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Biotransformation of gallic acid by *Beauveria sulfurescens* ATCC 7159

Feng-Lin Hsu · Li-Ming Yang · Shwu-Fen Chang · Li-Hsuan Wang · Chung-Yi Hsu · Pan-Chun Liu · Shwu-Jiuan Lin

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Abstract Preparative-scale fermentation of gallic acid (3,4,5-trihydroxybenzoic acid) (**1**) with *Beauveria sulfurescens* ATCC 7159 gave two new glucosidated compounds, 4-(3,4-dihydroxy-6-hydroxymethyl-5-methoxy-tetrahydro-pyran-2-yloxy)-3-hydroxy-5-methoxy-benzoic acid (**4**), 3-hydroxy-4,5-dimethoxy-benzoic acid 3,4-dihydroxy-6-hydroxymethyl-5-methoxy-tetrahydro-pyran-2-yl ester (**7**), along with four known compounds, 3-*O*-methylgallic acid (**2**), 4-*O*-methylgallic acid (**3**), 3,4-*O*-dimethylgallic acid (**5**), and 3,5-*O*-dimethylgallic acid (**6**). The new metabolite genistein 7-*O*-β-D-4″-*O*-methyl-glucopyranoside (**8**) was also obtained as a byproduct due to the use of soybean meal in the fermentation medium. The structural elucidation of the metabolites was based primarily on 1D-, 2D-NMR, and HRFABMS analyses. Among these compounds, **2**, **3**, and **5** are metabolites of gallic acid in mammals. This result demonstrated that microbial culture parallels mammalian metabolism; therefore, *B. sulfurescens* might be a useful tool for generating mammalian metabolites of related analogs of gallic acid (**1**) for complete structural identification and for further use in investigating pharmacological and toxicological properties in this series of compounds. In addition, a GRE (glucocorticoid response element)-mediated luciferase reporter gene assay was used to initially screen for the biological activity of the 6 compounds, **2**–**6** and **8**, along with **1** and its chemical *O*-methylated derivatives **9**–**13**. Among the 12 compounds tested, **11**–**13** were found to be significant, but less active than the reference compounds of methylprednisolone and dexamethasone.

Keywords Biotransformation · Gallic-acid · *Beauveria sulfurescens*

F.-L. Hsu
Graduate Institute of Pharmacognosy Science,
College of Pharmacy, Taipei Medical University,
250 Wu-Hsing St., Taipei 110, Taiwan

L.-M. Yang · L.-H. Wang · P.-C. Liu · S.-J. Lin (✉)
Department of Medicinal Chemistry, College of Pharmacy,
Taipei Medical University,
250 Wu-Hsing St., Taipei 110, Taiwan
e-mail: shwu-lin@tmu.edu.tw

L.-M. Yang (✉)
Division of Medicinal Chemistry,
National Research Institute of Chinese Medicine,
155-1, Li-Nong St., Sec. 2, Taipei 112, Taiwan
e-mail: lmyang@nricm.edu.tw

S.-F. Chang
Graduate Institute of Cell and Molecular Biology,
College of Medicine, Taipei Medical University,
250 Wu-Hsing St., Taipei 110, Taiwan

S.-F. Chang · C.-Y. Hsu
Graduate Institute of Neuroscience, College of Medicine,
Taipei Medical University,
250 Wu-Hsing St., Taipei 110, Taiwan

Introduction

Gallic acid (3,4,5-trihydroxybenzoic acid) (**1**) is one of the main endogenous phenolic acids found in plants in a free or esterified form. It is distributed in a large amount in tea, being present at about 5% of the dry weight (Harbowy and Ballentine 1997). Gallic acid (**1**) has been found to be pharmacologically active, which possesses antioxidative (Fukumoto and Mazza 2000), antimutagenic, anticarcinogenic (Akao et al. 2001; Saeki et al. 2000; Stich and Rosin 1984), antiinflammatory (Kawada et al. 1992), and

hepatoprotective activities (Anand et al. 1997). The metabolism and pharmacokinetics of **1** have been studied in human and various animal species, and 4-*O*-methylgallic acid (**3**) has been reported to be the major metabolite of **1** (Booth et al. 1959; Shahrazd and Bitsch 1998; Shahrazd et al. 2001; Yasuda et al. 2000; Zong et al. 1999). Other minor metabolites such as 3-*O*-methylgallic acid (**2**), 3,4-*O*-dimethylgallic acid (**5**), and pyrogallol (both the conjugated and unconjugated forms) have been reported as well. In addition, the metabolism of related substances such as black tea and tannic acid has also been studied, and it was revealed that **1** and its metabolites (**2**, **3**, and **5**) were detected in urine after given orally to humans and rats (Hodgson et al. 2000; Nakamura et al. 2003).

Inflammation is how the body copes with infections and tissue damage. Glucocorticoids (GCs) are the most effective drugs for preventing and suppressing inflammation caused by mechanical, chemical, infectious, and immunological stimuli. One major mechanism for GCs' to exert their activity is through binding to the glucocorticoid receptor (GR) resulting in either activation or repression of a large set of glucocorticoid responsive genes. The GR is a transcriptional regulator that upon binding to cognate ligands, occupies specific genomic glucocorticoid response elements (GREs) and modulates the transcription of nearby genes (Wang et al. 2006). Glucocorticoids such as dexamethasone and prednisolone have long been considered some of the most potent antiinflammatory agents. However, they have side effects due to inhibition of other steroid receptors (Necela and Cidlowski 2004). Thus, discovering glucocorticoid receptor agonists that exhibit a reduced incidence or reduced severity of side effects while maintaining potent antiinflammatory activity is currently a very demanding goal (Rosen et al. 2003).

Microorganisms and their enzymes are increasingly being used as biocatalysts to alter the structures of organic compounds. Utilization of microbes as models for mammalian metabolism of xenobiotics was postulated in the early 1970s (Smith and Rosazza 1974, 1975). Since then, the use of selected microorganisms as "models for xenobiotic metabolism" has been successfully exploited under similar circumstances in mammalian metabolism studies of diverse compounds to aid in predicting metabolic pathways where the direct isolation of mammalian metabolites proves difficult. Several reviews and updates on microbial models of mammalian metabolism have been published (Srisilam and Veeresham 2003; Venisetty and Ciddi 2003). To use microbial transformation to produce mammalian metabolites and/or novel analogs for biological screening (Hsu et al. 2002; Yang et al. 2004), the two-stage screening method routinely used in our laboratory was employed to identify microorganisms capable of biotransforming gallic acid (**1**). Among a range of microorganisms screened, *Beauveria sulfurescens* ATCC 7159 was proven to be able to generate several related biotransformation products. To the best of our knowledge, two reports on the microbial transformation of **1** have been conducted with *Pseudomonas putida* (Tack et al. 1972) and *Penicillium chrysogenum* (Rajakumar and Nandy 1986) which formed oxaloacetate, pyruvate, and oxalic acid. The aim of this work, therefore, was to identify a microorganism that can extensively metabolize gallic acid (**1**) and to chemically identify the metabolites. If the metabolic profile of gallic acid (**1**) by this microorganism is similar to that in mammals to some extent, then the microorganism might be used as a model in the studies of mammalian metabolism of other analogs of gallic acid and phenolic compounds in mammalian metabolism. Because gallic acid (**1**) possesses the antiinflammatory activity (Kawada et al. 1992), a GRE (glucocorticoid response element)-mediated luciferase reporter gene assay was used to initially screen to determine if **1** and related compounds are potential glucocorticoid agonists. This paper described the isolation and characterization of these metabolites produced by *Beauveria sulfurescens*. The GRE assay of **1** and related compounds are also presented.

Materials and methods

General

Melting points were determined using a Yanagimoto micromelting point apparatus and were uncorrected. Optical rotations were determined on JASCO DIP-1020 digital polarimeters. ^{1}H- and ^{13}C NMR analyses were performed on Bruker AM-500 spectrometers in CD_3OD and DMSO-d_6, using the corresponding solvent as the internal standard. NMR experiments included ^{1}H-^{1}H COSY, DEPT, NOESY, HMQC, and HMBC. Coupling constants (*J* values) were given in Hertz (Hz). Multiplicities of all carbon signals were verified through DEPT experiments. Low- and high-resolution FAB mass spectra were obtained using a JEOL JMX-HX 110 spectrometer. IR spectra were measured on a Perkin-Elmer spectrum GX/AutoImage microscope FT-IR spectrometer. Column chromatography was performed with MCI-gel CHP 20P (75–150 μm, Mitsubishi Chemical, Tokyo, Japan) and Kieselgel silica (230–400 mesh, Merck, Darmstadt, Germany). Compounds on TLC plates were visualized using $FeCl_3$ (5% *w*/*v* in EtOH) and 10% H_2SO_4 spray reagents.

Substrate

Gallic acid (**1**) was previously isolated from *Terminalia arborea*, and was characterized by ^{1}H- and ^{13}C NMR (Lin and Hsu 1996).

Microorganism

B. sulfurescens culture was maintained on Sabouraud–maltose agar slant in a sealed screw-cap tube in a refrigerator at 4°C until ready for use.

Fermentation procedure

B. sulfurescens ATCC 7159 was grown on medium comprising dextrose (20 g/l), yeast (5 g/l), NaCl (5 g/l), K_2HPO_4 (5 g/l), and soybean flour (5 g/l). The medium was adjusted to pH 7.0 before sterilization by autoclaving at 121°C for 15 min. The fermentation procedure was carried out by a previously described two-stage procedure (Yang et al. 2004). Gallic acid (**1**) (1.8 g) in dimethylformamide (DMF) (18 ml) was evenly distributed among 180 flasks on the fourth day after inoculation. Fermentation was further continued for 6 days. Culture control consisted of a fermentation blank in which microorganism was grown under identical conditions except for no substrate added. Substrate control consisted of sterile medium containing the same amounts of substrate and incubated under the same conditions. The incubation mixture was pooled and acidified with 6 N HCl and then filtered to remove cells. The filtrate was extracted three times with equal volumes of EtOAc-*n*-butanol (9:1). The combined extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated to give 6.9 g of a red residue.

Isolation of metabolites

The crude residue was chromatographed on a MCI-gel CHP 20P (75–150 μm, 4×60 cm) column eluted with H_2O–CH_3OH (stepwise gradient from 100% H_2O to 100% CH_3OH) and the eluates were monitored using benzene–ethyl formate–formic acid (5:4:1 and 2:7:1) as the TLC solvent system. In total, 35 fractions were combined based on the similar TLC profiles. Chromatography of the combined fractions 3–5 (1.2 g) on silica gel (230–400 mesh, 3×50 cm), eluted with CH_2Cl_2–CH_3OH (10:1), gave substrate **1** (410 mg, 22.8%). Further chromatography of the combined fractions 8 and 9 (0.7 g) over silica gel (230–400 mesh, 3×40 cm) eluted with a gradient of CH_3OH in CH_2Cl_2 gave **3**. After recrystallization with H_2O, 75 mg (3.8%) of **3** was obtained. Further column chromatography of the combined fractions 11–13 (0.5 g) over silica gel (230–400 mesh, 3×40 cm) using CH_2Cl_2 gradually enriched with CH_3OH yielded **2**. After recrystallization with H_2O, 70 mg (3.6%) of **2** was obtained as rectangle crystals. Fractions 14–18 (1.04 g) were repeatedly chromatographed on silica gel (230–400 mesh, 3×40 cm) eluted with a gradient of CH_3OH in CH_2Cl_2 to give **4**. After recrystallization with H_2O, 145 mg (3.8%) of **4** was obtained as a white amorphous powder. Further chromatography of fractions 20–22 (0.15 g) over silica gel (230–400 mesh, 1×30 cm) eluted with CH_2Cl_2–CH_3OH (12:1) yielded **5**. After recrystallization with H_2O, 64 mg (3.1%) of **5** was obtained. Fractions 24 and 25 (0.35 g) were chromatographed on silica gel (230–400 mesh, 1×40 cm) eluted with CH_2Cl_2–CH_3OH (12:1) to yield **6**. After recrystallization with H_2O, 112 mg (5.3%) of **6** was obtained as a white amorphous powder. Fractions 27 and 28 (100 mg) were chromatographed over silica gel (230–400 mesh, 1×20 cm) eluted with a gradient of CH_2Cl_2–CH_3OH [100 ml each of 15:1, 12:1, 9:1, 6:1:0.5 (H_2O)] to yield 8 mg (0.2%) of **7** as an oil. Further chromatography of fractions 31–33 (330 mg) over silica gel (230–400 mesh, 2×34 cm) eluted with a gradient of CH_2Cl_2–CH_3OH (10:1) yielded 28 mg (0.59%) of **8** as a white amorphous powder.

Compound **4**: White amorphous powder; $[\alpha]^{25}_D$–18.1 (*c* 1.1, MeOH); IR (KBr) ν_{max} 3467, 3419, 3235, 1715, 1698, 1598 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6) (see Table 1); LRFABMS *m/z* 361 $[M + H]^+$; HRFABMS *m/z* 361.1134 (calcd for $C_{15}H_{21}O_{10}$, 361.1135).

Compound **7**: Oil; $[\alpha]^{25}_D$ + 47.8 (*c* 1.0, MeOH); ^1H and ^{13}C NMR (CD_3OD) (see Table 1); LRFABMS *m/z* 375 $[M + H]^+$; HRFABMS *m/z* 375.1293 (calcd for $C_{16}H_{23}O_{10}$, 375.1291).

Compound **8**: White amorphous powder; $[\alpha]^{25}_D$–40.4 (*c* 1.0, MeOH); IR (KBr) ν_{max} 3416, 3281, 3147, 1656, 1612 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6) (see Table 1); LRFABMS *m/z* 447 $[M + H]^+$; HRFABMS *m/z* 447.1289 (calcd for $C_{22}H_{23}O_{10}$, 447.1291).

O-Methylation of gallic acid (**1**)

The synthesis of *O*-methylated gallic acid was prepared according to the procedures described previously (Hodgson et al. 2000; Luz Cardona et al. 1986). The identity of **9**–**13** was verified by ^1H and ^{13}C NMR, and by comparison with that described in the literature (Keck et al. 2000; Parmar et al. 1997; Wipf and Weiner 1999; Wu et al. 1992).

Transfection procedures and reporter gene assays

Twenty-four hours before transfection, about 1×10^5 mouse Raw 264.7 macrophage cells per well were seeded in 96-well white plates. The pGR-Luc plasmid and an internal control plasmid, the pGL-*hRluc*, were transfected into Raw 264.7 cells using the lipofectamine plus agent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. At 24 h posttransfection, final concentrations of 10 μM of each test compound including the reference compounds, methylprednisolone, and dexamethasone (Sigma, St. Louis, MO, USA), in DMSO were added to the cells. Cells were harvested 24 h after

Table 1 ^{1}H and ^{13}C NMR data of **4**, **7**, and **8** (DMSO-d_6, δ values in ppm)[a,b]

Position	**4**		**7**[c]		**8**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	126.5		125.9			
2	105.0	7.03 (*d*, 1.5)	106.4	7.16 (*d*, 1.8)	154.5	8.41 (*s*)
3	152.5		154.4		122.6	
4	137.4		142.6		180.5	
5	150.0		151.7		161.6	
5-OH						12.9 (*s*)
6	110.8	7.06 (*d*, 1.5)	112.5	7.17 (*d*, 1.8)	99.5	6.46 (*d*, 2)
7	166.9		166.5		162.9	
3-OCH_3	56.2	3.77 (*s*)	56.6	3.81 (*s*)		
4-OCH_3			61.0	3.77 (*s*)		
8					94.5	6.70 (*d*, 2)
9					157.2	
10					106.1	
1′	103.7	4.81 (*d*, 7.6)	96.2	5.57 (*d*, 8.5)	121.0	
2′	74.1	3.30 (*t*, 9)	74.1	3.42 (*t*, 9)		
2′,6′					130.1	7.38 (2H, *d*, 8.5)
3′	75.9	3.36 (*t*, 9)	78.1	3.48 (*m*)		
3′,5′					115.0	6.81 (2H, *d*, 8.5)
4′	78.9	3.96 (*t*, 10)	80.3	3.15 (*t*, 9)	157.4	
4′-OH						9.52 (*s*)
5′	75.8	3.15 (*ddd*, 10, 4, 1.5)	77.9	3.33 (*m*)		
6′	60.2	3.46 (*dd*, 12, 4) 3.56 (*d*, 12)	61.8	3.62 (*dd*, 12, 4) 3.74 (*d*, 12)		
4′-OCH_3	59.6	3.43 (*s*)	60.8	3.52 (*s*)		
1″					99.4	5.07 (*d*, 7.8)
2″					73.2	3.24 (*m*)
2″-OH						5.46 (*d*, 5)
3″					76.0	3.41 (*m*)
3″-OH						5.28 (*d*, 5)
4″					78.8	3.03 (*t*, 9)
4″-OCH_3					59.6	3.45 (*s*)
5″					75.6	3.48[d] (*m*)
6″					60.1	3.48[d] (*m*) 3.62 (*dd*, 10, 5)
6″-CH_2OH						4.70 (*br s*)

[a] Assignments based on DEPT, HMQC, and HMBC
[b] Signal multiplicity and coupling constants (Hz) are in parentheses.
[c] In CD_3OD
[d] Overlapping signals

treatment, and the reporter activity of firefly luciferase expressed from pGR-Luc, and *Renilla* luciferase from pGL-*hRluc* were assayed in a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA) using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical analysis

Data are from at least three individual experiments. The averages of the firefly/*Renilla* luciferase ratios were analyzed by two-tailed Student's *t* test for paired samples. Significance was accepted when p was <0.05.

Results

Fermentation of gallic acid (**1**) with *B. sulfurescens* ATCC 7159 for 10 days yielded four known *O*-methylated metabolites and three new glucosidated metabolites (Fig. 1) that were separated by column chromatography onto MCI gel and silica gel. The four known metabolites were characterized as 3-*O*-methylgallic acid (**2**), 4-*O*-methylgallic acid (**3**), 3,4-*O*-dimethylgallic acid (**5**), and 3,5-*O*-dimethylgallic acid (**6**) on the basis of 1D and 2D NMR (^{1}H, ^{13}C, HMBC, HMQC, and FABMS), and the experimental data for **2**, **3**, **5**, and **6** were in agreement with values published previously (Hattori et al. 1989; Inoshiri et

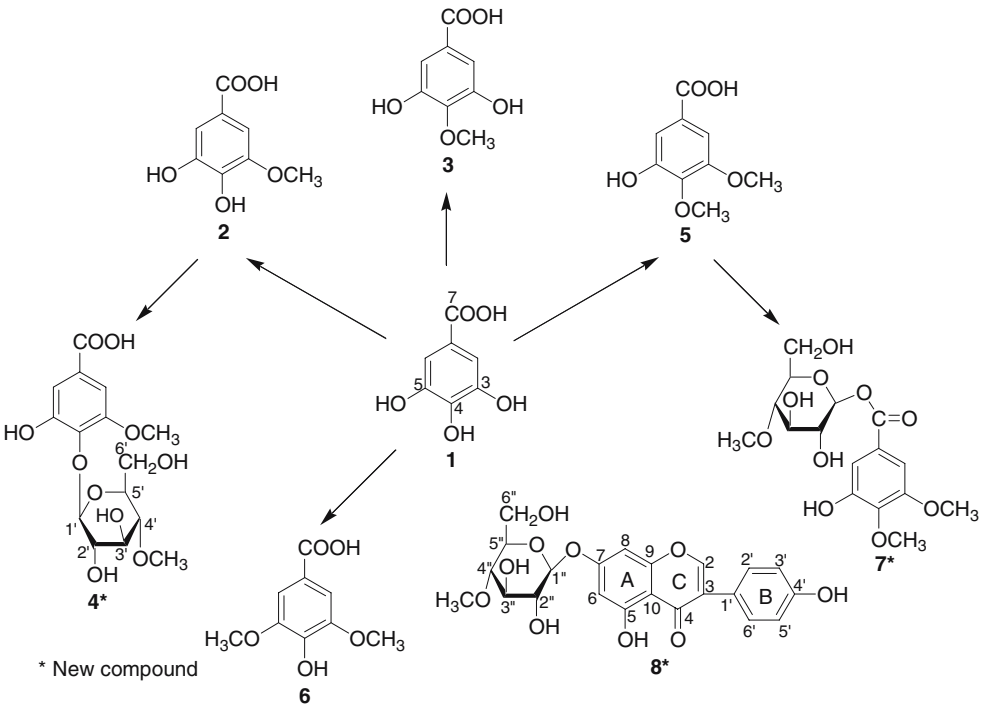

Fig. 1 Biotransformation of gallic acid (**1**) by *Beauveria sulfurescens*

al. 1987; Wu et al. 1992). Metabolite **4**, a white amorphous powder, was assigned the molecular formula $C_{15}H_{21}O_{10}$, as determined by a combination of positive ion HRFABMS (*m/z* 361.1134, calcd for $C_{15}H_{21}O_{10}$, 361.1135) and ^{13}C NMR analyses. In addition, signals for the gallic acid moiety, two methoxyl groups (δ_H 3.43, 3.77; δ_C 59.6, 56.2), and a glucopyranosyl unit (δ_C 103.7, 74.1, 75.9, 78.9, 75.8, and 60.2) were observed in the ^1H and ^{13}C NMR, and DEPT spectra. For the sugar unit, the COSY spectrum indicated a β-configuration at the anomeric position (δ_H 4.81, *d*, $J_{H\text{-}1'\text{–}H\text{-}2'}$=7.6 Hz) (Wei et al. 2004). Besides the sugar signals, the ^1H NMR spectrum also exhibited two *meta*-coupled aromatic signals (δ 7.03 and 7.06, *J*=1.5 Hz), which were assigned to the protons of the gallic acid residue. The catechol moiety was not retained as in gallic acid, as determined by the lack of the intense green coloration after $FeCl_3$ reagent spraying. This thus suggested that the two substituents were at C-3/C-4 or C-4/C-5 of gallic acid. The relative position of one methoxyl group at C-3 was established by the HMBC correlation observed between the methoxyl signal (δ_H 3.77, δ_C 56.2) and C-3 (δ_C 152.5). In addition, the anomeric proton signal (H-1′) at δ_H 4.81 showed a cross-peak with C-4 (δ_C 137.4) in the gallic acid moiety. This result indicates that the glucopyranosyl moiety is attached at the C-4 position of gallic acid. The location of a second methoxyl signal in **4** was deduced by correlating the methoxyl signal at δ_H 3.43 with C-4′ of the glucopyranosyl unit (δ_C 78.9). Based on the ^1H and ^{13}C NMR spectra with the aid of 2D-NMR experiments, metabolite **4** was identified as 4-(3,4-dihydroxy-6-hydroxymethyl-5-methoxy-tetrahydro-pyran-2-yloxy)-3-hydroxy-5-methoxy-benzoic acid.

The positive HRFABMS of metabolite **7** showed an $[M + H]^+$ ion at *m/z* 375.1293, consistent with the molecular formula of $C_{16}H_{23}O_{10}$, which was also deduced using ^{13}C- and DEPT NMR analysis. The ^{13}C NMR and DEPT spectra displayed three methyl, one methylene, seven methine, and five quaternary carbons. The ^1H and ^{13}C NMR spectra were very similar to those of **4**, except for the additional signal ascribable to a methoxyl group (δ_H 3.77, δ_C 61.0). In the HMBC spectrum, one of the methoxyl signals at δ_H 3.52 (δ_C 60.8) was correlated with C-4′ of glucopyranosyl unit (δ_C 80.3). Thus, a 4′-*O*-methoxy-glucosidic moiety also existed in metabolite **7**. The ^1H NMR spectrum additionally exhibited two *meta*-coupled aromatic signals (δ 7.16 and 7.17, *J*=1.8 Hz). Examination of the HMQC and HMBC spectra allowed us to assign an additional methoxyl group (δ_H 3.77) at C-4, which showed a cross-peak with a signal at δ_C 142.6. The sugar moiety was confidently linked to the carbonyl group of gallic acid (**1**) based on the HMBC correlation between δ_C 166.5 (C=O) and H-1′ (δ_H 5.57). The characteristic anomeric proton of the sugar was shown as a doublet with the coupling constant, *J*=8.5 Hz, indicating that the 4′-*O*-methoxy-glucosidic linkage has a β-D configuration (Wei et al. 2004). Hence, metabolite **7** was assigned as 3-hydroxy-4,5-dimethoxy-benzoic acid 3,4-dihydroxy-6-hydroxymethyl-5-methoxy-tetrahydro-pyran-2-yl ester.

Metabolite **8** showed a quasimolecular ion peak at *m/z* 447 $[M + H]^+$. The positive HRFABMS gave a quasimolecular ion peak at *m/z* 447.1289 $[M + H]^+$ corresponding to

the molecular formula $C_{22}H_{23}O_{10}$. The ^{1}H and ^{13}C NMR spectra also clearly showed the presence of a sugar moiety at δ_H 3.03–5.07 ppm and δ_C 60.1–99.4 ppm. The ^{13}C NMR spectrum displayed resonances for 20 carbons, while the DEPT spectrum showed the presence of one methyl, one methylene, ten methine, and eight quaternary carbons. The DEPT experiment of the sugar moiety displayed one CH_2 group at δ 60.1 ppm and five CH groups at δ 99.4, 78.8, 76.0, 75.6, and 73.2 ppm. These signals were the glucopyranosyl unit with the anomeric proton resonating at δ 5.07 ppm with J=7.8 Hz. The coupling constant agreed with a β–D configuration for the glucopyranosyl unit in the metabolite (Wei et al. 2004). ^{13}C NMR, DEPT, and HMQC measurements revealed an aglycone moiety having five methine, seven quaternary, and one carbonyl group carbons. A singlet signal at δ_H 8.41 (δ_C 154.5) suggested the presence of typical of an H-2 isoflavone proton (Erasto et al. 2004). Two *meta*-coupled aromatic signals (δ 6.46 and 6.70, J=2.0 Hz) and a chelated hydroxyl proton at δ 12.9 ascribable to the protons of the A ring of isoflavone skeleton. In the HMBC spectrum, the methine proton at δ 6.46 (d, J=2.0 Hz), corresponding to δ 99.5 in the ^{13}C NMR spectrum, showed connectivities with δ 94.5, 106.1, 161.6, and 162.9. The methine proton at δ 6.70 (d, J= 2.0 Hz), corresponding to δ 94.5 in the ^{13}C NMR spectrum, provided significant correlations with δ 99.5, 106.1, 157.2, and 162.9. The hydroxyl group at δ 12.9 (s) was correlated with δ 99.5, 106.1, 161.6, and 180.5. Moreover, the proton at δ 8.41, corresponding to δ 154.5 in the ^{13}C NMR spectrum, showed long-range correlation to a carbon signal at δ 157.2 together with δ 121.0, 122.6, and 180.5. Thus, the carbons at δ 94.5, 99.5, 106.1, 122.6, 157.2, 161.6, 162.9, and 180.5 could be assigned to C-8, C-6, C-10, C-3, C-9, C-5, C-7, and C-4 (C=O), respectively. The ^{1}H NMR revealed four aromatic protons (A_2B_2 system) at δ_H 6.81, 7.38 (each 2H, d, J=8.5 Hz), and δ_C 130.1, 115.0, which were assigned to a *p*-disubstituted benzene as found in ring B of genistein (an isoflavone). In the NOESY spectrum, the signal at δ 8.41 (H-2) showed a cross-peak with H-2′,6′ (δ 7.38), and the signal at δ 7.38 (H-2′,6′) showed cross-peaks with H-2 (δ 8.41) and H-3′,5′ (δ 6.81). On the other hand, the methoxyl signal at δ_H 3.45 was correlated with C-4″ of the glucopyranosyl unit (δ_C 78.8) in the HMBC spectrum. Moreover, a fragment ion at m/z 271 $[M + H]^+$ in the FABMS spectrum corresponded to the loss of a 4″-*O*-methoxy-glucosidic moiety. The anomeric proton at δ 5.07 was clearly correlated with C-5″ (δ 75.6) and δ 162.9 (C-7) indicating that 4″-*O*-methoxy-glucosidic moiety was attached to C-7. Except for the signal of 4″-OCH_3, the residual signals of ^{1}H and ^{13}C NMR spectra of **8** were in agreement with genistin in the literature (Lewis et al. 1998). Thus, metabolite **8** is characterized as genistein 7-*O*-β-D-4″-*O*-methyl-glucopyranoside. This metabolite might be a byproduct due to the use of soybean meal in the fermentation medium.

Discussion

Numerous fungi and other microorganisms metabolize foreign organic compounds in a manner similar to that of mammalian systems, and therefore can serve as microbial models for the study of mammalian metabolism (Rao and Davis 1997). The metabolism of gallic acid has been established in humans and animals (Hodgson et al. 2000; Yasuda et al. 2000; Zong et al. 1999), but this is the first report to produce methylated and glucosidated of gallic acid by the fungus. The fungus *B. sulfurescens* ATCC 7159 is one of the most frequently used microorganism for the preparative biotransformation of a variety of organic compounds, and its biocatalytic reactions were recently reviewed (Grogan and Holland 2000). The aim of the present study was to identify a microorganism capable of metabolizing gallic acid (**1**), and to explore the similarities in gallic acid metabolism between this microbial model and the reported mammalian systems. Such a microbial system could then be used as a model to predict and generate mammalian metabolites of other analogs of gallic acid and phenolic compounds. A scale-up fermentation of gallic acid (**1**) with *B. sulfurescens* ATCC 7159 gave two new glucosidated metabolites (**4** and **7**), four known *O*-methylated metabolites (**2**, **3**, **5**, and **6**), and a byproduct (**8**) (Fig. 1). *O*-Methylation of gallic acid (**1**) alters the chemical reactivity of its phenolic hydroxyl groups and enhances its lipophilicity. In mammals, *O*-methylation is a well-known detoxication pathway (Zhu et al. 1994). On the other hand, the formation of 4′-*O*-methyl glucosides allows these products to become more hydrophilic. The results suggested that *O*-methyltransferase is probably involved in the metabolic process. Metabolites **2**, **3**, and **5** are the reported metabolites of gallic acid in mammalian systems (Hodgson et al. 2000; Yasuda et al. 2000; Zong et al. 1999), and they have also been detected in urine and plasma after black tea and red wine consumption (Cartron et al. 2003; Hodgson et al. 2000). The formation of **8** was probably an artificial fermentation product. Previous study has been shown that

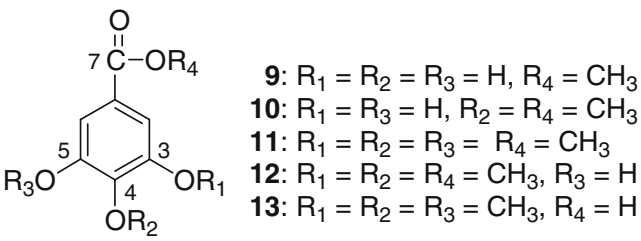

Fig. 2 Structures of compounds **9**–**13**

genistin is one of the components in soybean (Hosny and Rosazza 2002). Because the incubation medium contains soybean flour, metabolite **8** is probably derived from selective *O*-methylation of glucoside on genistin by *B. sulfurescens*. In addition, gallic acid (**1**) and its microbial metabolites, **2**–**6** and **8**, along with chemical *O*-methylated derivatives **9**–**13** were initially screened using a glucocorticoid receptor-mediated luciferase reporter gene assay to find potential novel glucocorticoid agonists that may provide antiinflammatory therapy for asthma and other chronic inflammatory and immune diseases (Fig. 2). Among the 12 compounds tested, **11**, **12**, and **13** with methoxyl group substituted at C-3, 4, 5, 7, C-3, 4, 7, and C-3, 4, 5, respectively, were found to activate glucocorticoid response reporter expression, but less active than the reference compounds of methylprednisolone and dexamethasone. The luciferase activities of **11**, **12**, and **13** were 1.656±0.224, 1.300±0.126, and 1.439±0.159, respectively, and the reference compounds, methylprednisolone and dexamethasone, were 2.050±0.298 and 1.988±0.219, respectively. The results suggest that lipophilicity seems to play an important role for the biological activity in this series of compounds.

In conclusion, two 4′-*O*-methylated glucosides of gallic acid and 4″-*O*-methylated genistin were generated by *B. sulfurescens* ATCC 7159, and were identified as new compounds. Metabolites **2**, **3**, and **5** are the metabolites of gallic acid in both mammals and this microbial model. The formation of the known mammalian metabolites **2**, **3**, and **5** suggests that *B. sulfurescens* has the potential to serve as a microbial model for generating mammalian metabolites of the related analogs of gallic acid for the structural identification and for further use in investigating pharmacological and toxicological properties of this series of compounds. The formation of new *O*-methyl glucosidated metabolites **4**, **7**, and **8** by *B. sulfurescens* well demonstrates the advantages and properties of microbial transformations, which may accomplish a reaction difficult to achieve through chemical synthesis. Thus, it appears that *B. sulfurescens* ATCC 7159 may be a useful tool to prepare various metabolites of bioactive phenolic xenobiotics. The biological screening obtained with regard to the structural characteristics of **1** and related compounds may provide preliminary information for the design of novel antiinflammatory agents. Work is also ongoing to evaluate these potential glucocorticoid agonists functioning on other transcriptional factor-regulated pathways, such as NF-κB and AP-1.

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