Microbial Mannosidation of Bioactive Chlorogentisyl Alcohol by the Marine-Derived Fungus *Chrysosporium synchronum*

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The biological transformation of the biologically active chlorogentisyl alcohol (1), isolated from the marine-derived fungus *Aspergillus* sp., was studied. Preparative-scale fermentation of chlorogentisyl alcohol with marine-derived fungus *Chrysosporium synchronum* resulted in the isolation of a new glycosidic metabolite, 1-*O*-(α-D-mannopyranosyl)chlorogentisyl alcohol (2). The stereostructure of the new metabolite obtained was assigned on the basis of detailed spectroscopic data analyses, chemical reaction, and chemical synthesis. Compounds 1 and 2 exhibited significant radical-scavenging activity against 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) with IC$_{50}$ values of 1.0 and 4.7 μM, respectively. The compounds 1 and 2 were more active than the positive control, L-ascorbic acid (IC$_{50}$, 20.0 μM).

Key words   marine-derived fungus; *Chrysosporium synchronum*; microbial mannosidation; 1-*O*-(α-D-mannopyranosyl)chlorogentisyl alcohol

The aim of our program is to explore the biological transformation of bioactive metabolites produced by microorganisms isolated from marine habitats. As part of this program, we identified the marine-derived ascomycete strains and actinomycete strains that regioselectively oxidize bioactive natural products to new and more active compounds: from terreusinone to the unsymmetrical alcohol derivative, terreusinol, by *Streptomyces* sp.,[1] from 6-*n*-pentyl-α-pyrone to two oxidized metabolites, 6-*n*-(4-oxopentyl)-α-pyrone and 6-*n*-[(S)-1-hydroxypentyl]-α-pyrone, by *Streptomyces* sp.,[2] from cyclonerodiol to three metabolites, 10(Z)-, 10(E)-cyclonerotriol, and cyclonerodiol mannopyranoside, by the genus of *Streptomyces* and *Penicillium*,[3] from geraniol to its 7-oxidized metabolite, 1,7-dihydroxy-3,7-dimethyl-(E)-oct-2-ene, by *Hypocrea* sp.,[4] and from (−)-mellein to (3R,4S)-4-hydroxymellein by *Stappia* sp.[5] In our continuing study of applications of microbial transformations,[6] we screened 50 cultures for their ability to biotransform chlorogentisyl alcohol (1). Chlorogentisyl alcohol was isolated from the marine algicolous fungus *Aspergillus* sp.[7] and was found to induce apoptosis in HeLa cells.[8] The marine-derived fungus *Chrysosporium synchronum* was selected as the biotransforming target strain.

Preliminary small-scale fermentations (100 ml) of *C. synchronum*, with the addition of monosaccharides (*e.g.*, glucose, galactose, mannose, rhamnose, or ribose) directly to the culture medium, respectively, did not provide promising result except for mannose. TLC analyses of the broth extracts confirmed that mannose only stimulated mannoside production relative to a control culture.

Biotransformation of chlorogentisyl alcohol was carried out using the marine-derived fungus *C. synchronum* according to a two-stage fermentation protocol.[9] The fermentations of 1 (20 mg) with *C. synchronum* were harvested after two weeks, and the filtered broths were extracted with EtOAc to afford crude extracts. The extract was purified by repeated silica gel flash chromatography (*n*-hexane in ethyl acetate) and HPLC (octadesyl silica (ODS), MeOH–H$_2$O) to yield a

new glycoside, 1-*O*-(α-D-mannopyranosyl)chlorogentisyl alcohol (2) (3.2 mg). The metabolite 2, [α]$_D$ +3.70 (*c*=0.3, MeOH), was isolated as a colorless oil. A molecular formula of C$_{13}$H$_{17}$ClO$_8$ was established by high resolution electrospray ionization mass spectrometry (HR-ESI-MS) and ^{13}C-NMR methods. The IR spectrum of 2 suggested the presence of hydroxyl (3400 cm^{-1}) and glycosidic (1018 cm^{-1}) groups. The ^{1}H- and ^{13}C-NMR data of 2 closely resembled those of 1 except for the appearance of additional proton signals corresponding to the five *sp*^{3} oxymethines and one oxymethylene. An extensive analysis of the distortionless enhancement by polarization transfer (DEPT) and 2D-NMR spectra, correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond coherence (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) experiments, suggested that 2 was a glycoside comprising 1 as an aglycone and one sugar unit. The hexopyranose unit was presumed to be mannose due to the *anti* diaxial orientations of H-3′/H-4′ ($J_{H3′–H4′}$=9.1 Hz) and H-4′/H-5′ ($J_{H4′–H5′}$=9.5 Hz) and the *syn* equatorial and axial orientation of H-2′/H-3′ ($J_{H2′–H3′}$=3.2 Hz) in the ^{1}H-NMR (CD$_3$OD). Acid hydrolysis of 2 furnished 1 and methyl mannose,[10] and the latter was identified on the basis of GC and GC-MS analyses of the trimethylsilyl derivative. Comparison of the ^{13}C-NMR data of 1 and 2, and the key HMBC correlation from H-1′ to C-1 were critical in establishing the position of mannose as attached at the 1-OH of 1. The stereochemistry of the anomeric position of the sugar moiety was assigned as the α-configuration on the basis of the anomeric carbon signal at δ 100.9 (d, C-1′) having a $^1J_{C–H}$ value of 175 Hz.[11] This value was deduced from DEPT nondecoupling measurements. The absolute configuration of the mannose unit was determined to be D from measurement of the optical rotation. Obtained from acid hydrolysis of 2 with 9% aq. HCl, the specific rotation of mannose, [α]$_D^{20}$ +28.8 (*c*=0.03, H$_2$O), indicated the presence of D-mannose in 2 [ref: [α]$_D^{20}$ +29.3].[12] These results led us to conclude that the structure of compound 2 was 1-*O*-(α-D-annopyranosyl)-

chlorogentisyl alcohol (Fig. 1). To clarify the structure of **2**, we synthesized **2** from **1** and 1-*O*-(2,3,4,6-tetra-*O*-acetyl-*α*-D-mannopyranosyl)trichloroacetimide. D-(+)-Mannose was acetylated by acetic anhydride in pyridine, followed by selective deacetylation of 1-acetyl group by piperidine to give 2,3,4,6-tetra-*O*-acetyl-D-mannopyranoside.[13] Treatment with trichloroacetonitrile of the C-1-unprotected mannopyranose, 2,3,4,6-tetra-*O*-acetyl-D-mannopyranoside, afforded 1-*O*-(2,3,4,6-tetra-*O*-acetyl-D-mannopyranosyl)trichloroacetimide.[14,15] Glycosidation of **1** with the above imidate in the presence of boron trifluoro-etherate[16] provided 1-*O*-(2,3,4,6-tetra-*O*-acetyl-*α*-D-mannopyranosyl)chlorogentisyl alcohols (**2a**) in 25% yield as a major product as well as two minor products, 4-*O*-(2,3,4,6-tetra-*O*-acetyl-*α*-D-mannopyranosyl)-chlorogentisyl alcohol (**2b**) and 7-*O*-(2,3,4,6-tetra-*O*-acetyl-*α*-D-mannopyranosyl)chlorogentisyl alcohol (**2c**), in trace amounts (Fig. 2). The *α*-configuration at C-1′ of **2a** was substantiated by the small value of the coupling constant [δ 5.49 (1H, br s, H-1′)] of the anomeric proton. Finally, the product **2a** was subjected to alkaline hydrolysis (NaOMe) to afford **2**. The spectroscopic data for the synthetic compound were identical to those of compound **2** in $[\alpha]_D$, ESI-MS, and ¹H-NMR. Although many examples of microbial glucosidation have been reported,[17—19] microbial mannosidation is very rare.[3] Thus, to the best of our knowledge, compound **2** is the second example of microbial mannosidation by the marine isolate of the microorganism. Compounds **1** and **2** exhibited significant radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), with IC$_{50}$ values of 1.0 and 4.7 μM, respectively. They were more active than the positive control, L-ascorbic acid (IC$_{50}$, 20.0 μM). We plan to intensively examine compounds **1** and **2** for inhibitory activity

against epidermal growth factor receptor (EGFR),[20,21] and the results will be reported in due course.

Experimental

General Optical rotation was determined on a Perkin Elmer model 341 polarimeter. UV/visible spectra were measured on a Hitachi U-2001 UV/Vis spectrometer. IR spectra were recorded on a Bruker Fourier transform (FT)-IR model IFS-88 spectrometer. ¹H- (400 MHz) and ¹³C-NMR (100 MHz) spectra were obtained on a JEOL JNM-ECP 400 NMR spectrometer, using tetramethylsilane (TMS) or solvent peaks [DMSO-d_6: ¹H (δ 2.50) and ¹³C (δ 39.5)] as reference standard. LC-MS and MS spectra were obtained on API 2000 (Applied Bio System, U.S.A.) and IT-TOF (Shimadzu, Japan) spectrometer, respectively. HPLC was performed on a Young Lin ACME HPLC system using a reversed-phase analytical column (Gemini C18, 4.6×250 mm, 5 μm) with UV detection. Incubations of microorganisms and biotransformations were performed on an Incubator Shaker JS-FS-2500 (Johnsam Co., Inchon, Korea).

Fungal Isolation The fungal strain was isolated from the surface of edible brown alga *Sargassum ringgoldium* (Korean name: KeunIp Mojaban) collected at Yokji Island of GyeongNam, Korea in 2009, and identified to be *C. synchronum* (EMBL/AM943023.1) on the basis of morphology and 18S ribosomal RNA (rRNA) analysis (SolGent Co., Ltd., Daejeon, Korea), identity of 98%. A voucher specimen is deposited at Pukyong National University with the code BAac049.

Biotransformation of 1 A two-stage fermentation protocol[9] was used for preparative scale formation of the metabolite of **1**. The modified medium contained soytone (0.1%), soluble starch (1.0%), mannose (0.1%), and seawater (100%), and it was autoclaved at 121 °C for 15 min. Preparative incubation was conducted in 1 l of sterile medium held in 3 l culture flask that was incubated on a rotary shaker (130 rpm) at 29 °C for 1 week. A 10% inoculum derived from one week old stage I culture was used to initiate stage II culture, which was incubated for 24 h more under the same condition before receiving 20 mg of **1** in 0.75 ml of *N*,*N*-dimethyl formamide (DMF), and incubation was continued at 29 °C for two weeks in the same manner to that described above. Substrate control consisted of sterile medium and substrate incubated under the same conditions but without microorganism. Also, culture control was composed of fermentation blanks in which the microorganism was grown under identical condition but without the addition of substrate. After two weeks of incubation, each control was harvested and analyzed by TLC. The culture was filtered through cheesecloth, and the filtrate was extracted with EtOAc. The organic layer was dried over anhydrous Na$_2$SO$_4$, filtered through sintered glass, and vacuum-concentrated to yield a crude extract (95 mg).

Isolation of the Metabolite (2) The crude extract (95 mg) was subjected to silica gel flash column chromatography. Elution was performed with *n*-hexane–EtOAc (stepwise, 0—100% EtOAc) to yield three fractions. Fractions 2 and 3 were separated by medium-pressure liquid chromatography (MPLC) (ODS) using a H$_2$O–MeOH gradient elution to afford crude **1** and **2**, respectively. These were further purified by HPLC (Gemini C18, 4.6×250 mm, 5 μm) utilizing a 30 min gradient program of 50 to 100% MeOH in H$_2$O to furnish the substrate, **1** (8.5 mg), and the metabolite (**2**) (3.2 mg), respectively.

1-*O*-(*α*-D-Mannopyranosyl)chlorogentisyl alcohol (**2**): A colorless oil; $[\alpha]_D^{20}$ +3.70 (*c*=0.3, MeOH); UV (MeOH) λ_{max} (log ε) 275 (3.3), 207 (0.6) nm; IR (KBr) ν_{max} 3400, 2924, 1627, 1241, 1018 cm^{+1}; ¹H-NMR (CD$_3$OD, 400 MHz) δ: 7.03 (2H, s, H-3, 5), 4.64 (2H, s, H$_2$-7), 5.37 (1H, d, *J*=1.5 Hz, H-1′), 3.98 (1H, dd, *J*=3.2, 1.5 Hz, H-2′), 3.87 (1H, dd, *J*=9.1, 3.2Hz, H-3′), 3.74—3.76 (3H, m, H-4′, 6′), 3.60 (1H, dt, *J*=9.5, 4.0 Hz, H-5′); ¹³C-NMR (CD$_3$OD, 100 MHz) δ: 150.9 (s, C-1), 132.0 (s, C-2), 117.6 (d, C-3), 146.7 (s, C-4), 116.3 (d, C-5), 121.6 (s, C-6), 60.9 (t, C-7), 100.9 (d, C-1′), 71.9 (d, C-2′), 72.2 (d, C-3′), 68.1 (d, C-4′), 75.3 (d, C-5′), 62.2 (t, C-6′); low resolution (LR)-ESI-MS: *m/z* 335 [M (³⁵Cl)−H] and 337 [M* (³⁷Cl)−H] (*m/z* 335:337=*ca.* 3:1); HR-ESI-MS: *m/z* 335.0538 [M−H] (Calcd for C$_{13}$H$_{16}$O$_8$Cl, 335.0539) (−0.30 ppm).

Acid Hydrolysis of 2, and Sugar Analysis A solution of **2** (0.5 mg) in 9% dry methanolic HCl (1.0 ml) was stirred at 80 °C for 1 h (N$_2$ atmosphere). The reaction mixture was neutralized with Ag$_2$CO$_3$ and filtrated. The residue, obtained by removal of the solvent, was dissolved in pyridine (0.5 ml) and treated with bis(trimethylsilyl)trifluoroacetamide (BSTFA) (0.5 ml) at rt for 30 min. Solvent was removed by a nitrogen stream and the residue dissolved in *n*-hexane was used for GC-MS analysis [(DB-1MS column, 60 m×0.32 mm); N$_2$ as a carrier gas at 0.7 ml/min; the program rate: 80—260 °C at 5 °C/min] showing peak at t_R 16.60 min, which corresponded to those of TMS/Me derivative of mannose (*m/z* 540 [M]⁺).
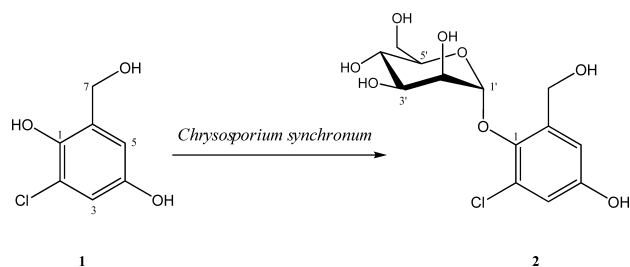

Fig. 1. Microbial Mannosidation of Chlorogentisyl Alcohol (**1**) and the Structure of Metabolite, 1-*O*-(*α*-D-Mannopyranosyl)chlorogentisyl Alcohol (**2**)
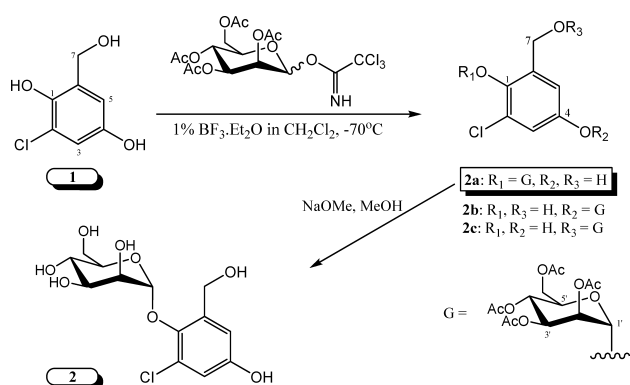

Fig. 2. Synthesis of 1-*O*-(*α*-D-Mannopyranosyl)chlorogentisyl Alcohol (**2**) from Chlorogensyl Alcohol (**1**)

Determination of the Stereochemistry of the Mannopyranose A solution of **2** (1.0 mg) in 9% aq. HCl (1.0 ml) was stirred at 80 °C for 1 h. The reaction mixture was treated as described above, and the residue thus obtained was partitioned into H_2O and CH_2Cl_2 to give the organic phase and the aqueous phase. The aqueous portion was applied to a Diaion HP-20 column (Mitsubishi Kasei, Japan, 20 ml of resin, 10×250 mm). The column was eluted with distilled water (100 ml) and MeOH (100 ml), successively. The elute with MeOH was concentrated to afford mannose (0.3 mg). The rotation recorded for mannose isolated in this study was $[\alpha]_D$ +28.8 (c=0.03, H_2O), which showed D-mannose in **2** [ref: $[\alpha]_D^{20}$ +29.3].[12]

Synthesis of 2 Molecular sieves 4A (60 mg) and 1-O-(2,3,4,6-tetra-O-acetyl-D-mannopyranosyl)trichloroacetimide (38 mg, 0.08 mmol) were added to a solution of **1** (15 mg, 0.08 mmol) in dry CH_2Cl_2 (7 ml) at rt, then the mixture was treated with boron trifluoro-etherate (1% solution in dry CH_2Cl_2, 0.5 ml) at −70 °C for 2 h. The reaction mixture was poured into ice water, and the whole was extracted with CH_2Cl_2. The organic phase was washed with aq. saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Removal of the solvent under reduced pressure gave a product that was purified by column chromatography (SiO₂, n-hexane–EtOAc=5:1) to afford not only 1-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)chlorogentisyl alcohol (**2a**) (9.0 mg, 0.02 mmol, 25%) as the major product, but also the minor products, 4-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)chlorogentisyl alcohol (**2b**) and 7-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)chlorogentisyl alcohol (**2c**) in trace amounts. A solution of **2a** (9 mg, 0.02 mmol) in MeOH (2 ml) was treated with 28% NaOMe (1 drop) at rt for 4 h. The reaction mixture was neutralized with Dowex [50W×8 (H⁺ form)], and the resin was removed by filtration. Removal of the solvent from the filtrate under reduced pressure gave a product (10 mg) that was purified by reversed-phase ODS-A gel (YMC Co., Japan) flash column chromatography using MeOH, followed by HPLC (ODS, MeOH–H_2O=5:1) to furnish **2** (4.8 mg, 0.01 mmol, 50%) (Fig. 2). The spectroscopic data ($[\alpha]_D$, ESI-MS, and ¹H-NMR) for the synthetic compound were virtually identical to those of the natural product (**2**).

Radical Scavenging Activity against DPPH[7] Samples (**1**, **2**, and positive control, L-ascorbic acid) to be tested were dissolved in MeOH and the solution (160 µl) was dispensed into wells of a 96-well microtiter tray. Forty microliters of the DPPH solution in MeOH ($1.5×10^{-4}$ M) was added to each well. The mixture was shaken and left to stand for 30 min, and the absorbance of the resulting solution was measured at 520 nm with microplate reader (Packard Co., U.S.A., Spectra Count™). The scavenging activity on DPPH radical was expressed as IC_{50}, which is the concentration of the tested compound required to give a 50% decrease of the absorbance from that of the blank solution [consisting of MeOH (160 µl) and DPPH solution (40 µl)].

References
1) Li X., Lee S. M., Choi H. D., Kang J. S., Son B. W., *Chem. Pharm. Bull.*, **51**, 1458—1459 (2003).
2) Li X., Kim S.-K., Jung J. H., Kang J. S., Choi H. D., Son B. W., *Bull. Korean Chem. Soc.*, **26**, 1889—1890 (2005).
3) Li X., Kim Y. H., Jung J. H., Kang J. S., Kim D.-K., Choi H. D., Son B. W., *Enz. Microbial. Technol.*, **40**, 1188—1192 (2007).
4) Leutou A. S., Yang G., Nenkep V. N., Siwe X. N., Feng Z., Khong T. T., Choi H. D., Kang J. S., Son B. W., *J. Microbiol. Biotechnol.*, **19**, 1150—1152 (2009).
5) Feng Z., Nenkep V. N., Yun K., Zhang D., Choi H. D., Kang J. S., Son B. W., *J. Microbiol. Biotechnol.*, **20**, 985—987 (2010).
6) Nenkep V. N., Yun K., Li Y., Choi H. D., Kang J. S., Son B. W., *J. Antibiot.*, **63**, 199—201 (2010).
7) Li Y., Li X., Son B. W., *Nat. Prod. Sci.*, **11**, 136—138 (2005).
8) Zhang Y., Ahn E.-Y., Jiang Y., Kim D.-K., Kang S.-G., Wu C., Kang S.-W., Park J.-S., Son B. W., Jung J. H., *Int. J. Oncology*, **31**, 1317—1323 (2007).
9) Smith R. V., Rosazza J. P., *J. Pharm. Sci.*, **64**, 1737—1759 (1975).
10) Stothers J. B., "Carbon-13 NMR Spectroscopy," Academic Press, London, 1972, p. 461.
11) Pretsch E., Buhlmann P., Affolter C., "Structure Determination of Organic Compounds, Tables of Spectral Data," Springer, Berlin, 2000, p. 153.
12) "The Merck Index, an Encyclopedia of Chemicals, Drugs, and Biologicals," Twelfth Edition; ed. by Budavari S., O'Neil M. J., Smith A., Heckelman P. E., Kinneary J. F., Merck & Co., New Jersey, 1996, p. 979.
13) van Boeckel C. A. A., Beetz T., Vos J. N., de Jong A. J. M., van Aelst S. F., van den Bosch R. H., Mertens J. M. R., van der Vlugt F. A., *J. Carbohydr. Chem.*, **4**, 293—321 (1985).
14) Zhou Y., Lin F., Chen J., Yu B., *Carbohydr. Res.*, **341**, 1619—1629 (2006).
15) Schmidt R. R., Michel J., *Angew. Chem. Int. Ed. Engl.*, **19**, 731—732 (1980).
16) Shibuya H., Kawashima K., Narita N., Ikeda M., Kitagawa I., *Chem. Pharm. Bull.*, **40**, 1154—1165 (1992).
17) Orabi K. Y., Clark A. M., Hufford C. D., *J. Nat. Prod.*, **63**, 396—398 (2000).
18) Shim H., Hong W., Ahn Y., *Bull. Korean Chem. Soc.*, **24**, 1680—1682 (2003).
19) Yim S.-H., Kim H. J., Lee I.-S., *Arch. Pharm. Res.*, **26**, 805—808 (2003).
20) Ranson M., *Br. J. Cancer*, **90**, 2250—2255 (2004).
21) Jorissen R. N., Walker F., Pouliot N., Garrett T. P., Ward C. W., Burgess A. W., *Exp. Cell Res.*, **284**, 31—53 (2003).