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Structural Determination of Oxidation Products of Flavonoids in Alcoholic Aqueous Solution with Reactive Oxygen Species

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Recently, much attention has been paid to the physiological functions of flavonoids associated with their antioxidant properties. However, there was a lack of information on the molecular mechanism at which flavonoids play the antioxidative role. We have already studied on the oxidation of quercetin with hydrogen peroxide and sodium hypochlorite in alcoholic aqueous solution and determined the oxidation products. Through the structural analysis of the oxidation products, it was clarified that the hydroxyl group at C-3 in the C ring plays the important role in the antioxidative action of quercetin. Successively, rutin and (+)-catechin were oxidized with sodium hypochlorite and their mono- and di-chlorinated derivatives were obtained. These facts indicate that these flavonoids can directly scavenge hypochlorous acid and the active site in this scavenging reaction is not the hydroxyl group at C-3.

Key words: flavonoids, antioxidative mechanism, sodium hypochlorite, oxidation product, structural determination, chlorinated derivative

INTRODUCTION

It is well known that reactive oxygen species induce oxidative damage to living cells, which often leads to the occurrence and/or the acceleration of life style-related diseases. Dietary antioxidants, such as vitamins, carotenoids and flavonoids, are therefore regarded as promising food factors for the prevention of life style-related diseases and health promotion [1]. Flavonoids have antioxidant properties and have been reported to prevent the development of free-radical induced diseases, including forms of cancer, coronary heart disease, and gastric mucosal injury [2]. It has been demonstrated that flavonoids play a role as hydrogen-donating free radical scavengers, and their function is strongly dependent on their structural properties, of which there are three key features: 1) the catechol group in the B ring, 2) a 2,3-double bond with the 4-oxo group in the C ring, and 3) a 3-(and 5-)hydroxyl group of the C (and A) ring [3]. Quercetin (**1**), one of the most abundant flavonoids in fruits and vegetables, satisfies all these structural criteria, and shows potent antioxidative activity. In spite of many studies, the precise oxidation mechanism by **1** is still a

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subject of argument. To clarify the chromophore responsible for the antioxidative activity, it is quite important to identify the oxidation products of **1**.

Previous studies demonstrated that both the catechol chromophore in the B ring and the enol moiety in the C ring of **1** are essential for exhibiting the antioxidative activity during lipid peroxidation [4, 5], whereas the enol moiety in the C ring only plays an important role in the antioxidation of **1** in alcoholic aqueous solution [6]. In this study, rutin (**2**) and (+)-catechin (**3**) were oxidized with sodium hypochlorite in alcoholic aqueous solution and the structures of the oxidaiton products were determined based on the spectral analyses and chemical evidence.

MATERIALS AND METHODS

Materials Flavonolids, shown in Fig. 1, were purchased from Sigma Chemical Co. and sodium hypochlorite was from Nakarai Chemicals. All these reagents were used without purification.

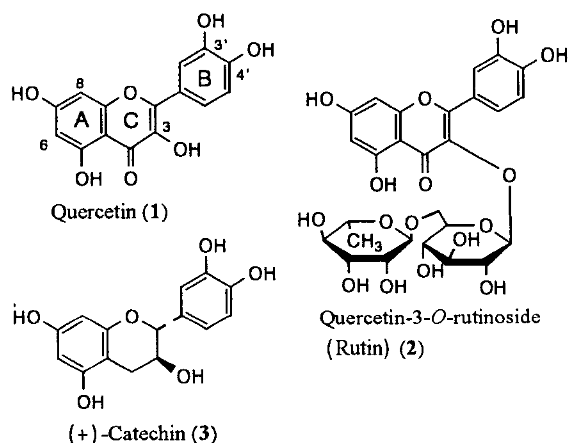

Figure 1. The structures of flavonoids

HPLC Analytical Conditions Reversed-phase HPLC for analysis was carried out with a YMC A-312 (ODS 6× 150 mm) column using solvent mixtures of H_2O/MeCN/AcOH as the mobile phase. The preparative reversed-phase HPLC was performed using a YMC D-ODS-5 (20×250 mm).

Oxidation of Rutin with Sodium Hypochlorite 300 ml of 10 mM sodium hypochlorite aq. soln. was added to 300 ml of 1mM **2** (915 mg) MeOH soln. with stirring. The reactant was immediately injected into the reversed-phase HPLC column, concentrated *in vacuo* and chromatographed by the preparative HPLC with H_2O/MeOH/AcOH (58:42:0.5 v/v/v) as the eluent. Removal of the solvent from the eluate gave two oxidation products, **R-1** (93 mg) and **R-2** (173 mg).

8-Monochlororutin (R-1) UV λ_{max} (MeOH) (nm) 257 (log ε=4.33), 358 (4.25); IR ν_{max} (cm^{-1}) 3400, 2910, 1650, 1600, 1064, 748; ^{1}H NMR (400MHz, MeOH-d_4) δ 7.83 (d, J=2.1 Hz, H2'), 7.78 (dd, J=8.5, 2.1 Hz, H6'), 6.89 (d, J=8.5 Hz, H5'), 6.33 (s, H6), 5.20 (d, J=7.7 Hz, H1"), 4.52 (d, J=1.3 Hz, H1'''), 3.82 (d, J=10.2 Hz, H6"), 3.63 (dd, J=3.4, 1.3 Hz, H2'''), 3.55~3.25 (8H), 1.12 (3H, J=6.2 Hz, H6'''); ^{13}C NMR (100 MHz, MeOH-d_4) δ 179.2 (C4), 162.0 (C7), 160.8 (C5), 159.0 (C2), 153.4 (C9), 150.2 (C4'), 145.9 (C3'), 135.8 (C3), 123.9 (C6'), 122.9 (C1'), 117.8 (C2'), 116.1 (C5'), 106.0 (C10), 104.6 (C1"), 102.4 (C1'''), 100.9 (C6), 99.5 (C8), 78.2 (C3"), 77.3 (C5"), 75.8 (C2"), 73.9 (C4'''), 72.3 (C3'''), 72.1 (C2'''), 71.4 (C4"), 69.7 (C5'''), 68.5 (C6"), 17.9 (C6''').

6,8-Dichlororutin (R-2) UV λ_{max} (MeOH) (nm) 283 (log ε=4.21), 382 (4.39); IR ν_{max} (cm^{-1}) 3415, 2915, 1645, 1590, 1064, 794, 749; ^{1}H NMR (400MHz, MeOH-d_4) δ 7.81 (d, J=1.7 Hz, H2'), 7.77 (dd, J=8.4, 1.7 Hz, H6'), 6.89 (d, J=8.4 Hz, H5'), 5.25 (d, J=7.6 Hz, H1"), 4.51 (s, H1'''), 3.81 (d, J=10.4 Hz, H6"), 3.60 (br, s, H2'''), 3.54~3.23 (8H), 1.10 (3H, J=6.2 Hz, H6'''); ^{13}C NMR (100 MHz, MeOH-d_4) δ 178.9 (C4), 159.5 (C2), 157.5 (C7), 156.4 (C5), 151.4 (C9), 150.4 (C4'), 146.0 (C3'), 135.7 (C3), 124.0 (C6'), 122.8 (C1'), 117.8 (C2'), 116.2 (C5'), 105.9 (C10), 105.6 (C6), 104.2 (C1"), 102.4 (C1'''), 100.5 (C8), 78.1 (C3"), 77.3 (C5"), 75.8 (C2"), 73.9 (C4'''), 72.3 (C3'''), 72.1 (C2'''), 71.5 (C4"), 69.7 (C5'''), 68.6 (C6"), 17.9 (C6''').

RESULTS AND DISCCUSSION

Product **R-1** and **R-2** were obtained in the form of a yellow amorphous powder. Product **R-1** has the molecular formula $C_{27}H_{29}O_{16}Cl$ based on the positive FAB-MS (*m/z*; 645[MH]$^+$) and HR-FAB-MS (*m/z*; 645.1230 [MH]$^+$, Calcd. for $C_{27}H_{30}O_{16}Cl$; 645.1456). The UV spectrum measured in MeOH showed a little shift from that of **2**. In the ^{1}H NMR spectrrum of **R-1**, the signals could be separated into four aromatic protons and fifteen sugar protons. The ^{13}C NMR and DEPT spectra showed 27 carbons including one methyl, one methylene, 14 methin and 11 quarternary carbon signals. All signals in the ^{1}H and ^{13}C NMR spectra were assigned by the HMQC and HMBC experiments. The proton signals for **R-1** were closely related to those of **2**, except a lack of one proton signal in the A ring of quercetin moiety. The carbon signals for C-5, 7 and 9 were shifted 2.2-5.1 ppm highfield against those of **2**, whereas that for C-8, which was converted into quaternary carbon from methin carbon, were shifted 4.6 ppm downfield. These facts could be deduced that an electron withdrawing group binds at the C-8 in the A ring. As a result, the structure of **R-1** was determined to be 8-monochlororutin, as depicted in Fig. 2.

Product **R-2** has the molecular formula $C_{27}H_{28}O_{16}Cl_2$ based on the positive FAB-MS (*m/z*; 679[MH]$^+$) and HR-FAB-MS (*m/z*; 679.0822 [MH]$^+$, Calcd. for $C_{27}H_{29}O_{16}Cl_2$; 679.1299). Acetylation of **R-2** with acetic anhydride/pyridine afforded deca-*O*-acetyl derivative. The NMR spectral data for **R-2** were closely related to those of **R-1**, except the disappearance of two aromatic protons in the A ring and the downfield shift of the carbon signal assigned to C-6 position. Based on these spectral evidences, **R-2** was identified to be 6,8-dichlororutin as shown in Fig. 2.

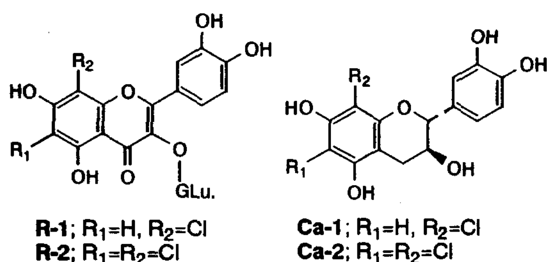

Figure 2. Products with Hypochlorous Acid

Similarly, (+)-catechin (**3**) was oxidized with sodium hypochlorite in alcoholic aqueous solution to give two chlorinated products, 8-monochloro-(+)-catechin (**Ca-1**) and 6,8-dichloro-(+)-catechin (**Ca-2**).

These oxidation products, mono- and di- chlorinated derivates of **2** and **3**, suggest that the oxidation reaction is an electrophilic aromatic substitution by chlorination. Therefore, their antioxidant mechanism in alcoholic aqueous solution is not similar to that of **1**. Further studies are required for the elucidation on the antioxidative mechanism of flavonoids *in vivo* which are already in progress.

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