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Metabolism of Gentiopicroside (Gentiopicrin) by Human Intestinal Bacteria[1)]

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As a part of our studies on the metabolism of crude drug components by intestinal bacteria, gentiopicroside (a secoiridoid glucoside isolated from *Gentiana lutea*), was anaerobically incubated with various defined strains of human intestinal bacteria. Many species had ability to transform it to a series of metabolites. Among them, *Veillonella parvula* ss *parvula* produced five metabolites, which were identified as erythrocentaurin, gentiopicral, 5-hydroxymethylisochroman-1-one, 5-hydroxymethylisochromen-1-one and *trans*-5,6-dihydro-5-hydroxymethyl-6-methyl-1*H*,3*H*-pyrano[3,4-*c*]pyran-1-one.

Keywords *Gentiana lutea*; gentiopicroside; gentiopicral; intestinal bacteria; metabolism

The microflora in the gastrointestinal tract play significant roles in the metabolic activation[2)] or inactivation[3)] of drugs, in the production of carcinogens, mutagens and promoters of carcinogenesis from dietary components or from secretion produced as a response to dietary components,[4,5)] and in the enterohepatic circulation of endogenous and exogenous substances.[6,7)]

In the course of our studies on the metabolic activation of crude drug components by intestinal bacteria, we have already reported the metabolism of swertiamarin,[8)] a bitter seco-iridoid glucoside from *Swertia japonica* MAKINO.[9)]; swertiamarin was demonstrated to be transformed to erythrocentaurin, its reduced product and a nitrogen-containing compound, gentianine, by intestinal bacteria.[8)]

In the present paper, we report the metabolism of another seco-iridoid glucoside, gentiopicroside (gentiopicrin, **1**), by defined strains isolated from human feces. Gentiopicroside (**1**) is one of the major bitter principles of common gentians[10,11)] which are widely used as stomachica and antiparasitica, but its pharmacological properties have not been extensively examined with the exception of antimicrobial activities against *Penicillium expansum*[12,13)] and *Staphylococcus aureus*[14)] in the presence of β-glucosidase and antitumor activity in mice bearing the experimental tumor leukemia P388.[15)]

Results

Screening of Defined Strains of Human Intestinal Bacteria for Metabolic Ability of Gentiopicroside (1) On anaerobic incubation, most of 24 strains of human intestinal bacteria transformed gentiopicroside (**1**) to metabolites G_1—G_5 in 22 h, when the reaction was monitored by thin layer chromatography (TLC). However, the amounts of the respective metabolites varied from one strain to other (Table I); metabolite G_1 was mainly produced by *Bifidobacterium breve*, *Peptostreptococcus anaerobius*, *Lactobacillus brevis*, *Lactobacillus xylosus*, *Lactobacillus plantarum* and *Fusobacterium nucleatum*, metabolite G_2 by *Clostridium innocuum* and *Lactobacillus acidophilus*, metabolite G_3 by *Veillonella parvula* ss *parvula*, *Proteus mirabilis* and *Bacteroides fragilis*, and metabolites G_4 and G_5 (which were not well separated on the TLC plate), by *V. parvula*. *V. parvula*, a gram-negative coccus, produced all five metabolites in appreciable amounts ($G_1:G_2:G_3:G_4+G_5=1:1:5:4$ in approximately relative ratio) under the same screening conditions (Fig. 1) and this bacterium was there-

TABLE I. Ability of Intestinal Bacterial Strains to Metabolize Gentiopicroside

Bacterial strain	G_1	G_2	G_3	G_{4+5}
Bifidobacterium bifidum a E319	+	−	++	−
B. adolesscentis	++	+	−	+
B. breve S-2 KZ 1287	+++	−	+	−
B. pseudolongum PNC-2-9-G	−	−	−	+
B. longum IV-55	−	−	++	−
Clostridium perfringens To-23	−	−	++	−
C. butyricum	−	−	−	++
C. innocuum ES 24-06	−	−	−	++
C. innocuum KZ 633	−	+++	−	+
Peptostreptococcus anaerobius 0240	+++	++	−	++
P. intermedius EBF 77/25	++	++	−	++
Veillonella parvula ss *parvula* ATCC 10790	++	++	+++	+++
Lactobacillus brevis II-46	+++	++	−	−
L. acidophilus ATCC 4356	++	+++	−	+
L. xylosus ATCC 155775	+++	−	++	+
L. fermentum ATCC 9338	++	+	−	++
L. plantarum ATCC 14917	+++	−	++	+
Streptococcus faecalis II-136	+	−	+	−
Escherichia coli 0-127	−	−	−	++
Klebsiella pneumoniae ATCC 13883	−	−	−	+
Proteus mirabilis S2	+	++	+++	+
Bacteroides fragilis ss *thetaotus*	+	−	+++	++
Fusobacterium nucleatum G-0470	+++	−	+	−
Gaffkya anaerobia G-0608	+	−	−	−

Each bacterium was cultured in the presence of gentiopicroside for 24 h at 37 °C. The products were extracted with EtOAc and analyzed by TLC-densitometry. The *Rf* values of the these metabolites on TLC with $CHCl_3$–MeOH (15 : 1) were as follows: G_1, 0.92; G_2, 0.85; G_3, 0.59; G_4 and G_5, 0.46—0.50.

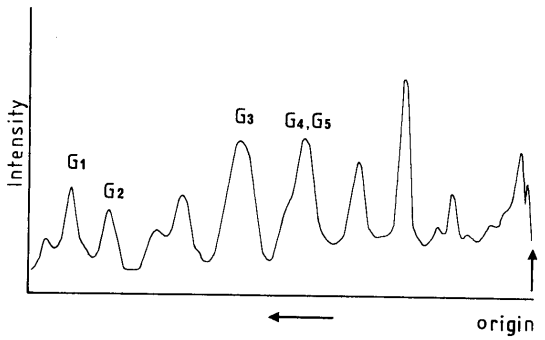

Fig. 1. A Typical TLC-Densitometric Profile of the Metabolites Formed by Anaerobic Incubation of Gentiopicroside with *V. parvula*

The products extracted with EtOAc were chromatographed on a TLC plate and analyzed by TLC-densitometry. The peaks corresponding to metabolites are represented by G_1—G_5; other peaks originate from the bacterial cells and culture broth.

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fore used for a large scale production of the metabolites, which enable us to isolate each metabolite and to determine the structures.

Structures of Metabolites A combination of solvent extraction and column chromatography of the products obtained by cultivation of *V. parvula* with gentiopicroside (**1**) led to the isolation of the above metabolites.

Metabolites G_1 and G_2 were identified as erythrocentaurin and gentiopicral (gentiogenal), respectively, by direct comparison of their mass spectra (MS), proton nuclear magnetic resonance (^{1}H-NMR) spectra and *Rf* values on TLC with those of authentic samples obtained by enzymic hydrolysis of swertiamarin by β-glucosidase.

Metabolite G_3, colorless powder, showed the molecular ion peak at *m/z* 176 in the MS, corresponding to the molecular formula $C_{10}H_8O_3$. The infrared (IR) spectrum of G_3 indicated the presence of hydroxyl and carbonyl groups (3460 and 1710 cm^{-1}). The ^{1}H-NMR spectrum showed aromatic proton signals at δ 8.29 (H-8), 7.52 (H-6) and 7.26 (H-7), among which the last signal was coupled with the former two with identical spin-spin coupling constants ($J_{6,7}=J_{7,8}=7.8$ Hz), and two doublet signals at δ 7.34 (H-3) and 6.78 (H-4), due to adjacent olefinic protons, besides a signal due to a hydroxymethyl group (δ 4.91). The structure of metabolite G_3 was finally concluded to be 5-hydroxymethylisochromen-1-one.

Metabolite G_4 was identical with 5-hydroxymethylisochroman-1-one, one of the metabolites of swertiamarin formed by human intestinal bacteria.[8]

Metabolite G_5 was obtained as a white powder, MS *m/z*: 196, corresponding to the molecular formula $C_{10}H_{12}O_4$. The IR spectrum of G_5 showed the presence of hydroxyl and conjugated carbonyl groups (3400 and 1685 cm^{-1}). In addition, the ^{1}H-NMR spectrum showed the presence of *sec*-methyl, hydroxymethyl and two olefinic protons. One of the olefinic protons (δ 5.52) coupled with methylene protons (δ 5.08 and 4.97) in the AMX type spin-spin coupling manner and the other resonated at low field (δ 7.55), similar to H-3 of gentiopicroside. The double-resonance experiments revealed the presence of a partial structure $-CH(CH_2OH)-CH(CH_3)-$; on irradiation at δ 2.44 (H-5), the double quartet (δ 4.61, H-6) and the two double doublets (δ 3.61 and 3.72, $HOCH_2$-5) became a quartet and two doublets, respectively. On irradiation at δ 4.61 (H-6), the multiplet (δ 2.44, H-5) and doublet (δ 1.31, Me-6) became a double doublet and a singlet. These findings led us to conclude the structure of metabolite G_5 to be 5,6-dihydro-5-hydroxymethyl-6-methyl-1*H*,3*H*-pyrano[3,4-*c*]pyran-1-one. Since the vicinal proton coupling value ($J_{5,6}=2.8$ Hz) in metabolite G_5 was quite similar to that of gentiopicroside (**1**) ($J_{1,9}=2.9$ Hz), the two adjacent substituents at C-5 and C-6 must be *trans*-oriented as in **1**. Contrary to the case of the metabolism of swertiamarin, no nitrogen-containing metabolite such as gentianine was detected in the culture.

Discussion

Anaerobic incubation of gentiopicroside (**1**) with each strain of human intestinal bacteria resulted in the transformation of gentiopicroside (**1**) to at least five metabolites in different ratios.

Chart 1 shows the possible metabolic processes leading to the various metabolites G_1 to G_5 from gentiopicroside (**1**) by intestinal bacteria. Gentiopicroside (**1**) is hydrolyzed by bacterial β-glucosidase to give an aglycone (**2**). The aglycone seems to be unstable in the hemiacetal form and is readily converted to two types of compounds, isochroman (G_1) and pyrano[3,4-*c*]pyran (**4** and G_2) derivatives. The aldehydes of both types are subsequently reduced to the respective alcohols (G_4 and G_5). The metabolic process involved in the formation of an isochromene-type metabolite (G_3) is not clear, but may involve dehydrogenation of the isochroman-type metabolite (G_4) at the C-3 and C-4 positions. Contrary to the case of metabolism of swertiamarin,[8] no pyridine monoterpene alkaloids were produced. This may be because the intramolecular cyclization

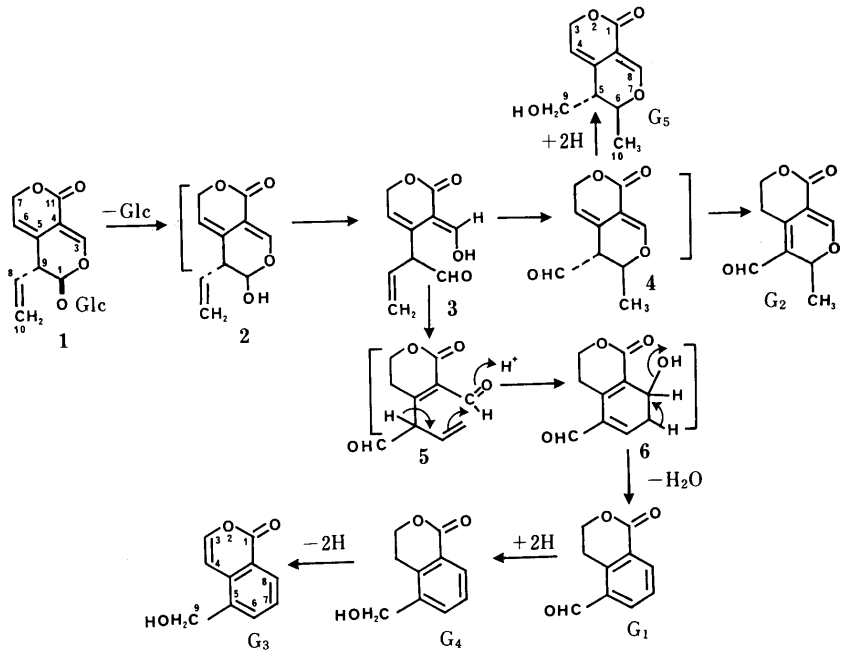

Chart 1. Possible Metabolic Processes of Gentiopicroside by Human Intestinal Bacteria

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of the dialdehyde intermediates (**3**→**4** and **5**→**6**) occurred more rapidly than the Schiff-base formation, as proposed in the metabolic processes of swertiamarin.[8]

Gentiopicral (G_2) has been reported to have antibacterial, autifungal and antitumor activities.[12-15] Our results suggest that orally administered gentiopicroside (**1**) is transformed to a bioactive substance, G_2, along with other substances whose biological activities are not yet clear, by intestinal flora in the gastrointestinal tract.

Experimental

Apparatus Ultraviolet (UV) spectra were taken on a Shimadzu UV-210A spectrometer. NMR spectra were measured with a JEOL JNM FX-270 (^{1}H, 270 MHz) spectrometer, and chemical shifts are presented as δ values ralative to tetramethylsilane as an internal standard. IR spectra were measured with a Hitachi 260-10 infrared spectrophotometer. MS were measured with a JEOL JMS DX-300 mass spectrometer at an ionization voltage of 70 eV. Densitometric profiles of thin-layer chromatograms were recorded on a Shimadzu CS-910 dual wavelength thin layer chromatoscanner (TLC scanner).

Chemicals Gentiopicroside (**1**) was isolated from the roots of *Gentiana lutea* by a similar method to that of Inoue *et al.*[9] Gentianine was prepared from swertiamarin by treatment with ammonia, followed by acid hydrolysis.[16] Gentiopicral (gentiogenal) was obtained by hydrolysis of swertiamarin with β-glucosidase. 5-Hydroxymethylisochroman-1-one was obtained by incubation of swertiamarin with *Lactobacillus brevis*, as reported in the previous paper.[8] β-Glucosidase from sweet almonds was purchased from Sigma Chem. Co. General anaerobic medium (GAM) was purchased from Nissui Co.

Microorganisms Intestinal bacterial strains were provided by Professor T. Mistuoka of the University of Tokyo.

Chromatography Wako gel (Wako Pure Chem. Co.) was used for column chromatography and Merck Kieselgel 60 F_{254} (layer thickness, 0.25 mm) was used for TLC. TLC plates were developed with $CHCl_3$–MeOH (15 : 1) and spots were detected under a UV lamp.

Screening of Intestinal Bacterial Strains for Ability to Metabolize Gentiopicroside (1) Each precultured bacterial strain (0.2 ml) was added to GAM broth (10 ml) and cultured for 24 h at 37 °C under anaerobic conditions. Gentiopicroside (**1**) was added to each culture, and the mixture was incubated for 22 h under the same conditions and then extracted with EtOAc (10 ml). After evaporation of the EtOAc, $CHCl_3$–MeOH (1 : 1, 0.5 ml) was added to the residue and an aliquot (50 μl) of the solution was applied to a silica gel TLC plate, which was developed with $CHCl_3$–MeOH (15 : 1). The metabolites separated on the plate were quantitatively analyzed with a TLC scanner at 230 nm relative to a reference wavelength of 550 nm.

Isolation of Metabolites A precultured bacterial suspension (100 ml) of *Veillonella parvula* ss *parvula* ATCC 10790 was added to GAM broth (900 ml) and incubated for 24 h at 37 °C in an anaerobic jar in which air had been replaced with O_2-free CO_2 in the presence of steel wool. Gentiopicroside (**1**, 1 g in 5 ml of 0.1 M phosphate buffer, pH 7.4) was then added, and the mixture was anaerobically incubated for 22 h at 37 °C and extracted 3 times with EtOAc (1000 ml each). The combined solutions were concentrated *in vacuo* to give an oily residue. The residue was applied to a column of silica gel, which was washed with hexane and eluted successively with benzene, benzene–$CHCl_3$ (3 : 1) and benzene–$CHCl_3$ (2 : 1) to afford five metabolites, G_1 (3 mg), G_2 (3 mg), G_3 (14 mg) and G_4+G_5 (12 mg). Repeated preparative TLC of a mixture of G_4 and G_5 gave two pure compounds.

Metabolite G_3: Colorless powder. MS *m/z* (rel. int.): 176 (100, M^+), 148 (51, M^+-CO), 119 (38), 120 (25), 91 (34). IR ν_{max}^{KBr} cm^{-1}: 3460 (OH), 1710 (C=O), 1635, 1600 (Ar). UV λ_{max}^{MeOH} nm: 243, 265, 317 (sh). ^{1}H-NMR (270 MHz, $CDCl_3$): δ 8.29 (1H, d, J=7.8 Hz, H-8), 7.76 (1H, d, J=7.8 Hz, H-6), 7.52 (1H, t, J=7.8 Hz, H-7), 7.34 (1H, d, J=5.9 Hz, H-3), 6.78 (1H, d, J=5.9 Hz, H-4), 4.91 (2H, d, J=5.4 Hz, H_2-9).

Metabolite G_5: White powder, $[\alpha]_D$ +2° (c=0.15, $CHCl_3$). MS *m/z*: (rel. int.): 196 (69, M^+), 181 (100, M^+-Me), 126 (91), 121 (94), 55 (52). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1685 (C=O), 1600 (Ar). UV λ_{max}^{MeOH} nm: 280. ^{1}H-NMR (270 MHz, $CDCl_3$): δ 7.55 (1H, s, H-8), 5.52 (1H, m, H-4), 5.08 (1H, dd, J=18.2, 2.7 Hz, H_b-3), 4.97 (1H, dd, J=18.2, 3.5 Hz, H_a-3), 4.61 (1H, dq, J=2.8, 6.6 Hz, H-6), 3.72 (1H, dd, J=11.0, 7.2 Hz, H_a-9), 3.61 (1H, dd, J=11.0, 6.7 Hz, H_b-9), 2.44 (1H, m, H-5), 1.31 (3H, d, J=6.6 Hz, H_3-10).

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References and Notes

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