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GLUCOSYLATION OF SILYBIN BY PLANT CELL CULTURES OF *PAPAVER SOMNIFERUM* VAR. *SETIGERUM*

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Key Word Index—*Papaver somniferum*; Papaveraceae; plant cell culture; silybin, silymarin; glycosylation; silybin-7-*O*-β-D-glucopyranoside.

Abstract—Cell cultures of *Papaver somniferum* var. *setigerum* were shown to carry out the biotransformation of silybin into silybin-7-*O*-β-D-glucopyranoside. © 1997 Elsevier Science Ltd

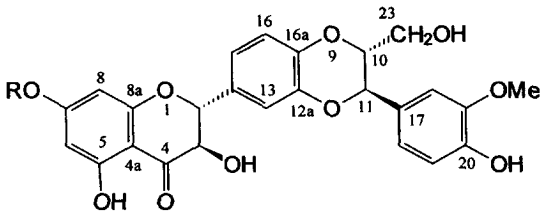
INTRODUCTION

Silybin (**1**), a natural flavonolignan isolated from the seeds of milk thistle (*Silybum marianum*), is an important hepatoprotective drug (Flavobion,™ Legalon™ used in the treatment of liver damage of various aetiology and as a liver protecting drug, by acting as a radical scavenger, removing the reactive radicals formed during the detoxification of xenobiotics by liver monooxygenase systems [1]. It stimulates metabolism of phospholipids as an anti-lipoperoxidant (probably at the RNA synthesis level) modulating the fluidity of the cell membranes. Silybin is also a very effective antidote in the treatment of severe intoxication caused by some poisonous mushrooms (e.g., by *Amanita phalloides*).

Glycosides may act as effective pro-drugs of the respective aglycones. No glycoside of silybin has been reported up to now.

Silybin is a rather complex target for glycosylation bearing as it does five OH groups of three different types. Selective glycosylation of a phenolic hydroxyl group in the presence of a primary alcoholic group by chemical means would involve complicated protection/deprotection procedures and is rather impracticle. Plant cell cultures offer an excellent tool for glucosylations of phenolic compounds [2–4], especially of the flavonoid type [5, 6].

Two cell cultures chosen for their potential glycosylation activity were tested for their ability to glucosylate silybin. This paper reports on the successful biotransformation of silybin into silybin-7-*O*-β-D-glucopyranoside by a plant cell culture of *Papaver somniferum* var. *setigerum*.



1 R = H

2 R = βGlc

RESULTS AND DISCUSSION

Suspension cultures of *Ajuga reptans* Bugle (Labiatae), known for its glycosylating activity [8, 9], *Papaver somniferum* var. *setigerum* Corb. were tested for their ability to glycosylate silybin (**1**). The last culture, which has been found to produce large amounts of β-glucosylesters [10], was expected to be able to glucosylate xenobiotics like silybin.

A methanolic solution of **1** was fed to 5-day-old cell suspension cultures which were then cultivated for a further 2 days. After extraction (see Experimental), the samples were analysed by TLC and HPLC. No transformation was observed with *A. reptans*. It was noted that silybin somehow inhibited its growth. In the *P. somniferum* cultures 12 h after administration of the silybin a new product with spectral properties similar to that of **1** was observed. The biotransfor-

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mation of **1** by *P. somniferum* was performed on a semipreparative scale. The product (**2**) was isolated by solid phase extraction on Amberlite XAD-2 and purified by column chromatography on a RP-18 stationary phase.

The UV spectrum of **2** in MeOH [λ_{max} 286 and 328(sh) nm] was typical of dihydroflavones and was very similar to the spectrum of compound **1**. Addition of MeONa to the solution of **2** caused a shift of the main absorption peak of only 6 nm (λ_{max} 292 nm) while that of compound **1** was shifted 36 nm. This suggested substitution of the 7-OH group [11]. Similarly, the UV spectrum of hesperidine, a flavanone having a substituted the 7-OH group, after addition of MeONa, shows a 3 nm shift only [11]. Addition of $AlCl_3$ to the methanolic solution of **2** caused a shift of the main absorption band from 286 nm to 310 nm (in compound **1** the same peak shifts to 315 nm). The spectral shift of about 24 nm was a proof of the integrity of the 5-OH group [11] participating together with the adjacent keto group in the Al^{3+} complexing. Addition of NaOAc caused a considerable bathochromic shift of the main band (286 nm) in the spectrum of **1** to the value of 325 nm. However, in the case of **2**, under the same conditions no change in the UV spectrum was observed. NaOAc, as a weak base, causes selective ionization of 7-OH groups in flavonoids. Therefore, the UV spectra of the new compound (**2**) suggested that only the 7-OH group was substituted and the rest of the flavonoid skeleton was unchanged.

The negative-ion FAB mass spectrum of **2** exhibited the $[M-H]^-$ ion at *m/z* 643 which fits well with the expected molecular composition of silybin hexoside (M-H = $C_{31}H_{31}O_{15}$). The aglycone anion *m/z* 481 was the base peak in the product ion spectrum of $[M-H]^-$. Elimination of water from this ion produced *m/z* 463 ion (20% rel. to *m/z* 481).

The ^1H NMR spectrum of **2** contained all the signals assignable to silybin (aromatic methoxyl, one AB system of two *meta*- disposed aromatic protons, two ABC systems due to 1,2,4-trisubstituted phenyl rings, an isolated AB system of two aliphatic protons, and a partial structure -CHCHCH$_2$OH). The newly introduced moiety was represented by a contiguous spin system, -OCH(O-)(CHO-)$_4$CH$_2$O-, i.e., a hexose unit. Four large vicinal couplings in the latter system were consistent with a six-membered ring adopting a chair confirmation and suggesting a glucopyranose moiety. According to $J_{1',2'} = 7.4$ Hz, it had a β-configuration. Comparison of the ^{13}C NMR spectra of silybin A (**1**) and **2** reveals six additional signals (one -OCHO-, four -OCH-, one -OCH$_2$-) that come from the attached sugar moiety. The largest chemical shift differences were observed for carbons 4*a*, 6, 7, and 8. Therefore, the product of the biotransformation of **1** is 7-*O*-β-D-glucopyranosylsilybin A.

The yield (HPLC) of **2** is nearly quantitative and therefore the biotransformation of **1** by *P. somniferum* var. *setigerum* is an excellent method for the production of its 7-*O*-β-D-glucopyranoside. To our knowledge, no glycosylation reaction catalysed by a cell culture of this plant has previously been reported.

EXPERIMENTAL

General. ^1H and ^{13}C NMR spectra were measured on a Varian VXR-400 spectrometer at 399.95 and 100.58 MHz, respectively, in CD_3OD at 25°. Residual solvent signal (δ_H 3.33, δ_C 49.3) served as an internal reference. Chemical shifts are given in the δ-scale; *J* values are given in Hz, digital resolution was 0.0002 and 0.0006 ppm, respectively. Carbon signal multiplicity was determined by an APT (Attached Proton Test) experiment. Manufacturer's software was used for 2D NMR (COSY, ROESY, HOM2DJ, HETCOR). Negative-ion FAB: Finnigan MAT 90 (Finnigan MAT, Bremen, Germany) double focusing instrument. The sample (0.2 mg) was dissolved in an α-monothioglycerol (Aldrich) matrix and bombarded with Xe atoms at 6 kV energy and 2 mA current. The products of collisionally-induced decompositions (He as a collision gas, 50% attenuation of the primary ion beam) in the first field-free region of the instrument were analysed by the daughter ion linked scan at constant B/E using manufacturer's software. Positive-ion electrospray spectra were measured on the same instrument: samples were dissolved in H_2O–MeOH mixt. (1:1) and introduced into the electrospray source (Finnigan MAT) via a linear syringe pump (10 μl min^{-1}).

Plant cell cultures. Cell suspension cultures of *Papaver somniferum* var. *setigerum* (Papaveraceae) and *Ajuga reptans* (Labiatae) [9, 10] are deposited at the Department of Pharmaceutical Sciences, University of Bologna, I. Cultures of *A. reptans* and *P. somniferum* were grown in a modified Gamborg medium [7] supplemented with NAA (1 mg ml^{-1}) and kinetin (1 mg ml^{-1}) and, when five-days-old, with 1 ml of 50% sucrose soln.

Silybin was obtained as a kind gift from Galena Pharmaceuticals, Opava, Czech Republic. Natural silybin is an equimolar mixt. of two diastereomers, A and B, having the configurations 2*R*, 3*R*, 10*R*, 11*R* and 2*R*, 3*R*, 10*S*, 11*S*, respectively. The absolute configuration of A and B is not known. We have used in our experiments pure silybin A that had been separated from natural silybin in our laboratory [12]. The use of the pure substance was necessary to enable spectral identification of the products. Biotransformation of natural silybin (mixt. of A and B) gives the same yields (HPLC).

Feeding experiments. Seven-day-old suspension cultures (50 ml) in 300 ml conical flasks cultivated on a rotary shaker at room temp. under normal day light were fed with compound **1** (5 mg) dissolved in MeOH (0.5 ml). After 2 more days of cultivation the dose of **1** was repeated. The cultures were harvested after 3 more days.

Assay of the samples. Samples of the culture (cells and medium) were taken immediately after addition of compound **1** (0 hr) and during the experimental period (4, 10, 24, 48 and 72 hr). Samples (10 ml) were extracted as reported in next section and the extracts loaded onto SM-2 prepacked columns (Bio-Rad, U.S.A.). The columns were washed with H_2O (20 ml) and eluted with EtOH (20 ml). The eluates were evapd to 2 ml and analysed by TLC (silica gel 60, F_{254}, Merck; mobile phase EtOAc–HCOOH–AcOH–H_2O 100:11:11:27). The spots were located under UV light and visualised by charring with 5% H_2SO_4 in EtOH. Silybin derivatives gave typical rusty spots. HPLC analyses were performed under the following conditions: SP 8800 ternary gradient pump, SP 8880 autosampler, Spectra Focus scanning UV/VIS detector, column 150 × 3.3 mm packed with Separon SGX C18, 7 μm (Tessek, Prague, Czech Republic). Isocratic elution was used; MeOH–H_2O = 37.9:62.1 (+1 ml l^{-1} AcOH), flow rate 0.6 ml min^{-1}, scan 210–360 nm, R_t of compound **2** = 5.25 min.

Isolation of products. The cultures from 10 flasks each containing 50 ml of culture supplemented with **1** were pooled, treated with an equal amount of MeOH, homogenised with an Ultra-Turrax homogeniser (Janke and Kunkel, Germany) and centrifuged. The pellet was re-extracted with Me_2CO (100 ml). Both extracts were pooled and evapd to less than 10% of the original vol. in order to remove all the organic solvents. The aq. phase was diluted with H_2O to about 500 ml and slowly loaded onto a column filled with non-ionic Amberlite XAD-2 (C. Erba, Italy) (600 g) in H_2O. The resin was washed extensively with H_2O (2 l) and then eluted with MeOH (600 ml). The eluate was evapd to a syrup. Compound **2** was isolated by MPLC on prepacked column Lobar (RP-18) size A (Merck, Germany). The crude sample containing **2** was dissolved in H_2O and loaded onto the column equilibrated with H_2O. Then it was washed with H_2O (300 ml) followed by a stepwise gradient of MeCN (1% increase/250 ml) in H_2O. Frs of 20 ml each were collected. Compound **2** was eluted in frs 135–150. 100 mg of **1** fed into 10 flasks yielded 60 mg of **2** (yield 45%).

Silybin A (**1**). Yellow microcrystalline powder, $[\alpha]_D^{23} = +6.09°$ ($c = 0.23$, Me_2CO), ^1H NMR (CD_3OD) δ 3.51 (1 H, *dd*, $J = 4.5$ and 12.3 Hz, H-23*u*), 3.72 (1 H, *dd*, $J = 2.5$ and 12.3 Hz, H-23*d*), 3.89 (3 H, *s*, OMe), 4.09 (1 H, *ddd*, $J = 2.5$, 4.5 and 8.1 Hz, H-10), 4.52 (1 H, *d*, $J = 11.5$ Hz, H-3), 4.92 (1 H, *d*, $J = 8.1$ Hz, H-11), 4.99 (1 H, *d*, $J = 11.5$ Hz, H-2), 5.91 (1 H, *d*, $J = 2.1$ Hz, H-6), 5.95 (1 H, *d*, $J = 2.1$, H-8), 6.86 (1 H, *d*, $J = 8.1$, H-21), 6.92 (1 H, *dd*, $J = 1.9$ and 8.1 Hz, H-22), 7.03 (1 H, *d*, $J = 8.3$, H-16), 7.03 (1 H, *d*, $J = 1.9$, H-18), 7.06 (1 H, *dd*, $J = 1.9$ and 8.3 Hz, H-15), 7.11 (1 H, *d*, $J = 1.9$, H-13); ^{13}C NMR (CD_3OD): δ 56.8 (*q*, OMe), 62.4 (*t*, C-23), 74.0 (*d*, C-3), 80.0 (*d*, C-11), 80.3 (*d*, C-10), 84.9 (*d*, C-2), 96.7 (*d*, C-6), 97.7 (*d*, C-8), 102.10 (*s*, C-4a), 112.3 (*d*, C-18), 116.6 (*d*, C-21), 117.9 (*d*, C-13), 118.2 (*d*, C-16), 122.0 (*d*, C-22), 122.4 (*d*, C-15), 129.7 (*s*, C-17), 131.8 (*s*, C-14), 145.4 (*s*, C-12a), 145.7 (*s*, C-16a), 148.6 (*s*, C-20), 149.5 (*s*, C-19), 164.7 (*s*, C-5), 165.55 (*s*, C-8a), 169.0 (*s*, C-7), 198.5 (*s*, C-4).

Silybin-A-7-O-β-D-glucopyranoside (**2**). Yellow amorphous solid, $[\alpha]_D^{23} = -29.8°$ ($c = 0.33$, MeOH). ^1H NMR (CD_3OD): δ 3.40–3.48 (4 H, *m*, H-4′, H-5′, H-3′, H-2′), 3.51 (1H, *dd*, $J = 12.4$ and 4.5 Hz, H-23*u*), 3.69 (1H, *dd*, $J = 12.0$ and 4.9 Hz, H-6′*u*), 3.73 (1H, *dd*, $J = 12.4$ and 2.5 Hz, H-23*d*), 3.88 (1H, *dd*, $J = 12.0$ and 1.7 Hz, H-6′*d*), 3.88 (3H, *s*, OMe), 4.10 (1H, *ddd*, $J = 8.1$, 4.5 and 2.5 Hz, H-10), 4.56 (1 H, *d*, $J = 11.6$ Hz, H-2), 4.94 (1 H, *d*, $J = 8.1$ Hz, H-11), 4.97 (1 H, *d*, $J = 7.4$ Hz, H-1′), 5.02 (1 H, *d*, $J = 11.6$ Hz, H-3), 6.24 (1 H, *d*, $J = 2.2$ Hz, H-8), 6.25 (1 H, *d*, $J = 2.2$ Hz, H-6), 6.86 (1 H, *d*, $J = 8.2$ Hz, H-21), 6.92 (1 H, *dd*, $J = 8.2$ and 1.9 Hz, H-22), 7.02 (1 H, *d*, $J = 8.4$ Hz, H-16), 7.03 (1 H, *d*, $J = 1.9$ Hz, H-18), 7.06 (1 H, *dd*, $J = 8.4$ and 1.9 Hz, H-15), 7.12 (1 H, *d*, $J = 1.9$ Hz, H-13); ^{13}C NMR (CD_3OD): δ 56.8 (*q*, OCH_3), 62.4 (*t*, C-23), 62.6 (*t*, C-6′), 71.4 (*d*, C-4′), 74.1 (*d*, C-3), 74.9 (*d*, C-2′), 78.0 (*d*, C-3′), 78.0 (*d*, C-11), 78.5 (*d*, C-5′), 80.3 (*d*, C-10), 85.1 (*d*, C-2), 97.3 (*d*, C-6), 98.7 (*d*, C-8), 101.6 (*d*, C-1′), 103.8 (*s*, 4a), 112.3 (*d*, C-18), 116.6 (*d*, C-21), 117.9 (*d*, C-13), 118.2 (*d*, C-16), 122.0 (*d*, C-22), 122.5 (*d*, C-15), 129.7 (*s*, C-17), 131.6 (*s*, C-14), 145.4 (*s*, C-12a), 145.8 (*s*, C-16a), 148.6 (*s*, C-20), 149.5 (*s*, C-19), 164.4 (*s*, C-5), 165.0 (*s*, C-8a), 167.6 (*s*, C-7), 199.4 (*s*, C-4).

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