Microbial Biotransformation of Gentiopicroside by the Endophytic Fungus *Penicillium crustosum* 2T01Y01

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Endophytic fungi are symbiotic with plants and possess multienzyme systems showing promising metabolite potency with region selectivity and stereoselectivity. The aim of this study was to use these special microorganisms as an *in vitro* model to mimic the potential mammalian metabolites of a natural iridoid gentiopicroside (GPS, compound 1). The fungi isolated from a medicinal plant, *Dendrobium candidum* Wall. ex Lindl., were screened for their biotransformation abilities with GPS as the substrate, and one strain with high converting potency was identified as *Penicillium crustosum* 2T01Y01 on the basis of the sequence of the internal transcribed spacer of the ribosomal DNA region. Upon the optimized incubation of *P. crustosum* 2T01Y01 with the substrate, seven deglycosylated metabolites were detected by ultraperformance liquid chromatography/quadrupole time of flight mass spectrometry (UPLC/Q-TOF MS). Preparative-scale biotransformation with whole cells of the endophytic fungus resulted in the production of five metabolites, including three novel ones, 5 α -(hydroxymethyl)-6 β -methyl-3,4,5,6-tetrahydropyrano[3,4-*c*]pyran-1(8*H*)-one (compound 2), (*Z*)-4-(1-hydroxybut-3-en-2-yl)-5,6-dihydropyran-2-one (compound 3), and (*E*)-4-(1-hydroxybut-3-en-2-yl)-5,6-dihydropyran-2-one (compound 4), along with two known ones, 5 α -(hydroxymethyl)-6 β -methyl-1*H*,3*H*-5,6-dihydropyrano[3,4-*c*]pyran-1(3*H*)-one (compound 5) and 5 α -(hydroxymethyl)-6 α -methyl-5,6-dihydropyrano[3,4-*c*]pyran-1(3*H*)-one (compound 6), aided by nuclear magnetic resonance and high-resolution mass spectral analyses. The other two metabolites were tentatively identified by online UPLC/Q-TOF MS as 5-hydroxymethyl-5,6-dihydroisochromen-1-one (compound 7) and 5-hydroxymethyl-3,4,5,6-tetrahydroisochromen-1-one (compound 8), and compound 8 is a new metabolite. To test the metabolic mechanism, the β -glucosidase activity of the fungus *P. crustosum* 2T01Y01 was assayed with *p*-nitrophenyl- β -D-glucopyranoside as a probe substrate, and the pathway of GPS biotransformation by strain 2T01Y01 is proposed. In addition, the hepatoprotective activities of GPS and metabolite compounds 2, 5, and 6 against human hepatocyte line HL-7702 injury induced by hydrogen peroxide were evaluated.

Microorganisms have been used to produce chemicals, pharmaceuticals, and perfumes for decades and also for pollutant degradation and recovery of the environment contaminated by chemicals (1). Another interesting use of microorganisms is for studying the metabolism of drugs and other chemicals. Smith and Rosazza, in the early 1970s, established the use of microbial models of mammalian metabolism (2, 3). It has been demonstrated that the microbial biotransformation system is very similar to the mammalian phase I metabolic reactions. Therefore, this *in vitro* biotransformation can be an attractive alternative for the metabolism of new drugs, making possible the scale production of metabolites and facilitating structure elucidation and toxicology tests (4). Other advantages of using microorganisms for drug metabolism studies include the low cost and the ease of experimental design in microbial transformation (5, 6, 7, 8).

Endophytes are bacterial or fungal microorganisms that colonize living internal tissues of plants without causing any disease symptoms (9). Endophytes can produce a great number of novel compounds with a broad spectrum of biological activities, such as antifungal, antibacterial, immunosuppressive, and antineoplastic activities (10). Endophytic fungi extensively transformed 2-hydroxy-1,4-benzoxazin-3(2*H*)-one and 2-hydroxy-7-methoxy-1,4-benzoxazin-3(2*H*)-one into less toxic metabolites, probably by using their oxidation and reductases. Agusta et al. reported the stereoselective oxidation at C-4 of flavans by the endophytic fungus *Diaporthe* sp. isolated from the tea plant *Camelia sinensis* (11). It has been documented that *Penicillium crustosum* could enantioselectively

metabolize albendazole to albendazole sulfoxide (12) and biotransform testosterone into five reduction products, 5 α -dihydrotestosterone, dihydrotestosterone, 3 α -hydroxy-5 β -androstane-17-one, 3 α -hydroxy-5 α -androstane-17-one, 4-androstene-3,17-dione, and 5 α -androstane-3,17-dione (13).

Therefore, endophytes have attracted more and more attention not only for producing novel compounds but also for transforming natural products to change their structures and bioactivities.

Gentiopicroside, or 5-ethenyl-6-(β -D-glucopyranosyloxy)-5,6-dihydro-1*H*,3*H*-pyrano[3,4-*c*]pyran-1-one (GPS, compound 1 [see Table 2]), a secoiridoid glucoside, is a principal bitter substance found in many gentianaceous plants, such as *Gentiana scabra* Gbe., *Gentiana lutea* L., *Swertia pseudochinensis* Hara., and *Swertia mussoitii* Franch, which are widely used as medicinal herbs in China and Europe (14, 15). GPS has been shown to exhibit a variety of pharmacological properties, including antibacterial, antiapoptotic, bitter stomachic, cholagogic, and hepatoprotective activities (16, 17). Nevertheless, like other iridoid glycosides, GPS

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normally acts as a prodrug and its activities are induced when the compound is activated by enzymes or nonenzymatically by acid hydrolysis. The hydrolytic β -glucosidases (EC 3.2.1.21) have been shown to convert the nonreactive iridoid glycosides into highly reactive aglycones (18, 19). As GPS belongs to the subclass of secoiridoids, the aglycone is not stable after hydrolysis, and as a result, no one has prepared its aglycone by using enzymes or acid hydrolysis to date.

Biotransformation of GPS by a strain of the human intestinal bacterium *Veillonella parvula* subsp. *parvula* produced five metabolites, erythrocentaurin, gentiopicral, 5-hydroxymethylisochroman-1-one, 5-hydroxymethylisochromen-1-one, and 5,6-dihydro-5-hydroxymethyl-6-methyl-1*H*,3*H*-pyrano[3,4-*c*]pyran-1-one (20). Wang et al. reported the biotransformation of GPS by asexual mycelia of *Cordyceps sinensis* yielding a new pyridine monoterpene alkaloid, (Z)-5-ethylidene-8-hydroxy-3,4,5,6,7,8-hexahydropyrano[3,4-*c*]pyridine-1-one (21).

In the present study, the biotransformation of GPS by an endophytic fungus isolated from an orchid medicinal plant, *Dendrobium candidum* Wall. ex Lindl., was done with the aim of discovering the reactive pharmacophore, and the metabolic pathway is proposed.

MATERIALS AND METHODS

Media and chemicals. The potato dextrose agar slants used and the preparation of the preculture were previously described (22). The liquid seed medium used contained glucose at 20 g/liter and 200 g potato water boiled for 30 min, filtered, and diluted to 1 liter with deionized water at pH 6.2. The biotransformation experiment was carried out with 100 ml of liquid seed medium and 1 ml of GPS substrate at 30 mg/ml. GPS (high-performance liquid chromatography [HPLC] purity, 98.5%) was purchased from the Shanghai R&D Center for Standardization of Chinese Medicine. Acetonitrile and methanol were HPLC grade and were purchased from Sigma-Aldrich (St. Louis, MO). All of the other chemicals used for extraction and isolation were analysis grade and commercially available. Deionized water was used throughout this study.

Analytical and instrumental methods. During the biotransformation process, 5 ml culture broth was taken from each flask at 1, 2, 4, and 6 days and an equal volume of acetonitrile was then added to the broth. The diluted solution was centrifuged at $12,000 \times g$ for 30 min to remove proteins. The supernatant was filtered with a 0.45- μ m micropore filter and transferred into a sampling vial for HPLC analysis. HPLC analysis was carried out with an Agilent 1200 series HPLC system equipped with a UV detector (Agilent Technologies). A Zorbax Bonus-RP [3.5 μ m, 75 mm by 4.6 mm [inside diameter (i.d.)]; Agilent Technologies] was used. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 1.5 ml/min. The gradient condition of the mobile phase was 5 to 20% B from 0 to 10 min, 20 to 95% B from 10 to 13 min, and 95% B from 13 to 15 min. The HPLC oven temperature was maintained at 40°C, and the detection wavelength was 225 nm.

Ultraperformance liquid chromatography/quadrupole time of flight mass spectrometry (UPLC/Q-TOF MS) analysis was carried out on a Waters ACQUITY Synapt G2 system (Waters Corp., Manchester, United Kingdom). The column effluent was monitored with a Q-TOF tandem mass spectrometer (Waters Co.) equipped with a LockSpray and an electrospray ionization (ESI) interface. High-purity nitrogen was used as the nebulizer and auxiliary gas. The ESI-tandem MS (MS/MS) experiment was performed in the positive mode with the following operating parameters. The capillary voltage was set at 3.0 kV, the sample cone voltage was set at 45 V, and the extracting cone voltage was set at 4 V. The source and desolvation temperatures were set at 150 and 450°C, respectively. The cone and desolvation gas flow rates were set at 50 and 850 liters/h, respectively. MassLynx 4.1 software (Waters Co.) was used to control the UPLC-

ESI-MS/MS system, as well as for data acquisition and processing. Chromatographic separation was done with a Waters Acquity UPLC T3 column (100 by 2.1 mm [i.d.], 1.8- μ m particle size; Waters Corporation, Milford, MA) kept at 40°C. The mobile phase consisted of 0.1% aqueous formic acid (A) and acetonitrile (B) at a flow rate of 0.5 ml/min. The gradient elution procedure was 5% B from 0 to 12 min, 5 to 90% B from 12 to 15 min, 90% B from 15 to 17 min, and 90 to 5% B from 17 to 18 min.

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were run on a Bruker AVANCE 400 FT NMR spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C , respectively, with deuterated dimethyl sulfoxide ($\text{DMSO}-d_6$; Sigma-Aldrich, St. Louis, MO) as the solvent. Coupling constants were expressed in hertz, and chemical shifts were reported on a parts-per-million scale with tetramethylsilane as an internal standard.

Microorganisms. (i) **Sampling.** *D. candidum* Wall. ex Lindl. plants were gathered from the Nanhui *D. candidum* artificial cultivation base, Shanghai, China. Plants with bulk soil and fresh humus soil were carefully packed and transferred to the laboratory within 48 h.

(ii) **Isolation and growth.** The endophytes were isolated from the stems of healthy *D. candidum* Wall. ex Lindl. plants. The stem was cut into pieces about 1 cm long and thoroughly washed with distilled water, followed by 75% (vol/vol) ethanol for 1 min and 5% sodium hypochlorite for 5 min, for surface sterilization. The pieces were then rinsed in sterile demineralized water three times for 1 min. Small pieces of the inner tissue of the stems were placed on potato dextrose agar petri plates pretreated with 0.1% chloramphenicol and incubated at $28 \pm 2^\circ\text{C}$ until fungal growth was initiated. The tips of the fungal hyphae were then removed from the aqueous agar and inoculated onto the mycological medium. A similar procedure, but without surface sterilization, was used as a negative control to check for surface-contaminated fungi. A total of 39 pure culture isolates were obtained. Each strain was aseptically transferred onto agar slants and allowed to grow for 4 days at 28°C, three tips of the slant endophytic fungi were subsequently inoculated into a 250-ml shake flask containing 100 ml of liquid seed medium, and the culture was incubated for 3 days at 28°C on a rotary shaker at 120 rpm. A 10-ml aliquot of liquid culture was then used for inoculation in the microorganism screening experiment as described below.

(iii) **Fermentation procedures.** Microbial metabolism studies were carried out by incubating cultures on an incubator shaker (ZHWY-211; Zhicheng Analytical Instrument Manufacturing Co., Shanghai, China) operated at 120 rpm and 28°C. The medium was sterilized at 121°C and 18 lb/in² for 20 min. Fermentations were carried out according to a standard two-stage protocol (23). Endophytic fungus stock inoculums were first prepared by suspending the fungus from one agar slant in 1 ml of sterile distilled water. Submerged stage I cultures were then initiated by adding 0.1 ml of the endophytic fungus stock inoculums to a 250-ml flask containing 50 ml of liquid medium. Following the incubation of stage I cultures for 2 days on the shaker, stage II cultures were initiated by inoculating 50 ml of fresh, sterile liquid medium with 1 ml of stage I culture broth. After the incubation of stage II cultures for 2 days, the complex medium was used for biotransformation of substrates.

(iv) **Microorganism screening.** To screen microorganisms for GPS biotransformation ability, a biotransformation experiment, a culture control, and a substrate control were run to identify the substrate metabolites, microorganism metabolites, and chemical degradation of substrates by chromatographic analysis. The biotransformation experiment was run through the substrates by the inoculation of microorganisms into a 250-ml shake flask containing 90 ml of the above-mentioned liquid medium and 10 ml of the above-mentioned liquid cultures. The above-mentioned experiments were allowed to proceed for 6 days at 28°C. Three shake flasks were taken out at each sampling time.

Twelve strains of endophytes were selected for incubation with the substrate according to the procedures described above. The GPS biotransformation abilities of the fungal strains tested were evaluated by measuring the consumption of GPS and the appearance of new products by HPLC and UPLC/Q-TOF MS/MS analyses.

Preparative-scale biotransformation. Preparative-scale biotransformation of GPS was carried out with 50 250-ml shake flasks, and each contained 100 ml of stage II cultures with 1 ml of GPS solution. A total of 1.5 g of GPS was used to prepare the biotransformed products. The incubation was continued for an additional 6 days. The other procedures were the same as those described previously for the strain-screening experiments.

Extraction, isolation, and purification. The cultures after incubation were filtered through four layers of gauze and washed with distilled water, and then the filtrations and washings (calculated 5 liters) were combined and extracted with three 5-liter volumes of *n*-butyl alcohol. The combined organic layers were concentrated under reduced pressure to yield 2.6 g of residue. The residue was first chromatographed on an MCI column (60 by 4 cm, 50 g of MCI gel; Mitsubishi Chemical Corporation) and eluted with water, 50:50 (vol/vol) water-methanol, and 20:80 (vol/vol) water-methanol to obtain three fractions. The 20:80 (vol/vol) water-methanol fraction was subjected to a Gilson 215 prep-HPLC system consisting of a Gilson 811D dynamic mixer and a UV detector (Gilson Corporation). Separations were run on a YMC-Pack ODS-A column (10 μ m, 250 by 20 mm [i.d.], 12 nm; YMC). The mobile phase consisted of 0.1% (vol/vol) aqueous trifluoroacetic acid (A) and 0.1% trifluoroacetic acid in acetonitrile (B). The gradient elution procedure was as follows: 0 to 25 min, 8% B; 25 to 30 min, 8 to 95% B; 30 to 35 min, 95% B. The flow rate was 10 ml/min, and monitoring was at 225 nm. The following five metabolites were prepared and purified: compound 2 (see Table 2), a brown powder with an HPLC purity of 98.5% (8.0 mg, 0.5% yield); compounds 3 and 4 (see Table 2), yellow powders with an HPLC purity of 97.0% (16.0 mg, 1.1% yield); compound 5 (see Table 2), a brown powder with an HPLC purity of 99.0% (19.2 mg, 1.3% yield); and compound 6 (see Table 2), a brown powder with an HPLC purity of 98.0% (8 mg, 0.5% yield).

Elucidation of the structures of compounds 2 to 6 was based on one-dimensional and two-dimensional NMR and high-resolution MS analyses.

Human hepatocyte-protective effect of GPS metabolites 2, 5, and 6. Human hepatocyte line HL-7702 was maintained in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum–100 U/ml penicillin–100 μ g/ml streptomycin–2 mM glutamine–10 mM HEPES buffer at 37°C in a humidified atmosphere (5% CO₂, 95% air). HL-7702 cells were pretreated with culture medium containing different concentrations of GPS compounds 2, 5, and 6 (5.0, 10.0, and 20 μ M, respectively) for 24 h and subsequently exposed to H₂O₂ (2.0 mM) diluted in culture medium for 1 h at 37°C (24, 25). Cell counting kit-8 (CCK-8) was then added to each cell culture and maintained in an incubator for 2.5 h before analysis. Five replicate wells were used for each concentration of GPS compounds 2, 5, and 6 in the experiments. Cell viability was measured spectrophotometrically at 450 nm with an enzyme-linked immunosorbent assay reader (26).

Assay of β -glucosidase activity. Determination of the β -glucosidase activity of the fungus was conducted by a modified form of the method of Otieno et al. (27). The organism was activated first as mentioned in the fermentation procedures described above. Subsequently, 5 ml of activated culture was inoculated into a 1,000-ml flask containing 500 ml of liquid medium and incubated at 28°C. After the incubation of stage II cultures for 2 days, 50-ml aliquots were taken aseptically from the liquid medium at 1, 2, 4, 5, 6, 7, and 8 days and the enzyme activity was determined immediately. β -Glucosidase activity was determined by measuring the rate of *p*-nitrophenyl- β -D-glucopyranoside (*p*-NPG) hydrolysis. One milliliter of 5 mM *p*-NPG prepared in 100 mM sodium phosphate buffer (pH 7.0) was added to 10 ml of each aliquot and incubated at 37°C for 24 h, and 0.5 ml of 1 M cold sodium carbonate (4°C) was added to stop the reaction. The absorbance at 420 nm of each mixture was measured with a spectrophotometer. The absorbances of a series of dilutions of *p*-nitrophenol were used to calculate the enzymatic activity.

Fungal 5.8S rDNA amplification, sequencing, phylogenetic analysis, and nucleotide sequence accession numbers. The identity of the organism was determined on the basis of partial or nearly full-length 5.8S ribo-

somal DNA (rDNA) gene sequence analysis. Fungal DNA was extracted from pure cultures by using a genomic DNA miniprep kit (Generay Biotechnology Corporation) according to the manufacturer's instructions. Primers ITS1 (5'-AACTCGGCCATTAGAGGAAGT-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for the amplification of *P. crustosum* 2T01Y01 5.8S rDNA. The PCR mixture (total volume, 50 μ l) contained 5 μ l of 10 $\times$ PCR buffer, 4 μ l of 25 mM Mg²⁺, 2 μ l of 10 mM deoxynucleoside triphosphates, 1 μ l of each primer (10 μ M), 2 μ l of the original template, 1 μ l of *Taq* polymerase, and 34 μ l of double-distilled water. Thirty-four cycles were run, with each cycle consisting of a denaturation step at 94°C (60 s), an annealing step at 53°C (45 s), and an extension step at 72°C (90 s). After the 34th cycle, a final 10-min extension step at 72°C was performed. The reaction products were separated on a 1.0% (wt/vol) agarose gel, and the amplicons were purified with a gel band purification kit (Generay Biotechnology Corporation).

The final sequence sets were then submitted to BLAST analysis, and identities of $\geq 99\%$ were considered conspecific. To verify the phylogenetic positions of genotypes, the sequences were aligned by the ClustalX 2.0.1 multiple-sequence alignment software and imported into MEGA 4.1. The evolutionary history was inferred by the neighbor-joining method. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in fewer than 50% of the bootstrap replicates were collapsed. The robustness of the tree topology was tested by bootstrap analysis (1,000 replicates).

Nucleotide sequence accession number. The 5.8S rDNA gene sequence of *P. crustosum* 2T01Y01 has been deposited in the GenBank database under accession number [KC193255](#).

RESULTS

Screening and identification of the microorganism strain. A total of 39 endophytic fungi were isolated from the stems of healthy *D. candidum* Wall. ex Lindl. plants. On the basis of the morphological features and genotypes of these fungi, 12 strains were screened, 3 showed the ability to metabolize GPS, and *P. crustosum* 2T01Y01 showed the highest transformation rate.

The 5.8S rDNA gene sequence of strain 2T01Y01 was determined and classified in the genus *Penicillium* on the basis of its phylogenetic affiliation. The length of the PCR product of strain 2T01Y01 was 507 bp. The G+C content of the DNA of strain 2T01Y01 was 58.2%. A Basic Local Alignment Search Tool (BLAST) search for the 5.8S rDNA sequence of strain 2T01Y01 revealed the highest degree of similarity to the *P. crustosum* strain with GenBank accession number [KC193255](#) (Fig. 1). As a result, strain 2T01Y01 was named *P. crustosum* 2T01Y01.

Identification of metabolites of GPS. Seven metabolites (compounds 2 to 8) of GPS were detected by HPLC and UPLC/Q-TOF MS/MS (Fig. 2), five of which (compounds 2 to 6) were isolated by repeated chromatographic separation and structurally elucidated by ¹H and ¹³C NMR and MS data. The ¹H and ¹³C NMR data for compounds 2 to 6 are summarized in Table 1. The structures of compounds 7 and 8 were tentatively identified by online Q-TOF MS/MS analyses (positive-ion mode). The retention times, maximum UV absorption wavelengths, and accurate measurements of compounds 2 to 8 are listed in Table 2.

Compound 2 was obtained as a powder. Its molecular formula (C₁₀H₁₅O₄) was deduced by HR-ESI-MS at *m/z* 199.0984 (calculated for [M + H]⁺: 199.0965) and confirmed by the ¹³C NMR data, indicating four degrees of unsaturation. The ¹H and ¹³C NMR and heteronuclear single quantum coherence spectral data revealed 10 carbon signals consisting of one methyl group, four methylenes (including three oxygen-bearing carbons), two me-

FIG 1 Phylogenetic tree of an endophyte strain (*P. crustosum* 2T01Y01) inferred on the basis of 5.8S rDNA sequences. Maximum-parsimony bootstrap values of 50% are indicated above the branch nodes. The number of bootstrap replicates was 1,000. The GenBank accession number of *P. crustosum* 2T01Y01 is in parentheses.

thines (including one oxygen-bearing carbon), two olefinic quaternary carbons, and one carbonyl quaternary carbon. There were no olefinic proton or aromatic proton signals in the low field of the ^1H NMR spectrum of compound 2, implying an olefinic bond between C-11 and C-12 and not between C-8 and C-11 or between C-4 and C-12 (Fig. 3). The ^1H - ^1H correlation spectroscopy (COSY) spectrum of compound 2 (Fig. 4A) implied connectivities of $\text{CH}_2(3)$ to $\text{CH}_2(4)$ and of H-C(5) to H-C(6). The heteronuclear multiple-bond correlation spectroscopy (HMBC) spectrum (Fig. 4A) showed

correlations between $\text{CH}_2(3)$ and C(1) and C(12), between H-C(6) and C(8), between H-C(8) and C(11) and C(12), between H-C(9) and C(6) and C(12), and between the methyl group and C(6) and C(5), respectively. With these correlations, the constitution of compound 2 could be deduced. The relative configuration of compound 2 was determined by nuclear Overhauser effect spectroscopy (NOESY) experiments. The H-C(5)-H-C(6) coupling constant was too small to be measured, indicating *ee* coupling between these two protons. The NOESY spectrum of compound 2 revealed enhance-

FIG 2 Total ion chromatograms, obtained by UPLC/Q-TOF MS in positive-ion mode, of the incubation solution with the substrate (A) and without the substrate (B).

TABLE 1 ^1H and ^{13}C NMR data for metabolites of GPS

Position	^{13}C ; ^1H (ppm) ^a data (signal, J [Hz]) ^b				
	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6
1	163.08	163.94	169.66	163.17	163.30
3	65.69; 4.38 (2H, m)	65.86; 4.29 (2H, t, $J = 6.0$)	67.71; 4.80 (2H, br)	68.81; 5.00 (2H, m)	68.81; 4.95 (2H, m)
4	25.38; 2.35, 2.65 (2H, m)	25.57; 2.41 (2H, t, $J = 6.0$)	32.39; 3.01 (2H, br)	114.45; 5.62 (1H, m)	113.02; 5.53 (1H, br)
5	46.73; 2.12 (1H, br)	162.19	134.74	43.96; 2.39 (1H, br)	41.79; 2.64 (1H, br, 5-H)
6	69.80; 3.73 (1H, m)	116.13; 5.79 (1H, s)	117.80; 5.76 (1H, s)	72.94; 4.60 (1H, m)	74.82; 4.44 (1H, m)
7		52.28; 3.12 (1H, dd, $J = 6.4, 6.8$)	50.85; 2.91 (1H, dd, $J = 6.4, 6.8$)		
8	62.05; 4.15 (2H, m)	135.67; 5.84 (1H, m)	136.93; 5.83 (1H, m)	150.61; 7.46 (1H, s)	152.24; 7.53 (1H, s)
9	58.21; 3.55 (2H, m)	117.62; 5.17 (2H, m)	116.57; 5.08 (2H, m)	60.52; 3.40 (2H, m)	58.02; 3.53 (2H, m)
10	19.03; 1.26 (3H, dd, $J = 6.4, 8.4$)	61.97; 3.57 (2H, d, $J = 6.4$)	62.14; 3.51 (2H, d, $J = 6.4$)	18.25; 1.22 (3H, d, $J = 6.6$)	15.53; 1.25 (3H, d, $J = 6.6$)
11	123.30			101.80	102.40
12	152.04			124.81	126.97

^a ^1H and ^{13}C NMR spectra were obtained with DMSO- d_6 .^b Abbreviations for NMR signals are as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad.

ments between H-C(5) and methyl protons H-6 and H-9, which demonstrated a *trans* configuration of the methyl at C-6 and the side chain at C-5. On the basis of all of the evidence, compound 2 was identified as 5 α -(hydroxymethyl)-6 β -methyl-3,4,5,6-tetrahydropyrano[3,4-*c*]pyran-1(8*H*)-one.

Compounds 3 and 4 gave the same retention time and the same molecular formula of $\text{C}_9\text{H}_{13}\text{O}_3$ by positive HR-ESI-MS (m/z 169.0882, calculated for $[\text{M} + \text{H}]^+$: 169.0859) (Table 2). The two metabolites showed closely similar ^1H and ^{13}C NMR spectral features (Table 1). Compared with the NMR spectral data of com-

TABLE 2 Retention times, maximum UV absorption wavelengths, and accurate measurements of elemental formulas of protonated molecules and product ions by Q-TOF MS/MS analysis of the parent drug and its metabolites

Compound(s)	t_R^a (min)	λ_{max} (nm)	Fragment ion, mol mass (Da) (relative intensity)	Elemental composition
1	12.56	205, 235, 270	$[\text{M} + \text{H}]^+$, 357.1190 (23) $[\text{M} + \text{H-Glc}]^+$, 195.0670 (66) $[\text{M} + \text{H-Glc-H}_2\text{O}]^+$, 177.0565 (100) $[\text{M} + \text{H-Glc-H}_2\text{O-CO}]^+$, 149.0612 (89) $[\text{M} + \text{H-Glc-H}_2\text{O-C}_2\text{O}_2]^+$, 121.0666 (44)	$\text{C}_{16}\text{H}_{21}\text{O}_9$ $\text{C}_{10}\text{H}_{11}\text{O}_4$ $\text{C}_{10}\text{H}_9\text{O}_3$ $\text{C}_9\text{H}_9\text{O}_2$ $\text{C}_8\text{H}_9\text{O}$
2	3.52	230	$[\text{M} + \text{H}]^+$, 199.0984 (52) $[\text{M} + \text{H-C}_2\text{O}_2]^+$, 155.0723 (100) $[\text{M} + \text{H-C}_2\text{O}_2\text{-H}_2\text{O}]^+$, 137.0618 (18) $[\text{M} + \text{H-C}_2\text{O}_2\text{-2H}_2\text{O}]^+$, 119.0511 (17) $[\text{M} + \text{H-C}_3\text{O}_3\text{-2H}_2\text{O}]^+$, 91.0548 (37)	$\text{C}_{10}\text{H}_{15}\text{O}_4$ $\text{C}_8\text{H}_{11}\text{O}_3$ $\text{C}_8\text{H}_9\text{O}_2$ $\text{C}_8\text{H}_7\text{O}$ C_7H_7
3, 4	5.72	225	$[\text{M} + \text{H}]^+$, 169.0882 (100) $[\text{M} + \text{H-H}_2\text{O}]^+$, 151.0759 (6) $[\text{M} + \text{H-CH}_2\text{O}]^+$, 139.0778 (43) $[\text{M} + \text{H-C}_2\text{H}_4\text{O}_3]^+$, 93.0720 (24)	$\text{C}_9\text{H}_{13}\text{O}_3$ $\text{C}_9\text{H}_{11}\text{O}_2$ $\text{C}_8\text{H}_{11}\text{O}_2$ C_7H_9
5	7.75	214, 245, 285	$[\text{M} + \text{H}]^+$, 197.0832 (52) $[\text{M} + \text{H-H}_2\text{O}]^+$, 179.0732 (17) $[\text{M} + \text{H-CH}_2\text{O}]^+$, 167.0726 (27) $[\text{M} + \text{H-CH}_2\text{O-H}_2\text{O}]^+$, 149.0617 (100) $[\text{M} + \text{H-CH}_2\text{O-H}_2\text{O-CO}]^+$, 121.0617 (50)	$\text{C}_{10}\text{H}_{13}\text{O}_4$ $\text{C}_{10}\text{H}_{11}\text{O}_3$ $\text{C}_9\text{H}_{11}\text{O}_3$ $\text{C}_9\text{H}_9\text{O}_2$ $\text{C}_8\text{H}_9\text{O}$
6	8.44	214, 245, 285	$[\text{M} + \text{H}]^+$, 197.0836 (80) $[\text{M} + \text{H-H}_2\text{O}]^+$, 179.0725 (12) $[\text{M} + \text{H-CH}_2\text{O}]^+$, 167.0726 (23) $[\text{M} + \text{H-CH}_2\text{O-H}_2\text{O}]^+$, 149.0621 (100) $[\text{M} + \text{H-CH}_2\text{O-H}_2\text{O-CO}]^+$, 121.0617 (45)	$\text{C}_{10}\text{H}_{13}\text{O}_4$ $\text{C}_{10}\text{H}_{11}\text{O}_3$ $\text{C}_9\text{H}_{11}\text{O}_3$ $\text{C}_9\text{H}_9\text{O}_2$ $\text{C}_8\text{H}_9\text{O}$
7	6.96	220, 285	$[\text{M} + \text{H}]^+$, 179.0729 (89) $[\text{M} + \text{H-CO}]^+$, 151.0775 (100) $[\text{M} + \text{H-CO-H}_2\text{O}]^+$, 133.0672 (12) $[\text{M} + \text{H-C}_2\text{O}_2\text{-H}_2\text{O}]^+$, 105.0718 (19)	$\text{C}_{10}\text{H}_{11}\text{O}_3$ $\text{C}_9\text{H}_{11}\text{O}_2$ $\text{C}_9\text{H}_9\text{O}$ C_8H_9
8	4.14	230	$[\text{M} + \text{H}]^+$, 181.0883 (52) $[\text{M} + \text{H-CH}_2\text{O}]^+$, 151.0773 (100) $[\text{M} + \text{H-CH}_2\text{O-H}_2\text{O}]^+$, 133.0670 (16) $[\text{M} + \text{H-CH}_2\text{O-CH}_2\text{O}_2]^+$, 105.0720 (65)	$\text{C}_{10}\text{H}_{13}\text{O}_3$ $\text{C}_9\text{H}_{11}\text{O}_2$ $\text{C}_9\text{H}_9\text{O}$ C_8H_9

^a t_R , retention time.^b λ_{max} , maximum UV absorption wavelength.

FIG 3 Key ^1H - ^1H COSY and HMBC correlations for compounds 2 (A), 3 (B), and 4 (C).

compound 2, each compound exhibited a carbonyl carbon [compound 3, $\delta(\text{C-1})$ 163.94; compound 4, $\delta(\text{C-1})$ 169.66] and two methylenes [compound 3, $\delta(\text{H-3})$ 4.29 (t, $J = 6.0$ Hz), $\delta(\text{H-4})$ 2.41 (t, $J = 6.0$ Hz); compound 4, $\delta(\text{H-3})$ 4.80, $\delta(\text{H-4})$ 3.01], which suggested that similar lactone rings were present in the two compounds. Furthermore, in the ^1H NMR spectra, a couple of olefinic signals [compound 3, $\delta(\text{H-6})$ 5.79, $\delta(\text{H-8})$ 5.84, $\delta(\text{H-9})$ 5.17; compound 4, $\delta(\text{H-6})$ 5.76, $\delta(\text{H-8})$ 5.83, $\delta(\text{H-9})$ 5.08] were observed in the two metabolites. These results suggested that the pyran ring from the parent compound (compound 1) was cleaved and further decarboxylated. Thus, a hydroxyl group in each me-

tabolite was formed and the resonance of hydroxymethylene at $\delta(\text{H-10})$ 3.57 (d, $J = 6.4$ Hz), $\delta(\text{C-10})$ 61.97 for compound 3 and $\delta(\text{H-10})$ 3.51 (d, $J = 6.4$ Hz), $\delta(\text{C-10})$ 62.14 for compound 4 were displayed. The ^1H - ^1H COSY spectra of compounds 3 and 4 (Fig. 3B and C) implied connectivities of $\text{CH}_2(3)$ to $\text{CH}_2(4)$, of $\text{H-C}(7)$ to $\text{CH}_2(10)$, and of $\text{H-C}(8)$ to $\text{CH}_2(9)$. The HMBC spectra of compounds 3 and 4 (Fig. 3B and C) show correlations between $\text{CH}_2(3)$ and $\text{C}(5)$, between $\text{CH}_2(4)$ and $\text{C}(6)$, between $\text{CH}(7)$ and $\text{C}(6)$, between $\text{CH}_2(9)$ and $\text{C}(7)$, and between $\text{CH}_2(10)$ and $\text{C}(5)$ and $\text{C}(8)$. From these data, the constitutional formulae of compounds 3 and 4 could be deduced.

(A)

(B)

FIG 4 MS/MS of the $[\text{M} + \text{H}]^+$ ion at m/z 179.0729 for compound 7 (A) and at m/z 181.0883 for compound 8 (B).

The relative configurations of compounds 3 and 4 were determined as follows. In the NOESY spectrum of compound 4, a correlation of H-C(6) with H-C(9) was observed, which indicated the *Z* stereochemistry of compound 4, whereas no correlation of H-C(6) and H-C(9) was displayed in the NOESY spectrum of compound 3, revealing the *E* stereochemistry of compound 3. On the basis of all of the evidence, compounds 3 and 4 were determined to be (*E*)-4-(1-hydroxybut-3-en-2-yl)-5,6-dihydropyran-2-one and (*Z*)-4-(1-hydroxybut-3-en-2-yl)-5,6-dihydropyran-2-one, respectively.

Compound 5 was obtained as a brown powder, and HR-ESI-MS showed its molecular ion at m/z 197.0832 (calculated for $[M + H]^+$: 197.0808) (Table 2), corresponding to the molecular formula $C_{10}H_{13}O_4$. The structure of compound 5 was identified on the basis of NMR and MS data to be 5 α -(hydroxymethyl)-6 β -methyl-1*H*,3*H*-5,6-dihydropyrano [3,4-*c*]pyran-1(3*H*)-one (Table 1), which was described in previous papers (20, 21).

Compound 6 gave the same molecular ion at m/z 197.0832 and exhibited 1H and ^{13}C NMR spectral patterns closely similar to those of compound 5, suggesting that the two compounds are a pair of geometric isomers. The relative configuration of compound 6 was determined as follows. In the 1H NMR spectrum, the H-C(5)–H-C(6) coupling constant was too small to be measured, suggesting *ee* coupling between these two protons. Furthermore, in the NOESY spectrum, correlations between H-C(5) and H-C(6) and between H-C(9) and methyl proton (H-10) were observed, confirming a *cis* relationship between the methyl group at C-6 and the side chain at C-5 of compound 6. On the basis of these observations, compound 6 was identified as 5 α -(hydroxymethyl)-6 α -methyl-5,6-dihydropyrano[3,4-*c*]pyran-1(3*H*)-one (20, 21).

Compounds 7 and 8 were eluted at retention times of 6.96 and 4.14 min and gave positive HR-ESI-MS values at m/z 179.0729 (calculated for $[M + H]^+$: 179.0708) and m/z 181.0883 (calculated for $[M + H]^+$: 181.0865) (Table 2), respectively. CID (collision-induced dissociation)-MS/MS of the precursor ion extracted from compound 7 gave three main product ions, $[M + H-CO]^+$ at m/z 151.0775, $[M + H-CH_2O_2]^+$ at m/z 133.0670, and $[M + H-CH_2O_2-CO]^+$ at m/z 105.0720 (Fig. 4A). MS/MS of compound 8 gave the product ions $[M + H-CH_2O]^+$ at m/z 151.0773, $[M + H-CH_2O-H_2O]^+$ at m/z 133.0676, and $[M + H-CH_2O-CH_2O_2]^+$ at m/z 105.0718 (Fig. 4B). By comparison with the MS/MS data of the parent drug (compound 1), compounds 7 and 8 were tentatively identified as 5-(hydroxymethyl)-5,6-dihydroisochromen-1-one and 5-(hydroxymethyl)-3,4,5,6-tetrahydroisochromen-1-one, respectively (20).

Hepatoprotective effects of GPS metabolites. The protective effects of GPS and its three available metabolites (compounds 2, 5, and 6) on the survival ability of HL-7702 cells were examined. The CCK-8 assay showed that the viability of HL-7702 cells was remarkably decreased by H_2O_2 . However, after pretreatment with GPS and compounds 2, 5, and 6, the metabolites (compounds 2, 5, and 6) could restore cell viability at concentrations of 20, 10, and 5 μ M, respectively, while GPS showed no activity at those concentrations, which indicated that the biotransformation products exhibit more potent protective effects than the substrate GPS.

DISCUSSION

P. crustosum has been found commonly in food, feed, and plants (28–30); has a wide range of biological functions; and possesses multienzyme systems with significant region selectivity and

FIG 5 Time-dependent release of p-NP after the incubation of p-NPG with *P. crustosum* 2T01Y01 ($n = 3$, mean $\pm$ SD).

stereoselectivity (13, 31), which have already been used to biotransform natural products (32, 33), chemicals (12, 34, 35), and endogenous materials (13) to change their structures and bioactivities.

GPS (compound 1), a secoiridoid glucoside, has been found as a principal component in many gentianaceous medicinal plants, and some of the species, such as *G. scabra* Gbe. and *G. lutea* L., are recorded in the official pharmacopoeias of China, Great Britain, and other European countries. GPS, like other iridoid glycosides, acts as a prodrug that needs to be activated by gut microbes to exert its biological activities, and the biotransformation pathway and the pharmacophore responsible for its activity remain to be evaluated.

In the present study, a *Penicillium* fungal strain isolated from a medicinal herb was found to show high GPS-transforming ability and identified as *P. crustosum* 2T01Y01 on the basis of the internal transcribed spacer of the rDNA region. Preparative-scale whole-cell incubation of GPS with this fungus resulted in the isolation and structural elucidation of five metabolites (compounds 2 to 6), while the other two metabolites (compounds 7 and 8) were identified tentatively by online UPLC-MS technology because of their limited concentrations in the incubation system. HPLC time course analysis revealed that the metabolites were detected on the second day and the substrate GPS was nearly completely consumed by the 6th day of incubation.

The biotransformations involved in drug metabolism in microorganisms are often hydrolysis, reduction, oxidation, and isomerization reactions (36). For the biotransformation of GPS by *P. crustosum* 2T01Y01, deglycosylation by the β -glucosidase that exists in the fungus might be the initiation step. In this study, the β -glucosidase activity of *P. crustosum* 2T01Y01 was determined with pNPG as the probe substrate and the result showed that the enzymatic activity of the fungus was activated on the 2nd day of incubation and reached the highest level on the 7th day (Fig. 5).

Therefore, the GPS metabolic pathway of *P. crustosum* 2T01Y01 can be proposed as shown in Fig. 6. GPS was first hydrolyzed by the fungal β -glucosidase to form an unstable hemiacetal aglycone, which was readily converted to the reactive intermediate aldehyde alcohol (compound Ia) or dialdehyde (compound Ib). Subsequently, compounds Ia and Ib underwent thorough intramolecular cyclization to produce pyrano[3,4-*c*]pyran (compound Ic) and isocoumarin (compound Id) derivatives, respectively. Compound Ic was further converted by reduction and hydrogenation to produce compounds 2, 5, and 6 or alternatively by oxi-

FIG 6 Proposed GPS metabolic pathway of *P. crustosum* 2T01Y01.

dation and decarboxylation to produce two pyran ring-opening products, compounds 3 and 4, a pair of *cis-trans* isomers. Simultaneously, compound Id was subjected to reduction to produce compound 7 or further hydrogenated to form compound 8.

It was obvious that the fungus has a multienzyme system and has more potent biotransformation activity than the single enzyme, as indicated in our previous paper (37), in which a single glycoside hydrolase was used for the biotransformation of GPS and only four metabolites, including the intermediate compounds Ic and Id, were identified.

The *in vitro* bioassay indicated that the three available metabolites

(compounds 2, 5, and 6) showed potent protective effects against HL-7702 cell injury induced by H_2O_2 , while the substrate GPS exhibited no activity at the concentrations tested, which is evidence that iridoid glycosides can only be biotransformed by enzymes into their aglycon derivatives to exert their broad spectral bioactivities.

The function of the metabolites of GPS for the fungus remains to be extensively investigated in the future.

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