Studies on the Constituents of the Leaves of Morus alba L.

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Two novel prenylflavanes (1, 2) and a glycoside (3) of 1 were isolated along with six known compounds, isoorientin (4), astragalin (5), scopolin (6), skimmian (7), roseoside II (8) and benzyl D-glucopyranoside (9), from the leaves of Morus alba. The inhibitory activities of compounds 1, 2 and 3 on the oxidation of human low density lipoprotein (LDL) were investigated.

Key words Morus alba; Moraceae; prenylflavane; antioxidative activity

The root bark of Morus alba L. has been used as a blood pressure depressant in China and Japan from old times. The constituents of the root bark were well investigated by T. Nomura and H. Hikino's groups and many flavones and their derivatives were isolated as active principles. The leaves of this plant have also been used as a blood pressure depressant, but the constituents of the leaves have not yet been thoroughly investigated. We have investigated the pharmacological activities of the leaves of this plant and found that the butanol extracts of the leaves inhibit the increase of serum cholesterol and prevent atherosclerosis.

Results and Discussion

Two novel prenylflavanes (1, 2) and a glycoside (3) of 1 were isolated along with six known compounds, isoorientin (4), astragalin (5), scopolin (6), skimmian (7), roseoside II (8) and benzyl D-glucopyranoside (9), from the leaves of M. alba.

Compound 1 was obtained as an amorphous powder, \([\alpha]_D^{15} = -5.5^\circ\) \((c = 1.0, \text{MeOH})\). Its high resolution EI-MS spectrum showed the \([M+H]^+\) ion peak at \(m/z\) 340.1684 corresponding to the molecular formula \(C_{21}H_{25}O_4\) \((340.1675)\). The \(^1H–1H\) shift correlation spectroscopy (COSY) spectrum of 1 suggested the presence of a prenyl group \([\delta 1.67, 1.86(3\text{H each, br s, H-4}', H-5')\], \(\delta 5.80\) \((1\text{H, br t, } J = 7.3\text{ Hz, H-2')}\), \(\delta 3.85, 3.91\) \((1\text{H each, d, } J = 7.3, 13.7\text{ Hz, H-1''}))\), a methoxyl group \([\delta 3.69(3\text{H, s})]\), and a sequence of C-2—C-4 \([\delta 2.71, 2.94(1\text{H each, d, } J = 2.71, 2.94\text{ Hz, H-2})\), a 1,2,3,4-tetrasubstituted benzene ring \([\delta 6.84, 6.91(1\text{H each, ABq, } J = 8.2\text{ Hz, H-6, H-5})\] and a 1,2,4-trisubstituted benzene ring \([\delta 6.66(1\text{H, d, } J = 2.4, 8.5\text{ Hz, H-5'\text{, H-3'}}), \delta 6.85(1\text{H, d, } J = 2.4\text{ Hz, H-3'}), \delta 7.70(1\text{H, d, } J = 8.5\text{ Hz, H-6'})\]).

The substitution pattern on the benzene rings was investigated by the analysis of heteronuclear multiple bond correlation spectroscopy (HMBC) spectrum of 1. As shown in Fig. 2, the methylene proton signal at \(\delta 3.85\) and 3.91 \((H-1')\) exhibited long range correlations with the carbon signal at \(\delta 116.5\) \((C-8)\) and two carbon signals \([\delta 155.4(\text{C-7}), \delta 154.7(\text{C-8a})]\) due to oxygen-bearing aromatic carbons, suggesting the presence of a prenyl group at C-8.

The proton signals \([\delta 2.71, 2.94(1\text{H each})]\) due to H-4 showed cross peaks with the carbon signals at \(\delta 127.3\) \((C-5), 113.4\) \((C-4a), \) and 154.7 \((C-8a)\). In addition, the H-2 signal at \(\delta 5.80\) showed long range correlations with the carbon signals due to C-1’ \((\delta 122.6), C-2’ (\delta 156.3), \) and C-6’ \((\delta 127.9)\) in the trisubstituted benzene ring. Finally, the location of the methoxyl group at C-4’ was confirmed by the nuclear Overhauser effect (NOE) experiments on 1. Irradiation at \(\delta 3.69\) due to the methoxyl protons produced significant enhancement of proton signals at \(\delta 6.85\) \((H-3')\) and \(\delta 6.66\) \((H-5')\) (Fig. 2). The results of the NOE experiments and the spectral data mentioned above were satisfactorily explained by the structure (1) shown in Fig. 2.

Compound 2 was obtained as an amorphous powder, \([\alpha]_D^{15} = -1.6^\circ\) \((c = 1.0, \text{MeOH})\). Its CI-MS spectrum showed the \([M+H]^+\) ion peak at \(m/z\) 341 corresponding to the molecular formula \(C_{21}H_{25}O_4\). Comparison of the \(^1H–13C\)-NMR spectra of 2 with those of 1 suggested that compound 2 is an isomer having the same framework as 1 and the structural difference should be the location of a methoxyl group. In the HMBC spectrum of 2, the methoxyl proton signal at \(\delta 3.74\) showed a cross peak with the carbon signal at \(\delta 157.0\) \((C-7)\) which exhibited long range couplings with the proton signals at \(\delta 3.63—3.74\) \((H-1'\text{})\) assignable to the methylene protons of a prenyl group. Furthermore, in the difference NOE experiments, irradiation of the methoxyl proton signal produced significant enhancement of the proton signal at \(\delta 6.57\) due to

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H-6. Thus, the structure of 2 was confirmed as shown in Fig. 1.

Compound 3 was obtained as an amorphous powder, [α]D
−91.1° (c=0.25, MeOH). Its FAB-MS spectrum showed the
[M+Na]+ ion peak at m/z 687 corresponding to the molecular
formula C33H44O14Na. Detailed analyses of the 1H- and
13C-NMR spectra of 3 suggested that 3 must be a glycoside of
1 (see Tables 1 and 2). Enzymatic hydrolysis of 3 with β-
glucosidase gave D-glucose and D-glucosidase gave D-glucose and
D-glucosidase gave D-glucose and D-glucosidase gave D-glucose.

Furthermore, long-range correlations were observed between
the signals at δ 5.59 (H-1 of 7-O-Glc) and δ 155.7 (C-7) in
the HMBC spectrum of 3. Thus, the structure of 3 was
confirmed as the 2',7-di-O-glucoside of 1.

Antioxidative Activities of New Compounds We have
reported that the butanol extracts of mulberry leaves and its
major component, isoquercitrin, exhibited inhibitory activity
on CuSO4-induced oxidation of low density lipoprotein
(LDL) and scavenging activity on 1,1-diphenyl-2-picrylhydrazyl
(DPPH) radical. We examined the antioxidative effects and radical scavenging activities of compounds 1, 2 and
3 on human LDL and DPPH radical. As shown in Table 3, 1
showed stronger antioxidative activity than quercetin used as
a positive control, while 2 exhibited almost the same activity
as that of quercetin. On the other hand, both 1 and 2 exhibited
very weak radical scavenging activity (Table 4). As expected, glycoside 3 showed hardly any antioxidative activi-

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Table 1. 1H-NMR (500 MHz, δ, CD3OD) Data for 1, 2, and 3

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<tr>
<th>Position</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>2</td>
<td>5.80 dd (2.1, 9.5)</td>
<td>5.84 dd (2.4, 9.5)</td>
<td>5.79 dd (2.2, 9.8)</td>
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<tr>
<td>3</td>
<td>2.08 m</td>
<td>2.14 m</td>
<td>1.89 m</td>
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<td>4</td>
<td>2.71 m</td>
<td>2.82 m</td>
<td>2.51 m</td>
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<tr>
<td>5</td>
<td>6.91 (8.2)</td>
<td>6.96 d (8.5)</td>
<td>6.82 d (8.2)</td>
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<td>6</td>
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<td>6.57 d (8.5)</td>
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<td>3'</td>
<td>6.85 d (2.4)</td>
<td>6.97 d (2.4)</td>
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<td>5'</td>
<td>6.66 dd (2.4, 8.5)</td>
<td>6.88 dd (2.4, 8.2)</td>
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<td>6'</td>
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<td>7.67 d (8.5)</td>
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<tr>
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<td>3.63—3.74 m</td>
<td>3.70 dd (7.3, 13.4)</td>
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<tr>
<td>2'</td>
<td>3.91 dd (7.3, 13.7)</td>
<td>3.91 dd (7.3, 13.7)</td>
<td>3.95 dd (7.3, 13.4)</td>
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<tr>
<td>4'</td>
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<td>1.63 br s</td>
<td>1.64 br s</td>
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<td>5'</td>
<td>1.86 br s</td>
<td>1.80 br s</td>
<td>1.78 br s</td>
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<td>OCH3</td>
<td>3.69 s</td>
<td>3.74 s</td>
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</table>

7-O-Glc

| G1c-1 | 5.59 d (7.3) |
| Glc-2 | 4.32—4.36 |
| Glc-3 | 4.39 dd (5.2, 11.9) |
| Glc-4 | 4.57 dd (2.7, 11.9) |

2'-O-Glc

| G1c-1 | 5.54 d (7.3) |
| Glc-2 | 4.27—4.32 |
| Glc-3 | 4.36 dd (5.2, 11.6) |
| Glc-4 | 4.55 dd (2.5, 11.6) |
| Glc-5 | 4.09 m |
| Glc-6 | 4.36 dd (5.2, 11.6) |

<table>
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<tr>
<th>Sample</th>
<th>Concentration</th>
<th>n</th>
<th>Relative lag time</th>
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<td>Quercetin</td>
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<tr>
<td>1</td>
<td>0.5 nmol/ml</td>
<td>4</td>
<td>1.70±0.25</td>
</tr>
<tr>
<td>2</td>
<td>0.5 nmol/ml</td>
<td>4</td>
<td>0.81±0.10</td>
</tr>
<tr>
<td>3</td>
<td>10.0 nmol/ml</td>
<td>3</td>
<td>0.85±0.07</td>
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</table>

Values are means±S.D. a Values are relative to the lag time of 0.5 nmol/ml quercetin assigned an arbitrary value of 1.0.

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Fig. 2. Selected HMBC (—) and NOE (—) on 1
ties or radical scavenging effect.

Experimental

The leaves of Morus alba L. were collected in Kanagawa prefecture and dried at 60—80 °C for 95 min with a tea manufacturing machine. The H and 13C-NMR spectra were measured on a JEOL JNM-Lambda-500 spectrometer in CDCl3 containing TMS as internal standard. The MS spectra were recorded on a Hitachi RMU-6M instrument. Optical rotation was measured on a JASCO DIP-370 polarimeter.

Isolation of Compounds 1—9 The dried leaves (2 kg) were extracted with hexane (2 l) and then 1-butanol (1 l). The 1-butanol solution was washed with water (500 ml x 3) and then concentrated at 40 °C under reduced pressure to give an oily material (80 g) which was chromatographed on a Diaion HP-20 column eluted successively with 1.5 l each of H2O, 20% MeOH, 30% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, MeOH and then acetone to afford eight fractions [fr. 1 (1.83 g), fr. 2 (1.75 g), fr. 3 (1.03 g), fr. 4 (1.90 g), fr. 5 (1.65 g), fr. 6 (0.74 g), fr. 7 (3.0 g), fr. 8 (1.82 g)]. The fr. 2 was separated into two fractions [fr. 2-1 (1.57 g), fr. 2-2 (0.17 g)] by Sephadex LH 20 column chromatography [H2O–MeOH, gradient with increasing of MeOH]. The fr. 2-1 was further separated by reversed phase HPLC [Inertsil prep ODS (GL Science Ltd.), 20 x 250 mm (column 1), H2O–MeOH (49 : 51), flow rate; 5.0 ml/min] to afford scopolin (1, 6 mg, retention time, 10 s on a vortex mixer, the solution was allowed to stand for 30 min, and then 1-butanol (1 l) was added. The mixture was extracted with ethyl acetate and then the organic layer and the water layer were concentrated in vacuo, respectively. From the ethyl acetate extract, compound 1 was identified by direct comparison with an authentic sample on TLC. Rf/0.60 (n-hexane : AcOEt 1:1). From the water layer residue, 1-glucose was identified by TLC direct comparison with an authentic sample. Rf 0.43 (n-BuOH : Me2CO : H2O = 4 : 5 : 1). Acid hydrolysis of 3 with 1 N HCl (110 °C, 1 h) gave 1-glucose, but compound 1 was not detected from the reaction mixture because of degradation of the aglycon during hydrolysis.

Assay for Antioxidative and Radical Scavenging Activities Materials Quercetin dehydrate was purchased from Extrasynthese (France). Disodium ethylenediaminetetraacetate (EDTA-2Na), butylated hydroxytoluene, copper sulfate, sodium azide and disopropyl fluorophosphate (DFP) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Isolation of Human LDL(11) Plasma was obtained after low-speed centrifugation of fresh blood collected in the presence of EDTA-2Na (0.2 %) and stored at −20 °C before use. Human LDL was isolated from plasma by preparative ultracentrifugation using Beckman model L-70 in a Beckman type 70.1 Ti rotor. The protein content in the LDL was measured by using a BCA protein kit (Pierce, IL, U.S.A.) and LDL concentration is expressed as protein content. The LDL was stored at 4 °C for later use. Human LDL was dialyzed three times in 24 h at 4 °C by a 100 fold volume of 10 mM deoxy-glycerol phosphate buffer containing 0.16 mM sodium chloride (PBS, pH 7.4) to remove EDTA-2Na.

Continuous Monitoring of Conjugated Diene Formation from LDL Dibenzylified LDL was diluted to 2.05 mg/ml with 10 mM PBS. Conjugated diene formation during oxidation of LDL was continuously monitored by the spectrophotometric method,(12) which is based on measurement of change in absorbance at 234 nm. The concentrations of LDL and copper were 0.05 mg/ml and 1.66 μM, respectively. Oxidation was initiated by addition of freshly prepared aqueous CuSO4 solution at 37 °C (final concentration: 1.66 μM) in the absence or presence of sample and continuously monitored by the absorbance at 234 nm using a Hitachi spectrophotometer (Tokyo, Japan).

Colorimetric Determination of the Scavenging Effect of Substances on DPPH Radical The scavenging effect of each substance on DPPH radical was measured by monitoring the decrease in absorbance at 517 nm.(13) The ethanolic solution of DPPH (final concentration of DPPH: 40 μM) in 0.1 N HCl was mixed with each sample solutions. After mixing for 10 s on a vortex mixer, the solution was allowed to stand for 30 min and the absorbance of the resulting solution at 517 nm was measured. The scavenging activity on DPPH radical was expressed as EC50.

References