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 H2O2/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-1H-furo[3,4-c][1]benzopyran-3(4H)-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-inflammatory activity than FA.1) We found that although MCP shared spectral and kinetic features with other peroxidases such as horseradish peroxidase,2) MCP had several unique characteristics, including enzyme pH stability and kinetic features with other peroxidases, the enzyme had several nonequivalent tripeptide have recently been yielded enzymically from FA by MCP and analyze FA dehydrotrimers, we have investigated the oxidative coupling of FA with H2O2/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-1H-furo[3,4-c][1]benzopyran-3(4H)-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, [α]D 10 +3.8° (c=0.12, MeOH), the high-resolution (HR) positive-ion ESI-MS gave the quasi-molecular ion peak at m/z 557.1418 [M+Na]+, indicating the molecular formula to be C29H26O10, which suggested the structure of FA dehydrotrimer. By 1H-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 1H 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 AX system, for which the 1H 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.13C-
NMR signals (Table 1) at $\delta$ 174.3 (9b) confirmed the presence of lactone group. Signals at $\delta$ 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 $\delta$ 87.2 (7a) and 75.1 (7b), two methylene carbons at $\delta$ 43.7 (8a) and 44.8 (8b) and three methoxy carbons at $\delta$ 56.3 (7a) and 56.5 were also detected.

Key heteronuclear multiple-bond connectivity (HMBC) (Fig. 1) led us to further conclude that: The methoxy group with $\delta$H 3.83, 3.90 (3H, s) were ascribed to aromatic fragments B, A and C, respectively, because the correlations of $\delta$H 3.83 to C-3b, $\delta$H 3.90 to C-3a and $\delta$H 3.90 to C-3c were shown. The di-substituted double bond with the chemical shift of the vinylic protons $\delta$H 7.45 (7c) and $\delta$H 6.29 (8c) was connected to aromatic fragment C, because the correlations of $\delta$H 7.45 to C-3b, $\delta$H 6.29 to C-3a and $\delta$H 6.29 to C-3c 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 $\delta$ 3.98 (8a) 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 $\delta$ 5.41 (7b) through 1b atom. The correlation of H-7b to C-4c and the chemical shift of the corresponding carbon atom ($\delta$C 75.1) indicated that the aromatic fragment C was also connected to the CH group through the phenolic oxygen atom; The correlations of H-8b (7b) to C-9b revealed that CH group with $\delta$ 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...
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-1H-furo[3,4-c][1]benzopyran-3(4H)-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-1H-furo[3,4-c][1]benzopyran-3(4H)-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 decarboxylated 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, [α]D 20 arc 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 C31H30O12, which suggested the structure of FA dehydrotrimer. By 1H-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 1H signals at δ 7.09 (dd), 6.95 (d) and 7.28 (d), fragment B was indicated by an ABX system, for which the 1H 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 1H 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 the molecule. Three methoxy groups at δ 3.79, 3.84 and 3.87 and one acetox group at δ 2.06 were characterized.

Key HMBC data (Fig. 4) provided further evidence: The methoxy groups with δH 3.79, 3.84 and 3.87 and one acetox group at δ 2.06 were characterized.

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

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

Fig. 4. The Main Correlations in the HMBC Spectrum of TriFA2 (2)
were connected to aromatic fragment A and B, respectively, 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 connected to the acetoxy group, because the correlation of H-7c to δ 169.9 (–OCOCH3) was exhibited and the chemical shift of the corresponding carbon atom (δc 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 JMS-700 spectrometer using m-nitrobenzyl alcohol as a matrix. Positive-ion HR-ESI-MS was recorded with Bruker APEX IV 7.0 TESLA spectrometer. UV and IR spectra were recorded with Shimadzu UV-1601PC and Nicolet Impact 410 spectrometers, respectively. 1H- and 13C-NMR, HSQC, HMBC and NOESY spectra were taken with a Bruker ACF-300 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 μm, Pharmacia) and ODS-C18 (100—200 μm, Waters).

Plant Material  Fruits of M. charantia were collected at a suburb of Nanjing, China, and identified by Dr. Mingjian Qin, China Pharmaceutical University.

Purification of MCP  MCF 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 filtration on sephadex G-150. The detailed method of the purification of DEAE-Sepharose FF, affinity chromatography on Con A sepharose and gel of ammonium sulfate fractionation, ion exchange chromatography on purified to electrophoretic homogeneity by combing consecutive treatment TESLA spectrometer. UV and IR spectra were recorded with Shimadzu UV-1601PC and Nicolet Impact 410 spectrometers, respectively. 1H- and 13C-NMR, HSQC, HMBC and NOESY spectra were taken with a Bruker ACF-300 MHz spectrometer using tetramethylsilane as an internal standard, and chemical shifts were recorded in δ values. Column chromatography was carried out with Sephadex LH-20 (20—100 μm, Pharmacia) and ODS-C18 (100—200 μm, Waters).

Biotransformation of FA in Aqueous Acetones  A solution of FA (4 g) in acetone (200 ml) and a solution of MCP (3.2 × 10^5 U) in buffer (100 mM NaAC-HAC, pH 5.0, 800 ml) were mixed and treated with hydrogen peroxide (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 Na2SO4 and evaporated to dryness at 40 °C under reduced pressure. The combined EtOAc layer was concentrated to a strong brown powder (3.5 g), which was subjected to column chromatography on ODS-C18 using an increasing of MeOH in H2O (3:7—6:4) as an eluent to yield four fractions (frs. I—IV). Fraction III [0.8 g from 500 ml, MeOH in H2O (5:5)] was chromatographed on sephadex LH-20 with methanol as eluent to yield 54 fractions (frs. III1—III54). Fractions III1—III54 [0.3 g from 30 ml] was chromatographed on sephadex LH-20 with CHCl3: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 (I): Amorphous powder; [α]D^20 +3.8° (c=0.12, MeOH); positive-ion HR-ESI-MS give [M+Na]+ m/z 557.1418 (Calcd for C31H31O12Na, 557.1415); UV 303 (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); 1H-NMR (acetone-d6, 500 Hz) δ: Table 1; 13C-NMR (acetone-d6, 125 Hz) δ: Table 1.

TriFA2 (2): Amorphous powder; [α]D^20 0° (c=0.535, MeOH); positive-ion HR-FAB-MS give [M+H]+ m/z 595.1832 (Calcd for C30H29O12, 595.1816), [M+Na]+ m/z 617.1619 (Calcd for C31H31O12Na, 617.1635); UV 281.2 (2.19); IR (KBr) cm⁻¹: 3414, 3178 (hydroxy), 1686 (ester carbonyl), 1632 (olefin), 1599, 1510 (phenyl); 1H-NMR (CD3OD, 500 MHz) δ: Table 1; 13C-NMR (CD3OD, 125 MHz) δ: Table 1.

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References