\[ \beta \text{-GALACTOSIDASE-INHIBITING NEW ISOFLAVONOIDS PRODUCED BY ACTINOMYCETES} \]

TADAHIKO HAZATO*, HIROSHI NAGANAWA, MICHIIHIKO KUMAGAI
TAKAAKI AOYAGI and HAMAO UMEZAWA

Institute of Microbial Chemistry, Kamiosaki 3–14–23, Shinagawa-ku, Tokyo, Japan
*The Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo, Japan

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Six isoflavonoids having \( \beta \)-galactosidase inhibiting activity were isolated from the culture filtrate of *Streptomyces xanthophaeus*. Their structures were determined by spectral analyses to be daidzein, daidzein 7-\( \alpha \)-L-rhamnoside, daidzein 4',7-di-\( \alpha \)-L-rhamnoside, genistein, genistein 7-\( \alpha \)-L-rhamnoside and genistein 4',7-di-\( \alpha \)-L-rhamnoside.

As reported previously, we have isolated isoflavonoids from the culture filtrate of *Streptomyces xanthophaeus*\(^1\) as active agents inhibiting \( \beta \)-galactosidase. In this paper, we report isolation, structure determination and inhibitory activities of these isoflavonoids.

The strain classified as *S. xanthophaeus* MD865-C3 was cultured as reported in a previous paper\(^1\). The purification procedures are shown in Fig. 1. Six inhibitors, II, III-1, III-2, III-3, IV-1 and IV-2 were isolated and their molecular formulae were determined by elemental analysis and the result of mass spectrometry. Their formulae, optical rotation values and color reactions on silica gel plates are listed in Table 1. The UV absorption maxima of these inhibitors in methanol under various conditions are presented in Table 2. These inhibitors were structurally related. Each of them had an isoflavone chromophore characterized by UV spectra and PMR spectra which showed characteristic signal of C-2 proton of isoflavone compounds as a singlet at \( \delta \) 8.05–8.45\(^2\),\(^3\),\(^7\).

Compounds III-1 and IV-1 were identified respectively as genistein and daidzein by comparison with authentic samples. In the PMR spectra of the other four compounds, doublets at \( \delta \) 1.14–1.20,
Table 1. The physicochemical data of isoflavonoids

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>[α]_D^20</th>
<th>Color reaction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>C_{27}H_{30}O_{12}</td>
<td>-175</td>
<td>+ + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-1</td>
<td>C_{15}H_{10}O_{5}</td>
<td>-146</td>
<td>+ + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-2</td>
<td>C_{21}H_{20}O_{8}</td>
<td>-169</td>
<td>+ + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-3</td>
<td>C_{21}H_{20}O_{8}</td>
<td>-130</td>
<td>+ + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV-1</td>
<td>C_{15}H_{10}O_{4}</td>
<td>-130</td>
<td>+ + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV-2</td>
<td>C_{21}H_{20}O_{9}</td>
<td>-130</td>
<td>+ + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Reagents:
- a) Anisaldehyde-sulfuric acid (green)
- b) Diphenylamine-aniline (yellow)
- c) 2,3-Dinitrophenylhydrazine (blue)
- d) 2,6-Dibromoquinonechloroimide (violet)

The PMR spectrum of III-2 indicated that it consisted of one molecule of daidzein (δ 8.35 1H, singlet, isoflavone C-2 proton) and one molecule of L-rhamnose (δ 5.62 1H, doublet J = 1.8 Hz, anomeric proton). The UV spectrum of III-2 at 262 nm showed a bathochromic shift in alkaline solution and the absorption maximum did not change following the addition of sodium acetate (Table 2). These results indicated that daidzein was linked to L-rhamnose at C-7<sup>6,7</sup>.) The high resolution mass spectrum of III-2 showed a molecular ion peak of m/e 400 (C_{21}H_{20}O_{8}), and fragments ions of m/e 283 corresponding to C_{16}H_{11}O_{5}, m/e 254 to C_{15}H_{10}O_{4}, m/e 137 to C_{7}H_{5}O_{3} and m/e 118 to C_{8}H_{6}O, respectively. The peak of m/e 283 suggested that rhamnose attached to 7-position of aglycone. The mass spectrum of tetraacetate of III-2 showed the peaks at m/e 568 (M<sup>+</sup>) and 296 (m/e 254 + 42). The peak at m/e 296 suggested the presence of acetyl group at 4' position. Thus, the structure of III-2 was determined as daidzein 7-L-rhamnoside.
Methanolysis of II gave daidzein and methyl L-rhamnoside. The PMR spectrum of II showed the presence of two anomic protons at $\delta$ 5.43 and 5.63. The mass spectrum of hexaacetyl derivative of II indicated the molecular ion at $m/e$ 798. The CMR spectrum of II in hexadeuterodimethylsulfoxide showed the presence of twelve carbon signals at $\delta$ 98.4, 98.5, 69.5, 69.9, 70.0, 70.2, 70.3, 70.4, 71.6, 71.8 and 17.9* which were attributed to the rhamnose moieties. The eight signals between $\delta$ 69.5 and 71.8 indicate no glycosidic linkage in the rhamnoses other than anomic carbons. The UV absorption maximum was not shifted by the addition of sodium acetate or dilute sodium hydroxide (Table 2). Therefore, both C-4' and C-7 hydroxyl groups of the aglycone should be substituted by the two rhamnoses.

Methanolysis of IV-2 or III-3 gave genistein (III-1) and methyl L-rhamnoside. From the ratio of intensities of the anomic proton and the C-2 proton of the isoflavone moiety, it was suggested that IV-2 consisted of one molecule of genistein and one molecule of L-rhamnose and III-3 contained one molecule of genistein and two molecules of L-rhamnose. The bathochromic shifts were observed in the UV spectra of IV-2 and III-3 after addition of aluminum chloride. However, the UV maxima did not change by the addition of sodium acetate. These results indicated that the C-7 hydroxyl group of genistein was substituted in both compounds. The UV maximum of IV-2 was shifted 12 nm by the addition of dilute sodium hydroxide but UV maximum of III-3 was not. The CMR spectrum of III-3 showed eight carbon signals at $\delta$ 69.5~71.8, and indicated that no glycosidic shift was present in the rhamnose moiety. Since the hydroxyl proton at C-5 of the genistein moiety of IV-2 and III-3 appeared at $\delta$ 12.95 and $\delta$ 12.80 in their PMR spectra,[2,7] hydrogen bondings were formed between the hydroxyl group on C-5 and the C-4 carbonyl group in these compounds. Thus, the structures of IV-2 and III-3 were determined to be genistein 7-L-rhamnoside and genistein 4',7-di-L-rhamnoside, respectively.

In the CMR spectra of IV-2 and III-3, the coupling constants between $^{13}$C-1 carbon and C-1 proton of the rhamnose were observed to be 171 Hz by application of gated decoupling method. This result indicated that both of the rhamnosides had $\alpha$ configurations[5]. Since all of these isoflavone rhamnosides were levorotatory, it was concluded that all of the L-rhamnosides described in this paper had $\alpha$ configurations. The structures of $\beta$-galactosidase inhibitors obtained from S. xanthophaeus are shown as follows:

The IC$_{50}$ (concentration required for 50% inhibition) and the Ki values of the six inhibitors are listed in Table 3. The Km of p-nitrophenyl $\beta$-d-galactopyranoside was about $3.7 \times 10^{-3}$ M.

* This signal contained two methyl carbons.
Table 3. Inhibition of $\beta$-galactosidase by isoflavonoids

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$IC_{50}$ ($\mu$g/ml)</th>
<th>$Ki$ ($\times 10^{-6}$ M)</th>
<th>Inhibition type</th>
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<tbody>
<tr>
<td>II</td>
<td>2.4</td>
<td>2.3</td>
<td>competitive</td>
</tr>
<tr>
<td>III-1</td>
<td>48</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III-2</td>
<td>3.6</td>
<td>7.1</td>
<td>competitive</td>
</tr>
<tr>
<td>III-3</td>
<td>1.2</td>
<td>1.3</td>
<td>competitive</td>
</tr>
<tr>
<td>IV-1</td>
<td>170</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IV-2</td>
<td>1.2</td>
<td>3.1</td>
<td>competitive</td>
</tr>
</tbody>
</table>

$: = $No determination

Experimental

Instruments
Melting points were determined by using Yanagimoto micro melting point apparatus and were not corrected. Optical rotation values were determined by using a Carl Zeiss 0.005 photoelectric polarimeter; IR spectra were taken by a Hitachi Model EPI-S2 infrared spectrometer; UV spectra were measured by a Hitachi Model EPS-3T spectrometer. PMR spectra were taken by a Varian XL-100 spectrometer. Abbreviations: s=singlet, d=doublet, t=triplet, dd=doublet of doublets, q=quartet, m=multiplet, dq=doublet of quartets. Gas chromatography was carried out by a Hewlett-Packard 402 high efficiency gas chromatograph with a hydrogen flame ionization detector. Mass spectra were taken by a Hitachi RMU-6M and RMU-7M mass spectrometer.

$\beta$-Galactosidase from bovine liver was purchased from Sigma Chemical Co. (St. Louis, U.S.A.), $p$-nitrophenyl $\beta$-d-galactopyranoside from BDH Chemical Ltd. (Pool, England).

Enzyme assay
The amount of hydrolysis of 5 mM $p$-nitrophenyl $\beta$-d-galactopyranoside in 0.05 M phosphate buffer pH 7.0, at 37°C was measured by the procedure reported previously. $Ki$ and $Km$ values were determined graphically by the method of LINEWEAVER and BURK.

Fermentation
Typical fermentation procedure was as follows: S. xanthophaeus was cultured in a medium containing lactose 2.0%, soybean meal 1.5%, NaCl 0.3%, $MgSO_4\cdot7H_2O$ 0.1%, $K_2HPO_4$ 0.1%, CuSO$_4\cdot5H_2O$ 0.00007%, FeSO$_4\cdot7H_2O$ 0.0001%, MnCl$_2\cdot4H_2O$ 0.0008%, ZnSO$_4\cdot7H_2O$ 0.0002% (pH 7.2). The fermentation was performed for 72 hours on a reciprocating shaking machine at 27°C.

Isolation procedure
A culture filtrate (28.5 liters, $IC_{50}=28 \mu$g/ml) was placed on an Amberlite XAD-2 column (10 x 28 cm). After washing the column with water, the active materials were eluted with methanol. The active eluate was evaporated under reduced pressure to a syrup 5.72 g, $IC_{50}=6 \mu$g/ml. This syrup was dissolved with methanol and chromatographed on a Sephadex LH-20 column (4 x 120 cm) and the column was developed with the same solvent. Four active peaks were obtained from Sephadex LH-20 chromatography; fraction I 280 mg, fraction II 227 mg, fraction III 653 mg, and fraction IV 290 mg. Fraction I was a minor component which has not yet been characterized. Fraction II was purified by florisil column chromatography (60 ml, 1.5 x 35 cm) with ethyl acetate - methanol (10:1) as a developing solvent to give compound II (15 mg). Three inhibitors, compound III-1 (28 mg), compound III-2 (300 mg), compound III-3 (108 mg) were obtained from silica gel column chromatography of fraction III. Fraction IV contained two active materials and they were purified by silica gel column chromatography (60 ml, 1.5 x 35 cm) using chloroform - methanol (10:1) as developing solvent. By this procedure compound IV-1 (25 mg) and compound IV-2 (153 mg) were obtained. The properties of these six inhibitors were as follows:


II: mp > 300°C; [z]_D^0 -175° (c 1, CH_3OH); Anal. Calcd. for C_{27}H_{30}O_{12}: C 59.35, H 5.51, O 35.13. Found: C 60.01, H 5.47, O 35.18. Mass spectrum (m/e): 254, 137, 118; PMR (d_6-DMF): δ 8.45 (1H, s, H-2), 7.55 (2H, H-2',6'), 7.11 (2H, H-3',5'), 8.09 (1H, d, J = 8.5 Hz, H-5), 7.18 (1H, dd, J = 1.8, 8.5 Hz, H-6), 7.28 (1H, d, J = 1.8 Hz, H-8), 5.63 and 5.43 (d, J = 1.8 Hz, rhamnose H-1X2), 5.0 ~ 3.30 (sugar protons), 1.15 (6H, J = 6 Hz, rhamnose C_5-CH_3).

III-2: mp 146°C; [z]_D^0 -146° (c 1, CH_3OH). Anal. Calcd. for C_{21}H_{20}O_8: C 62.91, H 5.04, O 31.97. Found: C 63.04, H 5.07, O 31.80. Mass spectrum (m/e): 400 (M+), 284, 283, 254, 137, 118; PMR (d_6-DMSO): δ 9.55 (1H, s, OH-4'), 8.35 (1H, s, H-2), 8.07 (1H, d, J = 8.5 Hz, H-5), 7.42 (2H, H-2',6'), 6.84 (2H, H-3',5'), 7.40 ~ 7.10 (2H, H-8, H-6), 6.50 (1H, d, J = 1.8 Hz, rhamnose H-1), 5.15 ~ 3.2 (sugar protons), 1.14 (3H, d, J = 6 Hz, rhamnose C_5-CH_3).

III-3: mp 232 ~ 257°C; [z]_D^0 -169° (c 1, CH_3OH). Anal. Calcd. for C_{27}H_{30}O_{13}: C 57.65, H 5.37, O 36.97. Found: C 57.81, H 5.25, O 36.68. Mass spectrum (m/e): 416, 270, 153, 118; PMR (d_6-DMSO): δ 12.80 (1H, s, OH-5), 8.05 (1H, s, H-2), 7.50 (2H, H-2',6'), 7.10 (2H, H-3',5'), 6.62 (1H, d, J = 2 Hz, H-8 or 6), 6.50 (1H, d, J = 2 Hz, H-6 or 8), 5.60 and 5.50 (d, J = 1.8 Hz, rhamnose H-1X2), 4.2 ~ 3.30 (sugar protons), 1.20 (6H, d, J = 6 Hz, rhamnose C_5-CH_3).

IV-2: mp 216 ~ 218°C; [z]_D^0 -130° (c 1, CH_3OH). Anal. Calcd. for C_{21}H_{20}O_9: C 60.57, H 4.84, O 34.58. Found: C 59.77, H 4.95, O 35.02. Mass spectrum (m/e): 416 (M+), 270, 153, 118; PMR (d_6-DMSO): δ 12.95 (1H, broad s, OH-5), 8.34 (1H, s, H-2), 7.40 (2H, H-2',6'), 6.85 (2H, H-3',5'), 6.70 (1H, d, J = 2 Hz, H-8 or 6), 6.48 (1H, d, J = 2 Hz, H-6 or 8), 5.56 (1H, d, J = 1.8 Hz, rhamnose H-1), 3.10 ~ 5.30 (sugar protons), 1.15 (3H, d, J = 6 Hz, rhamnose C_5-CH_3).

Methanolysis

Compound III-3 (100 mg) in 4 ml of 0.5 N dry methanolic hydrogen chloride was heated in a sealed tube at 80°C for 3 hours. After cooling, 10 ml of cold water was added to the reaction mixture to form a white precipitate. After the mixture was allowed to stand for 3 hours at 4°C, the precipitate was collected by filtration. Recrystallization of the precipitate from ethanol gave 42 mg of colorless crystals, whose IR and UV spectra were consistent with those of authentic genistein. After the filtrate was neutralized with Amberlite IR-45 (OH−), the resin was filtered off. The filtrate was concentrated to dryness under reduced pressure, and 45 mg of a crude anomeric mixture of a hexose was obtained. It was stored in vacuo on phosphorous pentoxide. The anomeric mixture (30 mg) was purified by silica gel column chromatography to give 25 mg of the pure hexose. It was recrystallized from ethyl acetate (yield 65%). mp 108 ~ 110°C. [z]_D^0 -62.5° (c 1, H_2O). PMR (D_2O) TMS (external): δ 1.75 (3H, d, J = 6 Hz, C_5-CH_3), 3.84 (3H, s, C_1-O-CH_3), 3.70 ~ 4.30 (3H, m, C_3,4,5-H), 4.37 (1H, dd, J = 2, 3.5 Hz, C_2-H), 5.18 (C_6-H). The data of methanolysis of III-2, II and IV-2 were as follows:

III-2 (100 mg): 52 mg daidzein, 31 mg hexose
II (100 mg): 38 mg daidzein, 47 mg hexose
IV-2 (100 mg): 51 mg genistein, 45 mg hexose

Methyl α-L-rhamnoside

A solution of 160 mg of L-rhamnose in 5 ml of 0.5 N dry methanolic hydrogen chloride was heated at 80°C for 3 hours. After cooling, 10 ml of cold water was added to the reaction mixture to form a white precipitate. After the mixture was allowed to stand for 3 hours at 4°C, the precipitate was collected by filtration. Recrystallization of the precipitate from ethanol gave 42 mg of colorless crystals, whose IR and UV spectra were consistent with those of authentic genistein. After the filtrate was neutralized with Amberlite IR-45 (OH−), the resin was filtered off. The filtrate was concentrated to dryness under reduced pressure, and 45 mg of a crude anomeric mixture of a hexose was obtained. It was stored in vacuo on phosphorous pentoxide. The anomeric mixture (30 mg) was purified by silica gel column chromatography (1.5 x 11 cm) to give 25 mg of the pure hexose. It was recrystallized from ethyl acetate (yield 65%). mp 108 ~ 110°C. [z]_D^0 -62.5° (c 1, H_2O). PMR (D_2O) TMS (external): δ 1.75 (3H, d, J = 6 Hz, C_5-CH_3), 3.84 (3H, s, C_1-O-CH_3), 3.70 ~ 4.30 (3H, m, C_3,4,5-H), 4.37 (1H, dd, J = 2, 3.5 Hz, C_2-H), 5.18 (C_6-H). The data of methanolysis of III-2, II and IV-2 were as follows:

III-2 (100 mg): 52 mg daidzein, 31 mg hexose
II (100 mg): 38 mg daidzein, 47 mg hexose
IV-2 (100 mg): 51 mg genistein, 45 mg hexose

Methyl α-L-rhamnoside

A solution of 160 mg of L-rhamnose in 5 ml of 0.5 N dry methanolic hydrogen chloride was heated at 80°C for 3 hours. This reaction mixture was neutralized with Amberlite IR-45 (OH−) resin, and evaporated to dryness in vacuo. The syrup thus obtained was purified by silica gel column chromatography to give methyl α-L-rhamnoside, which was recrystallized from ethyl acetate (yield 75%). mp 108 ~ 109°C. [z]_D^0 -62.5° (c 1, H_2O). PMR (D_2O) TMS (external): δ 1.25 (3H, d, J = 6 Hz, C_5-CH_3), 1.97, 2.03, 2.16 (3H, s, -OAcH_3), 3.40 (3H, s, -OCH_3). 3.87 (1H, dq, J=6, 9 Hz C_5-H), 4.63 (1H, broad s, C_6-H), 4.90 ~ 5.40 (3H, m, C_3,4,5-H).

G. C. analysis of sugar moiety

Hexamethyldisilazane (0.2 ml) and trimethylchlorosilane (0.1 ml) were added to the pyridine solution (1 ml) of sugar. The reaction mixture was warmed at 60°C for 2 minutes. After extraction
with 6 ml of chloroform, the chloroform layer was washed with water (3 ml x 2), and was evaporated under reduced pressure to give a yellow syrup. This material was dissolved in small amount of chloroform and subjected to gas chromatography. Gas chromatographic conditions were as follows: A long glass column (0.2 x 200 cm) was packed with 3% SE-30 supported on Chromosorb W, and was maintained at 150°C. The nitrogen gas flow rate was 40 ml/min. The retention time of the TMS-derivative of methyl L-rhamnoside was consistent with that of authentic methyl 2-L-rhamnoside (3.6 minutes).

Acknowledgments

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References

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