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Metabolism of daidzein by *Nocardia species* NRRL 5646 and *Mortierella isabellina* ATCC 38063

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Abstract

The phytoestrogen daidzein was metabolized by *Nocardia* species NRRL 5646 to give two metabolites obtained by hydroxylation and methylation. These metabolites were spectrally characterized as 7-methoxy-4′-hydroxyisoflavone (isoformononetin) and 7,8-dimethoxy-4′-hydroxyisoflavone. *Mortierella isabellina* ATCC 38063 was able to metabolize daidzein to the unusual metabolite daidzein-4′-rhamnopyranoside.

Keywords: Daidzein; Isoflavone; Isoformononetin; *Nocardia species*; *Mortierella isabellina*; Biotransformation; Microbial metabolism

1. Introduction

The phytoestrogen daidzein, 7,4′-dihydroxyisoflavone, is a major isoflavone component of soybeans, *Glycine max* (L.) Merr., Fabaceae (Hosny and Rosazza, 2002). Daidzein is consumed by humans as a component of soy and soy-based food products and as food supplement or additive to improve the nutritional value of the food. Daidzein could be also obtained following metabolism of the glycoside daidzin which occurs naturally in soy-based foods (Heinonen et al., 2002). Daidzein possesses a wide range of biological activities including antioxidant, antiinflammatory, antithrombotic, antiallergic, hypolipidemic and estrogenic properties and is considered as potential anticancer agent (Choo et al., 2002; Sugimoto and Yamaguchi, 2000; Keung and Vallee, 1998; Gooderham et al., 1996; Knight and Eden, 1996; Messina, 1995; Wong et al., 1998; Kaufman et al., 1997).

The anaerobic bacterium *Eubacterium limosum* ATCC 8486 was able to *O*-demethylate formononetin (4-methyldaidzein), glycitein (6-methyldaidzein) and biochanin A (5-hydroxy-4-methoxydaidzein) to produce daidzein, 6-hydroxydaidzein and genistein (5-hydroxydaidzein), respectively (Hur and Rafii, 2000).

The *Actinomycetes Nocardia* species NRRL 5646 is a prokaryotic organism which possesses an astonishing array of enzymes that catalyze numerous valuable biotransformation reactions with many natural product substrates (Hosny et al., 2002; Lin and Rosazza, 1998).

The fungus *Mortierella isabellina* ATCC 38063 is an eukaryotic organism commonly utilized as a microbial model for mammalian metabolism (Abourashed et al., 1999).

The aim of this work is to study the microbial metabolism of daidzein and to examine the capabilities of *Nocardia* species NRRL 5646 and *Mortierella isabellina* ATCC 38063 to transform daidzein for the purpose of converting this prototypic isoflavone to less available derivatives.

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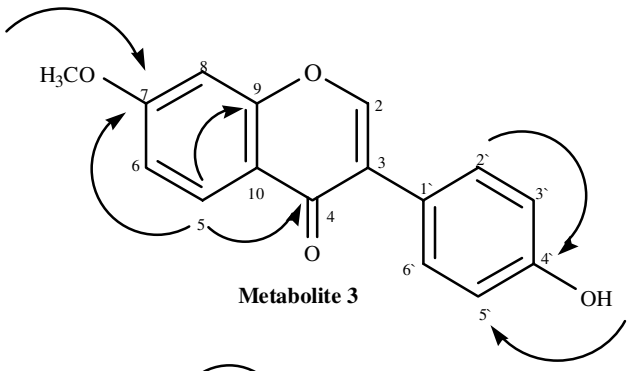
2. Results and discusions

The screening studies for the capabilities of several microorganisms, including some bacteria, fungi and yeasts, to metabolize daidzein revealed that the *Actinomycetes Nocardia* species NRRL 5646 and the fungus *Mortierella isabellina* ATCC 38063 were able to transform daidzein to different metabolites. Feeding *Nocardia species* NRRL 5646 with the phytoestrogen daidzein produced two metabolites less polar than the substrate. Metabolite **3** proved to be 7-methyldaidzein based on NMR analysis where a new proton singlet appeared at δ 3.91 was integrated for 3H and was assigned for a methoxyl group resonating at δ 56.2 in ^{13}C NMR spectrum. The location of this methoxyl group was confirmed to be at 7-position rather than 4′-position depending on the HMBC results (Fig. 1), where the proton singlet at δ 3.91, assigned for the new methyl group is correlated with the carbon signal at δ 163.7 which was assigned for C-7. The proton doublet at δ 8.02, assigned for H-5, is correlated with the carbon signals at δ 174.8, 163.7 and 157.5 assigned to C-4, C-7 and C-9, receptively. The proton doublet at δ 7.40, assigned to H-2′ and H-6′ is correlated with the carbon signal at δ 157.3 assigned to C-4′. The proton singlet at δ 9.57, assigned for the free OH group is correlated with the carbon signal at δ 115.0, assigned for C-3′ and C-5′, confirming the location of the free OH group at 4′-position. ESI-MS of metabolite **3** showed *m*/*z* 269 as $[M]^+ + 1$, 292 as $[M]^+ + Na$ and 268 as $[M]^+$ for $C_{16}H_{12}O_4$. These results confirm the structure of metabolite **3** as 7-methyldaidzein (isoformononetin).



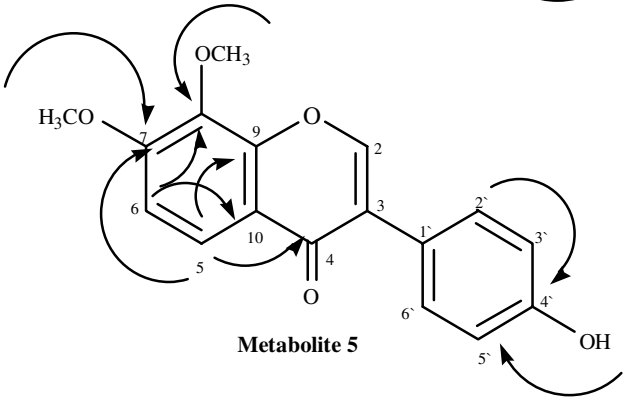


Fig. 1. Selected HMBC results for Metabolites **3** and **5**.

Spectral analysis proved that the structure of metabolite **5** has to be 7-methyl-8-methoxydaidzein. Two proton singlets appeared at δ 3.90 and 3.87, in ^{1}H NMR spectrum, each was integrated for 3H, were easily linked to the new carbon signals at δ 61.5 and 57.0, respectively. Based on HMBC correlations (Fig. 1), these signals were assigned to both methoxyl groups occurring at 7- and 8-positions of the isoflavone structure. The proton singlet at δ 3.90, assigned to the new methyl group, was correlated to the carbon signal at δ 156.5 (C-7). The proton doublet at δ 7.87, assigned to H-5, was correlated to the carbon signals at δ 175.4, 156.5 and 150.4, assigned to C-4, C-7 and C-9, respectively. The proton doublet at δ 7.30, assigned to H-6, is correlated with the carbon signals at δ 136.5 and 118.9, assigned to C-8 and C-10, respectively.

The location of the new methoxyl group was confirmed to be at 8-position based on HMBC correlations (Fig. 1), where the methoxyl proton singlet at δ 3.87 is correlated to the carbon signal at δ 136.5 (C-8). Further confirmation is evidenced by the absence of H-8 proton signal in ^{1}H NMR spectrum and desheilding of C-8 carbon signal by 33.7 ppm (from 102.8 to 136.5 ppm) which is consistent with C-8 hydroxylation. Furthermore, C-7 carbon signal is shielded by 7.1 ppm (from 163.7 to 156.6 ppm) which is consistent with an *o*-hydroxylation. Finally, the δ_c 61.5 value assigned for the new methoxyl group at 8-position is consistent with that of 7, 8-dimethoxy derivatives of isoflavone (Jha et al., 1980; Murthy et al., 1986). The proton doublet at δ 7.42, assigned to H-2′ and H-6′, is correlated with the carbon signal at δ 157.7 and is assigned to C-4′. The proton singlet at δ 9.57, assigned for the free OH group is correlated with the carbon signal at δ 115.5, assigned for C-3′ and C-5′, confirming the location of the free OH group at 4′-position.

High resolution ESI-MS gave *m*/*z* 298.0838 for $C_{17}H_{14}O_5$ (calc., 298.0841).

These results confirm the structure of **5** as 7-methyl-8-methoxydaidzein which is a new natural product.

Metabolite **6** was obtained as a single product of the metabolism of daidzein by *Mortierella isabellina* ATCC 38063. Spectrally, metabolite **6** was identified as daidzein-4′-rhamnopyranoside. The ^{1}H NMR spectrum of **6** is close to that of the substrate and showed some extra proton signals. These signals are consistent with those of rhamnose and represented by a doublet at δ 1.06 (J = 8 Hz), assigned to 3H-6 of rhamnose, a doublet at δ 5.43 (J = 2 Hz), assigned to the anomeric proton of rhamnose and a broad multiplets at δ 3.92–3.55, assigned for 4H2-5. These proton signals are easily linked to the new carbon signals at δ 98.0, 69.5, 67.3, 71.4, 67.2 and 16.5, assigned to C-1-6 of rhamnose, respectively. The location of the sugar is confirmed to be at 4′-posi-

tion based on HMBC results where the anomeric proton of the sugar at δ 5.43 is correlated with the carbon signal at δ 156.8, assigned to C-4′. ESI-MS gave *m/z* 423 for $[M]^+$ + Na and *m/z* 277 for aglycone + Na.

These results concluded the structure of **6** as daidzein-4′-rhamnopyranoside which is unusual new natural product.

In conclusion, daidzein was metabolized by *Mortierella isabellina* ATCC 38063 to daidzein-4′-rhamnopyranoside which is unusual glycosylated derivative of the isoflavone. The rhamnosylation of **1** by *Mortierella* is a one step process.

Nocardia species NRRL 5646 proved to introduce methoxylation by means of *S*-adenosylmethionine-dependant catechol-*O*-methyl transferase (Hosny et al., 2002). This *O*-methyltransferase was also detected and characterized in *streptomyces griseus* (Dhar and Rosazza, 2000; Hosney and Rosazza, 1999; Hosny et al., 2001).

Nocardia species NRRL 5646 afforded two different metabolites by *O*-methylation at position-7 and by both *O*-methylation at position-7 and hydroxylation and subsequent methoxylation of the resulting phenol at position-8 (Fig. 2). These metabolic reactions were likely catalyzed by *O*-methyltransferase enzymes similar to those found in other *Actinomycetes*. Our results suggest that *Nocardia species* NRRL 5646 may have two different types of *O*-methyltransferases, one requiring catechol, while the other requiring only a phenolic substituent.

The precise pathway for formation of metabolites **3** and **5** by *Nocardia* is unsure.

Direct methylation of **1**–**3** is certain. Formation of **5**, however, could involve hydroxylation of **3** to form **4** (8, 4′-dihydroxy-7-methoxyisoflavone) followed by methylation at position-8. Alternatively, direct hydroxylation of **1** to form catechol **2** followed by stepwise methylation to **5** can not be ruled out.

Soybean meal was used as a component of the growing cultures medium and many isoflavones are reported in soybean (Hosny and Rosazza, 2002). However, the isolated daidzein metabolites are different from those reported naturally and our results were verified by direct comparison with grown cultures under same conditions without adding the substrate.

3. Experimental

3.1. General

NMR spectra were obtained in DMSO-d_6 using TMS as the internal standard with chemical shifts expressed in δ and coupling constants (*J*) in Hz. Routine ^{1}H and ^{13}C NMR spectra were obtained with a Bruker NMR 400 (Bruker Instruments, Billerica, MA), operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). HMBC NMR experiments were carried out using a Bruker AMX-600 high-field spectrometer equipped with an IBM Aspect-2000 processor and with VNMR version 4.1 software.

Mass spectrometry was obtained as electrospray ionization spectra (ESI-MS) and high resolution electrospray ionization (HRES-ESI-MS) taken on a VG-ZAB-HF reversed-geometry (BE configuration, where B is a magnet sector and E is an electrostatic analyzer) mass spectrometer (MS) (VG Analytical Inc.).

Flash column liquid chromatography was performed using J.T. Baker glassware with 40 μSi gel (Baker) as the stationary phase. TLC was carried out on precoated Si gel 60 GF_{254} (Merck) plates. Developed chromatograms were visualized under UV light and by spraying developed plates with 0.01% vanillin/H_2SO_4, followed by heating at 100 °C for 10–20 s. Preparative scale TLC was carried out on 1-mm-thick Si gel 60 GF_{254} plates.

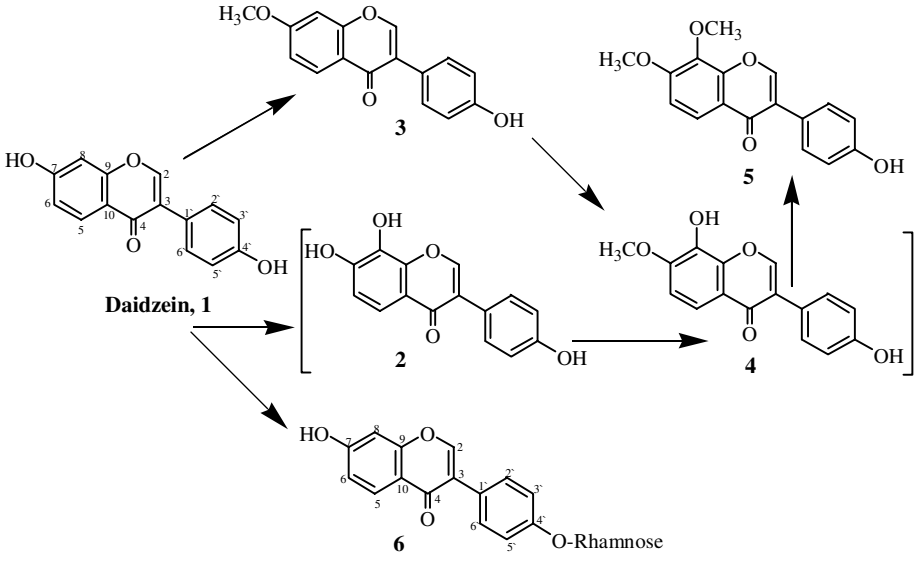


Fig. 2. Metabolism of daidzein by *Nocardia species* and *Mortierella isabellina*.

3.2. Microorganisms and substrate

Nocardia species NRRL 5646, *Mortierella isabellina* ATCC 38063, *Absidia pseudocylindrospora* ATCC 24169, *Aspergillus alliaceus* UI 315, *Aspergillus niger* ATCC 9142, *Bacillus cereus* NRRL B-14591, *Bacillus megaterium* ATCC 14581, *Beauveria bassiana* ATCC 7159, *Cunninghamella echinulata* ATCC 8688a, *Cunninghamella elegans* ATCC 9245, *Gliocladium virdi* ATCC 10097, *Comamonas testosterone* ATCC 11996, *Streptomyces griseus* ATCC 13273, *Thamnidium elegans* ATCC 18191, *Rhizopus stolonifer* NRRL 1478, *Cuvularia lunata* ATCC 38850, *Botrytis allii* NRRL 2502, *Candida tropicalis* UI 2312, *Aspergillus ochraceous* ATCC 1008, *Mucor mucedo* UI 5513, *Rhodtorula rubra* ATCC 20129 and *Amycolata autotrophica* ATCC 35203 were maintained at the University of Iowa, College of Pharmacy culture collection on slants of Sabouraud–dextrose agar or sporulation agar (ATCC medium #5; Gherna et al., 1982). Daidzein was obtained from Aldrich, Milwaukee, WI, USA. The purity of the substrate was determined by TLC and ^{1}H NMR spectrometry.

3.3. Analytical scale fermentation

The different microorganisms cultures were grown according to the standard two-stage fermentation protocol (Betts et al., 1974). Screening experiments were done in 125 ml DeLong culture flasks. The culture flasks held one fifth of their volume of the following medium: 2% glucose, 0.5% soybean meal, 0.5% yeast extract, 0.5% NaCl and 0.5% K_2HPO_4. The pH of the medium was adjusted to 7 using 6 N HCl before autoclaving for 20 min at 121 °C and 15 psi. After inoculation with the microorganism, stage I culture were incubated by shaking at 200 rpm and at 30 °C on New Brunswick Scientific, Innova 5000 Gyratory Tier, shaker (Edison, N.J.) for 72 h before being used to inoculate stage II culture flasks. Usually, 10% inoculum volumes are recommended. Two mg of daidzein, disolved in 20 μl dimethylformamide (DMF), was added to each flask of 24-h-old stage II cultures, which were incubated again and sampled periodically for analysis.

Samples of 1 ml each were taken after 12, 24, 36 and 48 h and every other day for 2 weeks following substrate addition. Each sample was extracted by shaking with 0.5 ml of 10% *n*-butanol/EtOAc and spun at 3.000*g* for 1 m in a desk-top centrifuge. EtOAc extract from all samples were spotted on Si gel GF_{254} TLC plates, developed in 10% or 15% MeOH/CH_2Cl_2 solvent systems, and visualized under UV light and after spraying with 0.01% vanillin/H_2SO_4, followed by heating for 5–10 s with a heat gun.

3.4. Preparative scale fermentation

Double sets of ten 125 ml DeLong culture flasks stage II cultures of *Nocardia species* NRRL 5646 and *Mortierella isabellina* ATCC 38063 were prepared. Each set re-

Table 1
^{1}H and ^{13}C NMR data for Daidzein and its metabolites[a]

	Daidzein		Metabolite **3**		Metabolite **5**		Metabolite **6**	
#	^{13}C	^{1}H (*J* = Hz)	^{13}C	^{1}H (*J* = Hz)	^{13}C	^{1}H (*J* = Hz)	^{13}C	^{1}H (*J* = Hz)
2	152.9	8.36, *s*	153.2	8.38, *s*	153.7	8.42, *s*	152.9	8.28, *s*
3	122.8		122.3		123.7		123.0	
4	177.2		174.8		175.4		174.5	
5	127.8	8.02, *d* (6.6)	127.0	8.02, *d* (6.6)	121.3	7.87, *d* (6.6)	129.0	7.90, br. *d*
6	115.0	7.24, *dd* (6.6)	117.6	7.08, *dd* (6.6)	111.4	7.30, *dd* (6.6)	112.3	6.92, br. *d*
7	162.6		163.7		156.5		157.7	
8	102.8	7.42, *d* (1.6)	100.6	7.16, *d* (1.6)	136.5		105.4	6.64, br. *s*
9	157.8		157.5		150.4		155.3	
10	116.9		114.8		118.9		122.2	
1′	123.6		123.7		122.7		125.5	
2′	130.2	7.39, *d* (6.3)	130.1	7.40, *d* (6.4)	130.5	7.42, *d* (6.6)	129.9	7.48 *d* (6.6)
3′	115.0	6.80, *d* (6.3)	115.0	6.81, *d* (6.4)	115.5	6.83, *d* (6.6)	116.3	7.08 *d* (6.6)
4′	158.1		157.3		157.7		156.8	
5′	115.0	6.80, *d* (6.3)	115.0	6.81, *d* (6.4)	115.5	6.83, *d* (6.6)	116.3	7.08 *d* (6.6)
6′	130.2	7.39, *d* (6.3)	130.1	7.40, *d* (6.4)	130.5	7.42, *d* (6.6)	129.9	7.48 *d* (6.6)
7-OMe			56.2	3.91, *s*	61.5	3.90, *s*		
8-OMe					57.0	3.87, *s*		
4′-OH		9.59		9.57		9.57		–
1″							98.0	5.43, *d* (2)
2″							69.5	3.92–3.55, 4H, br. *m* (H_2–H_5)
3″							67.3	
4″							71.4	
5″							67.2	
6″							16.5	1.06, *d* (8)

[a] In DMSO-d_6 at 400 MHz (^{1}H) or 100 MHz (^{13}C). Chemical shifts (δ) are expressed in ppm. Assignments based on HMBC.

ceived 100 mg daidzein in 1 ml DMF (400 μg substrate per ml of culture medium). After incubation for 7 days under the usual condition, the cultures of *Nocardia species* NRRL 5646 were combined, adjust pH to 3 and exhaustively extracted with 3 × 2 l of 10% *n*-butanol/EtOAc. The extract was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to yield a crude dark residue (187 mg). After incubation for 12 days (where maximum conversion was observed) under the usual condition the cultures of *Mortierella isabellina* ATCC 38063 were combined. The pH was adjusted to 3 and exhaustively extracted with 3 × 2 l of 10% *n*-butanol/EtOAc. The extract was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to yield a crude dark residue (233 mg).

3.5. Isolation and purification of the metabolites

Nocardia species extract (180 mg) was column chromatographed by the flash method, 50 g Si gel, 1 × 45 cm. The elution was achieved isocratically by Hexane–DCM–MeOH + HCOOH (5:5:0.5 + 0.01%) to afford one product (22 mg). Final purification achieved on prep. TLC using 5% MeOH/(Hex: DCM, 1:1) to afford 18 mg product (R_f = 0.37). This product was subjected to prep TLC using 20% IPA/Hexane to give **3** (5 mg) and **5** (9 mg), as white powders (R_f = 0.42 and 0.5, respectively). ESI-MS of **3** gave *m/z* 269 as $[M]^+ + 1$, 292 as $[M]^+ + Na$ and 268 as $[M]^+$ for $C_{16}H_{12}O_4$. High resolution ESI-MS of **5** gave *m/z* 298.0838 for $C_{17}H_{14}O_5$ (calc., 298.0841). The ^1H and ^{13}C NMR results of metabolites **3** and **5** are listed in Table 1.

Mortierella isabellina extract (230 mg) was column chromatographed by the flash method, 50 g Si gel 1 × 45 cm. The elution was achieved by DCM + 0.01%HCOOH with increasing the polarity with MeOH as 2%, 4%, ... 10% and 20%, for each 500 ml eluate. Fractions eluted with 10% contain the target metabolite. It was purified on 1-mm-thick TLC plates using 25% IPA/Hexane (3 developments) to afford 5 mg of metabolite **6** as white powder. ESI-MS gave *m/z* 423 for $[M]^+ + Na$ and *m/z* 277 for aglycone + Na. The ^1H and ^{13}C NMR results of metabolite **6** are listed in Table 1.

References

Abourashed, E.A., Clark, A.M., Hufford, C.D., 1999. Microbial models of mammalian metabolism of xenobiotics: an updated review. Curr. Med. Chem. 6, 359–374.

Betts, R.E., Walters, D.E., Rosazza, J.P.N., 1974. Microbial transformation of antitumor compounds. 1. Conversion of acronycine to 9-hydroxyacronycine by *Cunninghamella echinulata*. J. Med. Chem. 17, 599–602.

Choo, M.-K., Park, E.-K., Yoon, H.-K., Kim, D.-H., 2002. Antithrombotic and antiallergic activities of daidzein, metabolite of puerarin and daidzin produced by human intestinal microflora. Biol. Pharm. Bull. 25, 1328–1332.

Dhar, K., Rosazza, J.P.N., 2000. Purification and characterization of *Streptomyces griseus* Catechol *O*-methyltransferase. Appl. Environ. Microbiol. 66, 4877–4882.

Gherna, R., Pienta, P., Cote, R., 1982. ATCC Cataloge of Bacteria and Bacteriophages, 18th ed. American Type Culture Collection, Rockville MD, P 415.

Gooderham, M.H., Adlercreutz, H., Ojala, S.T., Wahala, K., Holub, B.J., 1996. A soy protein isolate rich in genistein and daidzein and its effects on plasma isoflavones concentration, platelet aggregation, blood lipids and fatty acid composition of plasma phospholipids in normal men. J. Nutr. 126, 2000–2008.

Heinonen, S.-M., Wahala, K., Adlercreutz, H., 2002. Metabolism of isoflavones in human subjects. Phytochem. Rev. 1, 175–182.

Hosney, M., Rosazza, J.P.N., 1999. Microbial hydroxylation and methylation of genistein by *Streptomycetes*. J. Nat. Prod. 62, 1609–1612.

Hosny, M., Rosazza, J.P.N., 2002. New isoflavone and triterpene glycosides from soybean. J. Nat. Prod. 65, 805–813.

Hosny, M., Dhar, K., Rosazza, J.P.N., 2001. Hydroxylations and methylations of quercetin, fisetin and catechin by *Streptomyces griseus*. J. Nat. Prod. 64, 462–465.

Hosny, M., Johnson, H.A., Ueltschy, A.K., Rosazza, J.P.N., 2002. Oxidation, reduction and methylation of carnosic acid by *Nocardia*. J. Nat. Prod. 65, 1266–1269, and references therein.

Hur, H.-G., Rafii, F., 2000. Biotransformation of the isoflavonoids biochanin A, formonoetin and glycitein by *Eubacterium limosum*. FEMS Microbiol. Lett. 192, 21–25.

Jha, H.C., Ziliken, F., Breitmaier, E., 1980. Carbon-13 chemical shift assignments of chromones and isoflavones. Can. J. Chem. 58, 1211–1219.

Kaufman, P.B., Duke, J.A., Brielmann, H., Boik, J., Hoyt, J.E., 1997. A comparative survey of leguminous plants as sources of the isoflavones, genisitein and daidzein: implications for human nutrition and health. J. Altern. Complem. Med. 3, 7–12.

Keung, W.-M., Vallee, B.L., 1998. Daidzein and its antidipostropic analogs inhibit serotonin and dopamine metabolism in isolated mitochondria. Proc. Natl. Acad. Sci. USA 95, 2198–2203.

Knight, D.C., Eden, J.A., 1996. A review of the clinical effects of phytoestrogens. Obstet. Gynecol. 87, 897–904.

Lin, S.-J., Rosazza, J.P.N., 1998. Microbial transformations of isocupressic acid. J. Nat. Prod. 61, 922–926.

Messina, M., 1995. Modern applications for an ancient bean: soybeans and the prevention and treatment of chronic disease. J. Nutr. 125, 5675–5695.

Murthy, M.S.R., Rao, V., Ward, R.S., 1986. Carbon-13 nuclear magnetic resonance spectra of isoflavones. Mag. Reson. Chem. 24, 225–230.

Sugimoto, E., Yamaguchi, M., 2000. Stimulatory effect of daidzein in oesteoblastic MC3T3-E1 cells. Biochem. Pharmacol. 59, 471–475.

Wong, W.W., Smith, E.O., Stuff, J.E., Hachey, D.L., Heird, W.C., Pownell, H.J., 1998. Cholesterol lowering effect of soyprotein in normocholesterolemic and hypercholesterolemic men. Am. J. Clin. Nutr. 68, 13855–13895.