H-7b to C-8a, H-7a to C-8b, H-8a to C-9b and H-8b to C-5c were detected. According to above analysis, the planar structure of triFA1 with a 3a,9b-dihydro-1*H*-furo[3,4-*c*][1]benzopyran-3(4*H*)-one ring system was concluded.

To confirm the relative stereo-structure of triFA1, nuclear Overhauser and exchange spectroscopy (NOESY) experiment was performed shown in Fig. 2. The NOESY correlations of H-8a to H-8b (6c) and H-7a to H-6c (7b) as well as the absence of the correlations of H-8a to H-7b and H-8b to H-7a suggested that the configurations of H-8a and H-8b should be α while the configurations of H-7a and H-7b should be β. From above results, the structure of triFA1 was assigned to be **1**. This is the first time that triFA1 possessing a 3a,9b-dihydro-1*H*-furo[3,4-*c*][1]benzopyran-3(4*H*)-one ring system has been identified from peroxidase-catalyzed oxidation of FA.

The proposed reaction mechanism for the formation of the dihydrotrimer with triFA1 (**1**) is shown in Fig. 3. It would appear to result from the combination of monomeric FA radicals (b) and (c) to decarboxylated dehydrodimer [8-5] radical couplings, and attack of monomeric FA radical (b) on the vinylic bond of the decaboxylated dehydrodimer leading to two free [8-5, 8-8] and [8-5, 8-7] dihydrotrimeric FA radicals (1-A) and (1-B). (1-A) through intra-molecular rearrangement, two stable [8-5, 8-8 and 7-O-4] dihydrotrimer FA radicals (1-C) and (1-D) are formed. One-electron oxidation of dihydrotrimer FA radical (1-D) leads to the corresponding carbocation (1-E). The latter then involve the cyclohydrogenation, giving triFA1 (**1**).

TriFA2 (**2**): Amorphous powder, $[\alpha]_D^{20}$ 0° (c=0.535, MeOH), the HR positive-ion FAB-MS give the quasi-molecular ion peaks at m/z 595.1832 $[M+H]^+$ and 617.1619 $[M+Na]^+$, indicating the molecular formula to be $C_{31}H_{30}O_{12}$, which suggested the structure of FA dehydrotrimer. By ^{1}H-NMR spectroscopy (Table 1), three different tri-substituted aromatic fragments (A, B, C) were revealed. Fragment A was shown by an AMX system, for which the ^{1}H signals at δ 7.09 (dd), 6.95 (d) and 7.28 (d), fragment B was indicated by an ABX system, for which the ^{1}H signals at δ 7.13 (dd), 7.05 (d) and 7.33 (d), and fragment C was suggested by an AMX system for which the ^{1}H signals at δ 7.04 (dd), 6.83 (d) and 7.24 (d). *trans*-Coupling of signals at δ 7.56 (d, J=16.0 Hz) and 6.41 (d, J=16.0 Hz) for one group and those at δ 7.58 (d, J=16.0 Hz) and 6.43 (d, J=16.0 Hz) for the other group identified two *E*-substituted vinyl groups. Vicinal protons at δ 6.15 (d, J=5.4 Hz) and 6.29 (d, J=5.4 Hz) identified one pair of nonequivalent CH fragments in the saturated part of

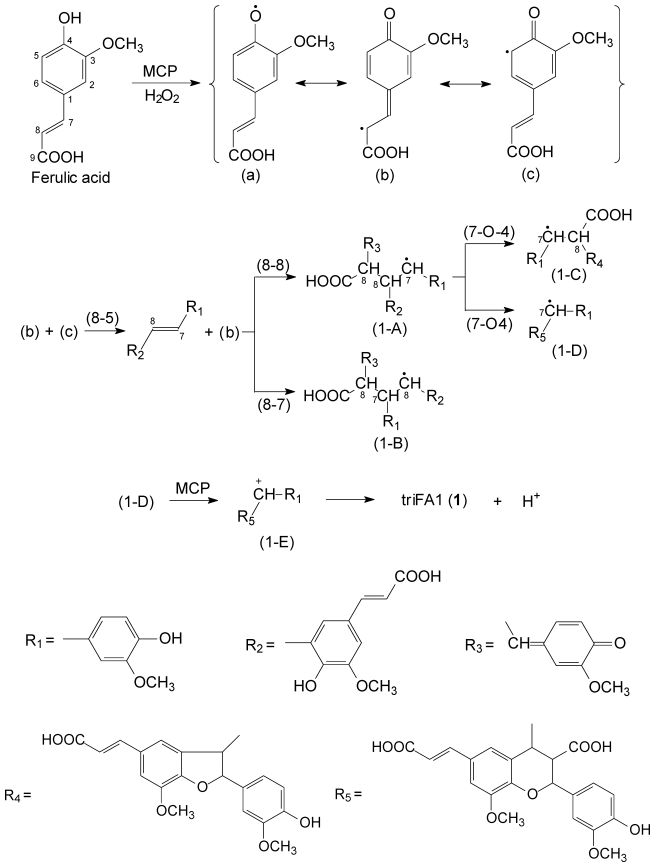

Fig. 3. The Proposed Mechanism for the Formation of TriFA1 (**1**)

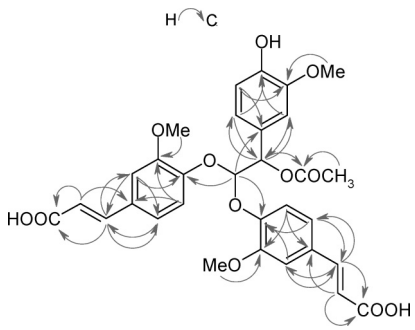

Fig. 4. The Main Correlations in the HMBC Spectrum of TriFA2 (**2**)

the molecule. Three methoxy groups at δ 3.79, 3.84 and 3.87 and one acetoxy group at δ 2.06 were characterized. ^{13}C-NMR signals (Table 1) at δ 169.9 and 21.0 confirmed the presence of one acetoxy group. Signals at δ 167.9 (9a), 145.1 (7a) and 118.2 (8a) for one group, and those at δ 167.9 (9b), 145.1 (7b) and 118.1 (8b) for the other group identified the presence of two anacrylic acid side chains. Among 18 aromatic carbons, six were oxygen-substituted, three were carbon-substituted, and the remaining aromatic carbons were methines. Two saturated carbons at δ 103.3 (8c) and 75.9 (7c) and three methoxy carbons at δ 56.4 (×3) were also detected.

Key HMBC data (Fig. 4) provided further evidence: The methoxy groups with δ_H 3.79, 3.84 and 3.87 were ascribed to aromatic fragments A, B and C, respectively, because the correlations of δ_H 3.79 to C-3a, δ_H 3.84 to C-3b and δ_H 3.87 to C-3c were detected. The two di-substituted double bonds

were connected to aromatic fragment A and B, respectively, because the correlations of H-7a to C-2a (6a), H-8a to C-1a, H-2a (6a) to C-7a, H-7b to C-2b (6b), H-8b to C-1b and H-2b (6b) to C-7b were observed. Aromatic fragment A and B were connected to the CH group with δ 6.29 (8c) through the O-4 oxygen atom, because the chemical shift of the corresponding carbon atom δ_C 103.3 was characteristic of the acetal carbon and the correlations of H-8c to C-4a (4b) also supported the conclusion. The CH group with δ 6.15 (7c) was connected to aromatic fragment C through 1c atom, because the correlations of H-7c to C-2c (6c), H-2c (6c) to C-7c and H-8c to C-1c were shown. The CH group with δ 6.15 (7c) was also connected to the acetoxy group, because the correlation of H-7c to δ_C 169.9 ($-OCOCH_3$) was exhibited and the chemical shift of the corresponding carbon atom (δ_C 75.9) also supported the conclusion. According to above analysis, the planar structure of triFA2 was assigned to be **2**.

In this compound, there was one chiral carbon (7c), which was located in side chain. The specific optical rotation of 0° indicated that triFA2 was a racemate of R/S (7c) stereo-isomers.

Experimental

General Methods Optical rotations were measured with a JASCO P-1020 digital polarimeter (cell length: 1.0 dm). Positive-ion HR-FAB-MS was recorded with a JEOL HX-110 spectrometer using *m*-nitrobenzyl alcohol as a matrix. Positive-ion HR-ESI-MS was recorded with Bruker APEXáV 7.0 TESLA 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 a Bruker ACF-500 MHz spectrometer with tetramethylsilane as an internal standard, and chemical shifts were recorded in δ values. Column chromatography was carried out with Sephadex LH-20 (20—100 μ, Pharmacia) and ODS-C_{18} (100—200 μ, Waters).

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 (No. 000804) was deposited in the Department of Nature Medicinal Chemistry, China Pharmaceutical University.

Purification of MCP MCP from fruits of *Momordica charantia* was purified to electrophoretic homogeneity by combing consecutive treatment of ammonium sulfate fractionation, ion exchange chromatography on DEAE-Sepharose FF, affinity chromatography on Con A sepharose and gel filtration on sephadex G-150. The detailed method of the purification of MCP has been reported previously.[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 FA in Aqueous Acetones A solution of FA (4 g) in acetone (200 ml) and a solution of MCP (3.2×10^4 U) in buffer (100 mM NaAC-HAC, pH 5.0, 800 ml) were mixed and treated with hydrogen peroxidase (3%, 20 ml) at room temperature and stirred for 8 h, during which the mixture was gradually turned reddish brown. The acetone was removed and the aqueous reaction mixture extracted with ethyl acetate. The extract was washed with water, dried with anhydrous Na_2SO_4 and evaporated to dryness at 40 °C under reduced pressure. The combined EtOAc layer was concentrated to afford a strong brown powder (3.5 g), which was subjected to column chromatography on ODS-C_{18} using an increasing of MeOH in H_2O (3 : 7—6 : 4) as an eluent to yield four fractions (frs. I—IV). Fraction III [0.8 g from 500 ml, MeOH in H_2O (5 : 5)] was chromatographed on sephadex LH-20 with methanol as eluent to yield 54 fractions (frs. III_1—III_{54}). Fractions $III_{23—27}$ [0.3 g from 30 ml] was chromatographed on sephadex LH-20 with $CHCl_3$: MeOH (1 : 1) as eluent to yield five fractions (frs. A—E). Fraction B [0.05 g from 10 ml] was chromatographed on sephadex LH-20 with acetone as eluent to give triFA1 (6 mg) and fraction C [0.1 g from 15 ml] was further chromatographed on sephadex LH-20 with acetone as eluent to give triFA2 (23 mg).

TriFA1 (**1**): Amorphous power; $[\alpha]_D^{20}$ +3.8° (c=0.12, MeOH); positive-ion HR-ESI-MS give $[M+Na]^+$ *m/z*: 557.1418 (Calcd for $C_{29}H_{26}O_{10}Na$, 557.1415); UV λ_{max} (MeOH) nm (log ε): 312.0 (1.01), 283.0 (1.67), 221.2 (2.43); IR (KBr) cm^{-1}: 3446, 3425 (hydroxyl), 1772 (lactone carbonyl), 1634 (olefin), 1607, 1520 (phenyl); ^{1}H-NMR (acetone-d_6, 500 Hz) δ: Table 1; ^{13}C-NMR (acetone-d_6, 125 Hz) δ: Table 1.

TriFA2 (**2**): Amorphous power; $[\alpha]_D^{20}$ 0° (c=0.535, MeOH); positive-ion HR-FAB-MS give $[M+H]^+$ *m/z*: 595.1832 (Calcd for $C_{31}H_{31}O_{12}$, 595.1816), $[M+Na]^+$ *m/z*: 617.1619 (Calcd for $C_{31}H_{30}O_{12}Na$, 617.1635); UV λ_{max} (MeOH) nm (log ε): 307.6 (1.57), 305.6 (1.57), 281.2 (2.19); IR (KBr) cm^{-1}: 3414, 3178 (hydroxyl), 1686 (ester carbonyl), 1632 (olefin), 1599, 1510 (phenyl); ^{1}H-NMR (CD_3OD, 500 MHz) δ: Table 1; ^{13}C-NMR (CD_3OD, 125 MHz) δ: Table 1.

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