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# Isolation and Identification of Chlorinated Genistein from *Actinoplanes* sp. HBDN08 with Antioxidant and Antitumor Activities

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A strain *Actinoplanes* sp. HBDN08 was screened by PCR-guided method using primers derived from conserved regions of halogenase genes. A new chlorinated isoflavone, 3′,8-dichlorogenistein (**1**), along with 8-chlorogenistein (**2**) were isolated from the fermentation broth of *Actinoplanes* sp. HBDN08. Their structures were elucidated on the basis of extensive 1D and 2D NMR as well as HRESI-MS, ESI-MS, UV, and IR spectroscopic analyses. The origin of the two compounds was also investigated by high-performance liquid chromatography (HPLC) analysis. The results demonstrated that they were not biosynthesized but derived from the biotransformation of genistein by *Actinoplanes* sp. HBDN08. The antioxidant activities of the isolated compounds **1** and **2** were evaluated by using the lipid peroxidation assay. Their antitumor activities were calculated according to the inhibitory rate of cell proliferation against the human breast cancer cell line MDA-MB-231. The results indicated that compounds **1** ($IC_{50}$ = 5.2 $\mu$M) and **2** ($IC_{50}$ = 7.5 $\mu$M) showed stronger antioxidant activities than genistein ($IC_{50}$ = 13.6 $\mu$M). In comparison with the antitumor activities of genistein, those of compounds **1** and **2** increased 7.7- and 2.6-fold, respectively. These results suggest that the PCR-guided screening strategy is a rapid method for obtaining halometabolite-producing strains. Moreover, these results reveal that chlorination has significant effects on the bioactivities of genistein. This could be important information for studying the structure−activity relationships of genistein.

**KEYWORDS:** ***Actinoplanes*** **sp. HBDN08; chlorination; antioxidant activity; antitumor activity; 8-chlorogenistein; 3′,8-dichlorogenistein**

## INTRODUCTION

Over 45,000 halogenated natural products are known to be produced by living organisms. These natural products include important antibiotics such as vancomycin and chloramphenicol as well as the antitumor agent rebeccamycin (*1*−*3*). Addition of electronegative chlorine often has significant effects on bioactivities. For example, the deschloro analogue of the antibiotic clorobiocin was 8-fold less active against *Bacillus subtilis* (*4*). Removal of the chlorine atoms from balhimycin, a glycopeptide antibiotic, resulted in an 8- to 16-fold reduction in activity against a variety of pathogenic bacteria (*5*). Salinosporamide A, a potent 20S proteasome inhibitor currently in phase 1 human clinical trials for the treatment of multiple myeloma and other cancers, is 500 times more active than its deschloro analogue salinosporamide B (*6*). Given the prominence of halogenation among natural products, there has been intense interest in understanding the mechanism by which these functional groups are incorporated during natural product biosynthesis. Since the first $FADH_2$-dependent halogenase is cloned from a chloritetracycline-producing strain by complementation of a chlorination-deficient mutant (*7*), a large number of halogenase genes involved in the biosynthesis of halogenated natural products are identified in many different microorganisms. These halogenases mainly carry out the chlorination of tryptophan, pyrrole and/or phenol derivatives, and activated aliphatic compounds (*8*, *9*). All halogenases, regardless of the structures of their organic substrates, share two conserved regions (GxGxxG and WxWxIP), which are essential criteria for the identification of $FADH_2$-dependent halogenases (*8*) and have been used for the construction of PCR primers to detect halogenase genes (*10*−*12*).

Genistein (4′,5,7-trihydroxyisoflavone) is a common precursor in the biosynthesis of antimicrobial phytoalexins and phytoanticipins in legumes, and an important nutraceutical molecule found in soybean seeds. In addition, it is a phytoestrogen with a wide variety of pharmacological effects in animal cells. Dietary genistein ingestion has been linked with a range of potential health beneficial effects. These include chemoprevention of breast and prostate cancers, cardiovascular disease, and postmenopausal ailments (*13*−*16*). Until now, many modified genistein, such as

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nitrated, chlorinated, and hydroxylated derivatives, have been isolated and showed altered biological properties compared to those of genistein (*17*−*21*). The different activities of these genistein derivatives have prompted us to obtain further genistein analogues to assess the structure−activity relationships of this class of compounds.

The potential of actinomycetes to produce natural halogenated products has attracted increasing attention because of the important features of halogenation for the biological activity of a large number of different natural products. In order to discover more halogenated compounds, a PCR-guided method using primers derived from conserved regions of halogenase genes was employed to obtain halometabolite-producing strains. The antioxidant and antitumor activities of the chlorinated metabolites isolated from the strain were examined.

## MATERIALS AND METHODS

**Reagents.** Dulbecco's modified Eagle's media (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Thiobarbituric acid (TBA) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). A cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals used were of analytical grade and were purchased from Nanjing Chemical Reagent Co., Ltd., Nanjing, China. Methanol (MeOH) of liquid chromatographic grade was obtained from Merck, Inc., Germany.

**Screening and Identification of the Strain HBDN08.** The actinomycete strains, isolated from soil samples collected in Harbin, China, were maintained on Gause's agar plates. The genomic DNA from the actinomycete was isolated by the standard procedures (*22*). The genomic DNAs were screened with degenerate primers derived from conserved regions of $FADH_2$-dependent halogenase sequences (*10*). The sequences of two sense primers are HA002 (5′-TCGGYGTSGGCGARGCGACCRTCCC-3′) and HA003 (5′-TSGGCGGCGGCACYGCSGGMTGGATG-3′). The two antisense primers are HA004 (5′-AGCATSGGRATCTTCCAGGTCCABCC-3′) and HA005 (5′-GCCGGAGCAGTCGAYGAASAGGTC-3′). PCR reaction conditions were as follows: 200 ng of genomic DNA was mixed with 30 pmol of each primer and 2.5 U Taq DNA polymerase (TaKaRa, Dalian, China) in a total volume of 50 $\mu$L containing 2 mM of each dNTP and 5% DMSO. The reaction was performed under standard conditions with an annealing temperature of 58 °C. The almost 16S rDNAs were PCR amplified using the primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1548r (5′-AAGGAGGTGATCCAGCCGCA-3′), which were complementary to the 5′-end and 3′-end of the prokaryotic 16S rDNA, respectively. The amplification reaction was performed as previously described (*23*). The PCR products were blasted against the latest GenBank database, using BLAST. Clustal X 1.81 order software was used to determine the matching alignment of multiple sequences, and MEGA 4.0 was used to calculate the evolutionary distance. The matrix distance through sequence data was calculated according to Kimura Two-Parameter. The phylogenetic tree was then constructed with the neighbor-joining (NJ) method of MEGA 4.0 software. The stability of the topology structure of the phylogenetic tree was appraised by using the bootstrap value with 1,000 repeats.

**Fermentation.** The seed for preculture was mycelia, which was subcultured on agar slants. The medium contained 10 g of soluble starch, 4 g of yeast extract, 3 g of casein hydrolysate, 0.5 g of $MgCl_2 \cdot 6H_2O$, and 0.5 g of $K_2HPO_4 \cdot 6H_2O$ in 1 L of water. The pH was adjusted to 7.0 with 1 M NaOH and 20 g of agar added, and this mixture was sterilized at 121 °C for 30 min. The mycelial suspension was prepared from the agar slants incubated at 28 °C for 6 days.

A mycelial suspension of the culture of strain *Actinoplanes* sp. HBDN08, 1 mL, was inoculated in a 250-mL Erlenmeyer flask that had 20 mL of the seed medium consisting of 0.48 g of corn starch, 0.02 g of glucose, 0.1 g of peptone, 0.1 g of yeast extract, 0.06 g of beef extract, and 0.08 g of $CaCO_3$. The inoculated flasks were incubated at 28 °C for 48 h on a rotary shaker at 250 rpm. Then 4 mL of the culture was transferred into a 250-mL Erlenmeyer flask containing 25 mL of the medium for fermentation consisting of 5% soluble starch, 2% glucose, 3% soybean meal, 0.6% $MgCl_2 \cdot 6H_2O$, 0.8% $CaCO_3$ at pH 7.0 before sterilization. Fermentation was carried out at 28 °C for 5 days on a rotary shaker at 250 rpm.

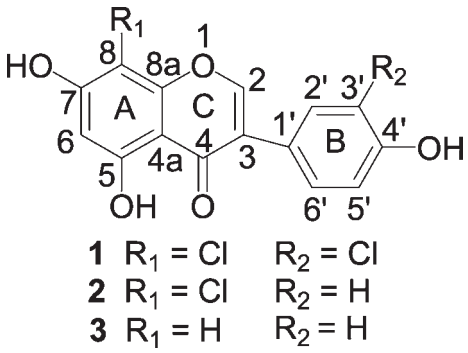


**Figure 1.** Structures of compounds **1** and **2** and genistein (**3**).

For the determination of the origin of compounds **1** and **2**, the fermentation broth (25 mL) was taken out and centrifugated. The mycelia cake was extracted with MeOH, and then the MeOH extract was analyzed using HPLC (LC-20A, SHIMADZU, Japan). HPLC was carried out with a Zorbax SB-C18 silica column (5 $\mu$m, 4.6 mm × 250 mm, Agilent) at 25 °C with an eluent of MeOH/$H_2O$ (47:53, v/v) at a flow rate of 1.0 mL/min, and detection was done by UV absorbance at 260 nm. The control was conducted as described above, except that $MgSO_4 \cdot 7H_2O$ in the fermentation medium was used instead of $MgCl_2 \cdot 6H_2O$.

**Isolation and Purification of Compounds 1 and 2.** Five liters of fermentation broth was filtered. The resulting cake was extracted with MeOH. The filtrate was extracted three times with equal volumes of ethyl acetate (EtOAc). The MeOH extract and EtOAc phase were mixed and then concentrated using a rotary evaporator to give a brown residue (10 g). The resulting residue was subjected to a silica gel (200−300 mesh, Qingdao Marine Chemical Factory, China) column and eluted with gradients of $CHCl_3$/MeOH (99:1−90:10, v/v) to afford fractions A−E on the basis of TLC ($GF_{254}$, Qingdao Marine Chemical Factory, China). Fraction C was subjected to a Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) column and eluted with ethanol (EtOH) to afford four fractions I−IV. Fraction IV was further separated by semipreparative HPLC (Agilent 1100 series, USA) using a Zorbax SB-C18 column (5 $\mu$m, 9.4 × 250 mm, Aglient) and a solvent of MeOH/$H_2O$ (55:45, v/v) with a flow rate of 1.5 mL/min at room temperature. The eluents were monitored with a photodiode array detector at 260 nm. Compounds **1** (25 mg) and **2** (90 mg) were obtained as offwhite amorphous solids.

**Spectrometry.** UV spectra were obtained on a Varian CARY 300 BIO spectrophotometer. IR spectra were recorded on a Nicolet Magna FT-IR 750 spectrometer ($\nu_{max}$ in $cm^{-1}$). $^1H$ and $^{13}C$ NMR spectra were measured with a Bruker DRX-400 (400 MHz for $^1H$ and 100 MHz for $^{13}C$) spectrometer. Chemical shifts were reported as parts per million ($\delta$), using the residual $CHCl_3$ ($\delta_H$ 7.26; $\delta_C$ 77.0) as an internal standard, and coupling constant ($J$) in Hz. $^1H$ and $^{13}C$ NMR assignments were supported by the HMBC experiment. The ESI-MS and HRESI-MS spectra were taken on a Q-TOF Micro LC-MS-MS mass spectrometer.

**Structural Elucidation of Compounds 1 and 2.** 3′,8-Dichlorogenistein (**1**, **Figure 1**), $C_{15}H_8Cl_2O_5$, offwhite amorphous solid. UV (EtOH) $\lambda_{max}$ nm (log $\varepsilon$): 267 (4.35). IR (KBr) $\nu_{max}$ $cm^{-1}$: 3271 (OH), 1655, 1579, 1503, 1424, 1369, 1287, 1248, 1068, 826; $^1H$ NMR (acetone-$d_6$, 400 MHz): $\delta$ 6.50 (1H, s, H-6), 7.10 (1H, d, $J$ = 8.4 Hz, H-5′), 7.45 (1H, dd, $J$ = 8.4, 2.0 Hz, H-6′), 7.68 (1H, d, $J$ = 2.0 Hz, H-2′), 8.41 (1H, s, H-2), 12.89 (1H, OH-5). $^{13}C$ NMR (acetone-$d_6$, 100 MHz): $\delta$ 98.5 (s, C-8), 100.2 (d, C-6), 106.9 (s, C-4a), 117.4 (d, C-5′), 120.9 (s, C-3′), 123.0 (s, C-3), 124.1 (s, C-1′), 129.7 (d, C-6′), 131.4 (d, C-2′), 154.0 (s, C-8a), 154.1 (s, C-4′), 155.0 (d, C-2), 160.8 (s, C-7), 161.7 (s, C-5), 181.4 (s, C-4). ESI-MS $m/z$ 337 $[M - H]^-$, HRESI-MS $m/z$ 336.9651 $[M - H]^-$ (calcd $C_{15}H_7Cl_2O_5$ for 336.9676).

8-Chlorogenistein (**2**, **Figure 1**), $C_{15}H_9ClO_5$, offwhite amorphous solid; $^1H$ NMR (acetone-$d_6$, 400 MHz): $\delta$ 6.48 (1H, s, H-6), 6.92 (2H, d, $J$ = 8.4 Hz, H-3′, 5′), 7.49 (2H, d, $J$ = 8.4 Hz, H-2′, 6′), 8.32 (1H, s, H-2), 13.00 (1H, OH-5). $^{13}C$ NMR (acetone-$d_6$, 100 MHz) $\delta$ 98.6 (s, C-8), 100.2 (d, C-6), 106.8 (s, C-4a), 116.1 (d, C-3′, 5′), 122.6 (s, C-1′), 124.4 (s, C-3), 131.3 (d, C-2′, 6′), 154.2 (s, C-8a), 154.4 (d, C-2), 158.7 (s, C-4′), 160.8 (s, C-7), 161.8 (s, C-5), 181.6 (s, C-4). ESI-MS $m/z$ 303 $[M - H]^-$.

**Antioxidant Activity.** The lipid peroxidation inhibiting activity was determined as described previously (*24*). Reaction mixtures contained

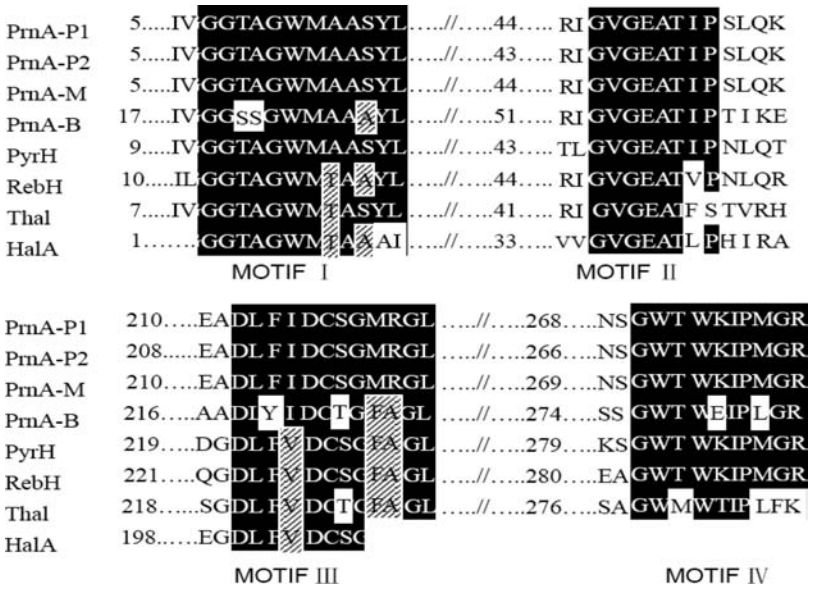


**Figure 2.** Sequence alignment of HalA with seven selected tryptophan halogenases. PrnA-P1, tryptophan 7-halogenase from *Pseudomonas aureofaciens*; PrnA-P2, tryptophan 7-halogenase from *Pseudomonas fluorescens*; PrnA-M, tryptophan 7-halogenase from *Myxococcus fulvus*; PrnA-B, tryptophan 7-halogenase from *Burkholderia cepacia*; PyrH, tryptophan 5-halogenase from *Streptomyces rugosporus*; RebH, tryptophan 7-halogenase from *Lechevalieria aerocolonigenes*; Thal, tryptophan 6-halogenase from *Streptomyces albogriseolus*; HalA, the amino acid sequence of PCR product *halA* amplified from *Actinoplanes* sp. HBDN08. The black boxes show conserved amino acid positions, and the gray boxes show similar amino acids.

80 $\mu$M potassium phosphate buffer (pH 7.4), microsomes (300−500 $\mu$g protein $mL^{-1}$), and test products in various concentrations. Peroxidation was started by adding vitamin C at a final concentration of 200 $\mu$M. The reaction mixture was incubated in an open Eppendorf tube for 90 min at 37 °C. The reactions were terminated by the addition of 250 $\mu$L of a 20% trichloroacetic acid solution followed by centrifugation at 10 000*g* for 3 min. The supernatant (600 $\mu$L) was boiled with 250 $\mu$L of a 0.67% TBA solution for 20 min. At room temperature, the amount of lipid peroxidation was determined by measuring the absorbance of the pink chromogen at 535 nm.

The antioxidant activity of each compound was expressed as the $IC_{50}$ value (the concentration in $\mu$L to inhibit 50% of the TBA reactive substances, calculated from the corresponding log-dose inhibition curve). Appropriate sample blanks were taken into account to calculate the $IC_{50}$ value.

**Antitumor Activity.** Human breast cancer cell line MDA-MB-231 was routinely cultured in DMEM supplemented 10% heat-inactivated fetal bovine serum at 37 °C for 4 h, in a humidified atmosphere of 5% $CO_2$ incubator. The adherent cells at their logarithmic growth stage were digested and were inoculated onto 96-well culture plates at a density of $1.0 \times 10^4$ cell/well for the determination of proliferation. Test samples were added to the medium, and incubation was continued for 72 h. Coloration substrate, cell counting kit-8 (CCK-8), was added to the medium followed by further incubation for 3 h. Absorbance at 450 nm with a 600 nm reference was measured thereafter. Medium and DMSO control wells, in which the compound was absent, were included in all of the experiments in order to eliminate the influence of DMSO. The inhibitory rate of cell proliferation was calculated by the following formula:

$$\text{Growth inhibition } (\%) = (OD_{control} - OD_{treated})/OD_{control} \times 100$$

The cytotoxicity of the compound on tumor cells was expressed as $IC_{50}$ values (the drug concentration to inhibit 50% of the cell viability, calculated from the corresponding log-dose inhibition curve) and was calculated by the LOGIT method.

**Statistical Analysis.** All of the experiments were conducted in triplicate. Data were expressed as the means ± standard deviations (SDs). Data were analyzed using one-way analysis of variance (ANOVA), followed by each pair of Student's *t* tests for multiple comparisons. Differences were considered significant if $p < 0.05$. All analyses were performed using SPSS for Windows, version 11.5 (SPSS, Chicago, IL).

## RESULTS AND DISCUSSION

**Screening and Identification of the Strain HBDN08.** The degenerate primers derived from conserved regions of $FADH_2$-dependent halogenase sequences were used for PCR screening of 57 strains, which were randomly selected from the soil samples. Ten PCR fragments were obtained with the expected size of approximately 500−700 base pairs. Among these screened strains, HBDN08 showed the highest antibacterial activities toward *Micrococcus luteus* ATCC 9341 and *Bacillus subtilis* ATCC 6633. Therefore, we sequenced the PCR product amplified from strain HBDN08, and the nucleotide sequence has been submitted to the GenBank database under accession number FJ875940. The sequence of *halA* showed a high identity of >90% to the genes of tryptophan halogenase PrnA from *Burkholderia cepacin*, *Burkholderia pyrrocinia*, and *Pseudomonas fluorescens* (the accession numbers of the *prnA* in GenBank are AF161183, AF161186, and AF161184, respectively). Comparison of the amino acid sequence of HalA with tryptophan 7-halogenase RebH from *Lechevalieria aerocolonigenes* (*25*) and PrnA from *Pseudomonas fluorescens* BL915 (*26*), with the tryptophan 5-halogenase PyrH from *Streptomyces rugosporus* (*10*) showed high degrees of sequence identity of 36%, 38%, and 35%, respectively. This result is consistent with the published sequence alignment (*10*). There are two subgroups of $FADH_2$-dependent halogenases which are both acknowledged to play a significant role in halometabolite biosynthesis (*2*, *3*, *10*). The one subgroup containing the tryptophan halogenase shows no overall sequence similarity to the other subgroup, which consists of the enzymes catalyzing the halogenation of phenol and/or pyrrole derivatives. All of the halogenases, regardless of the structures of their organic substrates, share two conserved regions. One is the flavin cofactor binding site (GxGxxG) at the amino terminal end and the other region, which is located near the middle of the enzymes, contains two tryptophan residues (WxWIP) (*8*). Comparison of published amino acid sequences of several tryptophan halogenases demonstrated that there were other two conserved motifs: motif II and motif III (*10*). From **Figure 2**, we concluded that the product of *halA* might be a part of a putative tryptophan halogenase because of the

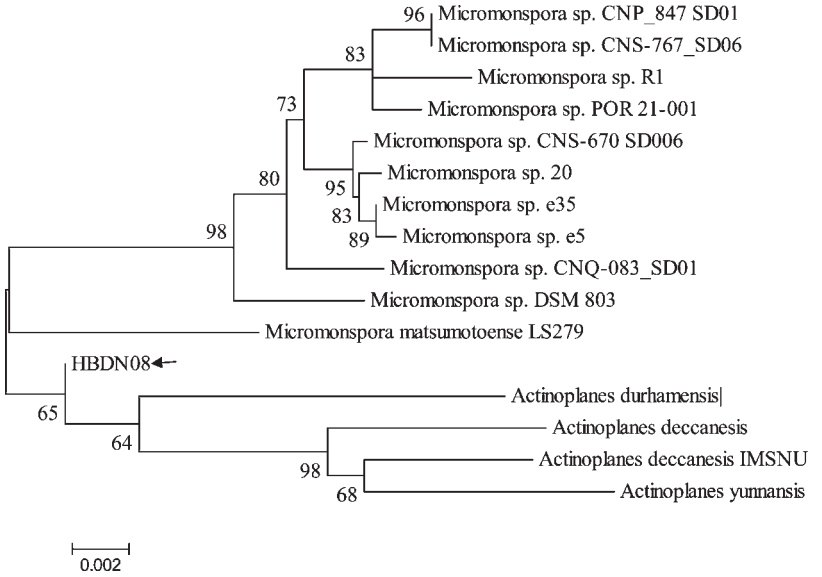


**Figure 3.** Phylogenetic analysis of strain HBDN08. HBDN08 was identified through NJ construction. Both the BLAST search and phylogenetic tree for strain HBDN08 clearly designated it as a novel strain of *Actinoplanes* sp.

presence of three motifs in HalA. Recently, the conserved regions mentioned above have been successfully employed for the construction of PCR primers to detect the putative halogenase genes and screen halometabolite-producing strains (*10*−*12*). On the basis of these findings, it was deduced that actinomycete HBDN08 had the potential to biosynthesize halogenated compounds.

The morphology of strain HBDN08 in agar medium is typical of *Actinoplanes* with an orange color of the mycelium. The phylogenetic analysis of the 16 (15 references and 1 clone) aligned sequences was conducted with 1 K bootstrap. These strains selected through a BLAST search showed maximum sequence homology percentage and query coverage as well as the lowest *E* value. The analysis of a 16S rDNA sequence homology report through BLAST and neighbor-joining (NJ) tree construction from aligned data identified the strain HBDN08 as a novel strain of *Actinoplanes* sp. (**Figure 3**). The 16S rDNA sequence of this strain has been submitted to the GenBank database under accession number FJ770217. The strain HBDN08 was deposited at the China General Microbiological Culture Collection Center (accession number CGMCC2944) and named as *Actinoplanes* sp. HBDN08.

**Structural Elucidation of Compounds 1 and 2.** Compound **1** (**Figure 1**) was isolated as an offwhite amorphous solid. Its molecular formula was established to be $C_{15}H_8Cl_2O_5$ by HRESI-MS *m*/*z* 336.9651 ([M − H]$^-$, calculated for 336.9676). The $^1$H and $^{13}$C NMR data revealed that compound **1** was an isoflavone. Its $^1$H NMR spectrum showed a hydrogen-bonded hydroxyl group at $\delta$ 12.89, due to the C-5 hydroxyl proton, and a characteristic singlet for isoflavone at $\delta$ 8.32 corresponding to H-2. The ABX spin system in $^1$H NMR $\delta$ 7.10 (1H, d, $J$ = 8.4 Hz), 7.45 (1H, dd, $J$ = 8.4, 2.0 Hz), and 7.68 (1H, d, $J$ = 2.0 Hz) indicated the presence of trisubstituted ring B of the isoflavone. Comparison of the $^1$H and $^{13}$C NMR data of compound **1** with those of compound **2** revealed that compound **1** was similar to compound **2** (*19*, *27*), except for the presence of a quarternary carbon at $\delta$ 120.9.

The observed HMBC correlated signal (**Figure 4**) of $\delta_H$ 7.10 and $\delta_C$ 120.9 assigned the quarternary carbon to C-3′ of ring B. The 34 unit enhancement of the molecular weight compared with that of compound **2** suggested a chlorine substituted at C-3′ in

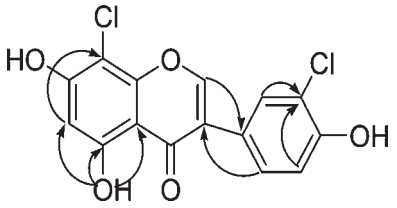


**Figure 4.** Key HMBC correlations of compound **1**.

compound **1**. The HRESI-MS gave the molecular formula $C_{15}H_8Cl_2O_5$, which further confirmed the presence of two chlorine atoms in compound **1**. Therefore, compound **1** was elucidated as 3′,8-dichlorogenistein. Compound **2** (**Figure 1**) was identified to be 8-chlorogenistein by the $^1$H, $^{13}$C NMR, and ESI-MS data and in comparison with those reported in the literature (*19*, *27*).

Previous studies showed the incorporation of a chlorine atom often occurs at the 6-, 8-, or 3′-position of genistein. These chlorinated genistein are biotransformed by bacteria, generated in human cells, or synthesized by chemical methods (*17*, *19*, *27*, *28*). However, to the best of our knowledge, there is no report so far about compound **1**, and the present result provided the first example that chlorination could be carried out simultaneously at the 8- and 3′-positions of genistein.

**Origin of Chlorinated Genistein.** Genistein is an isoflavone primarily contained in soybeans. In order to make clear whether or not compounds **1** and **2** were present in the soybean meal-containing medium, the above components in the medium were analyzed by HPLC before inoculation. The result demonstrated that except genistein, compounds **1** or **2** were not detected (**Figure 5b**). But after fermentation for 5 days, the contents of genistein in fermentation broth decreased, and compounds **1** and **2** appeared (**Figure 5c**). These results implied that compounds **1** and **2** were generated in the fermentation process. A few articles in the literature reported that some isoflavones were biotransformed by microorganisms during fermentation (*20*, *27*, *29*, *30*). In order to further confirm the origin of chlorinated compounds, we replaced $MgCl_2$ in the medium with $MgSO_4$. As we expected, there was no decrease of genistein, and also compounds **1** and **2** were not detected (**Figure 5d**). Therefore, the chlorine atoms of compounds **1** and **2** were proved to derive from the chloride in the

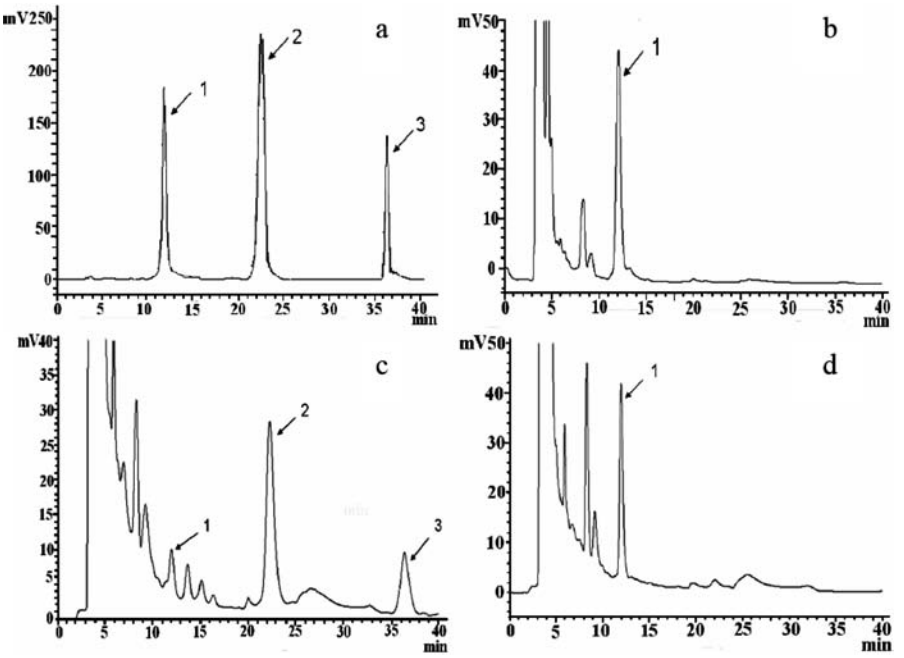


**Figure 5.** HPLC chromatograms of metabolites produced by *Actinoplanes* sp. HBDN08. (**a**) The standard samples; (**b**) fermentation for 0 day; (**c**) fermentation for 5 days; (**d**) fermentation for 5 days (the $MgCl_2$ in the fermentation medium was replaced by $MgSO_4$). The numbers 1, 2, and 3 of the peaks represent genistein, 8-chlorogenistein, and 3′,8-dichlorogenistein, respectively.

medium. These results indicated that compounds **1** and **2** were the biotransformation products of genistein by *Actinoplanes* sp. HBDN08. This is the first report of 3′,8-dichlorogenistein biotransformed by a microorganism.

Nowadays, biological halogenation has become attractive to organic and medicinal chemists because of substrate specificity and regioselectivity (*26*, *31*). Several types of halogenases including $FADH_2$-dependent halogenases are considered to play a major role in catalyzing the introduction of halogen atoms to organic compounds (*9*, *32*). They are involved in the biosynthesis of chlorinated natural products in many different bacteria. Most substrates of halogenases are tryptophan, pyrrole, and/or phenol derivatives, and activated aliphatic compounds (*8*, *9*). Genistein is a phenolic compound composed of two benzene rings (A and B) linked through a heterocyclic pyrane C ring (**Figure 1**). Furthermore, a homologous sequence of halogenase was identified in the genome of *Actinoplanes* sp. HBDN08 by a PCR-guided method using primers derived from conserved regions of the halogenase genes. Therefore, the biotransformation of chlorinated genistein by *Actinoplanes* sp. HBDN08 was deduced to be catalyzed by a halogenase.

Although no chlorinated metabolites were yet isolated from the fermentation broth of *Actinoplanes* sp. HBDN08, it did not mean that any bioactive compound was produced. The supernatant from fermentation broth showed high antibacterial activities toward *Micrococcus luteus* and *Bacillus subtilis*. However, the activities disappeared when the supernatant was processed under 45 °C for 1 h. This feature is similar to chlorinated glycopeptide antibiotics, such as vancomycin and teicoplanin (*33*, *34*). The further isolation and identification of the active components from the supernatant are still ongoing.

**Antioxidant and Antitumor Activities of Compounds 1 and 2.** The antioxidant and antitumor activities of genistein, **1**, and **2** were determined, and the results were collected in **Table 1**. Although they displayed a lipid peroxidation-inhibiting activity, their $IC_{50}$ values were lower than those of other polyphenolic antioxidants, such as kaempferol and quercetin (*24*). However, our results clearly indicate that the chlorination of genistein can enhance the antioxidant activity and can be useful information for studying the structure−antioxidant activity relationship of genistein.

**Table 1.** Antioxidant and Antitumor Activities of Genistein, **1**, and **2**

| | antioxidant activity | antitumor activity |
|---|---|---|
| compounds | $IC_{50}$ ($\mu$M) | $IC_{50}$ ($\mu$M) |
| genistein | $13.6 \pm 0.2$ | $71.1 \pm 0.6$ |
| 8-chlorogenistein (**2**) | $7.5 \pm 0.1$ | $23.7 \pm 0.5$ |
| 3′,8-dichloeogenistein (**1**) | $5.2 \pm 0.4$ | $9.2 \pm 0.3$ |

The inhibitory activities of genistein and the two chlorinated genisteins against the growth of human breast cancer cell line MDA-MB-231 were evaluated using the CCK-8 colorimetric method. Genistein, 8-chlorogenistein (**2**), and 3′,8-dichloeogenistein (**1**) dose-dependently inhibited the growth of MDA-MB-231 cells with $IC_{50}$ values of $71.1 \pm 0.6$ $\mu$M, $23.7 \pm 0.5$ $\mu$M, and $9.2 \pm 0.3$ $\mu$M, respectively. The results demonstrated that the addition of electronegative chlorine had significant effects on antitumor activities. Compared to genistein, chlorination can result in a 2.6- and 7.7-fold increase in the antitumor activity for 8-chlorogenistein (**2**) and 3′,8-dichloeogenistein (**1**), respectively. Chlorination of genistein can alter biological effects in comparison with those of parent compounds (*17*, *18*), such as estrogenic activity and antibacterial activity. Therefore, other biological effects of compounds **1** and **2** need further studies in our laboratory.

In conclusion, a PCR-guided screening strategy is a powerful tool for the rapid identification of halometabolite-producing strains. Moreover, the addition of chlorine can significantly enhance the bioactivities of genistein. The results suggest that these chlorinated compounds, especially 3′,8-dichlorogenistein (**1**), could be candidates for pharmaceutical use or as food additives.

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