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ISOFLAVONOIDS FROM STREPTOMYCETES: ORIGINS OF GENISTEIN, 8-CHLOROGENISTEIN, AND 6,8-DICHLOROGENISTEIN

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ABSTRACT.—Fermentations conducted with aureolic-acid producing and non-producing *Streptomyces griseus* strains in soybean-meal-containing medium yielded phenolic metabolites which were chomatographically and spectrally unrelated to either aureolic acid or its aglycone, chomomycinone. The yellow, phenolic compounds were isolated and characterized by ^{1}H nmr, ^{13}C nmr, and ms as the isoflavonoids genistein (5,7,4′-trihydroxyisoflavone) [**1**], 8-chlorogenistein [**2**], and 6,8-dichlorogenistein [**3**]. The presence of **1** in the soybean meal used in these fermentations was confirmed by extraction, isolation, and spectral identification. *S. griseus* grown in medium without soybean meal produced no isoflavonoids. Biotransformation of **1** yielded both chlorinated metabolites **2** and **3**, demonstrating that these isoflavonoids are products of microbial halogenation. Labelled isoflavonoids were not obtained when *S. griseus* was incubated with radiolabelled acetate or phenylalanine. These results demonstrate that isoflavonoids isolated from streptomycetes cultivated in media containing plant-derived nutrients such as soybean meal or cotton seed meal likely originate from the medium components and are not of microbial biosynthetic origin.

Isoflavonoids exhibit antimicrobial, insecticidal, estrogenic, and anticancer properties (1–6). The isoflavonoids are well known in members of the Leguminosae family (1), and numerous reports (7–13) claim the isolation of isoflavonoids from bacterial sources. However, the biosynthesis of these compounds by bacteria in general and streptomycetes in particular has not been well investigated. A common feature of all reports in which isoflavonoids are obtained from streptomycetes is that fermentations were conducted in media containing soybean or cotton seed meals as major organic medium components. Genistein (5,7,4′-trihydroxyisoflavone) and derivatives were isolated from fermentations when soybean meal was used, while narigenin (5,7,4-trihydroxyflavanone) and its derivatives were isolated when cottonseed was used in culture media. The amounts of isoflavonoids isolated appear to be directly proportional to the amounts of soybean or cottonseed meals contained in fermentation media.

Streptomycetes are extremely resourceful microorganisms. They are capable of the biosynthesis and/or biotransformation of many kinds of organic compounds (14,15). Well-known biotransformations include hydroxylations, glycosylations, deglycosylations, methylations, and halogenation reactions.

As a part of ongoing experiments concerned with the production and biogenesis of aureolic acid derivatives (16), we conducted fermentations with aureolic-acid producing and nonproducing strains of *Streptomyces* species in media with and without soybean meal. Yellow, phenolic compounds were observed only in extracts of cultures containing soybean meal. In this report, we describe the isolation and characterization of isoflavonoids and their derivatives and demonstrate with radioactive precursors that these compounds are not biosynthetically produced by our strains of *Streptomyces*.

RESULTS AND DISCUSSION

In a continuing search for new relatives of the aureolic acid family of antibiotics, fermentations were conducted using normal wild type and mutant *Streptomyces griseus* strains, and a strain of *Streptomyces plicatus* reported to produce mithamycin (17). Fermentations were grown according to the methods described by Montanari and Rosazza (16). The medium contained soybean meal, and Amberlite XAD-2 resin was added to

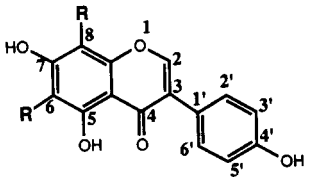
growing cultures to remove fermentation products. Solids from fermentations were removed by centrifugation, lyophilized, and extracted to afford three yellow substances with R_f values of 0.3, 0.2, and 0.1. Since these compounds all were phenolic and appeared to be yellow aureolic acid derivatives of interest, they were isolated and subjected to mass and nmr spectral analysis.

The metabolite with R_f value of 0.3 was the major compound (5 mg/3 liters culture) and was ultimately identified as genistein [**1**] based upon direct spectral and chomatographic comparison with authentic genistein, and the literature (18, 19). The cims gave *m/z* 271 $[MH]^+$, and hrfabms confirmed the molecular ion at *m/z* 271.0645 for $C_{15}H_{11}O_5$. The ^{13}C- and ^1H-nmr spectra confirmed the isolated compound structure as genistein [**1**].

A total of 3 mg of the compound at R_f 0.2 was obtained from 3 liters of *S. plicatus* fermentation and was identified as 8-chlorogenistein [**2**]. The cims of **2** gave *m/z* 305 and 307 for the $[MH]^+$ and $[MH + 2]^+$, ions respectively. These signals are typical for a chlorinated molecule where both isotopes of chlorine are evidenced in the mass spectrum. Hrfabms confirmed the molecular ion peak at *m/z* 305.0205 for $C_{15}H_{10}O_5Cl$. The ^1H-nmr spectrum was similar to that for **1**, except for the absence of singlets at 6.42 and 6.29 ppm and the appearance of a new, lone singlet at 6.52 ppm. This suggested that the chlorine atom was on the A ring at either position 6 or 8. Relative to **1**, the ^{13}C-nmr spectrum gave a new quaternary carbon signal at 99.8 ppm, while the methine carbon peak at 94.9 ppm (C-8) of **1** was absent and the C-6 carbon peak normally at 100.3 ppm was shifted downfield to 102.2 ppm. These data suggested that the chlorine atom was at the 8 position. The assignment was confirmed by an HMBC experiment (20) in which the lone proton signal at 6.52 ppm on the A ring showed long-range carbon couplings with signals at 162.5, 163.4, 99.8, and 107.9 ppm corresponding to C-5, C-7, C-8, and C-10, respectively. This result clearly indicates that the A-ring proton singlet can only be at the 6 position and the chlorine at the 8 position.

This is the first time that **2** has been identified as a halogenated genistein derivative in streptomycetes. Funayama *et al.* (21) used long-range selective proton decoupling (LSPD) experiments to make a similar assignment for 8-chloro-3′,4′,5′,7-tetrahydroxyisoflavone, which was isolated from a streptomycete grown on soybean-containing medium. König *et al.* (22) claimed to isolate 6-chlorogenistein from fermentations of *S. griseus* grown in a soybean-meal containing medium. However, structural assignments were based largely on the proximity of the single A ring proton signal at 6.50 ppm to the H-8 proton of **1** which resonates at 6.42 ppm, and were not confirmed by necessary proton-carbon correlation techniques such as HMBC or LSPD.

A 1-mg analytical sample of the metabolite at R_f 0.1 was isolated from a 3-liter *S. plicatus* fermentation grown in medium A and identified as 6,8-dichlorogenistein [**3**] by spectral analysis. The eims of **3** gave *m/z* 338, 340, and 342 for the M^+, $[M + 2]^+$, and $[M + 4]^+$ ions, respectively, for $C_{15}H_8Cl_2O_5$. The hreims confirmed the molecular ion peak at 337.97476. The ^1H-nmr spectrum was similar to those described above for



1 $R_6=R_8=H$
2 $R_6=H$, $R_8=Cl$
3 $R_6=R_8=Cl$

1 and **2**, except that proton signals for H-6 and H-8 were both absent in the spectrum. These data are sufficient to identify metabolite **3** as 6,8-dichlorogenistein. As was expected, the ^{13}C-nmr spectra of compounds **1–3** displayed similar carbon resonances for rings B and C.

Although **2** and **3** are new compounds, there have been two other reports (21,22) of chlorinated isoflavonoids being isolated from streptomycetes grown on soybean-meal containing media. These compounds were 6-chlorogenistein, 6,3-dichlorogenistein, and 8-chloro-3′,4′,5,7-tetrahydroxyisoflavone. While the mechanism of the chlorination reaction in streptomycetes is not clear and may involve the action of chloroperoxidases, the chlorine atom is believed to derive from NaCl, another component of the fermentation medium (22).

No isoflavonoids or related compounds could be detected by tlc or isolated from any of our cultures when they were grown on medium B, which contained no soybean meal, suggesting that the compounds isolated and identified above were derived from soybean meal. Soybeans contain the isoflavonoid glycosides genistin (genistein glucoside, 120 mg/100 g soybean meal), daidzin (daidzein glucoside), glycitein 7-β-glucoside, and traces of their aglycones (23–25). Cottonseed contains the flavonoid glycosides of quercetin and kaempferol (26,27). Amounts of isoflavonoids isolated from fermentations here and by others (Table 1) were essentially proportional to the amounts of soybean meal in the medium. We confirmed the presence of **1** in samples of soybean meal by extraction, hydrolysis, chromatography, and spectroscopic comparison with authentic genistein.

TABLE 1. Isoflavonoids Previously Isolated from Streptomycetes.

Reference	Medium Supplement	Isoflavonoid per vol Medium
Chimura *et al.* (7) . . .	2% Soybean meal	Genistein analogues 50 mg/400 liters
Komiyama *et al.* (8) . .	2% Soybean meal	Unreported amounts of genistein analogues in 200 liters
Hazato *et al.* (9)	1.5% Soybean meal	Genistein, daidzein and their glycosides 606 mg/28.5 liters
Umezawa *et al.* (11) . .	2% Soybean meal	Genistein and analogues 105 mg/9 liters
Nakayama *et al.* (12) . .	1% Pharmamedia (cotton seed flour)	3-Methylnaringenin 400 mg/150 liters
Satoshi *et al.* (34) . . .	2% Soybean flour	Genistein analogues 4.5 mg/20 liters

To examine the possibility that our *Streptomyces* cultures could achieve mono- and dichlorinations of isoflavonoids found in soybean meal, *S. plicatus* and *S. griseus* were incubated with **1** in medium B. Both cultures gave low yields of both **2** and **3** as characterized by chomatographic mobilities and by ms. Yields of chlorinated products were unexpectedly low, and it is possible that in normal fermentations, higher yields are obtained by initial microbial chlorination of the isoflavonoid glycosides found in soybean meal, followed by deglycosylation to the chlorinated isoflavone aglycones. Genistin or other genistein glycosides were unavailable to test this hypothesis.

Isoflavonoid biosynthesis in bacteria is not known. However, previous biosynthetic studies (1, 28–31) of isoflavonoids in plants demonstrate their common biogenesis from both acetate and phenylalanine as the usual precursors. We investigated the possibility that isoflavonoids could be formed biosynthetically by streptomycetes and not originate from plant-derived nutrients in the culture medium. Attempts to incorporate either radiolabelled sodium acetate or phenylalanine into genistein [**1**] or its metabolites using medium B were unsuccessful. Extracts of fermentations containing radioactive

precursors contained less than 1% of the total activity fed in both cases, and **1** could not be detected by chomatography. Radioactive extracts were subjected to tlc analysis, and elution of chomatographic zones scraped from tlc plates revealed little radioactivity associated with the zone for **1**. Extracts were also diluted with cold-carrier **1** and subjected to cc. In the ^{14}C-phenylalanine labelling experiment, a total of 4.4×10^6 dpm of radioactivity (1.1×10^{12} dpm/mM) was fed to the microorganism during fermentation. Only 366 dpm of radioactivity (3.9×10^4 dpm/mM) was recovered after extraction and one cc with cold-carrier **1**. Rechromatography of the initial column fractions yielded genistein fractions with no detectable radioactivity, showing that ^{14}C-phenylalanine was not incorporated into the isoflavonoid compounds. For the ^{14}C-acetate labelling study, a total of 2.77×10^6 dpm of radioactivity (1.24×10^{11} dpm/mM) was used in the fermentation. After extraction and only one cc with a cold-carrier **1**, only 65 dpm of radioactivity (2.28×10^4 dpm/mM) was recovered. The recovered radioactivity after only one cc contains less than 0.00002% of the starting specific activity of the ^{14}C-acetate. Neither radioactive acetate nor phenylalanine was incorporated into **1**. This work demonstrates that isoflavonoids are not biosynthesized from expected biosynthetic precursors by *S. plicatus*, and that the reported isolation of such compounds from other streptomycetes are largely fermentation medium artifacts.

EXPERIMENTAL

CHEMICALS.—L-Phenylalanine [^{14}C(U) (485.7 mci/mmol)] was purchased from Sigma Chemical Co., St. Louis, MO, and NaOAc [(1-^{14}C) (56 mci/mmol)] was purchased from New England Nuclear (NEN), Dupont, USA. Standard genistein was purchased from ICN Biomedicals Inc., Costa Mesa, CA. Liquid scintillation cocktail 3a70B was purchased from Research Products International, Mount Prospect, IL. Soybean meal (Victoria Feed Stock, Davenport, IA) and soybean flour (Sigma) are routinely used interchangeably in fermentation media.

GENERAL EXPERIMENTAL CONDITIONS.—^{1}H- and ^{13}C-nmr spectra were obtained on a Bruker WM 360 spectrometer equipped with Aspect 2000 software, operating at 360 and 90.5 MHz, respectively. HMBC experiments (20) were done on a Bruker AMX 600 spectrometer equipped with UXNMR software and operating at ^{1}H and ^{13}C frequencies of 600 and 150.9 MHz, repectively. Me_2CO-d_6 was used as the nmr solvent in all cases. Chemical ionizaton (NH_3) mass spectra of genistein and 8-chlorogenistein were determined on a Nermag R-10-10C (Delsi/Nermag, France) instrument using a direct inlet desorption probe. The eims of 6,8-dichlorogenistein was determined on a TRIO-1 mass spectrometer (VG Mass Laboratories, Ltd.). Hrfabms were obtained on a VG Analytical ZAB instrument with 3-nitrobenzyl alcohol (3-NBA) as the ionizing matrix. Radioactivity was determined by liquid scintillation counting (lsc) on a Beckman LS 8501 instrument with data calculated in cpm for a maximum of 10 min per sample.

CHROMATOGRAPHY.—For tlc, reversed-phased silica plates (RP-18F_{254}, Merck) and a solvent mixture of MeCN-H_2O-HCOOH (40:60:1) were used for all compounds. Developed plates were visualized by fluorescence quenching under 254 nm uv light and by spraying the developed plates with Pauley's reagent. Pauley's reagent consisted of solution A, $NaNO_2$ 0.5%; solution B, sulfanilic acid 0.5% in 2% HCl; and solution C, 5% NaOH in 50% EtOH. Equal volumes of a freshly prepared mixture of solutions A and B were sprayed onto developed tlc plates, followed by a separate spraying with solution C. Warming with a heat gun gave burnt orange colors for genistein [**1**], 8-chlorogenistein [**2**], and 6,8-dichlorogenistein [**3**] at R_f values of 0.3, 0.2, and 0.1 respectively.

Flash cc was used in the isolation of all compounds. Typical column dimensions were 2 × 20 cm, and Baker C-18 silica 40 mesh 60 gm (J.T. Baker, Phillipsburg, NJ) was used as the adsorbent. Columns were packed dry and pressurized with N_2 at 10–15 psi. The same solvent mixture used for tlc was used to elute the column at a rate of 4 ml/min, and 10-ml fractions were collected.

MICROORGANISMS.—*S. plicatus* ATCC 12957, *S. griseus* ATCC 13273, and an *S. griseus* AMY mutant strain (B.I. Dupont de Memours, Wilmington, Delaware) (32) were used in this work. Cultures were maintained on Sabouraud maltose agar slants and stored in a refrigerator at 4° prior to use.

FERMENTATIONS.—Cultures were grown in eithr medium A (containing soybean meal) glucose 2.5% (stage I), 5% (stage II), soybean meal 1.5%, $CaCO_3$ 0.3%, NaCl 0.3% (w/v) in distilled H_2O; or in medium B (no soybean meal) glucose 1%, yeast extract 0.3%, malt extract 0.3%, peptone 0.5% (all from Difco), NaCl 0.3% in distilled H_2O. Media were sterilized by autoclaving at 121°, 0.7 kg/cm^2 pressure for

20 min. Our standard two-stage fermentation protocol was used throughout (16), and cultures were grown in 200 ml of medium contained in 1-liter, stainless-steel-capped DeLong culture flasks. Stage I cultures were initiated by transferring cell suspensions of one slant of either *S. plicatus*, *S. griseus*, or the *S. griseus* AMY mutant to the sterile fermentation medium. Cultures were incubated on a gyrotary shaker (Model G-25, New Brunswick Scientific) operating at 250 rpm and 27° for 72 h. The 72 h Stage I culture was used as inoculum (10%) for Stage II cultures which were incubated under the same conditions. Amberlite XAD-2 resin (10 gm, Sigma) was added to each 200 ml 48-h-old Stage II culture to act as a solid state extraction system (16). Incubations were continued for an additional 5 days before being harvested.

ISOLATION OF THE METABOLITES.—In a typical workup, Stage II cultures (3 liters total) of *S. griseus* were centrifuged at 5000 rpm (4068 × *g*) (Sorvall, Model RC-5B Centrifuge) for 20 min, and the sedimented solids were collected, frozen, and lyophilized. The dry solids (150 gm) were extracted 3 times each with 600 ml of CH_2Cl_2-MeOH-HCOOH (80:20:1), and the extracts were dried by rotary evaporation to give 1.3 gm of crude extract. The crude brown extract was defatted with petroleum ether and dissolved in 50 ml of Me_2CO. The Me_2CO-soluble fraction (100 mg) of the crude extract was concentrated and purified by reversed-phase flash cc to give **1** (5 mg), **2** (3 mg), and **3** (1 mg), which were characterized as described below.

Genistein [**1**].—$C_{15}H_{10}O_5$, calcd for *m/z* 270; hrfabms 271.0645 for $C_{15}H_{11}O_5$; ^1H and ^{13}C nmr as in literature (18,19).

8-Chlorogenistein [**2**].—$C_{15}H_9O_5Cl$, calcd for *m/z* 304; hrfabms 305.0205 for $C_{15}H_{10}O_5Cl$; cims *m/z* (% relative abundance) $[MH]^+$ (^{37}Cl isotope) 307 (31.9%), $[MH]^+$ (^{35}Cl isotope) 305 (84.5%), 232 (51.1%), 218 (29.7%), 204 (64.7%), 153 (26.4%), 134 (100%), 117 (59.1%), 99 (28.6%); ^1H nmr (Me_2CO-d_6) ppm 8.30 (s, H-2), 7.50 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.98 (2H, d, J = 8.5 Hz, H-3′, H-5′), 6.52 (s, H-6); ^{13}C nmr (Me_2CO-d_6) ppm 182.8 (C-4), 163.4 (C-7), 162.5 (C-5), 159.8 (C-4′), 155.4 (C-2), 155.1 (C-9), 132.4 (C-2′, C-6′), 125.5 (C-1′), 123.5 (C-3), 117.1 (C-3′, C-5′), 107.9 (C-10), 102.2 (C-6), 99.8 (C-8); HMBC nmr ^1H signal at 8.30 ppm correlates with the following (^{13}C 125.5, 123.5, 155.1, 182.8), ^1H-signal at 7.50 ppm (^{13}C 117.1, 125.5, 132.4, 159.8), ^1H-signal at 6.98 ppm (^{13}C 117.1, 123.5, 159.8), ^1H signal at 6.52 ppm (^{13}C 99.8, 107.9, 163.4, 162.5).

6,8-Dichlorogenistein [**3**].—$C_{15}H_8O_5Cl_2$ calcd for *m/z* 338; hreims 337.97476; eims *m/z* (% relative abundance) $[M+4]^+$ ($2 \times {}^{37}Cl$ isotopes) 342 (8.66%), $[M+2]^+$ ($1 \times {}^{37}Cl$ isotope) 340 (68.94%), $[M]^+$ 338 (100%), 224 (5.40%), 222 (23.11%), 220 (37.88%), 149 (44.13%) 133 (20.45%), 129 (28.41%), 121 (33.52%), 118 (66.67%) 105 (25.19%); ^1H nmr (Me_2CO-d_6) ppm 8.35 (s, H-2), 7.50 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.98 (2H, d, J = 8.5 Hz, H-3′, H-5′); ^{13}C nmr (Me_2CO-d_6) ppm, 181.4, 158.7, 157.1, 154.6, 153.2, 131.2, 122.1, 115.4.

ISOLATION OF **1** FROM SOYBEAN MEAL.—Soybean meal (50 gm) was extracted by stirring 3 times with 400 ml of CH_2Cl_2-MeOH-HCOOH (80:20:1). The solvent was evaporated to give 100 mg of crude isoflavonoid glycosides. The crude extract was refluxed in 100 ml of 5% HCl in MeOH for 24 h. The solvent was evaporated, and the dark precipitate was dissolved in 50 ml H_2O. The solution was transferred to a 250 ml separatory funnel and extracted 3 times with 100 ml $CHCl_3$. The impure aglycone (10 mg) was obtained from the $CHCl_3$ layer. The aglycone was further purified by reversed-phase cc. A total of ca. 5 mg **1** was obtained, characterized by ^1H nmr and ms, and found identical to authentic genistein.

BIOCONVERSION OF GENISTEIN TO 8-CHLOROGENISTEIN AND 6,8-DICHLOROGENISTEIN.—Genistein (50 mg) dissolved in 1 ml of DMF was added to 200 ml of 48-h-old stage II *S. plicatus* and *S. griseus* cultures grown in medium B. The cultures were incubated as usual for 72 h, when tlc indicated that the substrate had been converted into the halogenated metabolites. Cultures were harvested by centrifugation (5000 rpm, 4068 × *g*), and the supernatant was collected, acidified to pH 3 with 88% HCOOH solution (19 M) (Mallinckrodt AR) and extracted 3 times with 200 ml of EtOAc. The EtOAc extracts were combined, evaporated to dryness, and washed with 50 ml of petroleum ether to remove contaminating lipids. After drying, the crude 60 mg extract was purified by reversed-phase cc to give a total of ca. 45 mg of unmetabolized **1** and less than milligram amounts of **2** and **3**, which were characterized by ms and chomatographically compared with **2** and **3** isolated earlier.

INCUBATION OF *S. PLICATUS* WITH RADIOLABELED PRECURSORS.—L-Phenylalanine [^{14}C(U), 100 μl, 1.84×10^6 cpm] and NaOAc ([1-^{14}C], 100 μl, 1.15×10^6 cpm) were separately added to duplicate 25 ml 48-h-old stage II cultures of *S. plicatus* growing on medium A. Control incubations included cultures without radioactive precursors and incubations containing radioactive precursors without microorganisms being added. The cultures and controls were incubated as usual for 7 days, and the incubator shaker was equipped with a saturated KOH solution trap to contain possible CO_2 emissions (33).

Culture and control duplicates were combined, acidified to pH 3.0 with 88% HCOOH solution

(Mallincrodt AR), and extracted three times with 50 ml of EtOAc. After combining extracts, the solvent was evaporated to obtain approximately 10–15 mg of crude extracts for each sample.

The crude extracts were dried and dissolved in 1 ml MeOH, and 50 μl samples were spotted on tlc plates along with 20 μg of standard **1**. Samples of acetate- and phenylalanine-containing cultures were also spiked with 20 μg of unlabelled **1**. Medium controls without cultures and containing radiolabelled phenylalanine or acetate were also extracted, and 50 μl of the radiolabelled precursor was also spotted. After development over a path of 10 cm, plates were divided into 10 × 1 cm sections, with section 1 representing the origin, and **1** and the chlorinated metabolites **2** and **3** were observed in sections 2 and 3. Si gel was carefully scraped from each section, and Si gel samples were eluted with 2 ml MeOH and added separately to 10 ml of scintillation cocktail for radioactivity determinations by liquid scintillation counting.

Standard **1** (5 mg) was added as cold carrier to 0.5 ml of the radiolabelled extracts. The mixture was purified by cc as before, at a rate of 4 ml/min, while 3-ml fractions were collected. Samples of 1 ml were withdrawn from each of the column fractions and analyzed by lsc for the radioactivity contents. Fractions containing **1** were determined by tlc. The concentration of isoflavones present in each fraction was determined by Beer's law and uv wavelength of 262 nm. The specific activities (dpm/mM) of the fractions were calculated from the concentrations and the radioactivity counts.

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