Microbial Metabolism of the Prenylated Chalcone Xanthohumol

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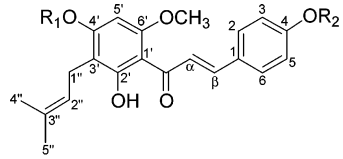
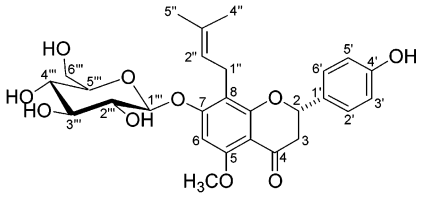
Microbial metabolism of xanthohumol (**1**), a prenylated chalcone isolated from hops, gave three novel glucosylated derivatives (**2**−**4**) and a known compound, isoxanthohumol (**5**). The structures of the new compounds were identified as xanthohumol 4′-*O*-*β*-glucopyranoside (**2**), xanthohumol 4,4′-*O*-*β*-diglucopyranoside (**3**), and 5-methoxy-8-prenylnaringenin 7-*O*-*β*-glucopyranoside (**4**) on the basis of spectroscopic methods.

The prenylated chalcone xanthohumol (**1**) (3′-[3,3-dimethylallyl]-2′,4′,4-trihydroxy-6′-methoxychalcone) is a major constituent isolated from the female inflorescences of *Humulus lupulus* L. (hops) (Cannabaceae), which are used to add bitterness and flavor to beer.[1,2] Various notable biological activities of **1** and its prenylated chalcone derivatives have been reported in recent years. Xanthohumol showed significant antioxidative and anti-inflammatory activities.[3,4] It has also been shown to be an effective antiproliferative[4,5] and cancer chemopreventive agent[4,6−8] in human cancer cell lines. It has also been reported to be a strong inhibitor of bone resorption[9] and a diacylglycerol acyltransferase inhibitor against arteriosclerosis.[10] Metabolism or biotransformation studies have been carried out in attempts to identify the metabolic fate of xanthohumol, and previous in vivo[11] and in vitro studies[12−14] provided several derivatives or metabolites including glucuronylated, hydroxylated, cyclized, and dehydrated metabolites. Microbial transformation studies of **1** with the fungi *Pichia membranifaciens* ATCC 2254 and *Cunninghamella echinulata* NRRL 3655 showed the presence of flavanone isomers and chalcone derivatives containing a dihydrofuran moiety.[15,16] Microbial transformation studies are useful to achieve selective conversions of compounds to derivatives that are difficult to produce synthetically, as well as to mimic mammalian metabolism.[17,18] In the present study of xanthohumol (**1**), a preparative-scale biotransformation by *Penicillium chrysogenum* and *Cunninghamella elegans* var. *elegans* afforded three novel glucosylated metabolites with chalcone or flavanone moieties. Production and structure elucidation of these metabolites (**2**−**4**) are reported herein.



1: R_1= H, R_2= H
2: R_1= β-glucopyranosyl, R_2= H
3: R_1= β-glucopyranosyl, R_2= β-glucopyranosyl



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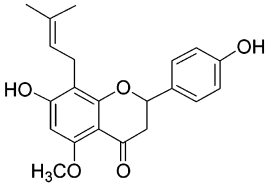

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Of 39 microbial cultures screened, *Rhizopus oryzae* KCTC 6946, *Penicillium chrysogenum* 6933, and *Cunninghamella elegans* var. *elegans* 6992 were selected for preparative-scale fermentation studies since they were found to be capable of biotransforming xanthohumol (**1**) on the basis of TLC analyses and control studies. The R_f values of the metabolites (**2**: R_f 0.22, **3**: 0.53, **4**: 0.57, and **5**: 0.21) were larger on RP-18 TLC in comparison with that of **1** (R_f 0.13), which indicated that more polar metabolites were produced. Substrate and culture controls confirmed that **2**−**5** were formed as a result of enzymatic activity, not as a consequence of degradation or nonmetabolic changes.

Metabolites **2** and **3** were produced by *P. chrysogenum* and isolated as yellow amorphous powders by column chromatography. Their UV spectra showed maximum absorption bands at 364 and 352 nm, respectively, similar to that of xanthohumol (**1**), which indicated that they were chalcone derivatives. IR spectra also showed close resemblance to those of **1**.

HRESIMS of metabolite **2** showed the $[M + H]^+$ peak at *m*/*z* 517.2072 (calcd for $C_{27}H_{33}O_{10}$, 517.2068), which established a molecular formula of $C_{27}H_{32}O_{10}$, indicating that it was a glycosylated derivative of **1**. Its ^{1}H NMR spectrum showed seven new characteristic signals typical of a sugar moiety, including a peak at δ_H 5.05 for an anomeric proton, in addition to the signals assignable to **1**. The ^{13}C NMR spectrum also exhibited six carbon signals between δ_C 62.9 and 101.9, which were not observed in **1**. The sugar was assigned to be glucopyranose on the basis of NMR data and the R_f value compared with that of glucose after acidic hydrolysis of **2**. The $J_{H,H}$ value (7.5 Hz) of the anomeric proton (H-1‴) at δ_H 5.05 indicated that this sugar had a *β*-configuration. The aromatic signals were shifted downfield, δ_C 162.8 (C-4′) as well as δ_H 6.44 (1H, s, H-5′) and δ_C 162.8 (C-5′), which suggested that the OH group at C-4′ was glucosylated by the fungus. These findings were confirmed by the HMBC correlation between H-1‴ and C-4′. Unambiguous assignment for both the aglycone and the sugar moiety was established by HSQC and HMBC experiments. The structure of metabolite **2** was established as xanthohumol 4′-*O*-*β*-glucopyranoside.

Metabolite **3** showed the $[M + H]^+$ peak at *m*/*z* 679.2580 (calcd for $C_{33}H_{43}O_{15}$, 679.2596), which established a molecular formula of $C_{33}H_{42}O_{15}$, indicating that it was a glycosylated derivative of **1**, with two sugar moieties. The ^{1}H and ^{13}C NMR spectra of **3** also showed seven and six characteristic signals of a sugar moiety, respectively, in addition to all the signals assignable to metabolite

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2. The two sugars were both identified as glucose on the basis of the large vicinal coupling constants ($J \approx 9$ Hz) and TLC identification pattern of monosaccharides after acidic hydrolysis. Compound **3** displayed aromatic signals downfield at δ_H 7.62 (2H, d, $J = 9$ Hz, H-2,6), 7.15 (2H, d, $J = 9$ Hz, H-3,5) and δ_C 131.2 (C-2,6), 118.2 (C-3,5) in the ^{1}H and ^{13}C NMR spectra, suggesting the glucosylated position to be at C-4. These findings were established by HMBC correlations between C-4′ and H-1‴ as well as between C-4 and H-1⁗. On the basis of these data, the structure of **3** was unambiguously assigned as xanthohumol 4,4′-*O*-*β*-diglucopyranoside.

Metabolite **4** was obtained as a white, amorphous powder by microbial transformation of **1** using *C. elegans* var. *elegans* with the glucosylated chalcone xanthohumol 4′-*O*-*β*-glucopyranoside (**2**). The UV spectrum of **4** showed a maximum absorption at 281 nm, which suggested that it was a flavanone derivative. HRESIMS of **4** showed the $[M + H]^+$ peak at *m*/*z* 517.2068 (calcd for $C_{27}H_{33}O_{10}$, 517.2068), which established a molecular formula of $C_{27}H_{32}O_{10}$, indicating that it was a glycosylated derivative. Several remarkable differences were shown in the ^{1}H and ^{13}C NMR spectra of **4**, when compared with those of **1**. While the ^{1}H and ^{13}C NMR signals due to the *trans*-olefinic bond disappeared, new signals typical of C-ring protons and carbons of a flavanone were observed at δ_H 5.36 (1H, dd, $J = 12.5$, 3 Hz, H-2), δ_H 2.99 (1H, dd, $J = 16.5$, 12.5 Hz, H-3), δ_H 2.65 (1H, dd, $J = 16.5$, 3 Hz, H-3), δ_C 78.0 (C-2), and δ_C 44.8 (C-2). The absolute configuration at the C-2 stereocenter was established to be *S* on the basis of the high-amplitude negative Cotton effect in the 270−300 nm region and the weak positive Cotton effect in the 325−350 region,[19] which indicated stereospecificity was achieved in the process of C-ring cyclization. In addition, seven proton and six carbon signals corresponding to a sugar were observed in the region ranging from δ_H 3.10 to 4.95 and from δ_C 60.9 (C-2) to δ_C 100.4 (C-2), respectively. The sugar was identified as glucose by TLC after acidic hydrolysis of **4**. The location of glucose was confirmed by HMBC correlation between H-1‴ and C-7. Unambiguous assignments for both the aglycone and the sugar moiety were established by HSQC and HMBC experiments. Thus, the structure of **4** was established as 5-methoxy-8-prenylnaringenin 7-*O*-*β*-glucopyranoside.

Metabolite **5** was produced by *R. oryzae* and was isolated as a white, amorphous powder. The UV spectrum of **5** showed a maximum absorption band at 290 nm, which suggested that it was a flavanone. On the basis of the spectroscopic data, **5** was identified as isoxanthohumol (5-methoxy-8-prenylnaringenin) by comparison with data in the literature.[1,4] Metabolite **5** did not show optical isomerism at C-2, which was confirmed by no absorption in its CD spectrum and no optical rotation.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP 1000 digital polarimeter. UV spectra were recorded on a JASCO V-530 spectrophotometer, and CD spectra were recorded on a JASCO J-715 CD/ORD spectropolarimeter. IR spectra were obtained on a JASCO FT/IR 300-E spectrometer. NMR experiments were recorded using a Varian Unity INOVA 500 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, and TMS was used as the internal standard. ESIMS and HRESIMS were determined on a Micromass QTQF2 mass spectrometer. EIMS was determined on a JEOL JMS-SX102A spectrometer. TLC was carried out on Merck silica gel F_{254}-precoated glass plates and RP-18 F_{254S} plates. Medium-pressure liquid chromatography (MPLC) was carried out with a Lobar column (10 × 240 mm) (Merck). HPLC was performed on a Waters 600E multisolvent delivery system connected to a UV detector using Supelco Supelcosil LC-SI (5 *μ*m, 10 × 250 mm) and Isco Allsphere ODS-2 (10 *μ*m, 10 × 250 mm) semipreparative columns.

Chemicals. Xanthohumol was isolated and purified from hop pellets, which were obtained from Hopsteiner in Germany, as described by Stevens et al.[1] The spectroscopic data of xanthohumol were in agreement with data in the literature.[1] Ingredients for microbial media including d-glucose, peptone, malt extract, yeast extract, and potato dextrose medium were purchased from Becton, Dickinson and Co.

Microorganisms and Fermentation of Cultures. All of the microorganisms were obtained from the Korean Collection for Type Cultures (KCTC). The cultures used for preliminary screening were as follows: *Absidia spinosa* KCTC 6588, *Alternaria alternata* 6005, *Aspergillus fumigatus* 6145, *Aspergillus niger* 6910, *Benisingtonia intermedia* 7207, *Candida albicans* 7965, *Candida famata* 7000, *Candida solani 7689*, *Cunninghamella elegans* var. *elegans* 6992, *Curvularia lunata* var. *lunata* 6919, *Debaryomyces hansenii* var. *hansenii* 7645, *Debaryomyces occidentalis* var. *occidentalis* 7194, *Debaryomyces robertsiae* 7299, *Filobasidium capsuligenum* 7102, *Filobasidium neoformans* 7902, *Fusarium oxysporum* 16325, *Gliocladium deliquescens* 6173, *Hormoconis resinae* 6966, *Kluyveromyces marxianus* 7155, *Metarhizium flavoviride* var. *minus* 6310, *Metschnikowia pulcherrima* 7605, *Microbacterium lacticum* 9230, *Mortierella ramanniana* var. *angulispora* 6137, *Monascus rubber* 6122, *Mycobacterium phlei* 3037, *Penicillium chrysogenum* 6933, *Pichia membranifaciens* 7006, *Pichia pastoris* 7190, *Polyporus arcularius* 6341, *Rhizopus oryzae* 6946, *Rhodotorula rubra* 7909, *Saccharomyces cerevisiae* 7904, *Saccharomycodes ludwigii* 7126, *Torulaspora delbrueckii* 7116, *Tremella mesenterica* 7131, *Trichoderma koningii* 6042, *Trichophyton mentagrphytes* 6085, *Trigonopsis variabilis* 7263, *Zygosaccharomyces rouxii 7191*.

Fermentation experiments were performed in three types of media; *F. oxysporum* f.sp. *lini*, *C. lunata* var. *lunata*, and *C. elegans* var. *elegans* were cultured on potato dextrose medium (24 g/L). *A. niger* was cultured on malt medium (Blekeslee's formula; malt 20 g/L, D-glucose 20 g/L, peptone 1 g/L). *A. alternata* and *P. membranifaciens* were incubated on malt medium (malt extract 20 g/L and peptone 5 g/L). Other microorganisms were cultured on yeast-malt medium (D-glucose 10 g/L, peptone 5 g/L, malt extract 3 g/L, and yeast extract 3 g/L).

Screening Procedures. Cultures for microbial transformation studies were grown according to the two-stage procedure. In the screening studies, the actively growing microbial cultures were inoculated in 100 mL flasks containing 20 mL of media and incubated with gentle agitation (200 rpm) at 25 °C in a temperature-controlled shaking incubator. The ethanolic solution (2 mg/0.1 mL) of **1** was added to each flask 24 h after inoculation and further incubated under the same conditions for 3 days. General sampling and TLC monitoring were performed on RP-18 TLC_{254S} (MeOH 70%) at 24 h intervals. UV light (254 and 356 nm) and anisaldehyde−sulfuric acid reagent were used for identification of metabolites. Substrate controls consisted of **1** and sterile YM medium incubated without microorganisms. Culture controls consisted of fermentation clutures in which the microorganisms were grown without addition of **1**.

Microbial Transformation of Xanthohomol (1) by *P. chrysogenum*. Preparative-scale fermentations were carried out under the same conditions with two 1 L flasks each containing 250 mL of medium and 50 mg of xanthohumol (**1**) for 6 days. The cultures were extracted with EtOAc two times, and the organic layers were combined and concentrated at reduced pressure. The EtOAc extract (850 mg) was subjected to silica gel (70−230 mesh, Merck) column chromatography with a $CHCl_3$−MeOH gradient solvent system (10:1→ 9:1→ 7:1→ 4:1→ 1:1) to provide seven fractions. Fraction 3 was further subjected to normal-phase HPLC with a $CHCl_3$−MeOH isocratic mixture (92:8, flow rate: 4 mL/min, UV detection: 360 nm) to give the metabolite **2** (8 mg, 5.5% yield). Fraction 6 was chromatographed on reversed-phase HPLC, eluting with 50% MeOH, to afford the metabolite **3** (2 mg, 1.0% yield).

Xanthohumol 4′-*O*-*β*-glucopyranoside (2): yellow, amorphous powder; $[\alpha]_D$ −153.2 (*c* 0.3, MeOH); UV λ_{max} (MeOH) (log ϵ) 364 (4.55) nm; IR (KBr) ν_{max} 3400, 2925, 1606, 1513, 1417, 1337, 1230, 1170, 1074, 832 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.79 (1H, d, $J = 15.8$ Hz, H-*β*), 7.72 (1H, d, $J = 15.8$ Hz, H-α), 7.52 (2H, d, $J = 8.5$ Hz, H-2,6), 6.84 (2H, d, $J = 8.5$ Hz, H-3,5), 6.44 (1H, s, H-5′), 5.22 (1H, br t, $J = 7.3$ Hz, H-2″), 5.05 (1H, d, $J = 7.5$ Hz, H-1‴), 3.97 (3H, s, OCH_3), 3.94 (1H, dd, $J = 12$, 2 Hz, H-6‴a), 3.67 (1H, dd, $J = 12$, 7.3 Hz, H-6‴b), 3.54 (1H, t, $J = 9$ Hz, H-2‴), 3.52 (1H, m, H-5‴), 3.49 (1H, t, $J = 9$ Hz, H-3‴), 3.41 (1H, dd, $J = 13.8$, 7.8 Hz, H-1″a), 3.35 (1H, t, $J = 9.5$ Hz, H-4‴), 3.24 (1H, dd, $J = 13.8$, 7 Hz, H-1″b), 1.78 (3H, s, H-5″), 1.64 (3H, s, H-4″); ^{13}C NMR (CD_3OD, 125 MHz)

δ 195.0 (C═O), 165.0 (C-2′), 162.8 (C-4′), 162.5 (C-6′), 161.4 (C-4), 144.3 (C-β), 131.8 (C-3″), 131.6 (C-2,6), 128.5 (C-1), 125.7 (C-α), 124.3 (C-2″), 117.1 (C-3,5), 112.2 (C-3′), 108.4 (C-1′), 101.9 (C-1‴), 91.7 (C-5′), 78.9 (C-5‴), 78.6 (C-3‴), 75.1 (C-2‴), 71.8 (C-4‴), 62.9 (C-6‴), 56.7 (O*C*H$_3$), 26.1 (C-5″), 22.6 (C-1″), 18.2 (C-4″); ESIMS *m*/*z* 517 [M + H]$^+$; HRESIMS *m*/*z* 517.2072 [M + H]$^+$ (calcd for $C_{27}H_{33}O_{10}$, 517.2068).

Xanthohumol 4,4′-*O*-β-diglucopyranoside (3): yellow, amorphous powder; [α]$_D$ −94.4 (*c* 0.2, MeOH); UV λ_{max} (MeOH) (log ϵ) 352 (4.52) nm; IR (KBr) ν_{max} 3423, 2924, 1618, 1509, 1232, 1074 cm^{-1}; ^{1}H NMR (CD$_3$OD, 500 MHz) δ 7.85 (1H, d, *J* = 15.5 Hz, H-β), 7.73 (1H, d, *J* = 15.5 Hz, H-α), 7.62 (2H, d, *J* = 9 Hz, H-2,6), 7.15 (2H, d, *J* = 9 Hz, H-3,5), 6.45 (1H, s, H-5′), 5.22 (1H, br t, *J* = 7 Hz, H-2″), 5.05 (1H, d, *J* = 7.5 Hz, H-1‴), 4.99 (1H, d, *J* = 7.5 Hz, H-1⁗), 3.97 (3H, s, OC*H*$_3$), 3.94 (1H, dd, *J* = 12, 2 Hz, H-6‴a), 3.91 (1H, dd, *J* = 12.3, 2.5 Hz, H-6⁗a), 3.71 (1H, dd, *J* = 12, 7 Hz, H-6⁗b), 3.67 (1H, dd, *J* = 12, 7 Hz, H-6‴b), 3.54 (1H, t, *J* = 9.5 Hz, H-2‴), 3.53 (1H, m, H-5‴), 3.51 (1H, m, H-5⁗), 3.49 (1H, t, m, H-3‴), 3.48 (1H, t, m, H-2⁗), 3.47 (1H, t, m, H-3⁗), 3.42 (1H, m, H-1″a), 3.40 (1H, t, m, H-4⁗), 3.35 (1H, t, *J* = 9.5 Hz, H-4‴), 3.24 (1H, m, H-1″b), 1.78 (3H, s, H-5″), 1.64 (3H, s, H-4″); ^{13}C NMR (CD$_3$OD, 125 MHz) δ 195.0 (C═O), 165.0 (C-2′), 162.9 (C-4′), 162.6 (C-6′), 161.1 (C-4), 143.3 (C-β), 131.8 (C-3″), 131.2 (C-2,6), 130.3 (C-1), 127.4 (C-α), 124.3 (C-2″), 118.2 (C-3,5), 112.2 (C-3′), 108.4 (C-1′), 102.1 (C-1⁗), 101.9 (C-1‴), 91.7 (C-5′), 78.9 (C-5‴), 78.6 (C-3‴), 78.4 (C-5⁗), 78.1 (C-3⁗), 75.1 (C-2‴), 75.0 (C-2⁗), 71.8 (C-4‴), 71.5 (C-4⁗), 62.9 (C-6″), 62.6 (C-6⁗), 56.7 (O*C*H$_3$), 26.1 (C-5″), 22.6 (C-1″), 18.2 (C-4″); ESIMS *m*/*z* 679 [M + H]$^+$, 701 [M + Na]$^+$; HRESIMS *m*/*z* 679.2580 [M + H]$^+$ (calcd for $C_{33}H_{43}O_{15}$, 679.2596).

Microbial Transformation of 1 by *C. elegans* var. *elegans*. Preparative-scale fermentations were carried out under the same conditions with two 1 L Erlenmeyer flasks each containing 500 mL of medium and 100 mg of xanthohumol (**1**) for 5 days. The cultures were extracted with EtOAc two times, and the organic layers were combined and concentrated at reduced pressure. The EtOAc extract (460 mg) was subjected to silica gel (70−230 mesh, Merck) column chromatography with a CHCl$_3$−MeOH gradient solvent system (10:1→ 9:1→ 8:1→ 7:1→ 6:1) to provide four fractions. Fraction 2 was further subjected to reversed-phase MPLC with 50% MeOH (2 mL/min) to give the metabolite **2** (35 mg, 12.0% yield). Fraction 3 was chromatographed on reversed-phase MPLC eluting with 53% MeOH (2 mL/min), to afford the metabolite **4** (27 mg, 9.3% yield).

5-Methoxy-8-prenylnaringenin 7-*O*-β-glucopyranoside (4): white, amorphous powder; [α]$_D$ −54.4 (*c* 0.2, MeOH); UV λ_{max} (MeOH) (log ϵ) 281 (4.41), 397 (3.77) nm; CD (MeOH) $\Delta\epsilon$ 283 (−30.9), 344 (+10.1); IR (KBr) ν_{max} 3421, 2922, 1652, 1601, 1520, 1345, 1279, 1099 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 9.54 (1H, s, 4′-O*H*), 7.30 (2H, d, *J* = 8.5 Hz, H-2′,6′), 6.79 (1H, d, *J* = 8.5 Hz, H-3′,5′), 6.48 (1H, s, H-6), 5.36 (1H, dd, *J* = 12.5, 3 Hz, H-2), 5.14 (1H, br t, *J* = 7.2 Hz, H-2″), 4.95 (1H, d, *J* = 7.5 Hz, H-1‴), 3.77 (3H, s, OC*H*$_3$), 3.74 (1H, dd, *J* = 12, 2 Hz, H-6‴a), 3.44 (1H, m, H-6‴b), 3.42 (1H, m, H-5‴), 3.34 (1H, m, H-1″a), 3.32 (1H, m, C-3‴), 3.31 (1H, m, C-2‴), 3.13 (1H, m, C-4‴), 3.08 (1H, dd, *J* = 13.5, 7 Hz, H-1″b), 2.99 (1H, dd, *J* = 16.5, 12.5 Hz, H-3a), 2.63 (1H, dd, *J* = 16.5, 3 Hz, H-3b), 1.59 (3H, s, H-5″), 1.56 (3H, s, H-4″); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 188.8 (C-4), 160.7 (C-7), 160.5 (C-8a), 159.6 (C-5), 157.4 (C-4′), 130.1 (C-3″), 129.5 (C-1′), 127.9 (C-2′,6′), 122.7 (C-2″) 115.1 (C-3′,5′), 109.8 (C-8), 106.1 (C-4a), 100.4 (C-1‴), 92.6 (C-6), 78.0 (C-2), 77.5 (C-5‴), 76.9 (C-3‴), 73.3 (C-2‴), 70.1 (C-4‴), 60.9 (C-6‴), 55.6 (O*C*H$_3$), 44.8 (C-3), 25.6 (C-5″), 21.7 (C-1″), 17.6 (C-4″); ESIMS *m*/*z* 517 [M + H]$^+$, 539 [M + Na]$^+$; HRESIMS *m*/*z* 517.2068 [M + H]$^+$ (calcd for $C_{27}H_{33}O_{10}$, 517.2068).

Microbial Transformation of 1 by *R. oryzae*. Scale-up fermentations were performed with six 1 L flasks each containing 250 mL of medium and 35 mg of xanthohumol (**1**) for 12 days under the same conditions. The cultures were extracted with EtOAc two times, and the organic layers were combined and concentrated at reduced pressure. The EtOAc extract (320 mg) was subjected to silica gel (70−230 mesh, Merck) column chromatography with a CHCl$_3$−MeOH gradient solvent system (30:1→ 20:1) to provide six fractions. Fraction 4 was further subjected to reversed-phase MPLC with 60% MeOH (2 mL/min) to give the metabolite **5** (89 mg, 42.3% yield).

Acid Hydrolysis of 2−4. Solutions of compounds **2**, **3**, and **4** (1−3 mg) in 2 N HCl were heated for 2 h. After cooling, each mixture was neutralized and partitioned between EtOAc and H$_2$O, respectively. The aqueous layer was concentrated and developed by cellulose TLC (BuOH−C$_6$H$_6$−C$_6$H$_5$N−H$_2$O = 5:1:3:3, R_f 0.15) in comparison with authentic glucose.

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