

β -GALACTOSIDASE-INHIBITING NEW ISOFLAVONOIDS
PRODUCED BY ACTINOMYCETES

TADAHIKO HAZATO*, HIROSHI NAGANAWA, MICHIIHIKO KUMAGAI
TAKAAKI AOYAGI and HAMA O 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

(Received for publication May 27, 1978)

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

As reported previously, we have isolated isoflavonoids from the culture filtrate of *Streptomyces xanthophaeus*¹⁾ as active agents inhibiting β -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 paper¹⁾. 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 δ 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 δ 1.14~1.20,

Fig. 1. Isolation of isoflavonoids from MD865-C3

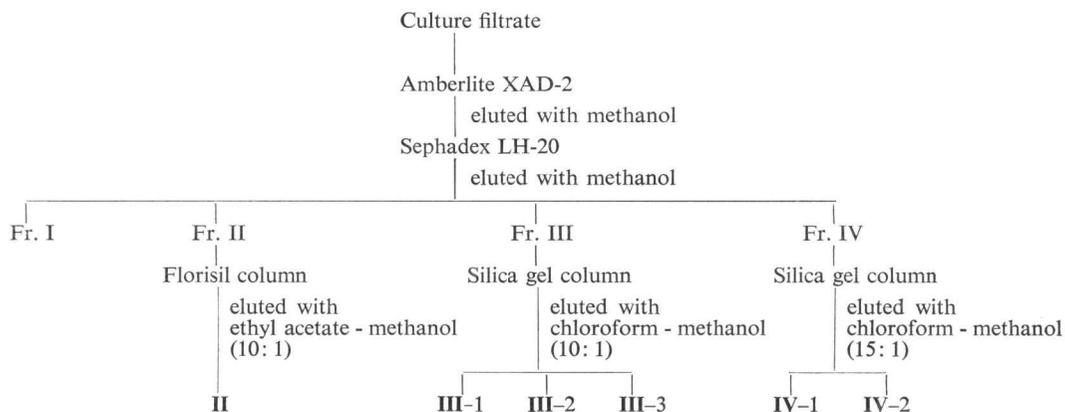


Table 1. The physicochemical data of isoflavonoids

Compound	Molecular formula	[α] _D ²⁰	Color reaction*			
			a	b	c	d
II	C ₂₇ H ₃₀ O ₁₂	-175	+	+	+	-
III-1	C ₁₅ H ₁₀ O ₅		-	-	+	+
III-2	C ₂₁ H ₂₀ O ₈	-146	+	+	+	+
III-3	C ₂₇ H ₃₀ O ₁₈	-169	+	+	+	-
IV-1	C ₁₅ H ₁₀ O ₄		-	-	+	+
IV-2	C ₂₁ H ₂₀ O ₉	-130	+	+	+	+

* Reagents;

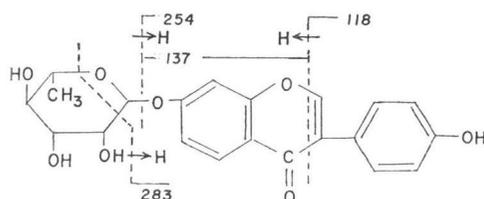
- Anisaldehyde-sulfuric acid (green)
- Diphenylamine-aniline (yellow)
- 2,3-Dinitrophenylhydrazine (blue)
- 2,6-Dibromoquinonechloroimide (violet)

Table 2. UV absorption maxima of isoflavonoids in methanol

Compound	λ_{max} (nm) Addition			
	None	NaOAc	AlCl ₃	NaOH
II	257	257	257	257
III-1	262	271	274	275
III-2	262	262	262	282
III-3	262	262	272	262
IV-1	250	260	250	259
IV-2	262	262	274	274

multiplets at δ 3.2~5.2 and doublets at δ 5.4~5.6 suggested the presence of 6-deoxyhexose in these compounds^{3,4}. Methanolysis of **III-2** afforded daidzein (**IV-1**) and a methyl glycoside. The PMR spectrum of this glycoside showed methyl and methoxyl groups as a doublet at δ 1.75 (J=6.0 Hz) and a singlet at δ 3.84. Acetylation of the methyl glycoside with acetic anhydride in pyridine afforded the methyl triacetyl glycoside, whose PMR spectrum showed methyl group (δ 1.23), methoxyl group (δ 3.40) and three acetyl groups (δ 1.97, δ 2.03, δ 2.16). From the spin decoupling experiment of the methyl triacetyl glycoside, the position of methyl group was shown to be C-6 in the pyranose ring. Specific rotation value of the methyl glycoside was -62.5° ⁵. The R_f values on paper chromatography and the retention time of its TMS derivative on gas chromatography agreed well with those of an authentic methyl α -L-rhamnoside. Therefore, the hexose was determined to be L-rhamnose.

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

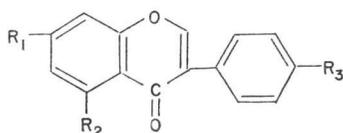


solution and the absorption maximum did not change following the addition of sodium acetate (Table 2). These results indicated that daidzein was linked to L-rhamnose at C-7^{6,7}. The high resolution mass spectrum of **III-2** showed a molecular ion peak of m/e 400 (C₂₁H₂₀O₈), and fragments ions of m/e 283 corresponding to C₁₆H₁₁O₅, m/e 254 to C₁₅H₁₀O₄, m/e 137 to C₇H₅O₃ and m/e 118 to C₈H₆O, 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⁺) 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 anomeric protons at δ 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 δ 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 δ 69.5 and 71.8 indicate no glycosidic linkage in the rhamnosides other than anomeric 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 rhamnosides.

Methanolysis of **IV-2** or **III-3** gave genistein (**III-1**) and methyl L-rhamnoside. From the ratio of intensities of the anomeric 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 δ 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 δ 12.95 and δ 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 α configurations⁸⁾. Since all of these isoflavone rhamnosides were levorotatory, it was concluded that all of the L-rhamnosides described in this paper had α configurations. The structures of β -galactosidase inhibitors obtained from *S. xanthophaeus* are shown as follows:



	R ₁	R ₂	R ₃	
II	O-Rha	H	O-Rha	daidzein 4',7-di- α -L-rhamnoside
III-1	OH	OH	OH	genistein
III-2	O-Rha	H	OH	daidzein 7- α -L-rhamnoside
III-3	O-Rha	OH	O-Rha	genistein 4',7-di- α -L-rhamnoside
IV-1	OH	H	OH	daidzein
IV-2	O-Rha	OH	OH	genistein 7- α -L-rhamnoside

The IC_{50} (concentration required for 50% inhibition) and the K_i values of the six inhibitors are listed in Table 3. The K_m of *p*-nitrophenyl β -D-galactopyranoside was about 3.7×10^{-3} M.

* This signal contained two methyl carbons.

Table 3. Inhibition of β -galactosidase by isoflavonoids

Inhibitor	IC ₅₀ (μ g/ml)	K _i ($\times 10^{-6}$ M)	Inhibition type
II	2.4	2.3	competitive
III-1	48	—	—
III-2	3.6	7.1	competitive
III-3	1.2	1.3	competitive
IV-1	170	—	—
IV-2	1.2	3.1	competitive

—: 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.

Enzyme and substrate

β -Galactosidase from bovine liver was purchased from Sigma Chemical Co. (St. Louis, U.S.A.), *p*-nitrophenyl β -D-galactopyranoside from BDH Chemical Ltd. (Pool, England).

Enzyme assay

The amount of hydrolysis of 5 mM *p*-nitrophenyl β -D-galactopyranoside in 0.05 M phosphate buffer pH 7.0, at 37°C was measured by the procedure reported previously¹³. *K_i* and *K_m* values were determined graphically by the method of LINEWEAVER and BURK⁹⁾.

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%, MgSO₄·7H₂O 0.1%, K₂HPO₄ 0.1%, CuSO₄·5H₂O 0.0007%, FeSO₄·7H₂O 0.0001%, MnCl₂·4H₂O 0.0008%, ZnSO₄·7H₂O 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, IC₅₀=28 μ g/ml) was placed on an Amberlite XAD-2 column (10 \times 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, IC₅₀=6 μ g/ml. This syrup was dissolved with methanol and chromatographed on a Sephadex LH-20 column (4 \times 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 \times 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 \times 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 $>300^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{20} -175^{\circ}$ (*c* 1, CH_3OH); Anal. Calcd. for $\text{C}_{27}\text{H}_{30}\text{O}_{12}$: C 59.35, H 5.51, O 35.13. Found: C 60.01, H 5.47, O 35.07. Mass spectrum (*m/e*): 254, 137, 118; PMR (d_6 -DMSO): 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 $\text{C}_5\text{-CH}_3\text{X}_2$).

III-2: mp 146°C ; $[\alpha]_{\text{D}}^{20} -146^{\circ}$ (*c* 1, CH_3OH). Anal. Calcd. for $\text{C}_{21}\text{H}_{20}\text{O}_8$: 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-6,8), 5.62 (1H, d, *J*=1.8 Hz, rhamnose H-1), 5.15~3.2 (sugar protons), 1.14 (3H, d, *J*=6 Hz, rhamnose $\text{C}_5\text{-CH}_3$).

III-3: mp $232\sim 257^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{20} -169^{\circ}$ (*c* 1, CH_3OH). Anal. Calcd. for $\text{C}_{27}\text{H}_{30}\text{O}_{13}$: 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_5 -dioxane): 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), 4.2~3.3 (sugar protons), 1.20 (6H, d, *J*=6 Hz, rhamnose $\text{C}_5\text{-CH}_3\text{X}_2$).

IV-2: mp $216\sim 218^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{20} -130^{\circ}$ (*c* 1, CH_3OH). Anal. Calcd. for $\text{C}_{21}\text{H}_{20}\text{O}_9$: 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 $\text{C}_5\text{-CH}_3$).

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 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 Amerlite 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 (1.5×11 cm) to give 25 mg of the pure hexose. It was recrystallized from ethyl acetate (yield 65%). mp $108\sim 110^{\circ}\text{C}$. $[\alpha]_{\text{D}}^{20} -62.5^{\circ}$ (*c* 1, H_2O). PMR (D_2O)_{TMS}(external): δ 1.75 (3H, d, *J*=6 Hz, $\text{C}_5\text{-CH}_3$), 3.84 (3H, s, $\text{C}_1\text{-O-CH}_3$), 3.70~4.30 (3H, m, $\text{C}_{3,4,5}\text{-H}$), 4.37 (1H, dd, *J*=2, 3.5 Hz, $\text{C}_2\text{-H}$), 5.18 ($\text{C}_1\text{-H}$). 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-rhamnoside⁵⁾

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 $108\sim 109^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{20} -62.5^{\circ}$ (*c* 1, H_2O).

Methyl 2,3,4-tri-O-acetyl- α -L-rhamnopyranoside¹⁰⁾

Melting point 88°C ; mass spectrum (*m/e*): 273 ($\text{M}^+ - \text{OCH}_3$), 244 ($\text{M}^+ - \text{CH}_3\text{COOH}$), 213, 171, 153, 111, 83. PMR (CDCl_3)_{TMS}: δ 1.25 (3H, d, *J*=6 Hz, $\text{C}_5\text{-CH}_3$), 1.97, 2.03, 2.16 (3HX3, s, $-\text{OAcX}_3$), 3.40 (3H, s, $-\text{OCH}_3$), 3.87 (1H, dq, *J*=6, 9 Hz $\text{C}_5\text{-H}$), 4.63 (1H, broad s, $\text{C}_1\text{-H}$), 4.90~5.40 (3H, m, $\text{C}_{2,3,4}\text{-H}$).

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 \times 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 \times 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 α -L-rhamnoside (3.6 minutes).

Acknowledgments

This work was partly supported by a contract from the Division of Cancer Treatment, the National Cancer Institute, NO1-CM-57009 and a Grant-in-Aid for Cancer Research 51-11 from the Ministry of Health and Welfare.

References

- 1) AOYAGI, T.; T. HAZATO, M. KUMAGAI, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Isoflavone rhamnoses, inhibitors of β -galactosidase produced by Actinomycetes. *J. Antibiotics* 28: 1006~1008, 1975
- 2) GANGULY, A. K. & O. Z. SARRE: Genistein and daidzein, metabolites of *Micromonospora rallophytica*. *Chem. & Ind.* 1970 (February): 201, 1970
- 3) MARKHAM, K. R. & T. J. MABRY: The identification of twenty-three 5-deoxy and ten 5-hydroxy-flavonoids from *Baptisia lecontei* (Leguminosae). *Phytochemistry* 7: 791~801, 1968
- 4) DE BRUYN, A.; M. ANTEUNIS, R. DE GUSSEM & G. G. S. DUTTON: $^1\text{H-N.m.r.}$ study of L-rhamnose, methyl α -L-rhamnopyranoside, and 4-O- β -D-galactopyranosyl-L-rhamnose in deuterium oxide. *Carbohydr. Res.* 47: 158~163, 1976
- 5) HASKINS, W. T.; R. M. HANN & C. S. HUDSON: The preparation of D-rhamnose from methyl α -D-mannopyranoside. *J. Am. Chem. Soc.* 68: 628~632, 1946
- 6) HOROWITZ, R. M. & L. JURD: Spectral studies on flavonoid compounds. II. Isoflavones and flavanones. *J. Org. Chem.* 26: 2446~2449, 1961
- 7) FUKUI, H.; H. EGAWA, K. KOSHIMIZU & T. MITSUI: A new isoflavone with antifungal activity from immature fruits of *Lupinus luteus*. *Agr. Biol. Chem.* 37: 417~421, 1973
- 8) BOCK, K. & C. PEDERSEN: A study of ^{13}C coupling constants in hexopyranoses. *J. Chem. Soc. Perkin Trans. 2*: 293~297, 1974
- 9) LINEWEAVER, H. & D. BURK: The determination of enzyme dissociation constant. *J. Am. Chem. Soc.* 56: 658~666, 1934
- 10) NESS, R. K.; H. G. FLETCHER & C. S. HUDSON: The reaction of tribenzol-2-L-rhamnopyranosyl bromide with methanol. Various benzoylated derivatives of L-rhamnose. *J. Am. Chem. Soc.* 73: 296~300, 1951