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Bioorganic & Medicinal Chemistry Letters 16 (2006) 790–793
Bioorganic & Medicinal Chemistry Letters

Microbial transformation of silybin by *Trichoderma koningii*

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Received 9 September 2005; revised 29 October 2005; accepted 8 November 2005
Available online 23 November 2005

Abstract—Microbial transformation of silybin A (**1**) and silybin B (**2**), the major hepatoprotective flavonolignan diastereomers from the fruits of *Silybum marianum*, with the culture broth of *Trichoderma koningii* gave two pairs of glucosylated derivatives. Their structures were identified as silybin A 3-*O*-β-D-glucopyranoside (**3**), silybin A 7-*O*-β-D-glucopyranoside (**4**), silybin B 3-*O*-β-D-glucopyranoside (**5**) and silybin B 7-*O*-β-D-glucopyranoside (**6**) by spectroscopic methods.

Silybin is a major hepatoprotective flavonolignan of silymarin, isolated from the fruits of milk thistle *Silybum marianum* (L.) Gaertn., which is used widely as a natural remedy (Legalon®, Madaus, Germany) in the supportive therapy of liver diseases.[1,2] Silybin is structurally a diastereoisomeric mixture of two flavonolignans, namely, silybin A (**1**) and silybin B (**2**), in approximately 1:1 ratio, and each of the silybin isomers was successfully isolated by preparative reversed-phase HPLC. Stereochemistry of the two diastereomers was determined as 2*R*, 3*R*, 7′R, 8′R for silybin A, and 2*R*, 3*R*, 7′S, 8′S for siybin B, respectively.[3,4] Silymarin and its major active constituent, silybin, have been reported to work as antioxidants, which prevent lipid peroxidation and scavenge free radicals, as well as increase hepatocyte protein synthesis and accelerate regeneration in damaged liver.[5] Unfortunately, very low solubility of silybin in water (430 mg/L) has been known to limit its bioavailability in oral administration of this drug.[2] Chemical and biochemical synthetic approaches to obtain new silybin derivatives have been performed for improvement of bioavailability and therapeutic efficiency of silybin. Its solubility was strongly improved by the preparation of silybin bishemisuccinate that enabled intravenous application of silybin (Legalon-SIL, Madaus, Germany).[6]

Previous studies showed several other forms of derivatives or metabolites with higher polarity which are modified at C-5, C-7, C-9′ and C-4″ of silybin by glycosylation,[7–9] glucuronidation,[10,11] oxidation,[12] or by phosphorylation.[13]

Microbial transformation studies have been known also to be useful methods to obtain more active or less toxic compounds and to achieve selective conversions of compounds to useful derivatives which are difficult to produce synthetically.[14] Biotransformation of silybin by microbes may offer probability to obtain some novel metabolites with higher water solubility. In the present study on the microbial transformation of two silybin isomers, silybin A (**1**) and silybin B (**2**), a preparative scale biotransformation by *Trichoderma koningii* KCTC 6042 afforded two pairs of glucosylated metabolites. We report unambiguous structure elucidation of these microbial metabolites herein.

The microorganisms[15] used for preliminary screening were obtained from Korean Collection for Type Cultures (KCTC). Cultures were grown according to the two-stage procedure.[16] The actively growing microbial cultures were inoculated in 250 mL flasks containing 50 mL of YM media,[17] and incubated with gentle agitation (200 rpm) at 25 °C in a temperature-controlled shaking incubator. The ethanolic solution (1 mg/mL) of silybin diastereomers[18] was added to each flask 24 h after inoculation and further incubated at the same condition for 4 days. General sampling and TLC monitoring (Si gel 60 F_{254}, $CHCl_3$–MeOH–H_2O = 70:30:4) were performed at 24 h intervals.

Keywords: Silybin; Microbial transformation; *Trichoderma koningii*; Silybin A 3-*O*-β-D-glucopyranoside; Silybin A 7-*O*-β-D-glucopyranoside; Silybin B 3-*O*-β-D-glucopyranoside; Silybin B 7-*O*-β-D-glucopyranoside.
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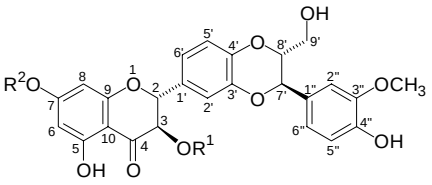
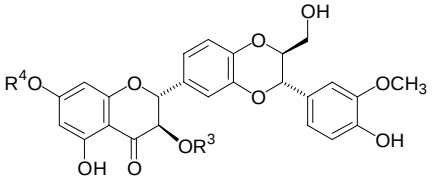
doi:10.1016/j.bmcl.2005.11.022



silybin A (**1**): R^1= H, R^2= H

silybin A 3-*O*-β-D-glucopyranoside (**3**): R^1= β-D-glucopyranosyl, R^2= H

silybin A 7-*O*-β-D-glucopyranoside (**4**): R^1= H, R^2= β-D-glucopyranosyl



silybin B (**2**): R^3= H, R^4= H

silybin B 3-*O*-β-D-glucopyranoside (**5**): R^3= β-D-glucopyranosyl, R^4= H

silybin B 7-*O*-β-D-glucopyranoside (**6**): R^3= H, R^4= β-D-glucopyranosyl

Of a total of 26 microbial cultures screened, *T. koningii* KCTC 6042 was found to be capable of biotransforming silybin based on the TLC analyses and control studies. R_f values suggested that the produced metabolite (R_f 0.22) is more polar compared with silybin (R_f 0.55). Further reversed-phase C_{18} TLC showed that this metabolite was composed of two types of silybin metabolites (R_f 0.74 and 0.50, MeOH 60%). Substrate and culture controls[19] exhibited that the metabolites were formed as a result of enzymatic activity, but not a consequence of degradation or non-metabolic changes. In scale-up studies, *T. koningii* was grown on a shaking incubator at 25 °C for 5 days in eighty 250 mL Erlenmeyer flasks, each containing 50 mL YM medium. Compounds **1** (50 mg) and **2** (50 mg) dissolved in EtOH were evenly distributed between flasks 1 day after incubation. The combined liquid culture filtrates were extracted with EtOAc (2 L × 2) and the organic layer was concentrated in vacuo. Two separate EtOAc extracts [632 mg for silybin A (**1**); 834 mg for silybin B (**2**)] were chromatographed on a silica gel column using a $CHCl_3$–MeOH–H_2O (100:20:1) isocratic eluent. The fractions containing microbial metabolites of **1** and **2** were rechromatographed on a Lobar® prepacked column using 32% MeOH to yield compounds **3** (12 mg, 18.0% yield) and **4** (5 mg, 7.5%) for silybin A and **5** (10 mg, 15.0%) and **6** (3 mg, 4.5%) for silybin B, respectively.

Metabolites **3**[20] and **5**[22] were obtained as a pale yellow amorphous powder by column chromatography. Close resemblances between **3** and **5** as well as between **4** and **6** were observed in their IR, UV, ^{1}H and ^{13}C NMR spectral data and reversed-phase TLC profiles. It was suggested that they are diastereoisomeric pairs of silybin metabolites. HRESIMS and HRFABMS spectra of **3** and **5** showed $[M+Na]^+$ peaks (calcd *m*/*z* 667.1639) at *m*/*z* 667.1595 and *m*/*z* 667.1602, respectively, which established a molecular formula of $C_{31}H_{32}O_{15}$ (MW 644). Their IR spectra exhibited absorption bands at ν_{max} 3400 (–OH), 1640 (–C=O), 1510 and 1275 cm^{-1}, and UV spectra in MeOH showed maximum absorption at λ_{max} 288 nm, which are similar to those of parent compounds. The ^{1}H and ^{13}C NMR spectra of **3** and **5** were remarkably different from those of **1** and **2** in several aspects. Each of the ^{1}H NMR spectra (Table 1) showed seven new characteristic signals typical with a sugar moiety between δ_H 3.03 and δ_H 3.96, in addition to all the signals assignable to **1** and **2**. The ^{13}C NMR spectra (Table 2) exhibited six novel carbon signals between δ_C 62.6 and δ_C 102.9, which were not observed in case

Table 1. ^{1}H NMR data of compounds **3**–**6**[a]

Position	3	4	5	6
2	5.28 (1H, d, 9.5)	5.03 (1H, d, 11.5)	5.31 (1H, d, 9.5)	5.03 (1H, d, 11.0)
3	4.95 (1H, d, 9.5)	4.57 (1H, d, 11.5)	4.94 (1H, d, 9.5)	4.57 (1H, d, 11.0)
6	5.89 (1H, br s)	6.22 (1H, br s)	5.90 (1H, br s)	6.22 (1H, br s)
8	5.89 (1H, br s)	6.23 (1H, br s)	5.90 (1H, br s)	6.23 (1H, br s)
2′	7.12 (1H, br s)	7.12 (1H, d, 2.0)	7.11 (1H, d, 2.0)	7.11 (1H, br s)
5′	6.99 (1H, d, 8.5)	7.01 (1H, d, 8.5)	6.99 (1H, d, 8.5)	7.02 (1H, d, 8.5)
6′	7.04 (1H, br d, 8.5)	7.05 (1H, dd, 8.5, 2.0)	7.05 (1H, dd, 8.5, 2.0)	7.05 (1H, br d, 8.0)
7′	4.92 (1H, d, 8.5)	4.93 (1H, d, 8.0)	4.92 (1H, d, 7.5)	4.93 (1H, d, 8.0)
8′	4.08 (1H, ddd, 8.0, 4.5, 2.5)	4.08 (1H, ddd, 8.0, 4.5, 2.0)	4.07 (1H, ddd, 8.0, 4.5, 2.5)	4.08 (1H, ddd, 8.0, 4.5, 2.5)
9′a	3.48 (1H, dd, 12.5, 4.5)	3.50 (1H, dd, 12.5, 5.5)	3.49 (1H, dd, 12.5, 4.5)	3.49 (1H, dd, 12.5, 4.5)
9′b	3.70 (1H, dd, 12.5, 2.5)	3.71 (1H, dd, 12.5, 2.5)	3.70 (1H, dd, 12.5, 2.5)	3.71 (1H, br d, 12.5)
2″	7.01 (1H, br s)	7.02 (1H, br s)	7.02 (1H, d, 1.5)	7.02 (1H, br s)
5″	6.83 (1H, d, 8.0)	6.84 (1H, d, 8.2)	6.83 (1H, d, 8.0)	6.84 (1H, d, 8.0)
6″	6.90 (1H, dd, 8.2, 1.5)	6.91 (1H, dd, 8.2, 2.0)	6.91 (1H, dd, 8.5, 2.0)	6.91 (1H, br d, 8.5)
1‴	3.94 (1H, d, 7.5)	4.96 (1H, d, 7.0)	3.96 (1H, d, 7.5)	4.96 (1H, d, 7.0)
2‴	3.23 (1H, br t, 8.5)	3.43 (1H, t, 8.5)	3.24 (1H, dd, 7.5, 9.0)	3.43 (1H, t, 8.5)
3‴	3.16 (1H, br t, 9.0)	3.44 (1H, m)	3.17 (1H, br t, 9.0)	3.44 (1H, m)
4‴	3.28 (1H, br t, 9.5)	3.39 (1H, d, 9.0)	3.30 (1H, br t, 9.5)	3.38 (1H, d, 9.5)
5‴	3.04 (1H, ddd, 9.5, 5.0, 2.0)	3.44 (1H, m)	3.03 (1H, ddd, 9.5, 5.5, 2.5)	3.44 (1H, m)
6‴a	3.63 (1H, dd, 12.0, 5.5)	3.68 (1H, dd, 12.5, 5.5)	3.60 (1H, dd, 12.0, 5.5)	3.67 (1H, dd, 12.5, 5.5)
6‴b	3.77 (1H, br d, 12.0)	3.88 (1H, dd, 12.0, 1.5)	3.75 (1H, dd, 12.0, 2.5)	3.86 (1H, br d, 12.0)
OCH_3	3.86 (3H, s)	3.87 (3H, s)	3.87 (3H, s)	3.87 (3H, s)

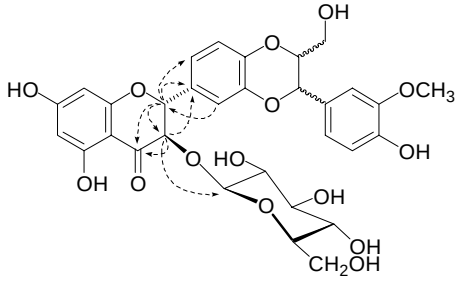
[a] In CH_3OH-d_4, δ in ppm (*J* in Hz), 500 MHz spectrometer; assignments confirmed by HMQC and HMBC experiments.

Table 2. ^{13}C NMR data of compounds **3**–**6**[a]

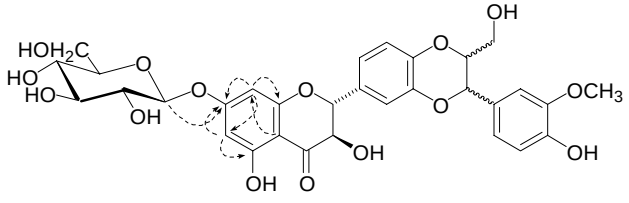
Position	3	4	5	6
2	83.3	85.0	83.2	84.8
3	77.2	74.0	77.4	73.9
4	195.8	199.3	195.6	199.2
5	165.6	164.3	165.6	164.2
6	96.7	97.1	96.7	97.0
7	170.1	167.5	170.0	167.4
8	97.7	98.6	97.7	98.4
9	164.1	164.9	164.1	164.8
10	102.4	103.7	102.5	103.5
1′	130.8	131.5	130.8	131.4
2′	117.9	117.8	117.9	117.7
3′	145.2	145.3	145.3	145.2
4′	145.6	145.7	145.6	145.6
5′	117.9	118.0	118.0	117.9
6′	122.4	122.4	122.2	122.3
7′	77.7	77.9	77.8	77.8
8′	80.0	80.2	80.1	80.1
9′	62.1	62.2	62.1	62.1
1″	129.4	129.5	129.4	129.4
2″	112.1	112.2	112.1	112.0
3″	149.2	149.4	149.3	149.3
4″	148.3	148.5	148.4	148.4
5″	116.3	116.4	116.3	116.3
6″	121.8	121.8	121.7	121.7
1‴	102.9	101.4	102.9	101.3
2‴	74.5	74.8	74.6	74.7
3‴	77.6	77.9	77.7	77.8
4‴	71.1	71.3	71.3	71.1
5‴	78.2	78.4	78.3	78.3
6‴	62.6	62.5	62.6	62.3
OCH_3	56.5	56.6	56.6	56.5

[a] In CH_3OH-d_4, δ in ppm (J in Hz), 125 MHz spectrometer.

of their parent compounds. The six-membered ring sugar was assigned a glucopyranose based on the spin-systems with consistently large vicinal coupling constants (J = 7.0–9.5 Hz). $J_{H,H}$ values (7.5 Hz) of anomeric protons (H-1‴) indicated that these sugars have β-configurations. Also, ^1H and ^{13}C NMR spectra of **3** and **5** exhibited the methine signals (C-3) shifted downfield at δ_H 4.95, δ_C 77.2, and δ_H 4.94, δ_C 77.4, which suggested that a hydroxyl group of C-3 was glucosylated by the fungus. These findings were further confirmed by the HMBC correlation between H-3 and C-1‴ (Fig. 1). Unambiguous assignments for both aglycone and the sugar moiety were established by HSQC and HMBC experiments. The structures of the two microbial metabolites **3** and **5** were established as silybin A 3-*O*-β-D-glucopyranoside and silybin B 3-*O*-β-D-glucopyranoside, respectively.



silybin 3-*O*-β-D-glucopyranosides (**3** and **5**)



silybin 7-*O*-β-D-glucopyranosides (**4** and **6**)

Figure 1. Key HMBC correlations of microbial metabolites **3**–**6.**

Metabolites **4**[21] and **6**[23] were obtained as a yellowish amorphous powder. The molecular formulae of **4** and **6** were assigned $C_{31}H_{32}O_{15}$ (MW 644) from their HRESIMS ($[M+Na]^+$ *m/z* 667.1642, **4**; *m/z* 667.1641, **6**) and ^{13}C NMR spectra, which also suggested that they are glycosylated derivatives of **1** and **2**. Their IR spectra showed that there are absorption bands very closely related with those of **3** and **5**, and the UV spectra (MeOH) showed maximum absorption at around 286 nm. The ^1H and ^{13}C NMR spectra (Tables 1 and 2) of **4** and **6** showed also seven and six additional characteristic signals of a sugar moiety, respectively, which were not observed in case of **1** and **2**. However, protons of the sugar were observed in the more downfield region ranging from δ 3.38 to 4.96, when compared with those of **3** and **5**. The sugar was assigned a glucopyranose based on the large vicinal coupling constants (J = 7.0–9.5 Hz), and it has β-configuration as suggested by $J_{H,H}$ values (7.0 Hz) of anomeric protons (H-1‴). Compounds **4** and **6** displayed the aromatic signals (C-6 and C-8) shifted downfield at δ_H 6.22, 6.23, δ_C 97.1, 98.6 and δ_H 6.22, 6.23, δ_C 97.0, 98.4, respectively. These findings indicated that the sugars were located at C-7 as *O*-glycoside forms, which was further established by HMBC correlations between C-7 and H-1‴ (Fig. 1). Therefore, unambiguous structures of **4** and **6** were assigned as silybin A 7-*O*-β-D-glucopyranoside and silybin B 7-*O*-β-D-glucopyranoside, respectively. It was noted that the presence of compound **4** was previously reported from the plant cell culture of *Papaver somniferum* var. *setigerum* with silybin A by Křen et al.[8]

Remarkable differences were not recognized between silybin A and silybin B in terms of the yield of each metabolite glucosylated at the same position. However, O-glucosylation of silybin isomers at C-3 was much preferred to O-glucosylation at C-7 by the fungus. It was interesting to note that production of 3-*O*-glycosides was readily achieved by microbial transformation, while the secondary alcohol at C-3 has been reported to be partially substituted by chemical synthetic methods only under prolonged reaction times with formation of small amounts of 3,9′-diglycosides.[7] Production of silybin analogues using microbial metabolic transformation has never been reported yet. This is the first report on the presence and unambiguous structure elucidation of silybin A 3-*O*-β-D-glucopyranoside, silybin B 3-*O*-β-D-glucopyranoside and silybin B 7-*O*-β-D-glucopyranoside.

Acknowledgments

The authors thank the Korea Basic Science Institute for running NMR and HRMS experiments. This work was supported by the Korea Research Foundation Grant funded by the Korean government (MOEHRD) (Grant No.: KRF-202-2003-F00008).

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15. The cultures used for preliminary screening are as follows: *Absidia spinosa* KCTC 6588, *Aspergillus fumigatus* 6145, *Candida albicans* 7965, *Candida famata* 7000, *Candida solani* 7689, *Debaryomyces hansenii* var. *hansenii* 7645, *Debaryomyces occidentalis* var. *occidentalis* 7194, *Debaryomyces robertsiae* 7299, *Filobasidium capsuligenum* 7102, *Filobasidium neoformans* 7902, *Gliocladium deliquescens* 6173, *Kluyveromyces marxianus* 7155, *Metarhizium flavoviride* var. *minus* 6310, *Metschnikowia pulcherrima* 7605, *Microbacterium lacticum* 9230, *Mortierella ramanniana* var. *angulispora* 6137, *Mycobacterium phlei* 3037, *Penicillium chrysogenum* 6933, *Pichia pastoris* 7190, *Saccharomyces cerevisiae* 7904, *Saccharomycod es ludwigii* 7126, *Torulaspora delbrueckii* 7116, *Tremella mesenterica* 7131, *Trichoderma koningii* 6042, *Trigonopsis variabilis* 7263, *Zygosaccharomyces rouxii* 7191.
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17. The liquid cultures consist of a basal medium of dextrose (10 g/L), peptone (5 g/L), yeast extract (3 g/L) and malt extract (3 g/L).
18. Silybin A (**1**) (t_R 35.17) and silybin B (**2**) (t_R 39.12) were separated by preparative reversed-phase HPLC using an isocratic MeOH–H_2O (42.5:57.5) solvent system from silybin, which was purchased from Sigma. Both compounds were authenticated by comparing their HPLC chromatogram, physical and spectral data with the literature.
19. Substrate controls consisted of silybin and sterile YM medium incubated without microorganisms. Culture controls consisted of fermentation clutures in which the microorganisms were grown without silybin addition.
20. Silybin A 3-*O*-β-D-glucopyranoside (**3**): pale yellow amorphous powder, $[\alpha]_D$ +22.2° (c = 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 288 (4.20); IR (KBr) ν_{max} cm^{-1}: 3400, 1640, 1510, 1275; ^1H and ^{13}C NMR (CH_3OH-d_4, 500 and 125 MHz) data, see Tables 1 and 2; HRESIMS *m/z* 667.1595 {calcd for [M ($C_{31}H_{32}O_{15}$)+Na]$^+$, 667.1639}.
21. Silybin A 7-*O*-β-D-glucopyranoside (**4**): yellowish amorphous powder; $[\alpha]_D$ −28.2° (c = 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 286 (4.23); IR (KBr) ν_{max} cm^{-1}: 3400, 1640, 1510, 1275; ^1H and ^{13}C NMR (CH_3OH-d_4, 500 and 125 MHz) data, see Tables 1 and 2; HRESIMS *m/z* 667.1642 {calcd for [M ($C_{31}H_{32}O_{15}$)+Na]$^+$, 667.1639}.
22. Silybin B 3-*O*-β-D-glucopyranoside (**5**): pale yellow amorphous powder; $[\alpha]_D$ +18.7° (c = 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 288 (4.01); IR (KBr) ν_{max} cm^{-1}: 3400, 1640, 1510, 1275; ^1H and ^{13}C NMR (CH_3OH-d_4, 500 and 125 MHz) data, see Tables 1 and 2; HRFABMS *m/z* 667.1602 {calcd for [M ($C_{31}H_{32}O_{15}$)+Na]$^+$, 667.1639}.
23. Silybin B 7-*O*-β-D-glucopyranoside (**6**): yellowish amorphous powder; $[\alpha]_D$ −26.6° (c = 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 285 (4.16); IR (KBr) ν_{max} cm^{-1}: 3400, 1640, 1510, 1275; ^1H and ^{13}C NMR (CH_3OH-d_4, 500 and 125 MHz) data, see Tables 1 and 2; HRESIMS *m/z* 667.1641 {calcd for [M ($C_{31}H_{32}O_{15}$)+Na]$^+$, 667.1639}.