Journal of Molecular Catalysis B: Enzymatic 102 (2014) 167–173

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Preparation of silybin phase II metabolites: *Streptomyces* catalyzed glucuronidation

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ARTICLE INFO

Article history:
Received 23 December 2013
Received in revised form 21 January 2014
Accepted 10 February 2014
Available online 20 February 2014

Keywords:
Silybin diastereomers
Silibinin
Silymarin
Glucuronidation
Glucuronyl transferase

ABSTRACT

Flavonolignan silybin is a major component of the silymarin complex isolated from seeds of the milk thistle (*Silybum marianum*) having strong antioxidant and hepatoprotective effects, and also anticancer, chemoprotective, dermatoprotective and hypocholesterolemic activities. Natural silybin (silibinin in pharmacological literature) is a mixture of two diastereomers: silybin A and silybin B. Their metabolism is strongly linked to Phase II biotransformations and respective conjugates are rapidly excreted in bile and urine. Conjugation reactions of both silybins are strictly stereoselective. Therefore, optically pure compounds must be used for metabolic studies. The aim of this study was to obtain the glucuronidated metabolites of both silybin A and B. *Streptomyces* sp. strain M52104 was found to be a highly effective tool for the preparation of silybin A-20-*O*-β-glucuronide, silybin B-20-*O*-β-glucuronide, silybin A-7-*O*-β-glucuronide and silybin B-7-*O*-β-glucuronide and minor amounts of silybin A and B-5-*O*-β-glucuronide. The glucuronides, which were thoroughly characterized by MS and NMR spectroscopy, can be used as invaluable authentic standards in metabolic studies of both silybin diastereomers.

1. Introduction

The flavonolignan silybin (CAS No. 22888-70-6; also denoted as silibinin in pharmacological literature) and its congeners such as 2,3-dehydrosilybin, isosilybin, silydianin and silychristin are important natural compounds obtained from silymarin, which is a complex extract from the seeds of *Silybum marianum* (L.) Gaertn. (Asteraceae; milk thistle) [1,2]. In the last decades, silymarin and silybin were in the focus mainly due to multiple beneficial biological activities. Originally known for its anti-phalloidin activity [3], and usable for the treatment of various liver disorders [4], silybin and its congeners, frequently as constituents of milk thistle extract, were later identified as antioxidants with cytoprotective effects and also potential hypocholesterolemic drugs [5,6]. Reports on the anticancer activities of silymarin components [7] triggered broad interest in the detailed study of these activities. Administration of silymarin (or silybin) proved to be often beneficial for cancer patients either by releasing negative side effects of chemotherapy or by potentiating anticancer treatment [2].

The molecular mechanism of silybin action is not completely clear. Only antioxidant and antiradical mechanisms were described in detail [8,9] showing that the C-20 OH is the most important moiety for antiradical activity and the C-7 OH possesses pro-oxidant activity, forming stable radicals.

Natural silybin is typically an approximately equimolar mixture of two diastereomers (Fig. 1), silybin A (**1a**)—(2*R*,3*R*)-2-[(2*R*,3*R*)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one—and silybin B (**1b**)—(2*R*,3*R*)-2-[(2*S*,3*S*)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one (Fig. 1) (usually **1b** is slightly prevalent in most preparations). Thanks to our recently developed enzymatic methods enabling effective separation of silybin A and B in the multigram quantities [10,11] we can perform now an array of chemical and biological

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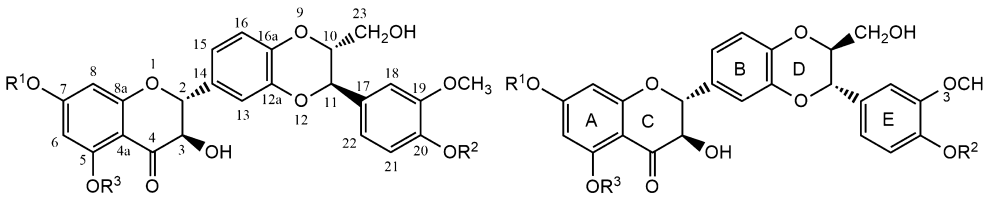
http://dx.doi.org/10.1016/j.molcatb.2014.02.008
1381-1177/



1a $R^1 = R^2 = R^3 = H$ silybin A (2*R*,3*R*,10*R*,11*R*)
2a $R^1 = \beta GlcA$, $R^2 = R^3 = H$
3a $R^2 = \beta GlcA$, $R^1 = R^3 = H$
4a $R^3 = \beta GlcA$, $R^1 = R^2 = H$

1b $R^1 = R^2 = R^3 = H$ silybin B (2*R*,3*R*,10*S*,11*S*)
2b $R^1 = \beta GlcA$, $R^2 = R^3 = H$
3b $R^2 = \beta GlcA$, $R^1 = R^3 = H$
4b $R^3 = \beta GlcA$, $R^1 = R^2 = H$

Fig. 1. Structures of silybin diastereomers and their glucuronides; βGlcA = β-D-glucuronyl.

experiments with pure silybin diastereomers at the preparative scale.

Silybin undergoes in human various biotransformation processes and is largely metabolized by both Phase I and II enzymes. Due to the complex nature of the silybin molecule bearing five OH groups and four stereogenic centers, its biotransformation processes and interactions are very complex. Stereochemistry of silybin is of the utmost importance, as it is now clear that silybin A (**1a**) and silybin B (**1b**) are metabolized in completely different ways, both in terms of pharmacokinetics[12,13] and pharmacodynamics, yielding different metabolites [14].

Beside sulfate ester formation [15], glucuronidation is one of the main conjugation reactions of flavonoids and polyphenols [16]: it is catalyzed by UDP-glucuronosyltransferases (UGTs), the microsomal enzymes encoded by a multigene family in humans.

Křen et al. [14] prepared the 7-*O*-β-glucuronide, 20-*O*-β-glucuronide, and 5-*O*-β-glucuronide of the optically pure "silybin A" [17] by enzymatic synthesis using ovine liver microsomes and uridine-5′-diphosphoglucuronic acid (UDPGA) as a donor, however only at the mg scale required for metabolic profiling.

These conjugates were formed *in vitro* in the proportion 7-*O* (62.5%), 20-*O* (27%), 5-*O* (2.5%), which reflects the approximate regio-preference of UGTs for silybin B molecule. This [14] and later the Han's paper [13] are probably the only studies so far describing the full spectral and, therefore, structural evidence (^{1}H, ^{13}C NMR and MS) for these important silybin conjugates/metabolites. Křen et al. [14] established that silybin B 20-*O*-β-glucuronide (**3b**) is the major silybin B conjugate in humans, while the C-7 regioisomer is also formed, but in a lower proportion. Han [13] demonstrated that silybin B (**1b**) was glucuronidated (using bovine microsomes + UDPGA) at *ca.* 2–3 × higher rate than its diastereomer (**1a**), and glucuronidation of silybin B was much preferred at the C-20, while that of silybin A was similar at both C-7 and C-20. In the mixture, both diastereomers influence the respective metabolism of each other [13]. Jančová [18] tested silybins (both **1a** and **1b**) metabolite formation *in vitro* by incubation with a human liver microsomal fraction, then with primary cultures of human hepatocytes and with twelve recombinant isoenzymes of UGT. Only three recombinant UGTs (UGT1A4, UGT2B4 and UGT2B17) do not participate in silybin glucuronidation processes. Chen et al. [19] investigated the glucuronidation of various flavonoids by recombinant UGT1A3 and UGT1A9 as well. Hoh et al. [20] identified four silybin monoglucuronides and two minor diglucuronides (HPLC–MS), however, without determining respective regioisomers, in patients with colorectal carcinoma after the administration of Silipide (Indena SpA, IT) containing silymarin (in complex with phospholipids).

Scarce attempts to test the biological activity of silybin glucuronides were limited by the paucity of the compounds and expensive method of their preparation. Radical scavenging activity of two silybin glucuronides (C-20 and C-7) was tested and proved that 7-*O*-silybin-β-glucuronide is a better antioxidant than free silybin [14] that is in good agreement with the finding that C-7 OH in the silybin molecule has prooxidative properties [9].

Metabolic profiles of resorbed silybin indicate that it mainly circulates in blood in the conjugated form [13,14]. Therefore, biological studies with the pure, well defined silybin conjugates are of utmost importance.

Typical method for preparation of silybin glucuronides using liver microsomes (with UGT activity) is quite feasible, but the amount of product is limited by extremely high price of UDPGA. Purification of the respective conjugates is labour and time consuming and usually involves preparative HPLC. Chemical glucuronidation *e.g.* using Koenigs–Knorr reaction is not easy as it involves complicated protection/deprotection methods. The use of heavy metals (Cd, Hg, Ag) is incompatible with silybin due to its strong complexation activity and the traces of metals are impossible to remove from the final preparations [21].

Recently, some of us [22] published a microbial transformation method enabling the glucuronidation of various polyphenolic dietary flavonoids, such as naringenin or quercetin and some stilbenoids, such as resveratrol, rhapontigenin or deoxyrhapontigenin. Therefore, we have decided to apply this methodology to the preparation of silybin glucuronides starting from the pure silybin diastereomers.

2. Materials and methods

2.1. Melting points, optical rotation

Melting points were measured on a capillary instrument (Büchi, CH) and are uncorrected. Optical rotations were measured in a 1 dm cell on a Perkin Elmer 321 spectropolarimeter (GB).

2.2. NMR

In Prague (Inst. Microbiol.) NMR spectra were recorded with a Bruker Avance III 400 MHz spectrometer (400.13 MHz for ^{1}H, 100.55 MHz for ^{13}C at 30 °C), a Bruker Avance III 600 MHz spectrometer (600.23 MHz for ^{1}H, 150.93 MHz for ^{13}C at 30 °C) and a Bruker Avance III 700 MHz spectrometer (700.13 MHz for ^{1}H, 176.05 MHz for ^{13}C at 30 °C) in DMSO-d_6 (99.9 atom% D, Sigma-Aldrich). Residual signals of the solvent were used as an internal standard (δ_H 2.500 ppm, δ_C 39.60 ppm). NMR experiments: ^{1}H NMR, ^{13}C NMR, *J*-resolved, COSY, HSQC, HSQC-TOCSY, HMBC, and 1D TOCSY were performed using the manufacturer's software. ^{1}H NMR and ^{13}C NMR spectra were zero-filled to fourfold data points and multiplied by a window function before Fourier transformation. A two-parameter double-exponential Lorentz–Gauss function was applied for ^{1}H to improve resolution, and line broadening (1 Hz)