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Preparation of silybin and isosilybin sulfates by sulfotransferase from *Desulfitobacterium hafniense*

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ABSTRACT

Flavonolignans silybin and isosilybin are major components of silymarin complex isolated from seeds of the milk thistle (*Silybum marianum*) featuring strong antioxidant and hepatoprotective effects, and also anticancer, chemoprotective, dermatoprotective and hypocholesterolemic activities. Natural silybin and isosilybin are mixtures of diastereoisomers: silybin/isosilybin A (**1a**, **1b**) and silybin/isosilybin B (**2a**, **2b**). The metabolism of these compounds is supposed to be strongly linked to Phase II of biotransformation and the respective conjugates are rapidly excreted in bile and urine. The aim of this study was to obtain optically pure sulfated metabolites of both silybins and isosilybins. Aryl-sulfate sulfotransferase (EC 2.8.2.22) from *Desulfitobacterium hafniense* was found to be a highly effective tool for the regiospecific enzymatic synthesis of silybin A-20-*O*-sulfate, silybin B-20-*O*-sulfate, isosilybin A-20-*O*-sulfate and isosilybin B-20-*O*-sulfate providing nearly quantitative yields and employing cheap *p*-nitrophenyl sulfate as sulfate donor. The isolated sulfated products will be used as authentic standards in metabolic studies of both silybins and isosilybins.

1. Introduction

Flavonolignan silybin (**1**, CAS No. 22888-70-6; also denoted as *silibinin*) and its congeners such as isosilybin (**2**, CAS No. 72581-71-6) (Fig. 1), 2,3-dehydrosilybin, silydianin and silychristin are important natural compounds with plethora of biological activities. Pure compounds are obtained from silymarin – a standardized seed extract of *Silybum marianum* (L.) Gaertn. (milk thistle), in which silybin occurs as approximately equimolar mixture of two diastereoisomers, silybin A (**1a**) and silybin B (**1b**). Analogously, isosilybin is naturally produced as a mixture (approx. 4/1) of two stereoisomers, isosilybin A (**2a**) and isosilybin B (**2b**).

Silybin is known for a number of health-beneficial hepatoprotective effects, such as anti-phalloidin activity [1], and thus, it is used in the treatment of various liver disorders [2]. Silybin and its congeners feature antioxidant, cytoprotective [3,4] and also hypocholesterolemic activities [5,6]. More recently, silybin derivatives gained attention because of its chemoprotective and anticancer activity [7,8]. Cytoprotective activity of silybin is thought to be mediated mainly by its antioxidative and radical-scavenging properties but new biological effects based on a specific receptor interaction were discovered [9]. Up to date, the molecular mechanism of silybin action is not completely understood and it is still a subject of extensive research.

Recent studies have demonstrated that isosilybin (**2**) is probably a more potent anticancer agent than silybin. Isosilybin was shown to possess *in vivo* anti-proliferative, anti-angiogenic, pro-apoptotic and cell-cycle modulatory properties [8]. Moreover, isosilybin and especially isosilybin B (**2b**) inhibited the growth of advanced human prostate cancer cells *in vivo* without any toxic effects.

Silybin and its congeners undergo complex biotransformation processes and they are largely metabolized mainly by Phase II enzymes although their interaction with cytochrome P450s cannot be completely excluded. Polyphenolic substances are easy targets for conjugation reactions and these are indeed the major biotransformation pathways leading to silybin excretion from the human body. Fundamental enzymes in the Phase II xenobiotic biotransformation reactions are UDP-glucuronosyltransferases, sulfotransferases, *N*-acetyltransferases, glutathione *S*-transferases, thiopurin *S*-methyltransferases, and *O*-methyl transferases. Silybin and isosilybin undergo quick conjugation, which is highly stereoselective, e.g. silybin A (**1a**) and silybin B (**1b**) were found to exhibit different metabolic profiles [10,11]. Biliary excretion of

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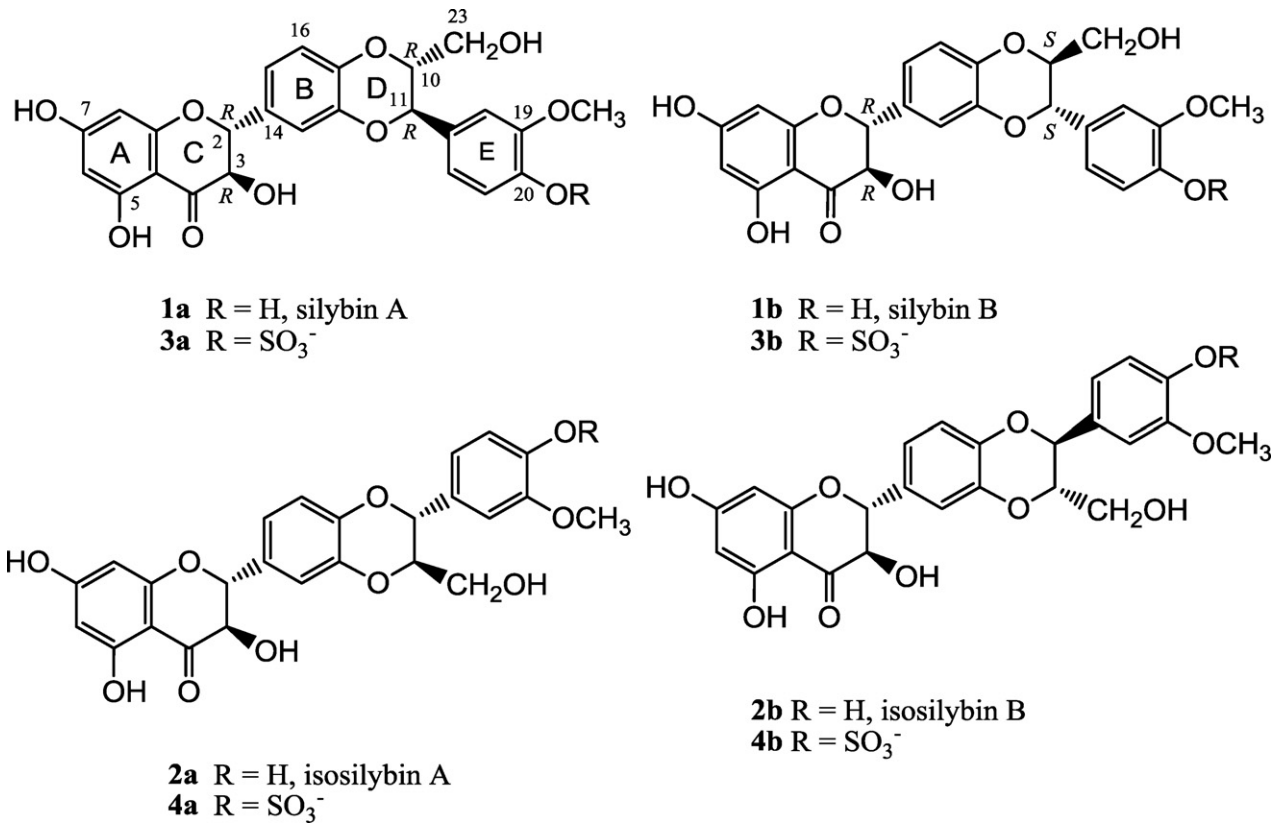

Fig. 1. Silybin, isosilybin and respective sulfates.

sulfate and/or glucuronates of silybin constitutes the major route of their elimination [12].

Sulfation converts xenobiotics into hydrophilic metabolites and facilitates their excretion. In humans, these processes are catalyzed by cytosolic sulfotransferases, a superfamily consisting of at least ten functional genes [13]. Sulfotransferases are present in various tissues including liver, kidney, brain, adrenals, gut and platelets. Aryl sulfotransferases are also able to reverse the process of sulfation in cells (desulfation) and consequently influence the process of sulfation [14,15].

Natural silybin (**1**) is an equimolar mixture of two diastereomers **1a** and **1b**. Even though the natural silybin (**1**) is an equimolar mixture of two diastereomers, most of the recent studies of silybin conjugation were accomplished only with the natural mixed silybin or even with crude silymarin, although some authors attempted to analyze metabolites of each diastereomers separately [10,11]. Miranda et al. [12] studied biliary excretion of glucuronide and sulfate conjugates of silymarin flavonolignans (silybin A and B, isosilybin A and B, silychristin and silydianin) in perfused rat livers. They found sulfation as the major metabolic pathway for **1b** (78%), **2a** (84%) and **2b** (85%). Silybin A is sulfated and glucuronidated in about the same ratio, however in total to a lower extent than silybin B. They concluded that all silymarin flavonolignans favor Phase II metabolism [12]. Silybin distribution and metabolism in patients with colorectal carcinoma was studied by Hoh et al. [16]. Using HPLC–MS three different silybin monosulfates were identified in plasma; however, due to absence of the respective reference materials it was impossible to elucidate their structures. This clearly demonstrates the need of optically pure authentic standards.

There is only scarce information on the preparation of sulfated silybin derivatives by chemical synthesis. Zarrelli et al. [17] described a procedure affording silybin-23-sulfate (mixture A and B). In this laborious procedure all remaining OH groups were protected selectively except C23-OH and this group was sulfated by SO_3•Et_3N. Sulfation of any other silybin position (or of its congeners), namely of the phenolic one, has not been described yet.

We have recently developed an enzymatic method for the separation of silybin diastereomers [18], which was scaled up to multigram scale [19]; consequently, a method for the preparation of optically pure isosilybin diastereomers was developed as well [20]. Moreover, we have successfully cloned and expressed a bacterial aryl-sulfate sulfotransferase (AST; 71.4 kDa) [21]. We found this enzyme to be able to efficiently transfer the sulfate moiety from *p*-nitrophenyl sulfate used as a donor to various acceptor molecules; e.g. 17-β-estradiol.

In the present study we used this arylsulfotransferase to prepare optically pure sulfated metabolites of silybins and isosilybins, which may be used as authentic reference material in metabolic studies.

2. Experimental

2.1. Aryl-sulfate sulfotransferase from Desulfitobacterium hafniense

Expression and purification of the AST enzyme was performed as described elsewhere [21]. The AST was stored as a stock solution of 9 μM (specific activity 10 units mg^{-1}) at −20 °C in 100 mM bis–tris methane buffer (pH 6.5). *p*-Nitrophenylsulfate (p-NPS) was obtained as K-salt from Sigma. 1 M tetrabutylammonium phosphate solution was from Aldrich [21].

2.2. General procedures

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 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) was used to obtain a better ^{13}C signal-to-noise ratio. Chemical shifts are given on a δ-scale with digital resolution justifying the reported values to three (δ_H) or two (δ_C) decimal places.

MALDI-MS spectra were measured on a MALDI-TOF/TOF ultraFLEX III mass spectrometer (Bruker-Daltonics, Bremen, DE). Positive/negative spectra were calibrated externally using the monoisotopic $[M+H]^+$ or $[M-H]^-$ ions of PepMixII calibrant (Bruker-Daltonics). α-Cyano-4-hydroxycinnamic acid (5 mg/mL) in 50% CH_3CN/0.1% TFA was used as a MALDI matrix. A 0.4 μL of sample dissolved in MeCN was premixed with 0.5 μL of the matrix solution on the target and allowed to dry at ambient temperature. The MALDI-TOF spectra were collected in reflectron mode. The exact masses were measured using LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source. The mobile phase consisted of methanol/water (4:1), flow rate 30 μL/min and the samples were injected using a 2-μL loop. Spray voltage, capillary voltage, tube lens voltage and capillary temperature were 4.5 kV, −40 V, −120 V and 275 °C, respectively. The mass spectra were internally calibrated using deprotonated stearic acid as lock mass.

2.3. HPLC

Enzymatic sulfations with acceptor substrates and *p*-NPS as donor were monitored by HPLC method. Typically, *p*-nitrophenyl sulfate K-salt (Sigma) (257 μg, 5 μmol) was dissolved together with 5 μmol of substrate in 1 mL of 50 mM Tris–glycine buffer (pH 9.0) in an Eppendorf tube and incubated at 20 °C or 30 °C. After 5 min AST (0.5–1.0 U) was added and samples were taken at regular time intervals. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmol *p*-NP per minute.

2.3.1. HPLC method A

Samples were diluted 20× with 50% CH_3CN (in H_2O) and 20 μL was injected into a Nucleosil 100-5 C-18 HD column equipped with guard column (all Macherey-Nagel, DE). Binary gradient elution at flow rate of 0.4 mL/min was used. Eluent A: 25 mM $NH_4H_2PO_4$ buffer (pH 6.3), 5 mM TBAP (tetrabutylammonium phosphate as an ion-pairing reagent) in H_2O and CH_3CN (95/5, v/v). Eluent B: CH_3CN/5 mM TBAP in H_2O (90/10, v/v). A gradient: 0–36 min, 0–60% B. The compounds were detected at 210 nm. The data collection and analysis was performed with the Agilent 1100 system and the chromatograms were analyzed with the AgilentChemStation program B.01.03.

2.3.2. HPLC method B

Analysis was carried out on the Shimadzu Prominence UFLC system (Kyoto, JP) consisting of a DGU-20A mobile phase degasser, LC-20AD solvent delivery unit, a SIL-20ACHT cooling autosampler, CTO-10AS column oven and SPD-M20A diode array detector equipped with Chromolith Performance RP-18e monolithic column (100 mm × 3 mm i.d., Merck). Isocratic elution with mobile phase $CH_3CN/CH_3OH/H_2O$/HCOOH (2/37/61/0.1, v/v/v/v) at the flow rate of 1.2 mL/min at 25 °C was used. The PDA (Photo Diode Array) data was acquired in the 200–450 nm range and the 285 nm signal was extracted.

2.4. Preparation of silybin sulfates

Optically pure silybin A or B [11,12] (**1a** or **1b** 99.5% purity; 30 mg, 0.062 mmol) were dissolved separately in 1.5 mL of acetone. This solution was mixed with 7 mL of 100 mM Tris–glycine buffer (pH 9.0) and *p*-nitrophenylsulfate (1 mL, 100 mM) was added to reach a final concentration of 10 mM. 500 μL of AST (9 μM) was added (final concentration 0.45 μM AST) and incubated at 20 °C. The reaction progress was monitored by HPLC method A.

After 24 h the mixture was concentrated by flushing with N_2 to final volume of 3 mL and the incubation was continued for another 24 h; at this stage the conversion was 80–85%. Sulfated product was isolated by extraction (3 × 3 mL *n*-butanol). *n*-Butanol phase contained mainly p-NPS and *p*-nitrophenol and small amount of product. The aqueous phase (2.8 mL) was acidified with 40 μL acetic acid to pH 4–5 and re-extracted (3 × 2 mL *n*-butanol). Organic phase containing the sulfated product was evaporated. The dry sample was dissolved in 2 mL of 80% methanol and loaded onto a Sephadex LH-20 column (30 g dry weight, 3 cm i.d.) packed and eluted with 80% methanol. The fractions were analyzed with HPLC method B and the fractions containing the product **3a** or **3b** were collected and evaporated *in vacuo* at 45 °C. Silybin A-20-*O*-sulfate (**3a**) was obtained as brownish solid (20 mg, 58%); (MALDI-TOF) *m/z* $C_{25}H_{22}O_{13}S$ $[M-H]^-$ calcd. 561.07; found 560.91. For ^{1}H, ^{13}C NMR data and HPLC – see Supplementary data. Silybin B-20-*O*-sulfate (**3b**) was obtained as a brownish solid (22 mg, 62%); (MALDI-TOF) *m/z* $C_{25}H_{22}O_{13}S$ $[M-H]^-$ calcd. 561.07; found 560.91. For ^{1}H, ^{13}C NMR data and HPLC – see Supplementary data.

2.5. Preparation of isosilybin sulfates

Isosilybin A [20] (**2a**, 28.2 mg, 96%) or isosilybin B [20] (**2b**, 25.2 mg 96%) were dissolved separately in 1 mL of DMSO. Each solution was mixed with 7 mL of 100 mM Tris–glycine buffer (pH 8.9) and 800 μL of 100 mM *p*-nitrophenyl sulfate and 250 μL of AST (final activity 0.23 U/mL and incubated at 30 °C). The reaction was monitored by HPLC method (B) as the release of *p*-nitrophenol from *p*-NPS and the substrate conversion (**2a** or **2b**) (Fig. 2). For the product purification we used an optimized solid phase extraction (SPE). The reaction mixture was diluted with 4 mL of 5% methanol in water and loaded on the column Chromabond C-18 SPE (3 mL, 500 mg; Macherey-Nagel, DE), which was preconditioned with 5 mL of methanol followed by 5 mL of water. The column was attached to a 12-port vacuum manifold (Supelco, USA). The column was then washed with 15 mL of 5% methanol and the product was eluted with 4 mL of 30% methanol. The solvent was evaporated *in vacuo* at max. 45 °C and the products were obtained as brownish solids: isosilybin A-20-*O*-sulfate **4a** (14.5 mg, yield 57%, HPLC purity > 95%) (HRMS-ESI) *m/z* $C_{25}H_{21}O_{13}S$ $[M-H]^-$ calcd. 561.07083; found 561.07060; For ^{1}H, ^{13}C NMR data and HPLC – see Supplementary data. Isosilybin B-20-*O*-sulfate **4b** (11.3 mg, yield 50%, HPLC purity > 95%); (HRMS-ESI) *m/z* $C_{25}H_{21}O_{13}S$ $[M-H]^-$ calcd. 561.07083; found 561.07074. For ^{1}H, ^{13}C NMR data and HPLC – see Supplementary data.

3. Results and discussion

New sulfated derivatives of the optically pure silybin A and B and isosilybin A and B, were successfully prepared using arylsulfotransferase from *D. hafniense*. The sulfated products were characterized by MS and the NMR data revealed esterification at position C20-OH as indicated by the up-field shift of the C-20 signal in case of both silybins and isosilybins, respectively. Isosilybin B sulfate (**4b**) tended to decompose/oxidize during the NMR measurement and

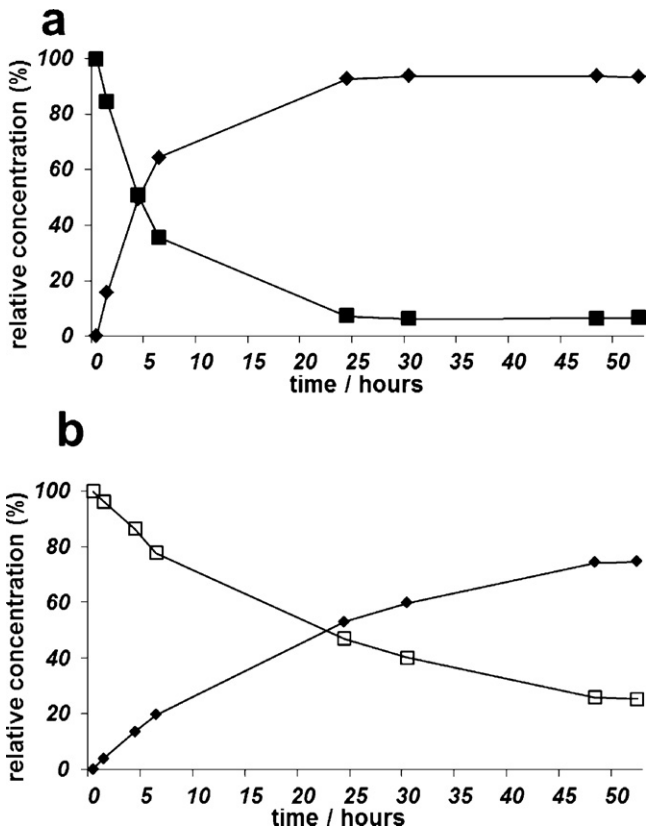

Fig. 2. Sulfation of isosilybin A (a) and isosilybin B (b) by *p*-nitrophenyl sulfate catalyzed by AST. Isosilybin A (■), isosilybin B (□) and *p*-nitrophenol (♦).

some amount of 2,3-dehydroisosilybin B sulfate was detected in the sample (data not given).

When the acceptor is depleted generation of *p*-nitrophenol (Fig. 2a) stops. Thus the arylsulfotransferase exhibits virtually quantitative sulfate transfer. It is quite clear that stereochemistry of respective silymarin congeners influences strongly its conjugation kinetics as clearly demonstrated in the case of sulfation of isosilybin A (**2a**), which is conjugated much faster than isosilybin B (**2b**) (Fig. 2).

During our work we have tested two purification methods, the first one based on the product extraction (for **3a**,**b**) and the other one based on the solid phase extraction (for **4a**,**b**). SPE extraction proved to be faster and more efficient, therefore it is proposed for the further work.

This study describes the first synthesis of optically pure silybin and isosilybin sulfates. No other aromatic OH groups at the ring A (C-7 and C-5) are sulfated neither in silybin and isosilybin thus the enzyme shows remarkable regioselectivity for the 20-OH position. It is obvious that this enzymatic procedure has great advantages over chemical ones since it is simple, direct, without the need of any protection/deprotection and it employs a cheap sulfate donor compared to, e.g., sulfotransferases that use excessively expensive and unstable 3′-phosphoadenosine-5′-phosphosulfate (PAPS).

4. Conclusions

The aim of this study was to obtain optically pure sulfated metabolites of both silybins and isosilybins. Aryl-sulfate sulfotransferase from *D. hafniense* was found to be a highly effective tool for the regiospecific enzymatic synthesis of silybin A-20-*O*-sulfate, silybin B-20-*O*-sulfate, isosilybin A-20-*O*-sulfate and isosilybin B-20-*O*-sulfate providing nearly quantitative yields and employing cheap *p*-nitrophenyl sulfate as sulfate donor. SPE extraction proved to be faster and efficient for the isolation of sulfated products. Sulfated flavonolignans will be used as authentic standards in metabolic studies of both silybins and isosilybins.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2012.12.005.

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Enzymatic preparation of silybin phase II metabolites: sulfation using aryl sulfotransferase from rat liver

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Abstract Aryl sulfotransferase IV (AstIV) from rat liver was overexpressed in *Escherichia coli* and purified to homogeneity. Using the produced mammalian liver enzyme, sulfation—the Phase II conjugation reaction—of optically pure silybin diastereoisomers (silybin A and B) was tested. As a result, silybin B was sulfated yielding 20-*O*-silybin B sulfate, whereas silybin A was completely resistant to the sulfation reaction. Milligram-scale sulfation of silybin B was optimized employing resting *E. coli* cells producing AstIV, thus avoiding the use of expensive 3′-phosphoadenosine-5′-phosphate cofactor and laborious enzyme purification. Using this approach, we were able to reach 48 % conversion of silybin B into its 20-sulfate within 24 h. The sulfated product was isolated by solid phase extraction and its structure was characterized by HRMS and NMR. Sulfation reaction of silybin appeared strictly stereoselective; only silybin B was sulfated by AstIV.

Keywords Silybin · Biotransformation · Diastereoisomers · Sulfation · Aryl sulfotransferase · Rat liver

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-4794-0) contains supplementary material, which is available to authorized users.

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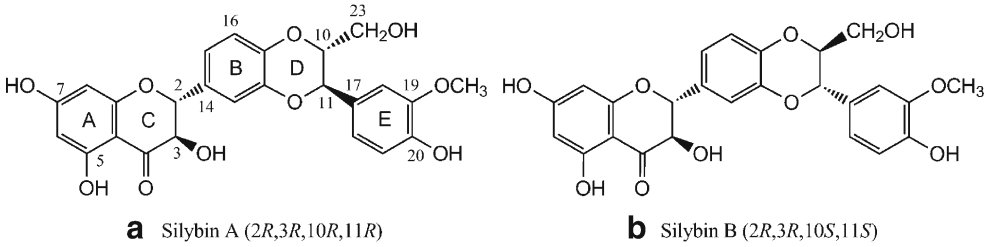
Introduction

Silybin is a major flavonolignan (30 %) of silymarin—a complex mixture extracted from the seeds of milk thistle (*Silybum marianum* (L.) Gaertn.) Besides silybin, silymarin also includes other flavonoids such as isosilybin, silydianin, silychristin, 2,3-dehydrosilybin, quercetin, taxifolin, and a polyphenolic fraction (Morazzoni and Bombardelli 1995; Wagner et al. 1968). Silybin consists of an approximate equimolar mixture of two diastereoisomers A (1a) and B (1b) (Scheme 1) (Šimánek et al. 2000; Gažák et al. 2007; Fraschini et al. 2002). Milk thistle has been used since ancient times for the treatment of gastrointestinal problems and liver and gall bladder diseases (Flora et al. 1998; De Groot and Rauen 1998). In vivo silybin protects the liver against various toxins, such as allyl alcohol, hepatotoxins of *Amanita phalloides* (Wieland et al. 1972; Wieland and Faulstich 1978; Vogel et al. 1984), CCl_4 (Hahn et al. 1968), and galactosamine or thioacetamide (Schriewer et al. 1973). Silybin is an effective antioxidant and a radical scavenger (Pietrangelo et al. 1995); it improves excretion of toxic substances from the body (Baer-Dubowska et al. 1998) and inhibits the formation of leukotrienes from polyunsaturated fatty acids by lipoxygenase inhibition (Fiebrich and

Scheme 1 Structures of silybin **a** and **b**



Koch 1979). It also acts as a chemoprotective agent inhibiting the carcinogenic effects of many chemicals (Dorai and Aggarwal 2004).

The first successful preparatory method yielding optically pure silybins based on the separation of silybin glycosides was published as early as 1997 (Křen et al. 1997). At that time, the absolute configuration of the respective silybins was not known; the pure silybins were denoted arbitrarily A and B. Later, Lee and Liu (2003) and Kim et al. (2003) used repeated high-performance liquid chromatography (HPLC) separation and determined the absolute configuration of silybins and isosilybins, but unfortunately, they used the opposite notation to the previous study (Křen et al. 1997)—as inferred from the optical rotations of silybin A and B. Therefore, in the literature before 2003, the nomenclature for silybin A and B is reversed. Optical rotation [α] is the easiest way to assign the absolute configuration of both compounds: natural silybin (a mixture of A and B, 1:1) has [α]=+11.4 (c=0.29, acetone) (Křen et al. 1997), silybin A has a higher [α]=+20 (c=0.21, acetone) than the mixture, and silybin B has a lower [α]=−1.07 (c=0.21, acetone) (Lee and Liu 2003).

Generally, polyphenolic substances such as silybin are easy targets for conjugation reactions and these are actually the major biotransformation pathway leading to excretion from the human body. Silybin undergoes quick conjugation, which is strictly stereoselective: silybin A and silybin B exhibit completely different metabolic (conjugation) profiles (Křen et al. 2000, Miranda et al. 2008; Weyhenmeyer et al. 1992; Wu et al. 2007). Unfortunately, most authors dealing with silybin metabolites identified these rather complex compounds using LC-MS techniques, which disregards stereochemistry and cannot determine exact regioisomeric structures (Jančová et al. 2007; Gunaratna and Zhang 2003).

So far, only a limited number of silybin sulfates have been prepared: 23-*O*-silybin sulfate by the chemical synthesis (Zarelli et al. 2011), and 7-*O*-silybin sulfate, via biotransformation using fungus *Cunninghamella blakesleeana* (Abourashed et al. 2012). Both silybin sulfates, prepared from the diastereomeric mixture of silybin A and B were tested only for their antioxidant activity by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay. 23-*O*-Silybin sulfate showed similar antiradical activity (Zarelli et al. 2011) as the silybin, whereas silybin sulfation at C-7 led to a substantial reduction in the radical scavenging activity (Abourashed et al. 2012). Recently, Biedermann (Agarwal et al. 2013) prepared silybin 7,23-disulfates from both optically pure silybins A and B.

Xenobiotics sulfation in mammals is catalyzed by cytosolic sulfotransferases (SULT; EC 2.8.2.), superfamily consisting in humans of at least ten functional genes (Strott 2002). Sulfotransferases occur in various tissues including liver, kidney, brain, adrenals, gut, and platelets (Wang and James 2006). Aryl sulfotransferase IV from rat liver is a well-known enzyme (EC 2.8.2.1), catalyzing the transfer of a sulfate group from phenolic sulfate esters to a phenolic acceptor substrate employing the 3′-phosphoadenosine-5′-phosphosulfate (PAPS) cofactor system (Sekura and Jakoby 1981, Burkart and Wong 1999). Moreover, aryl sulfotransferases are also able to reverse the process of sulfation in cells, which consequently influences the process of sulfation (Coughtrie et al. 1998; Kauffmann et al. 1991; Tan and Pang 2001).

Whole-cell biotransformation harboring recombinant protein is nowadays frequently applied in biocatalysis. Recently, resting microbial cells were found to be an effective biotransformation tool not only in cases when the classical enzymatic method failed, but also when overcoming the need of cofactors, low water solubility of substrates, and/or products as well as their inhibitory effects on the biocatalyst (Mao et al. 2012, Lee et al. 2012; Neunzig et al. 2012; Goretti et al. 2012).

To obtain silybin sulfate that can be considered as the silybin Phase II metabolite, the respective mammalian (preferably liver) enzyme should be used. We have decided to use aryl sulfotransferase IV from rat liver. We report here on the cloning, heterologous expression of aryl sulfotransferase IV from rat liver and its application in the enzymatic preparation of silybin sulfates.

Materials and methods

General methods

MALDI TOF/TOF

Negative mass spectra were measured in reflectron mode on a Matrix-assisted laser desorption ionization (MALDI)-TOF/TOF Ultraflex III mass spectrometer (Bruker Daltonics, Bremen, Germany) and externally calibrated using the monoisotopic $[M-H]^-$ ions of PepMixII calibrant (Bruker Daltonics).

A 5 mg/mL α-cyano-4-hydroxycinnamic acid in 50 % MeCN/0.1 % TFA was used as a MALDI matrix. Then, 0.4 μL of sample (1 mg/mL) dissolved in water was cleaned on graphite microcolumn and allowed to dry at ambient temperature. The dry sample was overlaid with 0.3 μL of the matrix solution on the target.

HRMS

High-resolution electrospray mass spectra were measured using a LTQ Orbitrap XL (Thermo Fisher Scientific). LTQ Orbitrap XL is a hybrid FT mass spectrometer combining a linear ion trap MS and the Orbitrap mass analyzer. The samples were dissolved in methanol (MeOH) and 80 % MeOH was used as a mobile phase at the flow rate of 100 μL/min. The following conditions were optimized for suitable ionization in the ESI source: sheath gas flow rate 35 AU, aux gas flow rate 10 AU of nitrogen, source voltage 4.3 kV, capillary voltage 40 V, capillary temperature 275 °C, and tube lens voltage 155 V.

HPLC

HPLC analyses were performed in a Shimadzu Prominence system (Kyoto, Japan) consisting of a DGU-20A mobile phase degasser, a LC-20AD solvent delivery unit, a SIL-20AC cooling auto sampler, a CTO-10AS column oven, and SPD-M20A diode array detector. Chromatographic data were collected and processed using Shimadzu Solution software (Kyoto, Japan) at a rate of 40 Hz and detector time constant of 0.025 s. The Chromolith Performance RP-18e monolithic column (100×3 mm i.d., Merck, Germany) coupled with a guard column (5×4.6 mm) (Merck, Germany) was used. Isocratic elution with mobile phase consisting of $CH_3CN/CH_3OH/H_2O/HCOOH$ (2/37/61/0.1: *v*/*v*/*v*/*v*) was employed in the analysis. The PDA data were acquired in the 200–450 nm range and the 285 nm signal was extracted. Flow rate was 1.2 mL/min at 25 °C.

Preparation of rat liver aryl sulfotransferase IV (EC 2.8.2.1)

Cloning of AstIV (Rattus norvegicus) into the E. coli expression vector pET-16b

For cytoplasmic expression of aryl sulfotransferase IV (AstIV) (GenBank accession no: P17988) in *E. coli* BL21(DE3), the gene was cloned into pET-16b vector of Novagen® (Darmstadt, Germany) using the *Nde*I and *Bam*HI restriction sites and N-terminal His_6-Tag for easier protein purification (see Supplementary data, Scheme S1). Therefore, the total RNA was isolated from 50 mg frozen rat liver tissue with the RNeasy Mini Kit from Quiagen® (Cologne, Germany). The rat liver tissue was kindly provided from the Institute of Pathology (University Hospital Aachen, Germany). The next step included the first strand cDNA synthesis with an anchored oligo $(dT)_{18}$ primer according to the instructions of the Transcriptor High Fidelity cDNA Synthesis Sample Kit from Roche® (Basel, Switzerland). The target gene was amplified using the forward primer 5′-ATGGAGTTCTCCCGTCC ACC-3′ and the reverse primer 5′-TCATAGTTCAC AACGAAACTTGAAGTCACAATC-3′. The insertion of the restriction sites *Nde*I and *Bam*HI was carried out with the forward primer 5′-CTATTTAGCCATATGGAGTTCTCCC GTCCACCGCTAGTG-3′ and the reverse primer 5′-ACACACACAGGATCCTCATAGTTCACAACGAAAC TTGAAGTC-3′. Ligation into the vector pET-16b resulted in the expression vector pET16b_*His_6AstIV*, which produces His_6AstIV (http://www.expasy.org/protparam.html) in the cytoplasm of *E. coli* after induction with isopropyl thiogalactoside (IPTG). The plasmids were purified with peqGold Plasmid Miniprep KitI from PEQLAB Biotechnologie GmbH® (Erlangen, Germany). Agarose gel extraction was carried out with NucleoSpin Extract II from MACHEREY-NAGEL® (Düren, Germany). The standard 1 kb DNA ladder, restriction enzymes, T4-ligase, and alkaline phosphatase FastAP were obtained from MBI Fermentas® (Vilnius, Lithuania). The integrity of the gene constructs was confirmed by sequencing (Sequiserve®, Vaterstetten, Germany).

E. coli strains, plasmids, and media

For plasmid propagation *E. coli* Nova Blue and for expression of *His_6AstIV*, *E. coli* BL21(DE3) (both strains Novagen®, Darmstadt, Germany) were used. Bacterial cultures were grown in lysogeny broth (LB) medium with ampicillin (Roth®, Karlsruhe, Germany) for selection and maintenance of plasmids.

Cultivation conditions

Terrific broth (TB) medium contained [g/L]: 12 pepton; 24 yeast extract; 12.5 K_2PO_4 ; 9.8 KH_2PO_4; and 4 mL glycerol. Conical flasks (500 mL) with 100 mL of TB medium and ampicillin (100 mg/L) were inoculated with an overnight culture of *E. coli* (0.5 mL) and cultivated on a rotary shaker at 200 rpm at 37 °C up to an OD_{600} of 0.6. Then, the cells were treated with 0.1 mM IPTG to induce expression of AstIV and subsequently grown at 16 °C. The harvest of the cells followed the next morning after induction (16 h). The *E. coli* cells were centrifuged 30 min at 3,360×*g* and 4 °C. An aliquot of the preserved cell pellets was frozen at −80 °C. For the cell disintegration a 60 % (*w*/*w*) cell suspension was made in 50 mMK phosphate buffer (pH7) from approximately 10 g of cells. The cells were disintegrated with ultrasound (0.5 cycle, 80 % amplitude) (Ultrasonic Processor UP50H 30 kHz, 50 W,

Hielsher, Germany) 4×1 min. Cell pellet and crude extract were used for the 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Laemmli 1970). The crude extract was used for subsequent purification steps.

Purification of AstIV by immobilized metal affinity chromatography

The crude extract was loaded in a loading buffer (50 mMK phosphate buffer; 150 mM KCl; 10 mM imidazole; pH7.5) onto the Ni^{2+}NTA agarose column (Qiagen, Germany) and incubated with Ni^{2+}NTA beads for 30 min. The column was washed with loading and washing buffer (50 mMK phosphate buffer; 150 mM KCl; 40 mM imidazole; pH7.5), and the enzyme was then eluted with elution buffer (50 mMK phosphate buffer, 150 mM KCl; 300 mM imidazole; pH7.5). The purity of the fractions was checked by 12 % SDS-PAGE and the concentration of proteins was assayed using Bradford method (Bradford 1976).

Purification of AstIV by gel permeation chromatography

After cell disintegration, the crude extract obtained was loaded on the Superdex column (Superdex 200, Amersham Biosciences, SE) in running buffer (50 mM KH_2PO_4; 300 mM NaCl; pH8.0) using Äkta Purifier protein chromatography system (Amersham Biosciences, SE). The purity of the fractions and protein concentration were assayed as above.

Enzyme activity assay

Standard enzymatic assay was performed at 25 °C. A 500 μL quartz cuvette contained typically 100 mM Bis-Tris propane (pH7); 0.25 mM dithiothreitol (DTT); 1 mM *p*-nitrophenyl sulfate as the sulfate donor, 50 μM of 2-naphthol as an acceptor and 20 μM PAP (3′-phosphoadenosine 5′-phosphate; Sigma). 100 μL of AstIV (concentration of protein 1.0 mg/mL) was added and release of *p*-nitrophenol was monitored at 400 nm spectrophotometrically (UV Pharmaspec-1700, Shimadzu, Japan). The rate of the enzymatic sulfate transfer was calculated from initial linear part of the kinetic curve, using molar extinction coefficient for *p*-nitrophenol (ε_{400}=0.2488 cm^2/μmol). One unit of activity is defined as the amount of the enzyme catalyzing the formation of 1 μmol of *p*-nitrophenol per minute.

The following reactions take place in the activity assay reaction mixture (Yang et al. 1996):

PAP+4-nitrophenyl sulfate ⇄ PAPS+4-nitrophenol [1]

PAPS+2-naphthol ⇄ PAP+2-naphthyl sulfate [2]

In addition, all activities were corrected for nonenzymatic release of *p*-nitrophenol in absence of enzyme and/or PAP as well.

Preparation of 20-*O*-silybin B sulfate—optimized methods

Preparation of 20-O-silybin B sulfate using the purified enzyme

Silybin B (1b, 0.369 μmol) dissolved in dimethyl sulfoxide (DMSO) (100 μL), *p*-nitrophenyl sulfate (1.1 μmol), DTT (0.25 mmol), PAP (40 nmol), 100 mM Bis-tris propane (pH 7, 476 μL), and pure AstIV (10×0.4 mL, 0.5 U/mL, every 12 h) were incubated in a Thermomixer (Eppendorf, Germany) at 37 °C for 136 h. The reaction progress was monitored by HPLC.

Preparation of 20-O-silybin B sulfate using the whole cells

E. coli cells expressing AstIV were prepared as described above but instead of disintegration, the cells were washed three times with 50 mMK phosphate buffer (pH7) and centrifuged 30 min at 3,158×*g* and 4 °C (5404 R, Eppendorf). Approximately 4 g (wet weight) of the washed cells were resuspended in 50 mMK phosphate buffer (40 mL, pH7) and directly used for the 20-*O*-silybin B sulfate preparation. Silybin B (1b, 40 mg, 83 μmol) and *p*-nitrophenyl sulfate (33 mg, 141.6 μmol) were dissolved in 4 mL of DMSO and slowly mixed with 40 mL of the cell suspension. The reaction was incubated at rotary shaker at 37 °C for 6 h then the cells were removed by centrifugation (30 min, 3,158×*g*, 5404 R Eppendorf) and a new portion of the fresh cells was added (40 mL, three times in 24 h). The reaction was monitored by HPLC and after 136 h, the product was purified by optimized solid phase extraction (SPE). The reaction mixture (after lyophilization) was diluted with 4 mL of 5 % methanol in water and loaded on the column Chromabond C18 SPE (3 mL, 500 mg; MACHEREY-NAGEL, Germany), which was preconditioned with 5 mL of methanol followed by 5 mL of water. The column was then washed with 6 mL of 10 % methanol and the product was eluted with 6 mL of 30 % methanol in water. The solvent was evaporated in vacuo at 45 °C maximum and the product was obtained as brownish solids. Structure was characterized by HRMS (*m*/*z* calc. for $C_{25}H_{21}O_{13}S$ 561.07083, found 561.07141) and ^{13}C, ^{1}H NMR (See Supplementary data Tab. S1).

Results

Expression, purification, and storage of recombinant aryl sulfotransferase IV

The gene of the AstIV from *Rattus norvegicus* was cloned into the pET16b expression vector via the 5′-

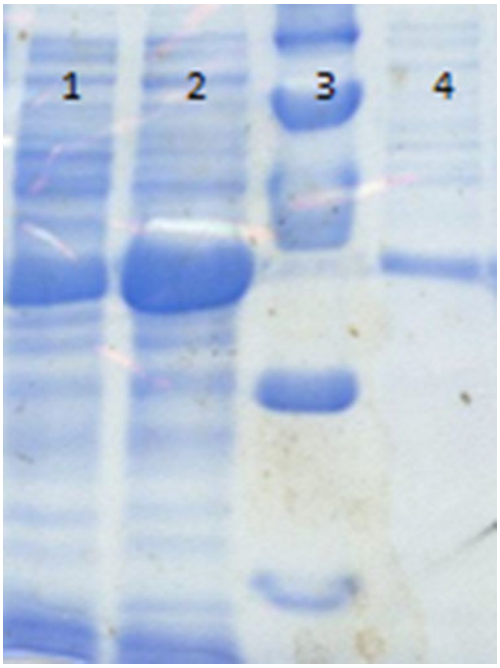


Fig. 1 10 % SDS-PAGE with Coomassie stain of AstIV after purification with gel permeation chromatography. **Lane 1** crude extract after 3 h (25 °C), **lane 2** crude extract after 16 h (16 °C), **lane 3** protein marker (97; 66; 45; 30; 20.1; 14.4 kDa), and **lane 4** purified enzyme

*Nde*I and 3′-*Bam*HI restriction sites and transformed into *E. coli* BL21(DE3) strain. The N-terminal His_6-Tag was included for the expected easier protein purification using metal affinity chromatography. The transformed *E. coli* clones were cultivated in liquid LB and TB media; apparently, the expression of protein was two times higher (according to SDS-PAGE, data not shown) in TB medium than in LB medium, moreover, a huge expression of AstIV was achieved even with quite a low concentration of inducer (0.1 mM IPTG). The cultivation temperature upon induction was very important: at 25 °C extensive expression of completely inactive enzyme was observed already after 3 h, while after 16 h at lower temperature (16 °C), we were able to obtain up to 3 U/mL of active AstIV (Fig. 1).

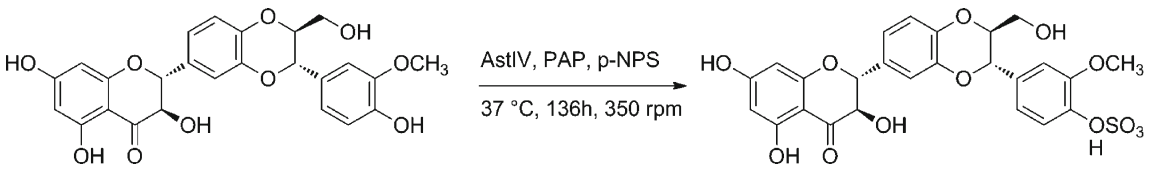
AstIV was purified from cell lysate to homogeneity by Ni^{2+}NTA-agarose, unfortunately, we found purification via His-Tag inefficient as the enzyme lost its initial activity (3 U/mL) probably due to high imidazole concentration (300 mM). Hence, the enzyme was obtained in a high yield amount and without activity loss using the gel permeation chromatography. The purity of the enzyme was confirmed by SDS-PAGE analysis, showing a single band of 33 kDa (Fig. 1). Aryl sulfotransferase IV from rat liver proved to be a very unstable enzyme quickly losing its activity: after 1 day storage at 4 °C or after 4 days at −20 °C the enzyme lost half of its original activity. Moreover, during the 6-h reaction at 37 °C, the enzyme lost its activity completely. We found that the addition of 10 % glycerol (*v*/*v*) can double the storage stability of the enzyme.

Preparation of 20-O-silybin B sulfate via enzymatic approach

Pure silybin A (1a), silybin B (1b), and mixed (A and B, natural) silybin (1) were used as substrates for the enzymatic reaction. This reaction afforded exclusively sulfate of silybin B (2, Scheme 2). Due to poor silybin solubility in water, cosolvent use was essential for the enzymatic reaction. Addition of 20 % ethanol substantially decreased the activity and stability of AstIV, and complete dissolution of silybin at 1 mg/mL concentration was impossible. Addition of 10 % DMSO notably increased silybin solubility (1 mg/mL) and did not negatively influence enzymatic reaction (Fig. 2). To overcome the enzyme's short lifetime, fresh enzyme (0.4 mL) was added every day, twice for 5 days to keep the reaction progress. After 5 days conversion of 92 % was reached (Fig. 2) scale-up of the sulfation reaction with the purified AstIV was seriously hampered by several factors: the extremely low solubility of the substrate, worse performance of the enzyme in reaction volumes over 1 mL, and last, but not least, by the need of the expensive cofactor PAP (3′-phosphoadenosine 5′-phosphate). Considering the above problems, we decided to perform and scale-up the reactions using resting *E. coli* cells expressing the rat liver AstIV.

Preparation and purification of 20-O-silybin B sulfate by whole-cell biotransformation

For the preparatory sulfation reaction, a 40 mL reaction mixture containing silybin B (40 mg), 4 g (wet weight) of



Scheme 2 Enzymatic preparation of 20-*O*-silybin sulfate B

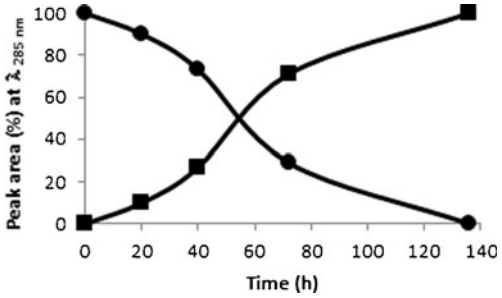


Fig. 2 Sulfation of silybin B by *p*-nitrophenyl sulfate catalyzed by purified enzyme (total volume 1.5 mL). Silybin B (●) and 20-*O*-silybin sulfate B (■)

freshly prepared and buffer-washed *E. coli* cells, *p*-nitrophenyl sulfate (33 mg) and 4 mL of DMSO was set up and incubated at 37 °C for 24 h; fresh cells were added three times during the reaction course (Fig. 3). Using this method, we reached sufficient conversion of silybin B (up to 48 %) in a reasonable time; moreover, we were able to recover the unreacted silybin B in the subsequent purification step. Due to the very polar properties of the product, the traditional purification processes such as flash chromatography, preparative TLC, or crystallization failed. Finally, solid phase extraction was chosen as the purification method of choice. The pure 20-*O*-silybin B sulfate was obtained in 25 % yield (10 mg), its structure was determined by high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR). Fragmentation in MALDI TOF/TOF MS/MS clearly demonstrated the presence of the sulfate in the position 20 or 23 (fragmentation at the dioxan moiety). The most abundant daughter ion (ΔM -80) suggested a loss of sulfate group. The second most abundant fragment with *m*/*z* value 259 $[M\text{-}H]^-$ corresponded to a coniferyl alcohol sulfate (see Supplementary data, Scheme S2.); NMR data corroborated proposed structure 20-*O*-silybin B sulfate. The sulfation at the C-20 has been unambiguously confirmed by both ^{13}C and ^{1}H NMR spectra, e.g., by the downfield shift of the respective signal (C-20) and upfield shifts of both C-21 and C-19 (in the *β*-position to the place of the substitution).

Discussion

All recent studies of silybin conjugation were accomplished only with natural silybin (mixture A and B) or even with the crude silymarin, and the products have never been directly identified; although some authors attempted to analyze separately metabolites of silybin A and silybin B (as free silybins after deconjugation). The sulfation was found to be the major metabolic pathway for silybin B (78 %—Miranda et al. 2008). Silybin A is sulfated and glucuronidated at about the same ratio, however, in total to a considerably lower extent than silybin B. Therefore, it is obvious that each silybin diastereomer has a specific route of Phase II metabolism (Miranda et al. 2008). Our study using for the first time optically pure silybins for the sulfation reactions by mammalian enzyme clearly confirmed these observations. Silybin B was sulfated in the position C-20, whereas silybin A was completely inert to the sulfation by rat liver aryl sulfotransferase. This has been proved also when the natural (mixed) silybin was used. Here, we observed conjugation of only silybin B. This experiment is also an indirect proof that silybin A is not an inhibitor of AstIV.

To obtain both conjugates of silybin A and B, we have recently used aryl sulfotransferase from *Desulfitobacterium hafniense* (Marhol et al. 2013). This bacterial enzyme was able to sulfate both silybins, although here, the reaction with the silybin B was approximately two times slower. It is interesting that also, in this case, the sulfation position occurred at C-20 in both silybins. We can speculate that the conjugation reaction in the case of silybin is probably dictated by the structure of the molecule itself and not entirely by the enzyme used. Use of the whole cells expressing AstIV substantially improved the procedure by skipping laborious enzyme purification (and loss of activity) and the addition of excessively expensive cofactor PAP.

We have clearly demonstrated that the conjugation reaction in the case of silybin diastereomers using authentic mammalian sulfotransferase is strictly stereoselective. This further corroborates the need to work with optically pure silybins in

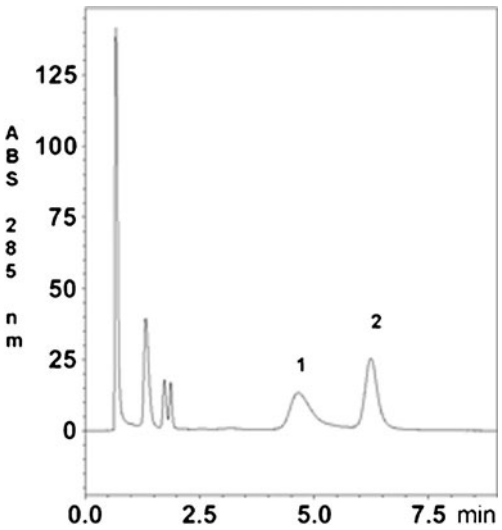


Fig. 3 HPLC chromatogram of whole cell biotransformation mixture after 24 h: silybin B-20-*O*-sulfate **2** (**peak 1**), silybin B **1b** (**peak 2**)

biological tests and disqualifies all mechanistic studies performed with mixed silybin or even with silymarin.

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Prokaryotic and Eukaryotic Aryl Sulfotransferases: Sulfation of Quercetin and Its Derivatives

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This manuscript is dedicated to Wolf-Dieter "Woody" Fessner on the occasion of his 60th birthday.

Two types of sulfotransferases, namely recombinant rat liver aryl sulfotransferase AstIV and bacterial aryl sulfotransferase from *Desulfitobacterium hafniense*, were used for the sulfation of quercetin, its glycosylated derivatives (isoquercitrin and rutin), and dihydroquercetin ((+)-taxifolin). The rat liver enzyme was able to sulfate only quercetin and taxifolin, whereas the quercetin glycosides remained intact. The *D. hafniense* enzyme sulfated isoquercitrin and rutin selectively at the C-4′ position of the catechol moiety with very good yields. Taxifolin was sulfated at the C-4′ position and a minor amount of the C-3′ isomer was formed. Sulfation of quercetin proceeded preferentially at the C-3′ position, but a lower proportion of the C-4′ isomer was formed as well. A detailed analysis of the kinetics of this reaction is provided and a full structural analysis of all products is presented.

Introduction

Flavonoids are naturally occurring polyphenols, which play a prominent role in the human diet and also in herbal medicines. Flavonoids are generally considered to be potent antioxidants and radical scavengers, but they have a plethora of other biological activities, such as antiallergic, anti-inflammatory, antimicrobial, or anticancer properties. For these reasons, they are now largely used for disease prevention and health improvement in dietary supplements and nutraceuticals.[1,2]

Flavonols are a major class of flavonoids in the human diet and, among them, quercetin[3] and its glycosides, rutin[4] and isoquercitrin,[5] are largely used in dietary supplements; therefore, they are in the focus of investigators. Taxifolin, also denoted as dihydroquercetin,[6] is also used in nutraceuticals, although not as frequently as quercetin.

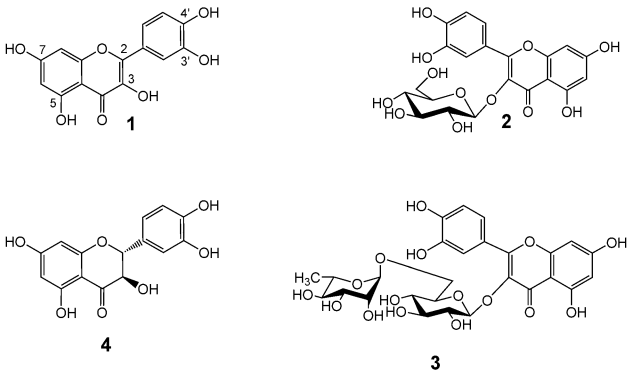

Figure 1. Quercetin (**1**), isoquercitrin (**2**), rutin (**3**), and taxifolin (**4**).

Quercetin (**1**; Figure 1) acts as a strong reducing agent to protect body tissues against oxidative stress and prevents the organisms from various diseases associated with oxidative and radical-mediated misbalances, such as cancer, cardiovascular diseases, or inflammation.[7–10] In nature, it often occurs in the form of glycoconjugates, for example, quercetin 3-*O*-β-D-glucopyranoside (**2**, isoquercitrin) or rutin (**3**, quercetin 3-*O*-rutinoside).

Rutin can be found in consumable parts of plants like apples, onions, berries, tea, wine,[11] red beans, or buckwheat (*Fagopyrum esculentum*);[12,13] it is also typically produced from the Brazilian tree Fava d'anta (*Dimorphandra mollis*). Rutin is widely utilized in the pharmaceutical, nutraceutical, and cosmetic industries as a stabilizer, preserver, and natural coloring agent.[14] The majority of its biological activities (vascular endo-

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thelium protection, anticancer, anti-inflammation, antiasthma, and antimicrobial) are considered to originate from the antioxidant and antiradical properties of rutin.[15] Isoquercitrin is widely distributed as well, but it could not until recently be prepared in sufficient amounts as a result of its low content in plant material.[16] Hence, a new and efficient method for the production of highly pure isoquercitrin (>95%) from rutin by the alkali-tolerant α-L-rhamnosidase from *Aspergillus terreus* was developed.[17–19] Isoquercitrin is a very efficient chemoprotective agent in both in vivo and in vitro experiments against cancer, allergy, cardiovascular diseases, diabetes, and oxidative stress and it displays better bioavailability than rutin and quercetin.[5]

(+)-Taxifolin, isolated from the bark of *Pinus roxburhii, Larix sibirica*, or *Taxus chinensis* var. *mairei*, plays a special role in keeping normal function of the circulatory system, improves immunity, and reduces cancer cell formation.[6]

During the last two decades, flavonoids have become popular as food adjuvants or dietary supplements and their daily intake has increased. As a result of this, their pharmacokinetics and metabolism have been extensively studied.[20,21] Flavonoids are preferably sulfated, glucuronidated, or methylated by sulfotransferases, uridine diphosphate glucuronyl transferases, *N*-acetyl transferases, glutathione *S*-transferases, thiopurin *S*-methyl transferases, and *O*-methyl transferases.[22] Metabolites of phase II of the biotransformation of polyphenols are often sulfates. Sulfation of xenobiotics and eobiotics usually leads to lower toxicity and improved elimination. However, sulfated small molecules could also be of value in therapeutics because of their hydrophilic nature and improved bioavailability. Sulfated quercetin derivatives are also important as authentic standards for metabolic studies. Besides this, some sulfated flavonols, including quercetin and rutin, possess anticoagulant or antiaggregant activity and also substantial antiviral activity.[23] Quercetin sulfates were shown to display some lipid peroxidation inhibitory effect[24] and antiradical activity (in the 2,2-diphenyl-1-picrylhydrazyl assay).[25] Quercetin 3′-*O*-sulfate demonstrated not only inhibition of cyclooxygenase-2 activity but also reduction of the expression of cyclooxygenase-2 messenger RNA.[26,27] Other interesting biological activities (antimicrobial, anti-inflammatory, anti-HIV, antitumor, etc.) of various quercetin sulfates and their derivatives were described in a recent review.[23]

The exact identification of the specific metabolites strongly depends on the analytical procedure used. Only HPLC–MS or MS analyses have been utilized so far for the conjugated-product characterization in the case of quercetin, rutin, and taxifolin. Up to now, no detailed identification of isoquercitrin metabolites (conjugates) has been accomplished.[28–30]

The preparation of authentic, pure, and fully structurally characterized metabolites of polyphenols is of utmost importance for the proper determination of their pharmacological profiles. Commonly used MS techniques seldom provide structural information that allows the determination of the exact structure. This is mainly important for polyphenols, for which regioisomers are usually hard to distinguish. NMR spectroscopy is the only reliable method (besides X-ray spectroscopy) to determine the site(s) of sulfation. However, even here, the task is not trivial because the place of sulfate substitution can be determined only indirectly (no couplings available) from the shifts of the carbon atoms in the aromatic ring. In the literature, there are numerous accounts of controversies concerning the distinguishing and proper assignment of 3′- and 4′-quercetin sulfates (at the catechol moiety). In this work, we have performed a rather complex structural study that has led to the unequivocal determination of the specific structures and possible re-evaluation of former studies.

We have recently developed preparatory methods for the chemoenzymatic synthesis of sulfated flavonoids[31] with two types of aryl sulfotransferases (ASTs). We used recombinant mammalian sulfotransferase IV from rat liver (EC 2.8.2.1) to catalyze the transfer of a sulfate group from phenolic sulfate esters to a phenolic acceptor substrate by employing the 3′-phosphoadenosine-5′-phosphosulfate (PAPS) cofactor system.[32,33] Fundamental problems, such as optimization of recombinant expression, poor stability of this enzyme, and PAPS regeneration, were addressed in detail in our study. The use of the mammalian liver enzyme should also lead to authentic mammalian metabolites, that is, the proper regioisomers.

Another sulfotransferase, namely the bacterial aryl sulfotransferase from *Desulfitobacterium hafniense,* previously proved to be suitable for the preparatory syntheses of certain aryl sulfates and its regioselectivity seems to be rather close to the mammalian enzyme.[31,34] In this case, a cheap sulfate donor, for example, *p*-nitrophenylsulfate (*p*-NPS), can be used.

We report herein the fully characterized sulfated flavonoids quercetin, taxifolin, isoquercitrin, and rutin, which were prepared by the bacterial aryl sulfotransferase from *D. hafniense*. Sulfated molecules prepared in this way can be considered as the phase II metabolites if the equivalent mammalian (preferably liver) enzyme is used. We have compared these sulfated products with the products obtained from aryl sulfotransferase IV from rat liver. A detailed NMR study supports the structure elucidations.

Results and Discussion

Polyphenolic substances are easy targets for conjugation reactions and these are the major biotransformation pathways leading to their excretion.[20] Quercetin is methylated, sulfated, and glucuronidated to form its major human metabolites: 3′-*O*-methylquercetin (isorhamnetin), quercetin 3-*O*-glucuronide, quercetin 3′-*O*-sulfate, and 3′-*O*-methylquercetin-3-*O*-glucuronide.[35,36] Isoquercitrin and rutin are deglycosylated to quercetin, conjugated and then methylated. There are, however, some indications that isoquercitrin can be absorbed intact and it could thus enter the circulation and undergo appropriate biotransformation reactions.[5]

Sulfation with aryl sulfotransferase AstIV from rat liver

Enzyme-activity optimization was tested with chaperone G7 (GroEL/GroES) coexpression in the strains BL21(DE3)Gold,

BL21(DE3)plyS, and Origami (DE3). However, no significant improvement of the enzyme activity and stability was reached. Therefore, we have followed our procedure[31] with whole-cell *Escherichia coli* BL21(DE3)Gold transformed with AstIV.

We tested the sulfation of all four quercetin derivatives with aryl sulfotransferase AstIV from rat liver, expressed recombinantly in *E. coli*, with the aim of verifying whether compounds **1**–**4** are substrates for this mammalian aryl sulfotransferase; if the results were positive, we wanted to isolate the respective sulfates, which should be identical to the authentic mammalian metabolites.

The sulfation reaction was tested with AstIV from rat liver by following a recently published procedure.[31] We have employed a modified semipreparatory sulfation method with transformed living *E. coli* cells expressing AstIV. This method overcame two major problems involved in the use of the isolated enzyme: 1) poor isolation yields and low stability of the pure enzyme and 2) the need for the expensive and labile cofactor 3′-phosphoadenosine-5′-phosphosulfate (PAPS), which is intrinsically present in the *E. coli* cells. AstIV is able to catalyze the parallel regeneration of PAPS by using *p*-NPS as a sulfate donor (see Figure 6).

Taxifolin was the only derivative that was accepted by AstIV as a substrate and the sulfated products were obtained. Isolated taxifolin monosulfate, tentatively assigned to be taxifolin 3′-*O*-sulfate (**4b**; see Figure 2 and 6), was characterized only by MS as a result of its paucity (1 mg). We proposed the structure of **4b** because another isolated product was identified (with MS and NMR spectroscopy) to be quercetin 3′-*O*-sulfate (**1b**), which is a presumed product of **4b** oxidation. Taxifolin is very sensitive to air oxidation at neutral and alkaline pH values (reaction at pH 7.5). Herein, we used metabolically active *E. coli* cells with preserved respiration involving cytochromes (and other redox enzymes), which may also cause enzymatic oxidation of taxifolin.

To avoid the oxidation potentially caused by cytochromes in the whole-cell catalyst, the respiration inhibitor sodium azide (1 mM) was added to the reaction mixture. We observed that the sulfation reaction was not affected by respiratory inhibition by the azide and both products were still formed. We could not distinguish whether the oxidation of the taxifolin skeleton occurred in the form of intact taxifolin or in the form of its sulfate. However, by taking into account sulfation experiments with pure quercetin, which gave no isolable product (see below), we assume that taxifolin was the primary substrate for the sulfation and only the sulfated product underwent oxidation to the respective quercetin sulfate. Sulfation at the 3′-position of the catechol moiety of quercetin and clearly also taxifolin seems to be typical for mammalian sulfotransferases because quercetin 3′-*O*-sulfate was isolated as a urinary metabolite, for example, in rats.[37]

If quercetin was tested as the substrate in the AstIV-catalyzed reaction, consumption of *p*-NPS accompanied by the formation of *p*-nitrophenol (*p*-NP) gave a clear indication that the sulfation reaction had occurred. However, despite our endeavors, no sulfated product was isolated. We assume that substrate **1** was probably sulfated but then was subsequently metabolized by the cells; hence, the sulfation reaction could only be proved indirectly.

Table 1. Rat liver AstIV substrate specificity towards quercetin derivatives.

Substance	Formation of *p*-NP	Isolated product(s)	Product structure
taxifolin	●[a]	●	taxifolin 3′-*O*-sulfate; quercetin 3′-*O*-sulfate
quercetin	●	not found	–
isoquercitrin	–[a]	–	–
rutin	–	–	–

[a] – indicates that this compound was not a substrate for AstIV; ● indicates isolated sulfated product(s) and/or the release of *p*-NP.

The quercetin glycosides rutin (**3**) and isoquercitrin (**2**) were not substrates for AstIV because no formation of *p*-NP was observed and also no sulfated products were identified (Table 1).

Sulfation with aryl sulfotransferase from *D. hafniense*

AstIV from rat liver indicated well the substrate specificity of the mammalian enzyme for phase II biotransformation. However, this did not afford preparatory amounts of the various metabolites. To overcome this, we used aryl sulfotransferase from *D. hafniense*, which is more stable, available in larger quantities, and has broader substrate specificity.

We performed a series of new transformations aimed at increasing the specific activity of the enzyme. The best results were obtained with BL21(DE3)Gold *E. coli* and yielded a specific activity of 28500 U mg^{-1}, which is approximately two orders of magnitude higher than the specific activity reported previously.[34] We found that, because of the high enzyme stability and lack of sulfatase activity, the purification of the protein, as in the work by Hartog and co-workers.[34], was not required. Therefore, we used a mere lysate of the transformed *E. coli*, which proved to be even better than the purified enzyme. As a result of the considerably higher activity of the AST used, we were able to substantially shorten (by approximately 20 times) the reactions times relative to those in the previous studies.[34]

With AST from *D. hafniense*, we were able to obtain sulfated products (Figure 2) in very good yields and in large amounts (Table 2). This enabled a detailed structural analysis of the products and also further biological tests.

Table 2. Analytical yields of sulfated products prepared by AST from *D. hafniense*.

Products[a]	Yield [%]
quercetin 3′-*O*-sulfate (**1b**) + quercetin 4′-*O*-sulfate (**1a**) (75:25)	47
taxifolin 4′-*O*-sulfate (**4a**) + taxifolin 3′-*O*-sulfate (**4b**) (80:20)	75
isoquercitrin 4′-O-sulfate (**2a**)	69
rutin 4′-O-sulfate (**3a**)	53

[a] Full details of the procedures for the sulfation of quercetin, taxifolin, isoquercitrin, and rutin with AST from *D. hafniense* are given in appropriate parts of the Experimental Section.

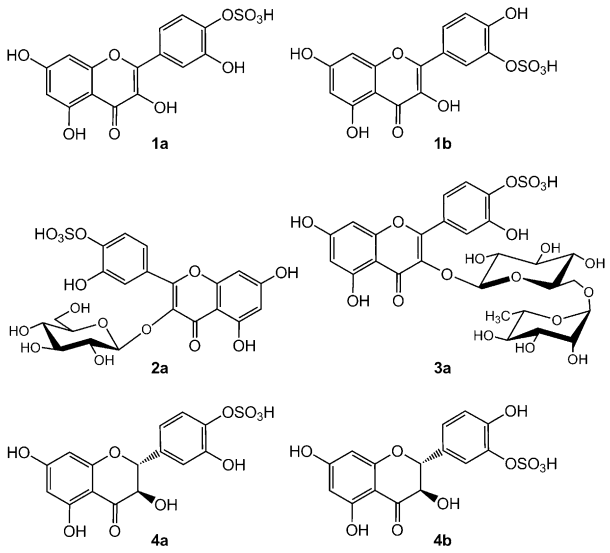

Figure 2. Isolated products sulfated by bacterial AST from *D. hafniense*.

For the preparatory reactions, we faced complex separation problems. The large amounts of *p*-nitrophenol that were formed complicated substantially the separations and the products were often contaminated by this toxic compound. To overcome this, we tried to replace the sulfate donor *p*-NPS with the alternative methyl sulfate, but unfortunately no reaction was observed.

We investigated the possibilities of bulk *p*-NP removal. We found that, in a very narrow pH range of 7.7–7.5, *p*-NP can be selectively removed by extraction with ethyl acetate, whereas the products and unused *p*-NPS remain in the aqueous phase. This pretreated mixture could then be separated by gel chromatography. Preparatory HPLC with an Asahipak column was also employed but was found to be less efficient; it provided lower yields and very poor purity of sulfated products (less than 50%), even after repeated injections. The separation by employing Sephadex LH-20 medium was found to be a convenient method that gave very pure products (>95%) in high yields (Table 2). There is only one slight disadvantage of this method, in that it is rather time demanding. Typically two to seven days were required for complete product purification. Isolated sulfated compounds **1a**–**4b** were fully characterized by MS and NMR spectroscopy analyses (see below and the Supporting Information).

At the pH value used (pH 8.9, as well as pH 7.0), taxifolin (**4**) is very sensitive to oxidation, which leads to the generation of the dehydro derivative (quercetin) and its sulfates. We observed that alkaline pH values and the presence of air oxygen led to the formation of the oxidized products. If the AST from *D. hafniense* is employed in the enzymatic reaction, the oxidation can be avoided by using a strictly inert atmosphere (N_2) and short reaction times. Unfortunately, this procedure cannot be used in the case of the whole-cell biotransformation. To avoid oxidation, the pure enzyme or crude lysate was applied under the inert atmosphere, but the requirement of the PAP(S) cofactor system and instability of AstIV limited the preparative reaction.

Table 3. Comparison of the two AST reactions and the catalyst properties.

Properties	AstIV rat liver	AST *D. hafniense*
PAP/PAPS	yes	no
catalyst	whole cells	lysate
optimum pH value	7	9
optimum temperature [°C]	37	30
reaction time	3 d	4 h
cosolvent	DMSO	acetone
typical yields	0–25% (10 mg)	50–80% (100 mg)
catalyst efficiency [mL of Luria–Bertani media for 1 mg of product]	450	0.1

Table 3 compares the reaction conditions, yields, and efficiency of the two enzymes studied. AstIV from rat liver is not a suitable catalyst for preparatory purposes because the reaction is time demanding, proceeds with low yields and poor efficiency, and causes substrate/product decomposition. On the other hand, this reaction provides important information on the authentic mammalian sulfated metabolites and the acceptance of substrates by this phase II biotransformation enzyme.

AST from *D. hafniense* was identified to be a suitable tool for the sulfation reaction; it is a highly efficient and highly yielding enzyme. Moreover, it was also found that it produces the same sulfated derivatives as the mammalian enzyme, which makes this enzyme applicable for production on a large scale.

Structure identification of the sulfated metabolites

Determination of the exact site of sulfation on the polyphenols is a fundamental issue; however, it is a very intriguing problem. Mass spectrometry can give only a limited amount of information, typically the number of the sulfates in the molecule. NMR spectroscopy does not allow a direct proof of the sulfation site because the sulfate group is not detectable with ^{1}H and ^{13}C NMR spectroscopy. Its position can be determined only indirectly through changes in the chemical shifts of attached and neighboring atoms. This situation is particularly complicated in the case of the catechol moiety that occurs in all of the substrates used. Unfortunately, in some literature sources, there are dubious interpretations of NMR data.[38]

Herein, we are reporting for the first time the complete NMR data of the sulfated compounds. The assignment of individual proton spin systems was accomplished by COSY and then transferred to the carbon atoms by HSQC analysis. HMBC experiments enabled us to assign quaternary carbon atoms and to link partial structures together. Quaternary carbon atoms in the catechol moiety showed the same HMBC coupling patterns in all samples under study (Figure 3). It is clear that the C-3′ and C-4′ carbon

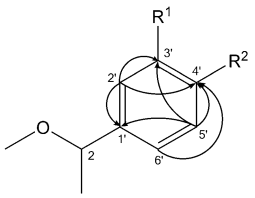

Figure 3. Diagnostic HMBC correlations in the catechol moiety of quercetin derivatives.

atoms can be differentiated by using a diagnostic correlation between the C-4′ carbon atom and the H-6′ proton; thus, all carbon atoms in the catechol moiety can be unequivocally assigned.

The fundamental question was to determine the position of the sulfate group(s). The 3′-OH and 4′-OH hydroxy groups resonate in the sulfates as broad singlets that give no correlation in the HMBC spectrum. Our novel approach for direct and unambiguous assignment was based on methylation of the isoquercitrin sulfate **2a**. The broad signal of the hydroxy group was replaced by an intensive methoxyl singlet that gave clear correlations with the C-2′ and C-3′ carbon atoms in the HMBC spectrum. The position of the methoxyl group in 5,7,3′-tri-*O*-methyl-4′-*O*-sulfo-3-*O*-β-D-glucopyranosyl quercetin (**5a**, prepared from **2a** by diazomethane methylation) was therefore directly assigned at the C-3′ carbon atom, which implied sulfate attachment at the C-4′ position. The parent isoquercitrin sulfate **2a** was thus definitely confirmed as the 4′-*O*-sulfate.

The ^{13}C NMR data of isoquercitrin 4′-*O*-sulfate (**2a**) were then compared with those of the mixture of two quercetin monosulfates (**1a** and **1b**). The chemical shifts of the minor component correspond to quercetin 4′-*O*-sulfate, whereas the major component agrees with the previously published quercetin 3′-*O*-sulfate (Table 4).[38] The mixture of quercetin sulfates **1a** and **1b** served also as a reference for the structure elucidation of the taxifolin and rutin sulfates (**4a**, **4b**, and **3a**; Tables 4 and 5). This simple and unequivocal method can be thus applied with certain care to the structural analysis of analogous compounds carrying a monosulfated catechol moiety.

Table 4. ^{13}C NMR data of flavonoid sulfates in CD_3OD (150.95 MHz, T = 25 °C).[a]

	4′-*O*-Sulfates [ppm]				3′-*O*-Sulfates [ppm]	
Atom	**5a**	**2a**	**1a**	**3a**	**1b**[38]	**1b**
2	156.22	158.46	146.97	158.65	147.0	147.45
3	138.65	136.61	138.56	136.50	137.6	137.80
4	175.95	179.95	177.88	179.85	177.4	177.70
5	162.37	163.42	162.81	163.37	162.5	162.74
6	97.61	100.33	99.68	100.43	99.3	99.64
7	166.85	166.60	166.13	166.68	165.7	165.94
8	94.18	95.10	94.79	95.25	94.4	94.82
9	160.70	158.91	158.62	158.96	158.2	158.51
10	109.88	106.20	104.92	106.11	104.5	104.86
1′	128.84	129.46	130.38	129.42	124.3	124.59
2′	115.76	119.62	118.00	119.81	123.8	124.07
3′	152.63	150.28	150.53	150.17	141.3	141.63
4′	145.36	143.98	143.04	144.01	152.6	152.86
5′	122.99	122.54	123.86	122.65	118.2	118.52
6′	122.67	123.56	121.00	123.48	127.4	127.72

[a] Data from carbohydrate moieties not given (for full data, see Tables S5 and S7 in the Supporting Information).

The comparison of the carbon chemical shifts of different flavonoids with their sulfates revealed several characteristic changes in the catechol moiety. All 4′-*O*-sulfates display a downfield shift for the C-3′ carbon signal and an upfield shift for the C-4′ signal relative to those for the parent compound; by contrast, an upfield shift of the C-3′ carbon signal and a downfield shift for the C-4′ signal were detected in the 3′-*O*-sulfates (Table 6).

Table 5. ^{13}C NMR data of flavonoid sulfates in [D_6]DMSO (150.95 MHz, T = 30 °C).

	4′-*O*-Sulfates [ppm]		3′-*O*-Sulfates [ppm]	
Atom	**1a**	**4a**	**1b**	**4b**
2	145.91	82.67	146.17	82.67
3	136.66	71.51	136.06	71.51
4	176.16	197.47	175.98	197.47
5	160.78	163.37	160.78	163.37
6	98.34	96.21	98.30	96.21
7	164.24	167.27	164.09	167.27
8	93.56	95.17	93.49	95.17
9	156.34	162.46	156.20	162.46
10	103.16	100.38	103.08	100.38
1′	126.89	133.89	122.29	133.89
2′	116.33	116.93	122.67	116.93
3′	148.55	148.87	140.88	148.87
4′	142.74	141.25	151.25	141.25
5′	122.12	122.65	117.26	122.65
6′	119.19	119.24	124.97	119.24

Table 6. Relative changes in the ^{13}C NMR chemical shifts in the catechol moieties, expressed as δ_C (sulfate)−δ_C (parent compound).

	3′-*O*-Sulfates [ppm]		4′-*O*-Sulfates [ppm]			
Atom	**1b**[a]	**4b**[a]	**1a**[a]	**4a**[a]	**3a**[b]	**2a**[b]
1′	0.28	0.36	4.88	5.80	6.02	6.11
2′	7.54	7.70	1.20	1.53	1.81	1.77
3′	−4.21	−4.40	3.46	3.88	4.08	4.09
4′	3.51	3.89	−5.00	−4.56	−6.06	−6.17
5′	−2.75	1.75	2.11	7.47	6.31	6.24
6′	9.33	5.51	3.55	−0.18	−0.40	0.07

[a] In [D_6]DMSO at T = 30 °C. [b] In CD_3OD at T = 25 °C.

Table 7. Relative changes in the ^{1}H NMR chemical shifts in the catechol moiety, expressed as δ_H (sulfate)−δ_H (parent compound).

	3′-*O*-Sulfates [ppm]		4′-*O*-Sulfates [ppm]			
Atom	**1b**[a]	**4b**[a]	**1a**[a]	**4a**[a]	**3a**[b]	**2a**[b]
2′	0.352	0.398	−0.001	0.100	0.104	0.029
5′	0.094	0.104	0.495	0.414	0.587	0.587
6′	0.308	0.367	0.037	0.144	0.035	0.049

[a] in [D_6]DMSO at T = 30 °C. [b] In CD_3OD at T = 25 °C.

Although the proton NMR spectra of individual flavonoids were not directly comparable, the common diagnostic features for their 3′- and 4′-*O*-sulfates were also observed. Regioisomers can be differentiated with downfield-shifted H-2′ and H-6′ proton signals in 3′-*O*-sulfates and a downfield-shifted H-5′ proton signal in 4′-*O*-sulfates (Table 7).

The proton chemical shifts of both quercetin 3′- and 4′-*O*-sulfates (Figure 4) are in accordance with the proton spectrum published by Jones et al.[37]

Our assignment of quercetin 4′-*O*-sulfate (**1a**) is in disagreement with the study of Duenas et al. in 2012.[38] Their structure assignment of the quercetin monosulfates in the chromato-

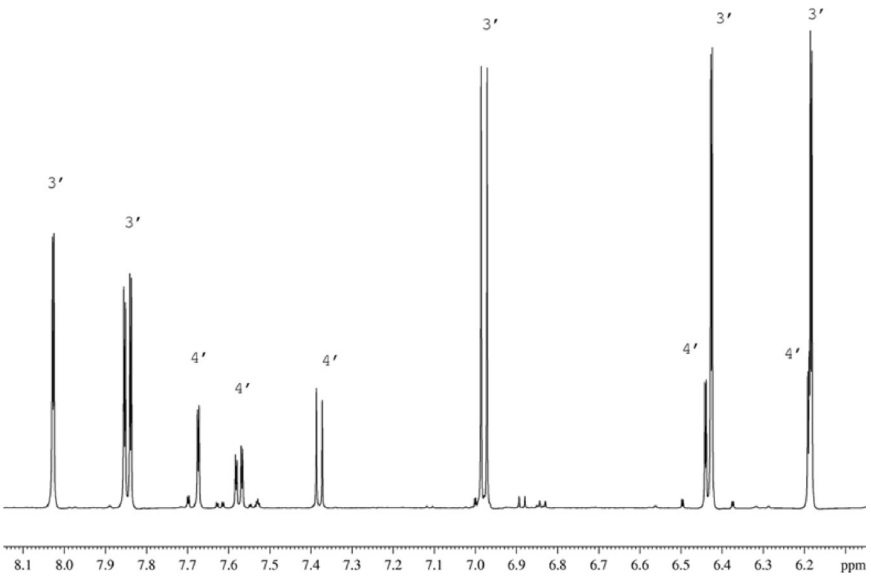

Figure 4. Expanded ^{1}H NMR spectrum of the mixture of quercetin 3′-*O*-sulfate (**1 b**) and quercetin 4′-*O*-sulfate (**1 a**) in [D_6]DMSO.

grams was based mainly on elution characteristics published by Jones et al.[37] The NMR data of the monosulfate that was assigned by Duenas et al.[38] as quercetin 4′-*O*-sulfate displayed neither up- nor downfield changes in the proton and carbon chemical shifts with respect to quercetin. This observation contradicts our carbon and proton NMR data and also the proton spectrum published in the original work by Jones et al.[37] Therefore, we regret to state that the structure assignment of quercetin 4′-*O*-sulfate in the study of Duenas et al.[38] was incorrect.

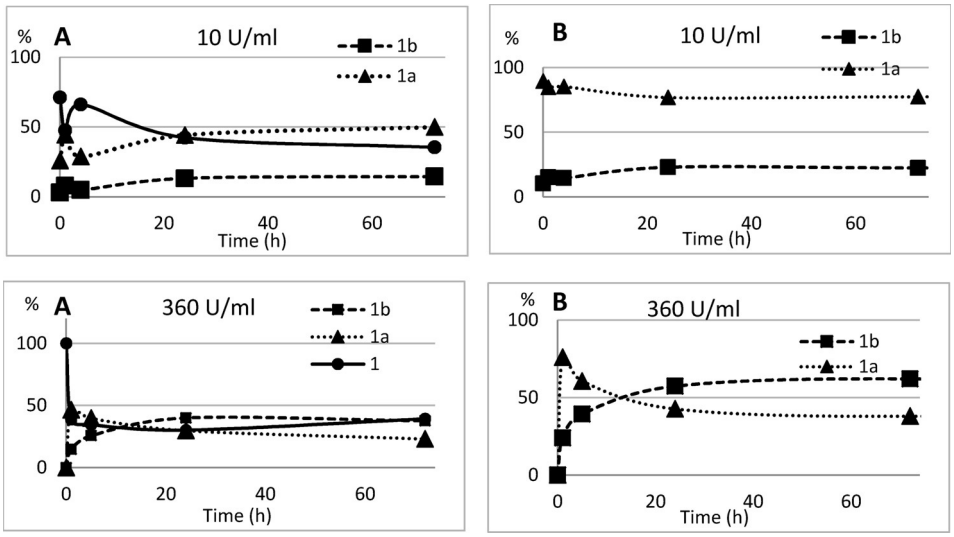

Figure 5. Kinetics of regioisomeric quercetin sulfate formation catalyzed by AST from *D. hafniense*. Quercetin (**1**; 100 mg, 0.332 mmol; Sigma) was dissolved in acetone (1.5 mL). *p*-NPS (5 mL, 100 mM), AST from *D. hafniense* (0.067 or 3 mL; corresponding to 10 or 360 U ml^{-1} of the reaction mixture), and tris(hydroxymethyl)aminomethane (Tris)–glycine buffer (to a final volume of 18 mL; 100 mM, pH 8.9) were added to the substrate solution and the mixture was incubated at $T = 30\,^{\circ}C$ under nitrogen. The reaction progress was monitored by HPLC method B and by NMR spectroscopy and expressed as A) a percentage of all quercetin derivatives in the reaction mixture and B) a percentage of the quercetin monosulfates. ■: Quercetin 3′-*O*-sulfate (**1 b**); ▲: quercetin 4′-*O*-sulfate (**1 a**); ●: quercetin (**1**).

Kinetic studies

During the preparation of this manuscript, the sulfation of quercetin (among other compounds) by AST from *D. hafniense* was published by van der Horst et al.[39] However, in their hands, the formation of only quercetin 4′-*O*-sulfate was observed. With the knowledge that they used a much less active enzyme, to deal this discrepancy, we performed a kinetic study of quercetin sulfation with reaction mixtures of 10, 36, 180, and 360 U mL^{-1} AST enzymatic activity for $t = 1$, 4, 24, and 168 h. With our original, optimized HPLC method B, we were able to separate the quercetin monosulfates and determine their proportion and also the relative conversion. We found that quercetin 3′-*O*-sulfate is the major reaction product only with 360 U mL^{-1} enzymatic activity and reaction times greater than 4 h. Low enzymatic activity (for example, 10 U mL^{-1}; Figure 5) always led to preferential production of quercetin 4′-*O*-sulfate and lower conversion, but both regioisomers were always found in the reaction mixture (Figure 5 and Figures S2 and S14 in the Supporting Information).

On the other hand, careful examination of the NMR data (chemical shifts and signal integration) in the supplementary material to the work of van der Horst et al.[39] revealed that approximately 15% of quercetin 3′-*O*-sulfate was also present in their product, which was declared in the paper to be pure quercetin 4′-*O*-sulfate. The signals of the quercetin 3′-*O*-sulfate in their spectra were erroneously ascribed to *p*-NP and *p*-NPS, which were, however, absent in the spectra.

The kinetic study was also performed in the same manner with taxifolin, but in this case, taxifolin 4′-*O*-sulfate was the major reaction product, together with less than 20% of the 3′-*O*-sulfate, irrespective of the reaction length and enzyme activity (data not shown).

To elucidate whether the enzyme from *D. hafniense* was able to catalyze the transfer of the sulfate group between the 3′- and 4′-positions of the quercetin molecule, we incubated a mixture of quercetin 3′-*O*-sulfate (**1b**) and quercetin 4′-*O*-sulfate (**1a**; 75:25 ratio) in Tris–glycine buffer in the presence of the enzyme for 24 h. The proportion of the regioisomers did not change during whole incubation time (samples at $t=0$, 1, 4, and 24 h; data not shown). Subsequently, this mixture of quercetin sulfates (as potential sulfate donors) was incubated in the presence of AST from *D. hafniense* with an equimolar amount of pyrocatechol (as a potential sulfate acceptor). In this case, we found, by using HPLC method C, that quercetin sulfates and pyrocatechol quickly disappeared from the reaction mixture, whereas quercetin and sulfated pyrocatechol were formed. Already after 1 hour, 65% of the quercetin sulfates were desulfated and 61% of the pyrocatechol was sulfated. After one week, only 6% of the initial amount of quercetin 3′-*O*-sulfate and 12.5% of the 4′-*O*-sulfate remained in the reaction mixture (Figure S15 in the Supporting Information). The proportion of the regioisomers in this reaction mixture progressively changed from 70:30 ($t=0$), through 64:36 ($t=1$ h) and 66:34 ($t=4$ h), to 52:48 ($t=166$ h). This result suggests that quercetin 3′-*O*-sulfate is desulfated slightly preferentially. The various reactants were determined by co-chromatography with authentic samples, by UV spectra of respective peaks and by NMR spectroscopy confirmation.

Conclusions

We have tested two types of sulfotransferases, namely recombinant rat liver aryl sulfotransferase AstIV and bacterial aryl sulfotransferase from *Desulfitobacterium hafniense*, for the sulfation of quercetin (**1**), its glycosylated derivatives (isoquercitrin (**2**) and rutin (**3**)), and dihydroquercetin ((+)-taxifolin (**4**)). The rat liver enzyme was able to sulfate only quercetin and taxifolin, whereas the quercetin glycosides remained intact. The *D. hafniense* enzyme sulfated isoquercitrin and rutin selectively at the C-4′ position of the quercetin moiety in very good yields. Taxifolin was sulfated at the C-4′ position and a minor amount of the C-3′ isomer was formed. The sulfation of quercetin proceeded preferentially at the C-3′ position, but a lower proportion of the C-4′ isomer was formed as well.

We propose the methylation of flavonoid sulfates as a novel approach for the direct and unequivocal determination of the position of sulfates (and possibly also other groups that lack direct interactions) in polyphenols. The replacement of the hydroxy group signals by easy detectable methoxyl singlets enables the methoxyl groups to be located based on the HMBC correlations and allows clear deduction of the site of sulfate attachment.

The AST from rat liver is not a convenient catalyst because it is time demanding and gives poor yields with a limited spectrum of substrates. Isoquercitrin and rutin were not substrates for the AST from rat liver. On the other hand, the bacterial AST from *D. hafniense* was identified as a perfect tool for the biotransformation of the whole array of flavonoids; it is stable, highly efficient, and high yielding (50–80%). The isolation and full characterization of the sulfated products from both ASTs indicated that their sulfated products were identical. The sulfates can, therefore, be used as authentic standards in further metabolic studies.

Experimental Section

NMR spectroscopy

NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (600.23 MHz for ^{1}H, 150.94 MHz for ^{13}C) and Bruker Avance III 700 MHz spectrometer (700.13 MHz for ^{1}H, 176.05 MHz for ^{13}C; compound **5a**). The residual signals of solvents (DMSO: $\delta_H=2.500$ ppm, $\delta_C=39.60$ ppm; CH_3OH: $\delta_H=3.330$ ppm, $\delta_C=49.30$ ppm) were used as internal standards. NMR experiments: ^{1}H NMR, ^{13}C NMR, gradient COSY, gradient HSQC, and gradient HMBC were performed by 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 NMR spectra to improve the resolution and line broadening (1 Hz) was applied to get a better ^{13}C NMR signal-to-noise ratio. Chemical shifts are given in the δ scale with digital resolution that justifies the reported values to three (δ_H) or two (δ_C) decimal places, respectively.

Mass spectrometry

Mass spectra in the negative-ion mode were measured with the LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source. The samples were dissolved in methanol and introduced into the mobile-phase flow (methanol/water, 4:1; 100 μL min^{-1}) by using a 2 μL loop. The spray voltage, capillary voltage, tube lens voltage, and capillary temperature were 4.0 kV, −16 V, −120 V, and 275 °C, respectively.

The ESI mass spectrum of trimethyl derivative **5a** was acquired with a Waters Micromass ZMD mass spectrometer (Micromass UK Ltd., Manchester, UK; direct inlet; cone voltage: 20 V; source voltage: 4.3 kV).

Analytical HPLC–photodiode array (PDA)

All analytical HPLC analyses were performed with the Shimadzu Prominence System (Shimadzu, Kyoto, Japan) consisting of a DGU-20A mobile-phase degasser, two LC-20AD solvent delivery units, a SIL-20AC cooling autosampler, a CTO-10AS column oven, and the SPD-M20A diode-array detector. Chromatographic data were collected and processed with Shimadzu Solution software at a rate of 40 Hz and a detector time constant of 0.025 s.

Method A: The Chromolith Performance RP-18e monolithic column (100×3 mm internal diameter; Merck, Germany) coupled with a guard column (5×4.6 mm; Merck, Germany) was used. Mobile phases: acetonitrile/water/formic acid (80/20/0.1, v/v/v; phase A) and acetonitrile/water/formic acid (5/95/0.1, v/v/v; phase B). Gradient: 0–4 min 7→40% A, 4–6 min 40% A, 6–7 min 40→7% A, 7–8 min 7% A. Flow rate: 1.5 mL min^{-1} at 25 °C. The PDA data were acquired in the 200–450 nm range and the 285 nm (**4**) and 370 nm (**1**, **2**, **3**) signals were extracted, respectively.

Method B: Separation of quercetin sulfates was achieved on a Kinetex 5 μm pentafluorophenyl core–shell silica column (150×

4.6 mm; Phenomenex, USA) thermostated at $T=40\,^{\circ}C$ and equipped with a guard column (5×4.6 mm; Merck, Germany). Mobile phases: water/trifluoroacetic acid (100/0.1, v/v; phase A) and methanol/water (80/20, v/v; phase B). Linear gradient: 0–25 min 40→80% B. Flow rate: 0.8 mL min^{-1}. The PDA data were acquired in the 200–450 nm range and the 370 nm signal was extracted.

Method C: Separation of taxifolin sulfate(s) was achieved on a Kinetex 5 μm pentafluorophenyl core–shell silica column (150×4.6 mm; Phenomenex, USA) equipped with a guard column (5×4.6 mm; Merck, Germany). Mobile phases: water/trifluoroacetic acid (100/0.1, v/v; phase A) and methanol/water (80/20, v/v; phase B). Binary gradient elution: 0–16 min 45→50% B, 16–18 min 50→70% B, 18–22 min 70% B. Flow rate: 0.8 mL min^{-1}. The mobile phase was filtered through a 0.45 μm nylon membrane filter (Whatman, USA). The PDA data were acquired in the 200–450 nm range and the 285 nm signal was extracted.

Preparatory HPLC was performed on a 20×300 mm Asahipak GS-310 20F column (Shodex, USA). The system consisted of a Shimadzu LC-8A pump, a SPD-20 A dual wavelength detector, a FRC-10A fraction, and a CBM-20A controller connected to a computer. Separations were performed with methanol at $T=25\,^{\circ}C$. Flow rate: 5 mL min^{-1}. Detection was at 254 and 369 nm.

Aryl sulfotransferase from rat liver

AstIV from rat liver (the plasmid bearing *astIV* was kindly provided by Prof. L. Elling, RWTH Aachen University, Germany) was expressed as described in our previous work[31] with the following modifications. To support the enzyme folding and to enhance the activity of AstIV, the cotransformation with the chaperone G7 (GroEL/GroES) was applied for the *E. coli* strains BL21(DE3)Gold, BL21(DE3)plyS, and Origami (DE3). However, no significant improvement of the enzyme activity and stability was observed. Freshly prepared whole cells, as well as crude lysate or purified AST enzyme, were prepared with some modifications in accordance with Ref. [31] and tested in the biotransformation experiments. Only the freshly prepared whole cells were able to catalyze the sulfation reaction (see Figure 6).

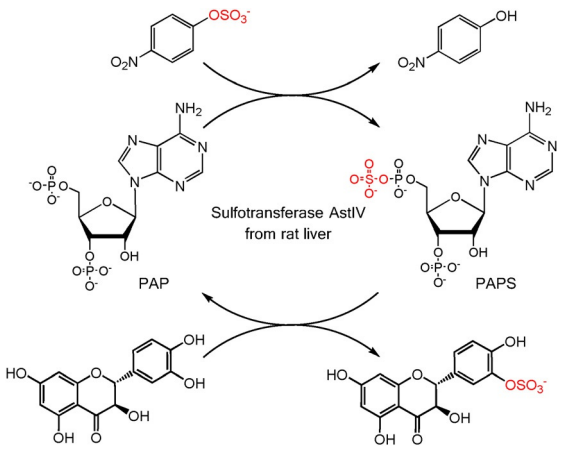

Figure 6. Sulfation of (+)-taxifolin (**4**) and PAPS regeneration, (both) catalyzed by sulfotransferase AstIV from rat liver.

Taxifolin sulfation by aryl sulfotransferase from rat liver

(+)-Taxifolin (**4**; 52 mg, 0.171 mmol; Amagro, Czech Republic) and *p*-NPS (35 mg, 0.136 mmol) were dissolved in DMSO (3 mL). Whole *E. coli* cells (2 g wet weight) expressing recombinant aryl sulfotransferase from rat liver resuspended in potassium phosphate buffer (pH 7.5, 17 mL) were added. The reaction mixture was incubated in the bench-top 5404 R rotary shaker at $T=37\,^{\circ}C$ for 24 h, then the cells were centrifuged (30 min, 5000 rpm; Eppendorf, Germany) and a new portion of fresh cells (2 grams, wet weight, resuspended in potassium phosphate buffer) was added (15 mL; 3 times in 3 days). The reaction was monitored by HPLC method A and the product was purified after 72 h by preparative HPLC by employing an Asahipak GS-310 20F column (Shodex, USA) with isocratic elution with 100% methanol. The fraction containing the putative sulfated product(s) was evaporated, dissolved in 80% methanol (1 mL), and loaded onto a Sephadex LH-20 column (15 g dry weight; 12 mm internal diameter) packed and eluted with 80% methanol. The fractions were analyzed by HPLC method A and the fractions containing the products **4b** and **1b** were collected and evaporated in vacuo at $T=45\,^{\circ}C$. Taxifolin sulfate (**4b**) was obtained as a yellowish solid (1 mg, 1.5%). The structure was characterized only by HRMS (*m/z* calcd for $C_{15}H_{11}O_{10}S$: 383.00784; found: 383.00677; see Figure S11 in the Supporting Information). Quercetin 3′-*O*-sulfate (**1b**) was obtained as a yellowish solid (8 mg, 12%). The structure was characterized by HRMS (*m/z* calcd for $C_{15}H_9O_{10}S$: 380.99219; found: 380.99126; see Figure S10 in the Supporting Information) and by NMR spectroscopy (For ^{1}H and ^{13}C NMR data, see Tables S1 and S2 in the Supporting Information). Complete assignment of all NMR signals was accomplished by a combination of gradient COSY, gradient HSQC, and gradient HMBC experiments.

To avoid substrate oxidation (of taxifolin to quercetin), tentatively thought to be caused by cytochromes, an inhibitor of the respiration, NaN_3 (1 mM final concentration), was added to the reaction mixture and the reaction was repeated, which yielded the same products.

Substrates **1**, **2**, and **3** were subjected to the above-described procedure for taxifolin; however, no sulfated products were obtained.

Aryl sulfotransferase from *D. hafninense*

Expression of the AST enzyme was performed as described by van der Horst et al.[34] with the following amendments. The plasmid containing the AST gene (kindly provided by Dr. van der Horst, University of Amsterdam, The Netherlands), was retransformed into various *E. coli* strains: BL21, BL21(DE3)Gold, and BL21(DE3)plyS (Table 8). Bacterial cultures were grown in Luria–Bertani medium with kanamycin and appropriate second antibiotics (according to the *E. coli* strain used) for the better selection and maintenance of the plasmids. The purity of the enzyme was confirmed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis analy-

Table 8. Transformation of AST from *D. hafninense* into various expression systems.

Strain	Activity [U mL^{-1}] at $T=37\,^{\circ}C$	at $T=30\,^{\circ}C$
BL21	0	N.D.[a]
BL21(DE3)plyS	12	N.D.[a]
BL21(DE3)Gold	105	2170

[a] N.D.: not determined.

sis,[40] which showed a single band of 70 kDa (Figure S13 in the Supporting Information). The crude lysate of the most active clone (*E. coli* BL21(DE3)Gold) was used in the enzymatic reactions. The AST was always prepared fresh and standard enzymatic assays were performed at $T=37$ or 30 °C (Table 8). The activity of AST was expressed as released *p*-NP at 400 nm (extinction coefficient at 400 nm: 0.2488 $cm^2\,\mu mol^{-1}$). One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 mmol *p*-NP per minute. The activities were corrected for nonenzymatic *p*-NP release. The specific activity of the recombinant AST from *D. hafniense* was 28500 U mg^{-1} (with 2-naftol as the acceptor).

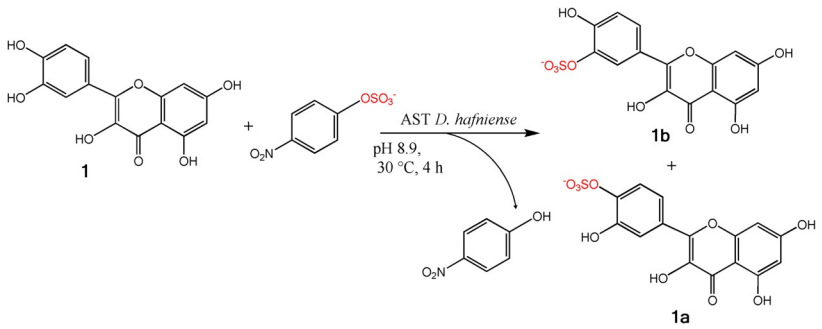

Figure 7. Sulfation of quercetin (**1**) catalyzed by sulfotransferase from *D. hafniense*.

Quercetin sulfation with aryl sulfotransferase from *D. hafniense*

Quercetin (**1**; 150 mg, 0.498 mmol; Sigma) was dissolved in acetone (2 mL). 100 mM Tris–glycine buffer (pH 8.9; 20 mL), *p*-NPS (Sigma–Aldrich; 130 mg, 0.50 mmol), and AST from *D. hafniense* (2 mL, 180 U ml^{-1} of the reaction mixture) were added to the substrate solution and the mixture was incubated for 4–5 h at $T=$ 30 °C under nitrogen. The reaction progress was monitored by HPLC or by TLC (ethyl acetate/methanol/HCO_2H, 4:1:0.01). The reaction was terminated by short heating to > 90 °C and the reaction mixture was halved by evaporation in vacuo so that all organic solvents were removed. The pH value was adjusted to 7.5–7.7 and then *p*-NP and residual starting materials were removed by extraction (3×50 mL EtOAc). The aqueous phase (15 mL) containing the sulfated product was evaporated and the residue was dissolved in 80% methanol (2 mL) and loaded onto a Sephadex LH-20 column (30 g dry weight, 3 cm internal diameter) packed and equilibrated with 80% aqueous methanol. The elution typically takes 2–7 days. The fractions were analyzed by TLC (EtOAc/MeOH/HCO_2H, 4:1:0.01, v/v/v) and the fractions containing the respective products were collected and evaporated in vacuo at $T=45$ °C.

The mixture of quercetin 3′-*O*-sulfate (**1b**) and quercetin 4′-*O*-sulfate (**1a**, side product, 25%) was obtained as a yellowish solid (90 mg; 47.5% yield), the identity of which was confirmed by HRMS (*m/z* calcd for $C_{15}H_9O_{10}S$: 380.99219; found: 380.99126; Figure S1 in the Supporting Information). These two compounds were not separable by preparatory HPLC and only partial separation was achieved by analytical HPLC method B (Figures 7 and 8); both regioisomers could be distinguished in the NMR spectra (see Tables S1 and S2 for **1b**, Tables S3 and S4 for **1a**, and Figure S2 in the Supporting Information). The complete assignment of all NMR signals was accomplished by the use of a combination of gradient COSY, gradient HSQC, and gradient HMBC experiments.

Isoquercitrin sulfation with aryl sulfotransferase from *D. hafniense*

Isoquercitrin (**2**; 150 mg, 0.323 mmol), prepared as described previously,[18] was sulfated by using the same procedure as that for quercetin. The reaction progress was monitored by TLC and by HPLC method A. Isoquercitrin 4′-*O*-sulfate (**2a**) was obtained as a yellowish solid (120 mg; 69% yield). The structure was determined by HRMS (*m/z* calcd for $C_{21}H_{19}O_{15}S$: 543.04501; found: 543.04367; Figure S3 in the Supporting Information) and by NMR spectroscopy (see Table S5 and Figure S4 in the Supporting Information).

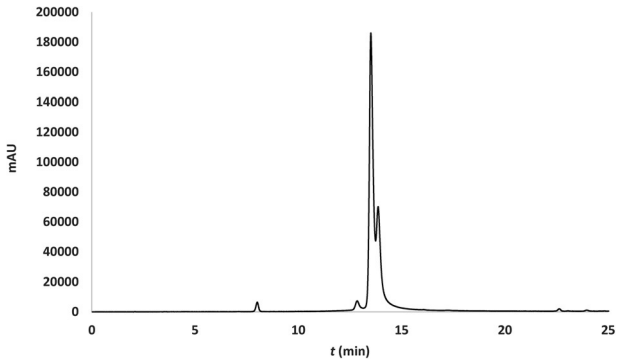

Figure 8. HPLC analysis of quercetin 3′-*O*-sulfate (**1b**; $t_R=13.76$ min), quercetin 4′-*O*-sulfate (**1a**; $t_R=14.07$ min); method B.

Rutin sulfation with aryl sulfotransferase from *D. hafniense*

Rutin (**3**; 150 mg, 0.246 mmol; Sigma–Aldrich) was sulfated by using the same procedure as that for quercetin. The reaction was monitored by TLC and by HPLC method A. Rutin 4′-*O*-sulfate (**3a**) was obtained as a yellowish solid (90 mg; 53% yield). The structure was determined by HRMS (*m/z* calcd for $C_{27}H_{29}O_{19}S$: 689.10183; found: 689.10079; Figure S6 in the Supporting Information) and by NMR spectroscopy (see Table S7 and Figure S7 in the Supporting Information).

Taxifolin sulfation with aryl sulfotransferase from *D. hafniense*

Taxifolin (**4**; 200 mg, 0.66 mmol; Amagro, Czech Republic) was sulfated ($t=4$ h) by using the same procedure as that for quercetin. The mixture of taxifolin 4′-*O*-sulfate (**4a**) and taxifolin 3′-*O*-sulfate (**4b**, side product, approximately 20%) was obtained as a yellowish solid (190 mg; 75.4% yield). The structure was determined by HRMS (*m/z* calcd for $C_{15}H_{11}O_{10}S$: 383.00784; found: 383.00677; Figure S8 and S9 in the Supporting information) and by NMR spectroscopy (see Table S8 for **4a** and Table S9 and Figure S12 for **4b** in the Supporting Information).

Kinetics of regioisomer sulfate formation of taxifolin and quercetin catalyzed by aryl sulfotransferase from *D. hafniense*

Quercetin (**1**; 100 mg, 0.332 mmol; Sigma) and taxifolin (**4**; 100 mg, 0.33 mmol; Amagro, Czech Republic) were separately dissolved in acetone (1.5 mL). *p*-NPS (5 mL, 100 mM), AST from *D. hafniense* (0.067, 0.3, 1.5, or 3 mL; corresponding to 10, 36, 180, or 360 U ml^{-1} of the reaction mixture, respectively), and Tris–glycine buffer (to a final volume of 18 mL; 100 mM; pH 8.9) were added to the substrate solution and the mixture was incubated at $T=30\,^{\circ}C$ under nitrogen. The reaction progress was monitored by HPLC method B and by NMR spectroscopy. The reaction was stopped after 1, 4, and 24 h and, in selected cases, also after 72, 168, and 336 h.

Investigation of potential trans-sulfation activity of aryl sulfotransferase from *D. hafniense*

Two experiments were performed to evaluate the potential trans-sulfation activity of the aryl sulfotransferase from *D. hafniense* owing to its regiospecificity in quercetin sulfation. First, the quercetin sulfates **1b** and **1a** (75:25; 1 mg, 2.5 μmol in 100 mM Tris–glycine buffer (130 μL) at pH 8.9) were incubated with and without the enzyme (180 U ml^{-1} of the reaction mixture). The reaction was stopped at $t=0$, 1, 4, and 24 h and the potential reaction progress was monitored by HPLC method B. Second, a sample of quercetin sulfates **1b** and **1a** (75:25; 3.6 mg, 9 μmol in 100 mM Tris–glycine buffer (600 μL) at pH 8.9) was incubated with an equimolar amount of pyrocatechol (**6**; 1 mg, 9 μmol) and with the enzyme (180 U ml^{-1} of the reaction mixture). The reaction was stopped at $t=0$, 1, 4, 24, and 166 h and the potential reaction progress was monitored by HPLC method C.

Preparation of 5,7,3′-tri-*O*-methyl-4′-*O*-sulfo-3-*O*-β-D-glucopyranosyl quercetin (5 a)

To enable exact structure determination by NMR spectroscopy, compound **2a** was methylated at all free phenolic groups. Isoquercitrin 4′-*O*-sulfate (**2a**, 9 mg) was dissolved in dry methanol and cooled to $T\approx 5\,^{\circ}C$, then a fresh solution of diazomethane (0.7 mL in diethyl ether) was added. The reaction mixture was kept at $T=5\,^{\circ}C$ for 20 min and then evaporated in vacuo with a bath temperature not exceeding $T=35\,^{\circ}C$. 5,7,3′-Tri-*O*-methyl-4′-*O*-sulfo-3-*O*-β-D-glucopyranosyl quercetin (**5a**) was obtained as a yellowish solid (8 mg, 77%). The structure was characterized by MS (ESI$^-$; found: *m/z* 585.82 (100%) [(*M*−H)]$^-$) and by NMR spectroscopy (for ^{1}H and ^{13}C NMR data, see Table S6 and Figure S5 in the Supporting Information). The complete assignment of all NMR signals was accomplished by the use of a combination of gradient COSY, gradient HSQC, and gradient HMBC experiments.

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Keywords: biotransformations · enzyme catalysis · flavonoids · sulfur · transferases

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Sulfation modulates the cell uptake, antiradical activity and biological effects of flavonoids in vitro: An examination of quercetin, isoquercitrin and taxifolin

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ABSTRACT

Quercetin 3′-*O*-sulfate is one of the main metabolites of the natural flavonoid quercetin in humans. This study was designed to prepare quercetin 3′-*O*-sulfate (**1**), isoquercitrin 4′-*O*-sulfate (**2**) and taxifolin 4′-*O*-sulfate (**3**) by the sulfation of quercetin, isoquercitrin (quercetin 3-*O*-glucoside) and taxifolin (2,3-dihydroquercetin) using the arylsulfate sulfotransferase from *Desulfitobacterium hafniense*, and to examine the effect of sulfation on selected biological properties of the flavonoids tested. We found that flavonoid sulfates **1**–**3** were weaker DPPH radical scavengers than the corresponding nonsulfated flavonoids, and that **1**–**3**, unlike quercetin, did not induce the expression of either heme oxygenase-1 in RAW264.7 cells or cytochrome P450 1A1 in HepG2 cells. In both cell types, the cell uptake of compounds **1**–**3** was much lower than that of quercetin, but comparable to that of the glycoside isoquercitrin. Moreover, HPLC/MS metabolic profiling in HepG2 cells showed that flavonoid sulfates **1**–**3** were metabolized to a limited extent compared to the nonsulfated compounds. We conclude that sulfation of the tested flavonoids reduces their antiradical activity, and affects their cell uptake and biological activity in vitro.

1. Introduction

Natural flavonoids are common components of human diet and despite rather low doses, they are considered to be beneficial for human health.[1] In general, the biological activity of individual flavonoids may be affected by their metabolic transformation, which mainly occurs in the intestine and liver. As dietary flavonoids are mostly glycosylated, the biotransformation processes involve deglycosylation and subsequent conjugation of the released aglycones to glucuronides, sulfates and methyl derivatives. Although the metabolites may undergo deconjugation in vivo, human tissues are primarily exposed to a mixture of flavonoid conjugates, and hence the conjugates should be included in in vitro studies carried out to assess the molecular effects of flavonoids.[2]

The flavonol quercetin (3,5,7,3′,4′-pentahydroxyflavone; Fig. 1) is mainly ingested in the form of various 3-*O*-glycosides such as isoquercitrin (quercetin 3-*O*-β-D-glucopyranoside; Fig. 1) and rutin (quercetin 3-*O*-rutinoside),[3] but the major forms of quercetin found in human plasma have been identified to be quercetin 3-*O*-glucuronide, 3′-*O*-methylquercetin 3-*O*-glucuronide and quercetin 3′-*O*-sulfate.[4] Unconjugated quercetin has been the focus of a large number of biological studies. It has been primarily recognized to have antioxidant and anti-inflammatory effects.[5] At the molecular level, quercetin has been shown, for instance, to induce the expression of genes encoding the xenobiotic-metabolizing enzyme cytochrome P450 1A1 (CYP1A1)[6] and the cytoprotective enzyme heme oxygenase-1 (Hmox1/HO-1).[7] In contrast, much less is known about the bioactivities of quercetin conjugates, with the sulfates being the least extensively investigated.[8] Nonetheless, various quercetin sulfates containing one or more sulfate groups have been

Abbreviations: AhR, aryl hydrocarbon receptor; AST, arylsulfate sulfotransferase; CYP1A1, cytochrome P450 1A1; DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; GAPDH/Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Hmox1, heme oxygenase-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Nrf2, nuclear factor erythroid 2-related factor 2; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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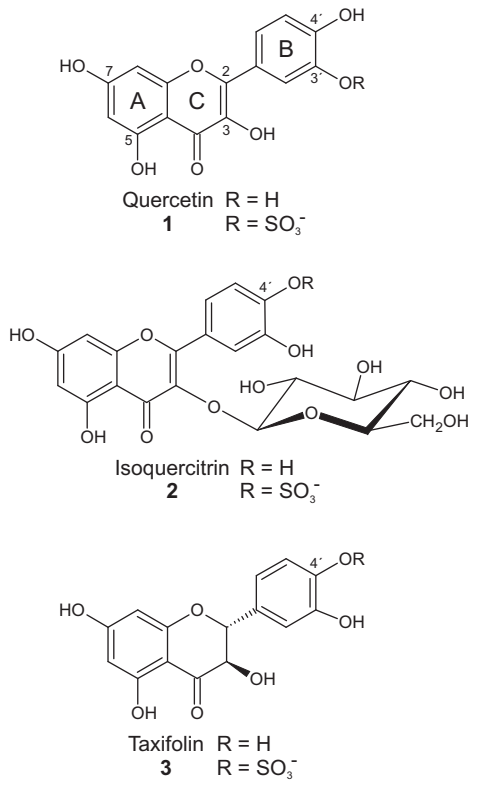


Figure 1. Structures of tested flavonoids and their sulfates (**1**–**3**).

prepared chemically or enzymatically to date[9] and existing research shows that the biological effects of quercetin may increase, decrease or remain unchanged after the sulfation.[8,9] For instance, quercetin 7-*O*-sulfate binds to bovine serum albumin with similar affinity to quercetin, whereas sulfation at the 3-OH and double sulfation at 4′-OH/7-OH decreases its affinity to albumin.[10] Since the consequences of conjugation cannot be easily predicted, the biological activity of conjugated flavonoids has to be investigated on a case-by-case basis. The aim of our study was to prepare sulfates of quercetin, isoquercitrin and taxifolin (2,3-dihydroquercetin; Fig. 1) using the arylsulfate sulfotransferase from *Desulfitobacterium hafniense*, and to examine (a) the radical scavenging effect, (b) the effect on the expression of CYP1A1 and Hmox1 in appropriate cell models, and (c) the metabolic fate of prepared flavonoid sulfates under the given experimental conditions.

2. Results and discussion

2.1. Sulfation of flavonoids by arylsulfate sulfotransferase (AST) from *D. hafniense*

Quercetin sulfates may be prepared chemically using various sulfating reagents such as sulfamic acid, chlorosulfonic acid, tetrabutylammonium hydrogen sulfate and complexes of sulfur trioxide with tertiary amines or amides.[9,11–14] In addition, enzymatic synthesis of quercetin 3,3′-di-*O*-sulfate and 3,7,3′-tri-*O*-sulfate has been reported using AST isolated from the human intestinal bacterium *Eubacterium* A-44.[15] All these studies succeeded in preparing analytical amounts (LC/MS) of the respective sulfates for the identification of authentic mammalian quercetin metabolites.[13] In some studies, the structure determination and/or NMR signal assignment of respective regioisomers[12] seem to be questionable.

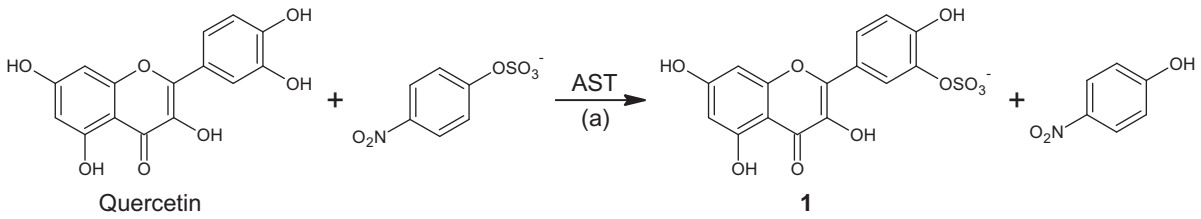
In this study, we aimed to prepare quercetin 3′-*O*-sulfate (a quercetin metabolite in mammals) and sulfates of analogous flavonoids in amounts enabling biological testing. We used the AST from *D. hafniense* for the sulfation of three natural flavonoids including quercetin (flavonol), isoquercitrin (flavonol glycoside) and taxifolin (flavanonol). The AST from *D. hafniense* catalyzes the transfer of a sulfate group from *p*-nitrophenyl sulfate to various phenolic compounds, including flavonoids as well as non-phenolic alcohols.[16–18] The flavonoids used in our study were sulfated by AST, specifically at the B ring. The sulfation of quercetin preferentially yielded quercetin 3′-*O*-sulfate (**1**) (Scheme 1); isoquercitrin and taxifolin were sulfated to isoquercitrin 4′-*O*-sulfate (**2**) and taxifolin 4′-*O*-sulfate (**3**), respectively (Fig. 1).

The product of quercetin sulfation always contained some amounts of the inseparable positional isomer, quercetin 4′-*O*-sulfate, due to incomplete regioselectivity of the enzyme. Both quercetin sulfates were identified and fully structurally characterized by a detailed NMR study (Supplementary Tables S1 and S2). The major product of quercetin sulfation was identified as 3′-*O*-sulfate (**1**) mainly by comparison of ^{13}C NMR data of the catechol moiety with the data reported by Duenas et al.[12] Similarly, the structure of quercetin 4′-*O*-sulfate was assigned using the data recently published by van der Horst et al.[17] These structure assignments were also supported by HMBC correlations in the catechol moiety. In addition, proton chemical shifts of both quercetin 3′-*O*-sulfate (**1**) and 4′-*O*-sulfate were in accordance with the proton spectral data reported in the literature.[13,14] Although ^{1}H NMR spectra of individual flavonoids were not directly comparable, the common diagnostic features for the 3′- and 4′-*O*-sulfates were observed. Regioisomers can be differentiated using down-field shifted protons H-2′ and H-6′ in 3′-*O*-sulfates and down-field shifted proton H-5′ in 4′-*O*-sulfates. The positions of sulfate groups in compounds **2** and **3** were also identified by careful comparison of their NMR data with those of quercetin and by detailed evaluation of HMBC interactions.

To our knowledge, the preparatory separation of **1** and quercetin 4′-*O*-sulfate has not been achieved so far—some authors succeeded in the separation of small amounts for spectral investigation[12,13] but none of these studies reported the yields. This (see Supplementary Fig. S1) and other studies[12,13] demonstrate the problematic separation of the two isomers even when using sophisticated gradient elution.

2.2. Antiradical activity of flavonoid sulfates

The study compared the antiradical effect of sulfated and non-sulfated flavonoids using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. This assay is based on evaluating the reducing ability of compounds toward the stable DPPH radicals.[19] The antiradical activity of nonsulfated flavonoids decreased in the order of quercetin > isoquercitrin > taxifolin with the EC_{50} values of 3.8, 4.1 and 6.1 μM, respectively (Table 1). These findings are consistent with our previous results showing that aglycones have a slightly higher antiradical/antioxidant activity than flavonoid glycosides, that is, quercetin versus isoquercitrin.[20] The decrease in the antiradical activity of taxifolin compared to that of both flavonols is attributed to the absence of the C2–C3 double bond, as proposed at the theoretical level[21] and confirmed experimentally.[20,22] We found that sulfation at the B ring significantly diminished the DPPH scavenging effect of all three flavonoids. Compound **1** retained some antiradical activity, with an EC_{50} value of 23.5 μM. In contrast, the antiradical effect of **2** and **3** was only mild or negligible. For instance, at the highest concentration tested (50 μM) the DPPH scavenging effect of isoquercitrin and taxifolin exceeded 80%, but their 4′-*O*-sulfates **2** and **3** only reduced 10% and 2% of the DPPH radicals, respectively (Table 1). This attenuation of the DPPH radical scavenging effect by sulfation is consistent with other



Scheme 1. Sulfation of quercetin by arylsulfate sulfotransferase (AST) from *D. hafniense*. Reagents and conditions: (a) AST from *D. hafniense*, pH 8.9, 30 °C, 4 h.

Table 1
DPPH radical scavenging activity of flavonoids and their sulfates (**1**–**3**)

Compound	EC_{50} (μM)	DPPH scavenging activity (%)	
		10 μM[a]	50 μM[a]
Quercetin	3.8 ± 0.1	83.4 ± 3.2	86.6 ± 2.4
1	23.5 ± 0.5***	28.7 ± 1.8***	86.4 ± 3.6
Isoquercitrin	4.1 ± 0.1	82.6 ± 3.3	83.7 ± 2.7
2	>50	1.5 ± 0.3***	9.6 ± 1.7***
Taxifolin	6.1 ± 0.2	69.2 ± 2.1	84.6 ± 2.0
3	>50	1.2 ± 0.2***	2.0 ± 0.6***

Data are means ± SD of three experiments.
[a] Concentrations of tested compounds in the reaction mixture.
*** p <0.001, significantly different from the value obtained with a corresponding nonsulfated flavonoid.

Table 2
Effect of flavonoid sulfates (**1**–**3**) on the viability, CYP1A1 mRNA level and CYP1A1 activity in HepG2 cells

Compound	Concentration	Viability (% of control)	CYP1A1 mRNA (fold of control)	CYP1A1 activity (fold of control)
DMSO	0.1%	100	1	1
TCDD	5 nM	n.d.	694.7 ± 17.4**	30.1 ± 7.3*
Quercetin	50 μM	94.3 ± 10.5	168.6 ± 45.9*	1.4 ± 0.5
1	10 μM	90.6 ± 6.9	1.0 ± 0.1	1.0 ± 0.2
1	50 μM	91.9 ± 6.9	1.1 ± 0.0	1.2 ± 0.3
2	10 μM	94.1 ± 7.7	0.8 ± 0.3	1.0 ± 0.2
2	50 μM	94.2 ± 7.6	0.9 ± 0.2	1.1 ± 0.1
3	10 μM	91.7 ± 7.5	0.9 ± 0.2	1.0 ± 0.2
3	50 μM	96.3 ± 2.9	1.0 ± 0.2	1.0 ± 0.2

HepG2 cells were treated for 24 h with 0.1% DMSO (control), 5 nM TCDD (positive control), 50 μM quercetin or with the tested flavonoid sulfates (**1**–**3**). The cell viability was determined by the MTT assay, the levels of CYP1A1 mRNA were determined by quantitative real-time PCR with results normalized to GAPDH mRNA, and the enzymatic activity of CYP1A1 was evaluated as 7-ethoxyresorufin *O*-deethylase activity in intact cells. Data are means ± SD of three experiments. n.d., not determined.
* p <0.05, significantly different from control.
** p <0.01, significantly different from control.

Table 3
Effect of flavonoid sulfates (**1**–**3**) on the viability and Hmox1 mRNA level in RAW264.7 cells

Compound	Concentration	Viability (% of control)	Hmox1 mRNA (fold of control)
DMSO	0.1%	100	1
Quercetin	50 μM	87.1 ± 2.8*	6.8 ± 1.5*
1	10 μM	83.3 ± 7.4	0.9 ± 0.1
1	50 μM	81.8 ± 6.4*	1.0 ± 0.0
2	10 μM	78.3 ± 5.6*	0.9 ± 0.1
2	50 μM	87.3 ± 1.9**	0.9 ± 0.1
3	10 μM	88.3 ± 4.2*	0.9 ± 0.1
3	50 μM	88.7 ± 3.1*	1.0 ± 0.0

RAW264.7 cells were treated for 6 h with 0.1% DMSO (control), 50 μM quercetin (positive control) or with the tested flavonoid sulfates (**1**–**3**). The cell viability was determined by the MTT assay and the levels of Hmox1 mRNA were determined by quantitative real-time PCR with results normalized to Gapdh mRNA. Data are means ± SD of three experiments.
* p <0.05, significantly different from control.
** p <0.01, significantly different from control.

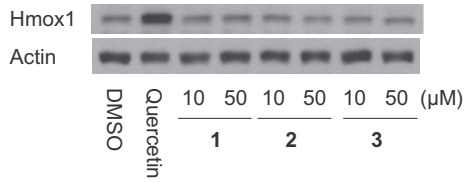


Figure 2. Effect of flavonoid sulfates **1**–**3** on heme oxygenase-1 (Hmox1) protein levels in RAW264.7 cells. Cells were treated for 6 h with 0.1% DMSO (control), 50 μM quercetin or with 10 and 50 μM **1**–**3**. After treatment, the protein levels of Hmox1 and actin in the whole cell lysates (20 μg/lane) were analyzed by Western blotting.

reports[9] and confirms the importance of the *o*-dihydroxy (catechol) group for the antiradical activity of flavonoids.[23]

2.3. Flavonoid sulfates do not induce the expression of CYP1A1 in HepG2 cells

The biological part of the study was designed to examine whether the prepared flavonoid sulfates could mimic the in vitro bioactivity of quercetin. Compounds **1**–**3** were tested at concentrations of 10 and 50 μM. The lower concentration was selected as one that was physiologically relevant[2] and the higher one was used because at that concentration quercetin exerts a number of biological effects in vitro.[24,25] Using human hepatoma HepG2 cells, we examined the possible modulation of CYP1A1 expression. CYP1A1 is an inducible enzyme, which is for instance responsible for the metabolic activation of various procarcinogens including polycyclic aromatic hydrocarbons. The expression of CYP1A1 is regulated by the aryl hydrocarbon receptor (AhR).[26] Quercetin, in contrast to isoquercitrin and taxifolin, has been shown to activate the AhR and to induce *CYP1A1* gene expression in several cell models including HepG2 cells.[25,27] In this study, HepG2 cells were fully responsive to 5 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent AhR activator,[26] as documented by increased mRNA and activity levels of CYP1A1 (Table 2). Consistent with our previous results, 50 μM quercetin after 24 h of exposure significantly elevated the CYP1A1 mRNA level to 169-fold compared to the control. The activity of CYP1A1 was analyzed in intact cells after 6, 12 and 24 h of exposure, but a small (non-significant) increase in its activity with 50 μM quercetin was found after 24 h (Table 2). Flavonoid sulfates **1**–**3** at concentrations of 10 and 50 μM were found to be non-cytotoxic in HepG2 cells, with cell viability higher than 90% in all treatments after 24 h of incubation (Table 2). Compounds **1**–**3** also appeared to have a mild or negligible effect on both mRNA and the activity levels of CYP1A1 in HepG2 cells. After 24 h of incubation, CYP1A1 mRNA levels induced by **1**–**3** reached

Table 4
Qualitative LC/MS parameters and relative amounts of flavonoid sulfates **1**–**3** and their metabolites in HepG2 cells and culture medium

Metabolite	*m/z*	t_R	Cells		Medium	
			6 h	24 h	6 h	24 h
*Quercetin 3′-O-sulfate (**1**)*						
Parent (compound **1**)	380.991	9.8	67.2	86.0	79.2	81.8
Parent (quercetin 4′-*O*-sulfate)	380.991	9.6	16.3	14.0	19.5	15.0
Loss of sulfate	301.035	12.0	16.5	n.d.	0.5	0.4
Methylation	395.007	10.6	n.d.	n.d.	0.3	0.8
Methylation	395.007	10.8	n.d.	n.d.	0.5	2.0
*Isoquercitrin 4′-O-sulfate (**2**)*						
Parent (compound **2**)	543.044	6.4	100.0	100.0	98.6	94.8
Loss of sulfate	463.087	8.5	n.d.	n.d.	1.4	5.2
*Taxifolin 4′-O-sulfate (**3**)*						
Parent (compound **3**)	383.009	5.3	100.0	100.0	78.8	77.6
Taxifolin sulfate	383.009	5.6	n.d.	n.d.	16.0	14.7
Taxifolin sulfate	383.009	6.0	n.d.	n.d.	4.3	5.0
Loss of sulfate	303.051	6.8	n.d.	n.d.	0.2	0.4
Desaturation (quercetin 4′-*O*-sulfate)	380.991	9.6	n.d.	n.d.	0.7	2.3

HepG2 cells were treated with 50 μM flavonoid sulfates **1**–**3**. Cells and culture medium were analyzed after 6 or 24 h of incubation by HPLC/MS. Σ (parent compound + predominant metabolites) = 100%. Data are means of three experiments. n.d., not detected.

0.8-fold to 1.1-fold, and the activity levels of CYP1A1 varied between 1.0-fold and 1.2-fold compared to the control (Table 2).

2.4. Flavonoid sulfates do not induce the expression of Hmox1 in RAW264.7 cells

The study also investigated the effect of flavonoid sulfates on the expression of Hmox1 in murine macrophage RAW264.7 cells. Hmox1 is a cytoprotective enzyme producing the antioxidant biliverdin and the anti-inflammatory agent carbon monoxide. The expression of the *Hmox1* gene is regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2), and is inducible under conditions of oxidative or electrophilic stress.[28,29] Flavonoid sulfates **1**–**3** exhibited a weak cytotoxicity in RAW264.7 cells, nonetheless the cytotoxic effect was comparable to that of quercetin. After 6 h of exposure, the viability of RAW264.7 cells was between 78% and 89% in response to 10 and 50 μM **1**–**3**, while 50 μM quercetin reduced the viability to 87% (Table 3). Quercetin, unlike isoquercitrin and taxifolin,[24] induces the expression of Hmox1 via activation of the extracellular signal-regulated kinases, which in turn activate the transcription factor Nrf2.[7,30] Our results confirmed the ability of quercetin to stimulate Hmox1 expression in RAW264.7 cells. After 6 h of exposure, 50 μM quercetin increased the level of Hmox1 mRNA to 6.8-fold compared to the control (Table 3) and this effect was also accompanied by upregulation of the Hmox1 protein (Fig. 2). In contrast, no increase in the expression of Hmox1 at both the mRNA and protein levels was found in cells treated for 6 h with 10 and 50 μM **1**–**3** (Table 3, Fig. 2).

2.5. Stability and cell uptake of flavonoid sulfates

The interpretation of biological results requires knowing the biotransformation patterns of the tested compounds under the given experimental conditions. The study therefore examined the stability, cell uptake and biotransformation of sulfated flavonoids using an HPLC/MS method with chromatographic separation on a phenyl-based stationary phase, which enables simultaneous detection of conjugated and unconjugated flavonoids.[31,32] Using MS with negative electrospray ionization, quercetin (*m/z* 301.034 $[M-H]^-$), isoquercitrin (*m/z* 463.088) and taxifolin (*m/z* 303.051)

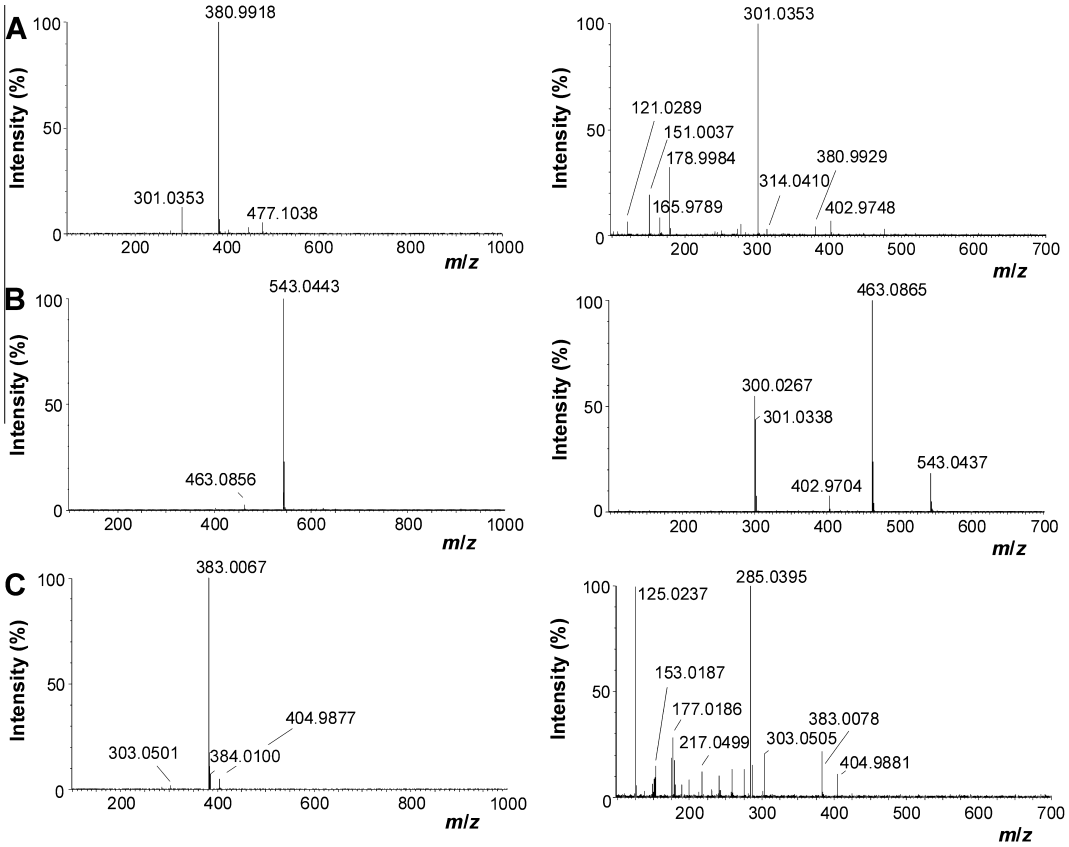


Figure 3. Full MS spectra (left panels) and corresponding fragmentation spectra (right panels) of (A) compound **1**, (B) compound **2**, and (C) compound **3**.

gave chromatographic peaks at 12.0, 8.5 and 6.8 min, respectively. Flavonoid sulfates **1**–**3** were eluted at lower retention times. The compounds **1** (*m*/*z* 380.991), **2** (*m*/*z* 543.044) and **3** (*m*/*z* 383.009) gave peaks at 9.8, 6.4 and 5.3 min, respectively (Table 4). The full MS spectra and corresponding fragmentation spectra of flavonoid sulfates **1**–**3** are shown in Fig. 3.

To assess the stability of flavonoid sulfates, the compounds were dissolved in DMSO, diluted with the complete culture medium to a concentration of 50 μM and incubated for up to 24 h at 37 °C. We found that quercetin sulfate **1** was less stable than compounds **2** and **3**. The initial concentration of **1** decreased to 82% and 59% after 6 and 24 h of incubation, respectively. In contrast, the concentrations of **2** and **3** found after 24 h of incubation were 99% and 90% of the initial value, respectively (data not shown).

The cell uptake of the tested compounds was evaluated by determining the total amount of parent flavonoids extracted from cells. For this purpose, HepG2 cells were incubated with 50 μM of individual flavonoids and analyzed after 6 and 24 h of incubation. The highest cell uptake was found for quercetin. The yield of quercetin was 1.14 nmol per 10^6 cells after 6 h of incubation, but it decreased to 4 pmol per 10^6 cells after 24 h due to metabolic transformation. The sulfation of quercetin produces a negative charge in the molecule and increases its polarity and water solubility.[9] Consistently, quercetin sulfate **1** was absorbed much less than quercetin, with the yield of **1** being 38 and 33 pmol per 10^6 cells after 6 and 24 h, respectively. Isoquercitrin, whose molecule contains a polar sugar moiety, was only found in HepG2 cells after 24 h of incubation (31 pmol per 10^6 cells) and taxifolin, unlike its metabolites, was not detected. Compounds **2** and **3** were found in HepG2 cells after both 6 and 24 h. The intracellular levels of **2** and **3** were higher after 6 h of exposure and reached 19 and 62 pmol per 10^6 cells, respectively. The extracts of RAW264.7 cells were analyzed after 3 and 6 h of incubation and the results exhibited similar trends to those observed in HepG2 cells. For instance, the yield of quercetin after 3 h was 1.15 nmol per 10^6 cells, while the yields of isoquercitrin, **1**, **2** and **3** after 6 h reached only 16, 29, 8 and 7 pmol per 10^6 cells (data not shown). The chromatographic records of flavonoid sulfates **1**–**3** extracted from HepG2 and RAW264.7 cells are shown in Fig. 4. Our results showed that the cell uptake of the tested flavonoids corresponded with their lipophilicity. Not surprisingly, the highest cell uptake was found for quercetin whose amphipathic molecule, consisting of hydrophobic aromatic rings and hydrophilic hydroxyl groups, may readily penetrate the cell membrane.[33] Our results also confirmed that both the hydrophilic sugar moiety[34,35] and the charged sulfate group make the passage through the cell membrane difficult.[33]

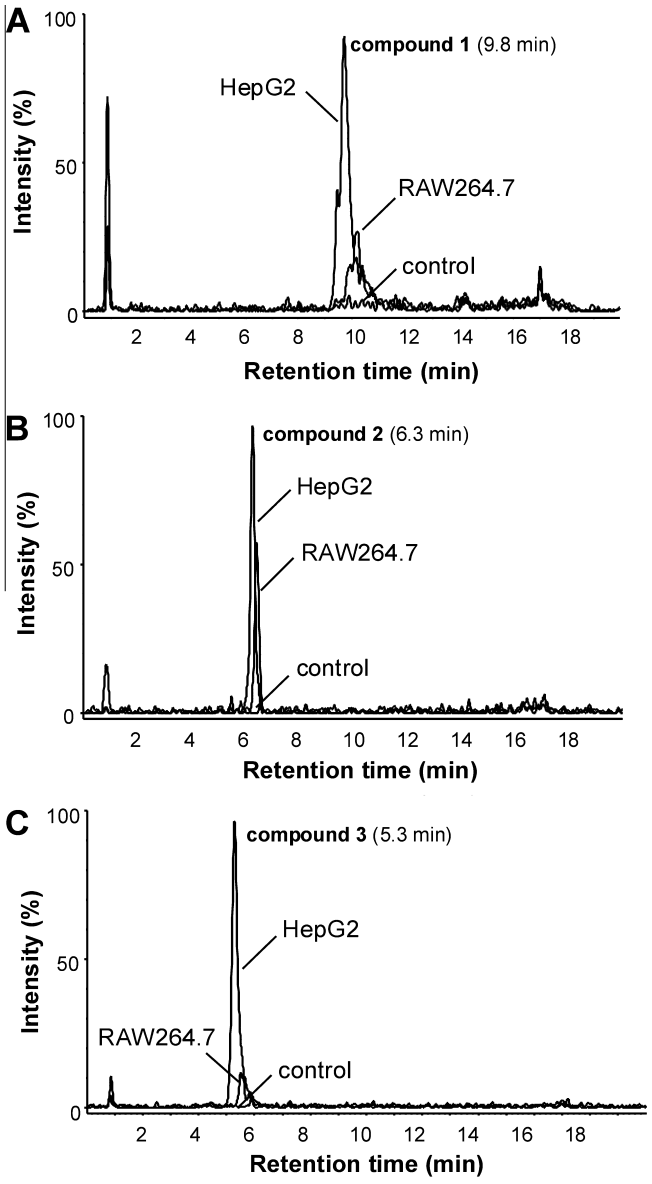


Figure 4. HPLC/MS chromatograms of flavonoid sulfates **1**–**3**. HepG2 cells or RAW264.7 cells (as indicated) were treated for 6 h with 0.1% DMSO (control) or with 50 μM compound **1** (A), compound **2** (B) or compound **3** (C), and the cell extracts were analyzed by HPLC/MS. The *m*/*z* values of 381, 543 and 383 were monitored using selected-ion monitoring mode.

2.6. Biotransformation of flavonoid sulfates

Finally we examined the biotransformation of flavonoid sulfates **1**–**3** in HepG2 and RAW264.7 cells. HepG2 cells are metabolically less competent than human hepatocytes, nonetheless these cells retain the activity of phase II conjugation enzymes.[25,36] As shown in Supplementary Table S5, analysis of the biotransformation products of quercetin, isoquercitrin and taxifolin confirmed the ability of HepG2 cells to produce methylated, sulfated and glucuronidated conjugates. One of the quercetin metabolites was identified as quercetin 3′-*O*-sulfate, that is, compound **1**. Taxifolin, unlike isoquercitrin, was also sulfated in HepG2 cells. Two monosulfates of taxifolin were produced, but neither of the metabolites were sulfated at the 4′-OH group (Supplementary Table S5). After the treatment of HepG2 cells with 50 μM **1**, we found that the cells contained the parent compound **1**, the parent positional isomer quercetin 4′-*O*-sulfate and unconjugated quercetin, a product of the loss of the sulfate group. In addition, two methyl derivatives of **1** were found in the culture medium (Table 4). After 6 h of incubation, 16.5% of the total amount of intracellular quercetin was desulfated (Table 4), nonetheless the biological results indicate that the intracellular concentrations of the unconjugated quercetin do not reach the values required for efficient biological response, that is, CYP1A1 induction. When HepG2 cells were incubated with 50 μM **2** or **3**, the parent compounds but no metabolites were detected in the cells (Table 4). However, analyses of the culture medium showed that both **2** and **3** were at least partially desulfated and compound **3** was also oxidized to quercetin 4′-*O*-sulfate. Moreover, taxifolin produced by the desulfation of **3** was presumably resulfated at OH groups other than 4′-OH, since two taxifolin monosulfates were found in the medium (Table 4). As expected, RAW264.7 cells were found to exhibit negligible metabolic activity compared to HepG2 cells. After the treatment of RAW264.7 cells for 3 or 6 h with 50 μM of flavonoid sulfates **1**–**3**, no metabolites were found in either cells or the medium (data not shown).

3. Conclusion

In summary, using the arylsulfate sulfotransferase from *D. hafniense*, we have prepared monosulfates of quercetin, isoquercitrin and taxifolin bearing the sulfate group on the B ring. We have shown that such sulfation reduces the DPPH radical scavenging activity and that the prepared flavonoid sulfates, in contrast to quercetin, do not induce the expression of either Hmox1 in RAW264.7 cells or CYP1A1 in HepG2 cells. HPLC/MS analyses have also demonstrated that the cell uptake of flavonoid sulfates is much lower than that of quercetin but comparable to that of the quercetin glycoside isoquercitrin. We have also found that flavonoid sulfates are metabolized in HepG2 cells to fewer biotransformation products than the corresponding nonsulfated flavonoids.

We may suppose that the weaker in vitro bioactivity of sulfated flavonoids is associated with (i) lower cell uptake or (ii) lower affinity of the compounds to target biomolecules. For instance, it is clear that the affinity of quercetin sulfates to AhR, which controls the expression of CYP1A1,[26] is lower than that of quercetin since classic AhR ligands are hydrophobic and planar or coplanar molecules.[26] In humans, sulfation is known to play a major role in detoxification and/or inactivation of both endogenous and exogenous compounds such as steroid hormones and drugs.[9,37] Accordingly, we conclude that the sulfation of flavonols and flavanonols on the B ring may substantially modulate their cell permeability and in vitro biological activity.

4. Experimental

4.1. General

NMR spectra were recorded in a Bruker Avance III 600 MHz spectrometer (600.23 MHz for ^{1}H, 150.94 MHz for ^{13}C) at 30 °C in DMSO-d_6 and at 25 °C in CD_3OD (Sigma–Aldrich, Steinheim, Germany). Residual signals of the solvents (DMSO: δ_H 2.500 ppm, δ_C 39.60 ppm; CD_3OD: δ_H 3.330 ppm, δ_C 49.30 ppm) were used as internal standards. NMR experiments included ^{1}H NMR, ^{13}C NMR, COSY, gHSQC, and gHMBC. ^{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) was applied to obtain a better ^{13}C signal-to-noise ratio. Chemical shifts are given on the δ-scale, with digital resolution justifying the reported values to three (δ_H) or two (δ_C) decimal places.

Mass spectra in negative ion mode were measured using a LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source. The samples were dissolved in methanol and introduced into the mobile phase flow (methanol/H_2O, 4:1; 100 μL/min) using a 2-μL loop. Spray voltage, capillary voltage, tube lens voltage and capillary temperature were 4.0 kV, −16 V, −120 V and 275 °C, respectively.

HPLC analyses, used to monitor the reaction progress, were performed using a Prominence system (Shimadzu, Kyoto, Japan) consisting of a DGU-20A mobile phase degasser, two LC-20AD solvent delivery units, a SIL-20AC cooling autosampler, a CTO-10AS column oven and SPD-M20A diode array detector. Chromatographic data were collected and processed using Shimadzu Solution software (Shimadzu) at a rate of 40 Hz and detector time constant of 0.025 seconds. The separation was performed in a Chromolith Performance RP-18e monolithic column (100 × 3 mm i.d.; Merck, Darmstadt, Germany) coupled with a guard column (5 × 4.6 mm; Merck). Mobile phase A, acetonitrile/H_2O/HCO_2H (80:20:0.1), and mobile phase B, acetonitrile/H_2O/HCO_2H (5:95:0.1) were employed in the analyses; gradient: 0–4 min 7–40% A; 4–6 min 40% A, 6–7 min 40–7% A, 7–8 min 7% A. The flow rate was 1.5 mL/min at 25 °C. The PDA data were acquired in the 200–450 nm range, the 285 nm and 360 nm signals were extracted.

Optical rotation was measured in methanol at 22 °C with a Rudolph Autopol polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA) and electronic circular dichroism (ECD) spectrum was recorded on a Jasco-815 spectrometer (Jasco, Easton, MD, USA) using a 2 mm quartz cell in methanol at 200–400 nm with scanning speed of 20 nm/min and time response of 8 s. IR spectra were measured using a FT-IR Nicolet 205 spectrometer (Nicolet, Madison, WI, USA) in KBr tablet.

4.2. Preparation of arylsulfate sulfotransferase (AST) from *Desulfitobacterium hafniense*

The expression, purification and characterization of AST from *D. hafniense* were performed as described by van der Horst et al.[18] The enzymatic activity of AST was measured spectrophotometrically at 400 nm and at 30 °C as the release of *p*-nitrophenol (ε_{400} = 0.2488 cm^2/μmol). One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of *p*-nitrophenol per min. The activities were corrected for the non-enzymatic release of *p*-nitrophenol.

4.3. Sulfation of quercetin by AST from *D. hafniense*

Quercetin (103 mg, 0.323 mmol; Sigma) was dissolved in 2 mL of acetone. Tris–glycine buffer (20 mL, 100 mM, pH 8.9), *p*-nitrophenyl sulfate (130 mg, 0.5 mmol; Sigma) and AST from *D. hafniense* (2 mL, 2170 U/mL; prepared as described in Section 4.2) were added to the substrate solution and the mixture was incubated under nitrogen at 30 °C. The reaction progress was monitored by HPLC as mentioned above or by TLC, with the conversion being ca. 60–90% after 4 h. TLC was performed on Silica Gel 60 F254 plates (Merck) using a mobile phase of ethyl acetate/methanol/HCO_2H (4:1:0.01); the spots were visualized by UV light (254 nm) and/or by spraying with 5% H_2SO_4 in ethanol and charring. The reaction mixture was evaporated (max. 40 °C) in vacuo to half its volume to remove all organic solvents. The pH was adjusted by HCl to 7.5–7.7, and *p*-nitrophenol and residual starting material were removed by extraction with ethyl acetate (3 × 50 mL). The aqueous phase (15 mL) containing the sulfated product was evaporated (max. 40 °C), the residue was dissolved in 2 mL of 80% methanol and loaded onto a Sephadex LH-20 column (30 g dry weight, 3 cm i.d.) packed and equilibrated with 80% methanol. The fractions were analyzed by TLC as mentioned above, and the fractions containing the respective product were collected and evaporated in vacuo at 45 °C. The enzymatic procedure yielded quercetin 3′-*O*-sulfate (**1**, 90 mg), yield: 47.5% related to sulfate donor; HRMS calcd for $C_{15}H_9O_{10}S$: *m*/*z* 380.99219, found: 380.99126 (Supplementary Fig. S2). The product of quercetin sulfation contained an inseparable positional isomer quercetin 4′-*O*-sulfate, HRMS calcd for $C_{15}H_9O_{10}S$: *m*/*z*: 380.99219, found 380.99126. The contents of 3′-*O*-sulfate (**1**) and 4′-*O*-sulfate in the product were 78% and 22%, respectively (for ^{1}H and ^{13}C NMR data of 3′-*O*-sulfate, see Supplementary Table S1 and those of 4′-*O*-sulfate Supplementary Table S2; for HPLC see Supplementary Fig. S1 and for IR data see Supplementary Fig. S3).

4.4. Sulfation of isoquercitrin by AST from *D. hafniense*

Isoquercitrin (quercetin 3-*O*-β-D-glucopyranoside, 150 mg, 0.323 mmol; prepared as described previously[38]) was sulfated using the same procedure as for quercetin. Isoquercitrin

4′-*O*-sulfate (**2**) was obtained as a yellowish solid (120 mg). Yield: 69%, purity (HPLC) was over 96%, HRMS calcd for $C_{21}H_{19}O_{15}S$: *m*/*z* 543.04501, found 543.04367 (Supplementary Fig. S5, for ^{1}H and ^{13}C NMR data see Supplementary Table S3, for HPLC see Supplementary Fig. S4, for IR data see Supplementary Fig. S6, and for ECD and UV spectra see Supplementary Fig. S7). $[\alpha]_{589}^{22}$ −14.7 (*c* 0.00046 mol/L, methanol).

4.5. Sulfation of taxifolin by AST from *D. hafniense*

Taxifolin (200 mg, 0.66 mmol; Amagro, Prague, Czech Republic) was sulfated using the same procedure as for quercetin. Taxifolin 4′-*O*-sulfate (**3**) was obtained as a yellowish solid (190 mg). Yield: 75%, purity (HPLC) was over 96%, HRMS calcd for $C_{15}H_{11}O_{10}S$: *m*/*z* 383.00784, found 383.00677 (Supplementary Fig. S9, for ^{1}H and ^{13}C NMR data see Supplementary Table S4, for HPLC see Supplementary Fig. S8 and for IR data see Supplementary Fig. S10).

4.6. Reagents for biological testing

Isoquercitrin (98% purity, HPLC) was prepared by the enzymatic procedure as described previously.[38] Taxifolin (96% purity) was obtained from Amagro (Prague, Czech Republic). Quercetin (98% purity), dimethyl sulfoxide (DMSO) and solvents for HPLC were purchased from Sigma-Aldrich. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Ultra Scientific (North Kingstown, RI, USA).

4.7. DPPH radical scavenging assay

The antiradical activity of the tested compounds was assessed using a DPPH (1,1-diphenyl-2-picrylhydrazyl radical) assay.[19] In brief, the tested compounds were dissolved in methanol and incubated in the presence of 34 μM DPPH (Sigma) for 30 min at room temperature. After incubation, the absorbance at 517 nm was measured and used to calculate their DPPH scavenging activity. The EC_{50} values (concentrations required to obtain a 50% scavenging effect) were calculated using the software OriginPro 8 (OriginLab, Northampton, MA, USA).

4.8. Cell cultures and treatments

Human hepatocyte carcinoma HepG2 cell line (No. 85011430, ECACC, Salisbury, UK) and murine macrophage RAW264.7 cell line (No. 91062702, ECACC) were cultured at 37 °C in Dulbecco's modified Eagle's medium (No. D5796, Sigma) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (Biochrom, Berlin, Germany) in a humidified atmosphere containing 5% CO_2. For all experiments, both HepG2 cells and RAW264.7 cells were seeded in the complete culture medium into multiwell plates at a density of 1×10^5 cells/cm^2. After overnight stabilization, HepG2 cells were treated in fresh culture medium with the tested compounds or with 0.1% (*v*/*v*) DMSO (control). Experiments on RAW264.7 cells were performed in serum-free medium. The culture medium was replaced with the serum-free medium 8 h after seeding. Following overnight incubation, RAW264.7 cells were treated in fresh serum-free medium with the tested compounds or with 0.1% (*v*/*v*) DMSO (control).

4.9. Cell viability assay

After the treatment of HepG2 cells and RAW264.7 cells with 0.1% DMSO (control), the tested compounds or 1.5% (*v*/*v*) Triton X-100 (positive control), the cell viability was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. In brief, cells were washed with PBS and incubated for 2 h at 37 °C in fresh serum-free medium containing 0.5 mg/mL MTT (Sigma). After this, the medium was removed and the formazan produced by active mitochondria was dissolved in DMSO. The absorbance at 540 nm was measured on a spectrophotometric plate reader and used to calculate relative cell viability, where cells treated with DMSO alone represented 100% viability.

4.10. Reverse transcription and quantitative real-time PCR

After treatment, total RNA was extracted using TRI Reagent Solution (Applied Biosystems, Foster City, CA, USA). RNA samples (2 μg) were reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and real-time PCR was performed in a LightCycler 480 II system (Roche Diagnostics, Mannheim, Germany) using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays consisting of specific primers and FAM dye-labeled TaqMan minor groove binder probes (Applied Biosystems). The assay ID for human CYP1A1 was Hs00153120_m1; for human GAPDH, Hs99999905_m1; for mouse Hmox1, Mm00516005_m1; and for mouse Gapdh, Mm99999915_g1. Amplification conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Crossing point values, equivalent to C_T, were determined using the second derivative maximum analysis. Relative changes in gene expression were calculated by the comparative C_T method using the equation, $2^{-\Delta\Delta C_T}$ with results normalized to Gapdh mRNA levels.

4.11. Western blot analysis

After treatment, cells were washed with cold phosphate-buffered saline (PBS; pH 7.4), scraped from the plates, pelleted by centrifugation for 3 min at 1500 *g* and 4 °C, and lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors Complete (Roche Diagnostics), 0.2% (*m*/*v*) sodium dodecyl sulfate (SDS), 1% (*v*/*v*) Nonidet-P40, 1% (*v*/*v*) Triton X-100, pH 7.4). After incubation on ice for 30 min, whole cell lysates were centrifuged for 10 min at 16,000 *g* and 4 °C, and the supernatants were collected. Aliquots containing an equal amount of protein were subjected to electrophoresis through 10% SDS-polyacrylamide gel, proteins were transferred to polyvinylidene difluoride membrane by electroblotting, and the membranes were probed with appropriate primary antibodies. Rabbit polyclonal heme oxygenase-1 (H-105) and goat polyclonal actin (I-19) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies were visualized with rabbit anti-goat or goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies using a chemiluminescent reaction.

4.12. CYP1A1 activity assay

The CYP1A1 enzymatic activity in intact HepG2 cells was evaluated as 7-ethoxyresorufin *O*-deethylase activity as described previously.[39] After treatment, cells were washed with PBS and incubated for 30 min at 37 °C in serum-free medium containing 8 μM 7-ethoxyresorufin and 10 μM dicumarol (Sigma) used to inhibit cytosolic diaphorase. After this, the fluorescence of resorufin released into the medium was measured on a spectrophotometric plate reader at excitation and emission wavelengths of 530 and 585 nm, respectively. The fluorescence values were standardized against cellular protein content and used for calculations of fold changes versus controls.

4.13. HPLC/MS analysis of biotransformation products

After treatment, cells were scraped from the plates, collected by gentle centrifugation, washed twice with PBS, resuspended in 0.4 mL of methanol containing 5% (*v*/*v*) acetic acid and sonicated 10 times at 50% amplitude with a cycle set at 0.5 s using a UP200s Ultrasonic Processor equipped with a Sonotrode Microtip S2 sonicator probe (Hielscher, Teltow, Germany). Afterward, the cell lysates were centrifuged for 2 min at 14,000 *g* at room temperature and the supernatants were analyzed by HPLC/MS. Aliquots of culture medium were diluted (1:1, *v*/*v*) in methanol containing 5% (*v*/*v*) acetic acid, centrifuged for 2 min at 14,000 *g* and the supernatants were analyzed by HPLC/MS.

A Waters ACQUITY I-Class UPLC system (Waters, Milford, MA, USA) equipped with binary solvent manager, sample manager, column manager and PDA detector was used and the chromatographic separation was performed in an Agilent Zorbax Eclipse XDB-Phenyl column (100 mm × 2.1 mm i.d., 5 μm; Agilent Technologies, Palo Alto, CA, USA). The injection volume was 5 μL. Solvent A was composed of 0.2% acetic acid in an aqueous 10% methanol solution. Solvent B was composed of methanol containing 0.02 M ammonium acetate. The linear gradient profile was as follows: 0–14 min 10–50% B, 14–16 min 50–100% B, 16–18 min 100–10% B, 18–20 min 10% B. The mobile phase flow rate was 0.4 mL/min, the temperature of the autosampler was 4 °C and the column oven was set to 35 °C.

A Waters Synapt G2-S Mass Spectrometer (Waters, Manchester, UK) was connected to the UPLC system via an electrospray ionization (ESI) interface. The ESI source operated in negative mode with the capillary voltage at 2.1 kV and the sampling cone at 40 V. The source temperature and the desolvation temperature were set at 120 °C and 300 °C, respectively. The cone and desolvation gas flows were 0 L/h and 500 L/h, respectively. Data were acquired from 50 to 1000 Da with a 0.1 s scan time. The mass spectrometer was calibrated across the mass range 50–1000 Da using a solution of sodium formate in acetonitrile. Data were automatically centroided and mass corrected during acquisition using a leucine-enkephalin external reference (20 μg/L in a mixture of water/acetonitrile/formic acid (100:100:0.2), flow rate of 10 μL/min). Data acquisition was achieved using two interleaved scan functions (MS^E experiments), which enabled simultaneous acquisition of both low-collision-energy (CE) and high-collision-energy mass spectra from a single experiment. The low trap CE was set to 5 V and the low transfer CE was set to 2 V for Function 1. For Function 2 (high CE), the trap CE was set to 4 V and the transfer lenses were ramped CE in the range of 15–30 V. Post-acquisition processing of the data was performed using the software Metabolynx V4.1 (Waters).

4.14. Statistical analysis

Results were expressed as means ± standard deviation (SD). The differences in mean values were analyzed by Student *t*-tests. A *p* value of less than 0.05 was considered to be statistically significant.

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Supplementary data

Supplementary data (HPLC chromatograms, MS spectra, and ^{13}C and ^{1}H NMR data of compounds **1**–**3**, ECD and UV spectra of compound **2**, and qualitative LC/MS parameters and relative amounts of quercetin, isoquercitrin, taxifolin and their metabolites in HepG2 cells and culture medium) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.07.055.

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Prokaryotic and eukaryotic aryl sulfotransferases: Sulfation of unexplored flavonolignans from silymarin.

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Abstract: Natural flavonoids and flavonolignans feature beneficial properties for living organisms such as antioxidant and hepatoprotective effects, anticancer, chemoprotective, dermatoprotective and hypocholesterolemic activities. Their metabolism in mammals is complex, the exact structure of their metabolites still remain partly unclear and the standards are usually not commercially available. Hence, this work focused on the preparation of potential and defined biotransformation Phase II sulfated metabolites of unexplored silymarin flavonolignans 2,3-dehydrosilybin, silychristin and silydianin. Pure sulfated derivatives were prepared using aryl sulfotransferase from *Desulfitobacterium hafniense* and aryl sulfotransferase from rat liver. Using heterologously arylsulfotransferase from *D. hafniense* and cheap *p*-nitrophenyl sulfate as sulfate donor, sulfated flavonolignans were obtained almost in quantitative yields. Silymarin flavonolignans yielded exclusively monosulfates at the position C-20 (C-19 in the case of silychristin), except 2,3-dehydrosilybin that gave also the 7,20-*O*-disulfated derivative. On the contrary, recombinant mammalian PAPS-dependent aryl sulfotransferase was less efficient and had a narrower substrate specificity. The sulfated products prepared by both aryl sulfotransferases, were fully characterized by HRMS and NMR methods and will be used as authentic standards for *in vivo* metabolic experiments.

Keywords: silymarin; silychristin; silydianin; flavonoid; sulfotransferase; biotransformation.

1. Introduction

Silymarin is a crude extract from milk thistle (*Silybum marianum*), which has been used for various human ailments since middle ages [1]. Fruits of this notoriously-known herb contain flavonolignans such as silybin (**1**), 2,3-dehydrosilybin (**2**), isosilybin, silychristin (**3**), silydianin (**4**), 2,3-dehydrosilychristin (**5**) and 2,3-dehydrosilydianin (**6**, Figure 2). Most of the above flavonolignans exist as mixtures of two diastereoisomers, e.g., silybin A (**1a**) and B (**1b**) (Figure 1), 2,3-dehydrosilybin A and B, isosilybin A and B, silychristin A and B; only silydianin exists as a single stereomer [2]. Flavonolignans from silymarin feature strong antioxidant and hepatoprotective effects, and also have anticancer, chemoprotective, dermatoprotective and hypocholesterolemic activities [3]. Although silychristin and silydianin were described more than 40 years ago [4], their biological properties were not studied (only as constituents of silymarin complex) probably due to their poor availability. Recently, new preparatory method using Sephadex LH-20 was developed, which provides an access to multigram amounts of pure silychristin A, silydianin, taxifolin and a fraction containing silybin and isosilybin [5].

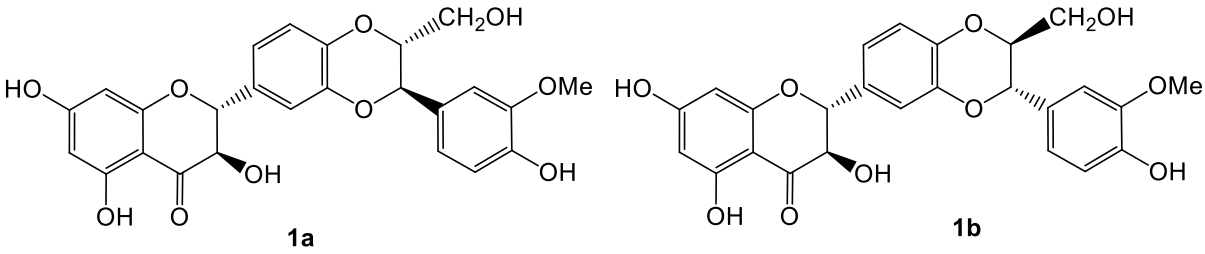


Figure 1: Structures of silybin A (1a) and silybin B (1b).

In last two decades flavonoids became highly popular as food adjuvants, dietary supplements and their daily intake increased; therefore, their pharmacokinetics and metabolism are now extensively studied [6, 7]. Flavonoids are typically metabolized by Phase II biotransformation enzymes, they are sulfated, glucuronidated or methylated by sulfotransferases (SULT), UDP-glucuronyl transferases (UTGs), *N*-acetyltransferases, glutathione *S*-transferases, thiopurin *S*-methyltransferases and *O*-methyltransferases [6]. Identification of respective metabolites strongly depends on analytical

procedure. Often only HPLC-MS or MS analysis were applied for characterization of conjugated products [8]. To date, no detailed structural identification of silychristin and silydianin metabolites (conjugates) was performed.

We report here the first fully characterized sulfated metabolites of unexplored silymarin flavonolignans silychristin, silydianin and 2,3-dehydrosilybin, which were prepared at the semipreparatory scale by bacterial aryl sulfotransferase from *Desulfitobacterium hafniense* (AST DH). We have compared these sulfated products with products obtained from aryl sulfotransferase IV (AST IV) from rat liver. Sulfated molecules prepared in this way may be considered as the Phase II metabolites, when the respective mammalian liver enzyme is used.

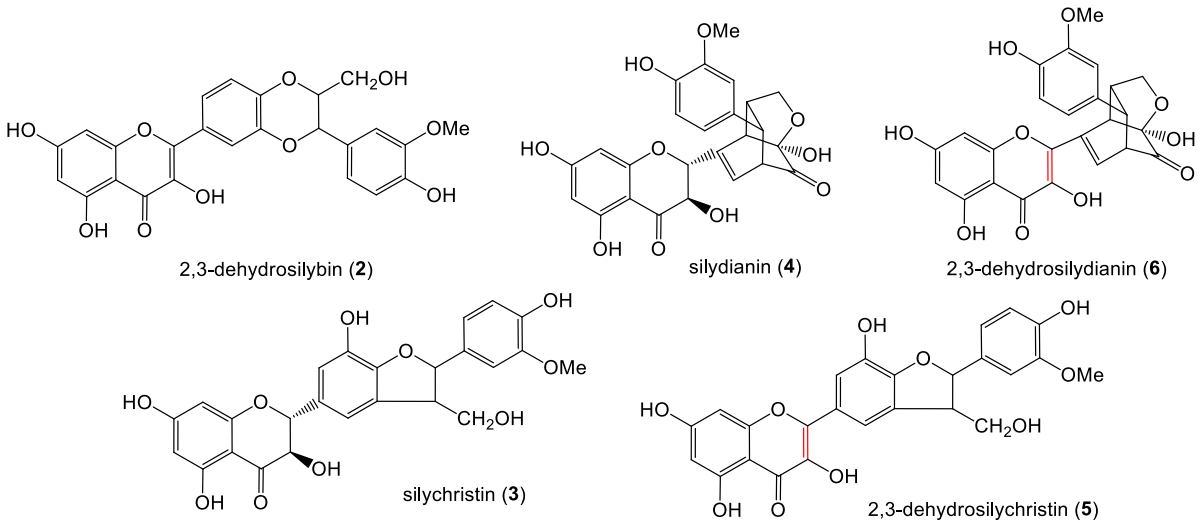


Figure 2: Structures of unexplored silymarin flavonolignans.

2. Results and Discussion

Virtually all metabolic studies performed so far with crude silymarin or with silybin A&B mixture reported several glucuronidated and sulfated derivatives of particular flavonoid components but their exact structures were not determined [8]. Silychristin is glucuronidated and sulfated in approximately equal ratio, whereas silydianin is rather glucuronidated (80.5%) than sulfated [8]. Recently reported sulfates of silybin A and B and isosilybin A and B were obtained by bacterial AST from *Desulfitobacterium hafniense*, which catalyzed selectively C-20 sulfation of all above

components, however in various proportions [9]. However, mammalian enzyme aryl sulfotransferase IV from rat liver heterologously expressed in *E. coli*, used for the same reactions, exhibited strictly stereoselective sulfating reaction of silybin isomers. Silybin B was sulfated yielding 20-*O*-silybin B sulfate, whereas silybin A proved to be completely resistant to the sulphating reaction [10]. It is obvious that the conjugation reaction is dictated by the structure of the substrate and not entirely by the enzyme used [9, 10]. So far only silybin and isosilybin sulfation was published but other sulfated metabolites of less explored silymarin components and derivatives are required to provide authentic metabolites for *in vivo* pharmacological studies.

2.1. Sulfation catalyzed by recombinant AST IV

Silybin A (**1a**), silybin B (**1b**), as well as their natural mixture (**1**) were the first flavonolignan substrates sulfated by purified AST IV. However, this method suffered from several disadvantages: need for DMSO as co-solvent, for fresh enzyme supplemented twice a day, long reaction time (5 days) and addition of PAP as a cofactor [11]. Problems with purification and stability of AST IV, regeneration of the cofactor (PAP) and scale-up prompted us to develop an entirely new concept employing whole *E. coli* cells transformed with the respective gene. The reaction mixture (preparatory scale) typically contained the substrate (40 mg), 4 g (wet based) of freshly prepared and buffer-washed *E. coli* cells (BL21(DE3)Gold strain), *p*-NPS and 4 mL of DMSO. The reaction mixture was incubated at 37 °C for 24 hours; the fresh cells were added three times during the reaction course to keep the reaction progress [11].

2.2. Sulfation by AST DH

Due to the enhanced sulfation activity of AST DH crude extract the reaction time was reduced from one week to ca 4 hours reaching nearly quantitative conversion [12]. Acetone was found to be most promising co-solvent in this case as it did not significantly reduce the activity of the lysate [13], it dissolves all silymarin flavonolignans, and replacing DMSO by acetone also enabled easier TLC monitoring. At pH 8.9 silymarin flavonolignans were apt to air oxidation forming thus respective 2,3-

dehydro-derivatives and their sulfates. Oxidation can be limited by using inert atmosphere (Ar) and shorter reaction time.

2.3. Isolation of sulfated products

We found that in a narrow pH range 7.7-7.5 the *p*-NP can be easily removed by extraction with ethyl acetate, while the products remained in the water phase. This pretreated mixture was separated by gel chromatography. Preparative HPLC using Asahipak column was found less efficient providing lower yields and very poor purity of sulfated products (less than 50%) even after repetitive injections. The column chromatography employing Sephadex LH 20 was found to be a convenient method giving very pure products (>95 %) in high yields (see table 1). There is only one slight disadvantage of this method as it is rather time demanding. Typically 2 to 7 days are required for product purification. Isolated sulfated compounds **18-21** (scheme 2) were fully characterized by MS and NMR analyses (see supplementary data). At applied pH (pH 8.9 as well as at pH 7.0) the substrates **3** and **4** are more sensitive to oxidation, so 2,3-dehydro-derivatives and their sulfates, are formed. We investigated that alkaline pH and air oxygen cause easy formation of oxidized products. When the AST from *D. hafniense* is employed in enzymatic reaction, the oxidation can be easily avoided by using strict inert atmosphere (Ar). Unfortunately, this procedure cannot be used in case of whole cell biotransformation. To avoid oxidation, the pure enzyme or crude lysate were applied under the inert atmosphere, but the requirement of PAP and instability of AST from rat completely limited the preparative reaction.

2.4. Sulfation of silybin synthetic derivatives catalyzed by AST DH

Not only natural substrates such as silybin [9] can be utilized by this enzyme, but also some selectively substituted silybin derivatives can be converted to their sulfated forms. Silybin 20-*O*-sulfate (**7**), silybin 7-*O*-butyratel (**8**), silybin 7-*O*-glucuronide (**9**), silybin 7,23-*O*-disulfate (**10**), 20-*O*-methyl silybin (**11**), silybin 23-*O*-monovinyldodecanoate (**12**), silybin 23-*O*-acetate (**13**) and silybin 23-*O*-butyrate (**14**, Figure 3) were sulfated according to the described sulfation procedure. Reactions were monitored by HPLC for 24 hours. Preliminary results suggest that substituents at C-7-OH of

silybin do not influence the reaction at all: silybin 7-*O*-butyrate or silybin 7-*O*-glucuronide were fully converted to their sulfated form. Free C-20 OH of silybin was found to be crucial in sulfation reaction. Its substitution blocked sulfation of the molecule (substrates silybin 20-*O*-sulfate and 20-*O*-methyl silybin). Not entirely uniform results were achieved with silybin substituted at 23-OH. Silybin 23-*O*-acetate and silybin 23-*O*-butyrate were sulfated but no sulfation of silybin 7,23-*O*-disulfate or silybin 23-*O*-monovinyldodecanoate was observed.

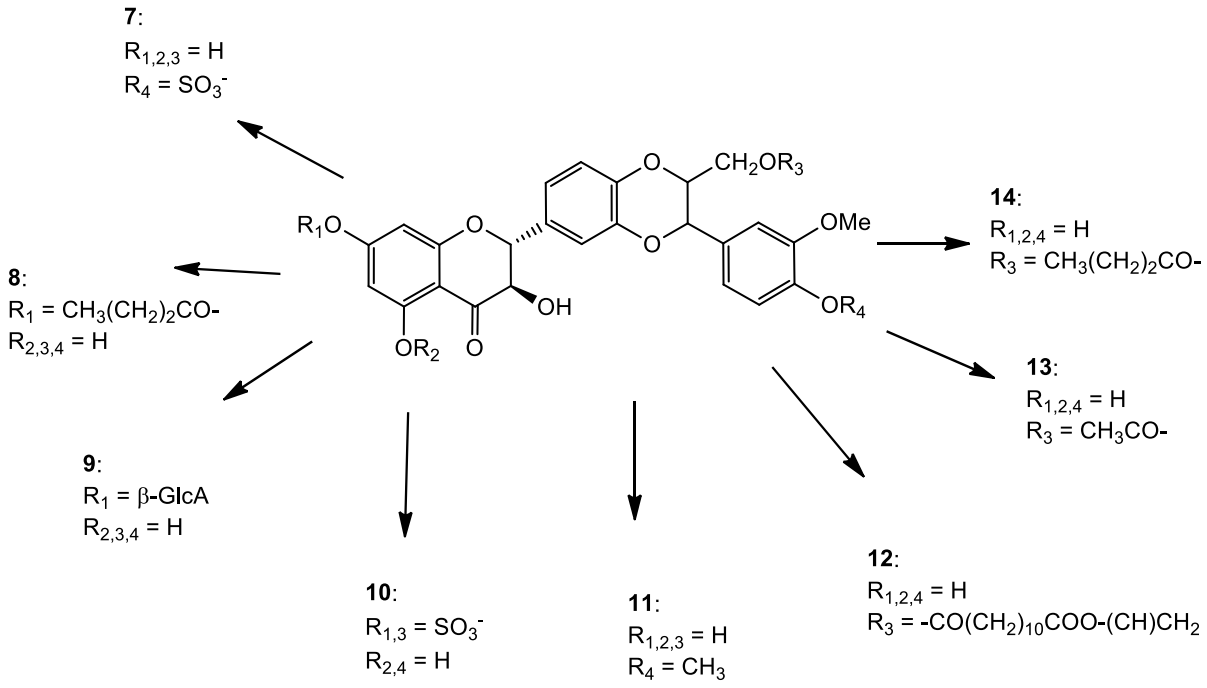


Figure 3: Semisynthetic silybin derivatives

Table 1. Detected sulfates of selectively protected silybin (**7-14**), resorcinol (**15**), catechol (**16**) and naringin (**17**) by HPLC and MS.

substrate	Sulfation (HPLC)	Sulfation (MS)
silybin 20-*O*-sulfate (**7**)	-	-
silybin 7-*O*-butyratel (**8**)	•	??
silybin7-*O*-glucuronide (**9**)	•	N.D.
silybin 7,23-*O*-disulfate (**10**)	-	??
20-methyl silybin (**11**)	-	-
23-*O*-monovinyldodecanoyl silybin (**12**)	-	??
23-*O*-silybin acetate (**13**)	•	-
23-*O*-butanoyl silybin (**14**)	•	??
resorcinol (**15**)	•	•
catechol (**16**)	•	•
naringin (**17**)	•	•

The AST DH was found as an enzyme with very broad substrate specificity (Figure 3). Not only substrates **1-6** can be utilized by this enzyme forming respective sulfates (**18-21**) but also some of selectively protected silybin derivatives **7-14**, resorcinol (**15**), catechol (**16**) and naringin (**17**, Table 1) can be converted to their sulfated form (according to HPLC and/or MS analysis). 20-C-OH position of silybin plays a crucial role in sulfation reaction, because once the position is occupied no further sulfation could be observed as in the case of compounds **7** and **11**. A bulky substituent at position 23 C-OH negatively affects the sulfation (**12**, **13**). Generally, blocking of the position 7-C-OH do not influence the reaction at all (**8**, **9**). Surprisingly, no formation silybin tri sulfate was observed (**10**).

2.5. 2,3-Dehydrosilybin

Sulfation by AST IV was not tested for sulfation using AST IV due to its paucity. Using catalysis by crude lysate of AST DH, two sulfated products were successfully isolated: 2,3-dehydrosilybin 20-*O*-sulfate (**18**, 10 mg, 25.6 %) and 2,3-dehydrosilybin 7,20-*O*-disulfate (**19**, 9 mg, 28.6 %).

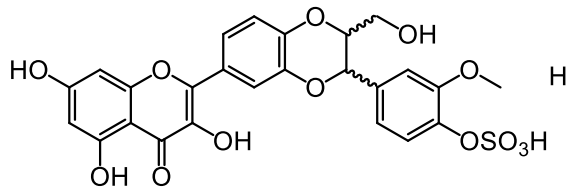
2.6. Silychristin and silydianin

For this purpose we decided to prepare potential sulfated metabolites of remaining silymarin flavonoids by aryl sulfotransferase from *D. hafniense* and compare them with true metabolites obtained by aryl sulfotransferase from rat liver. When silychristin (**3**) or silydianin (**4**) were tested as substrate in this AST IV catalyzed reaction both consumption of *p*-NPS and formation of *p*-nitrophenol (*p*-NP) were observed. This finding clearly proved the sulfation reactions. Despite our attempts no sulfated products were isolated and recovery of the substrate was impossible. We assume that these substrates were probably sulfated but immediately metabolized by the cells, hence the sulfation products could not be isolated.

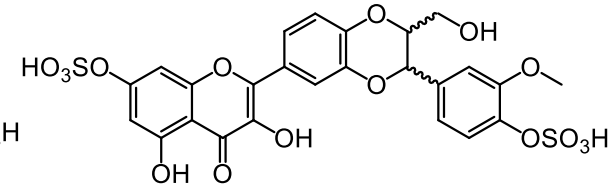
Employing aryl sulfotransferase from *D. hafniense* we obtained hundreds of milligrams of sulfated products **18-21** in very good yields (See Table 1, Figure 4).

Table 2. Sulfated product yields.

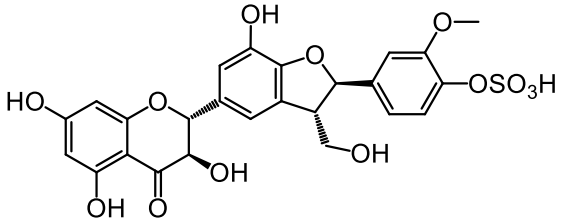
Products	Yield (%)	Yield (mg)
2,3-Dehydrosilybin 20-*O*-sulfate (**18**)	29	9
Dehydrosilybin 7,20-*O*-disulfate (**19**)	26	10
Silychristin 19-*O*-sulfate (**20**)	57	100
Silydianin 20-*O*-sulfate (**21**)	10	42



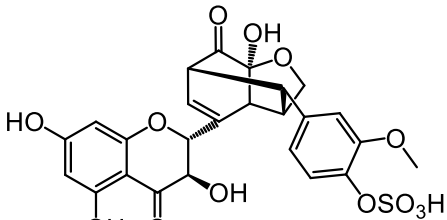
2,3-Dehydrosilybin 20-*O*-sulfate (**18**)



2,3-Dehydrosilybin 7,20-*O*-disulfate (**19**)



Silychristin-19-*O*-sulfate (**20**)



Silydianin-20-*O*-sulfate (**21**)

Figure 4. Isolated sulfated products **18-21** by general procedure A

There is lack information about flavonoid metabolites (sulfates) identification in the literature. From above flavonoids only quercetin metabolites were better described, however no ^{13}C NMR data have been ever published. Here for the first time we are reporting complete NMR data of sulfated compounds **18-21**.

The assignment of individual proton spin systems was achieved by COSY, and then transferred to carbons by HSQC. HMBC experiment enabled us to assign quaternary carbons and join partial structures together. Quaternary carbons in the catechol moiety evinced the same HMBC coupling patterns in all samples under study (see Figures S1-S5). It is obvious that carbons C-3'and C-4'can be differentiated using a diagnostic correlation between the carbon C-4' and the proton H-6'.

The comparison of carbon chemical shifts of different flavonoids and flavonolignans with their sulfates revealed several characteristic changes in the catechol moiety. All 4'-O-sulfates display up-field shift of the carbon C-3'and down-field shift of C-4'compared to the original (parent??) compound; oppositely down-field shift of the carbon C-3'and up-field shift of C-4' were detected in 3'-O-sulfates (see Table 3).

Table 3: Relative changes of the carbon chemical shifts in the catechol moiety (expressed as a difference between sulfate and parent compound)

	analogs of 4'-sulfate			3'-sulfates [12]		4'-sulfates [12]			
Atom	20-DHS	20-SDS	19-SCHS	3'-QS	3'-TS	4'-QS	4'-TS	4'-RS	4'-IQS
1'	3.93	4.30	4.17	0.28	0.83	4.88	6.30	6.02	6.11
2'	0.30	0.35	0.12	7.54	7.66	1.20	1.46	1.81	1.77
3'	2.70	2.88	2.94	-4.21	-4.45	3.46	3.84	4.08	4.09
4'	-3.88	-3.78	-3.92	3.51	3.76	-5.00	-4.72	-6.06	-6.17
5'	5.19	5.55	5.56	-2.75	1.71	2.11	7.43	6.31	6.24
6'	-1.00	-0.68	-0.99	9.33	5.43	3.55	-0.28	-0.40	0.07

3. Conclusions

AST from rat liver is not convenient catalyst because it is time demanding and giving poor yields with limited spectrum of substrates. Isoquercitrin and rutin were not substrates for AST from rat liver. Silychristin, silydianin and quercetin sulfates prepared by AST from rat liver could not be isolated probably due to their further metabolism. On the other hand bacterial AST from *D. hafniense* was

identified as a perfect tool for the biotransformation of the whole array of the flavonoids and flavonolignans. It is stabile highly efficient and high yielding (50-80%). When we isolated and fully characterized sulfated products from both ASTs we found that they their sulfated products are identical and so that proved sulfates might be used as an authentic standards in further metabolic studies.

4. Experimental

4.1. General methods

4.1.1. NMR

NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (600.23 MHz for ^{1}H, 150.94 MHz for ^{13}C) at 30 °C in DMSO-d_6 and at 25 °C in CD_3OD (for particular details see respective data). Residual solvent signals (DMSO: δ_H 2.500 ppm, δ_C 39.60 ppm; CD_3OD: δ_H 3.330 ppm, δ_C 49.30 ppm) served as internal standards. NMR experiments: ^{1}H NMR, ^{13}C NMR, gCOSY, gHSQC, and gHMBC 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 window function before Fourier transformation. Two-parameter double-exponential Lorentz-Gauss function was applied for ^{1}H to improve resolution and line broadening (1 Hz) was applied to get better ^{13}C signal-to-noise ratio. Chemical shifts are given in δ-scale with digital resolution justifying the reported values to three (δ_H) or two (δ_C) decimal places.

4.1.2. Mass Spectroscopy

Mass spectra in the negative ion mode were measured using LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source. The samples were dissolved in methanol and introduced into the mobile phase flow (methanol/water 4:1; 100 µL/min) using a 2-µL loop. Spray voltage, capillary voltage, tube lens voltage and capillary temperature were 4.0 kV, -16 V, -120 V and 275 °C, respectively.

4.1.3. Analytical HPLC-PDA

All analytical HPLC analyses were performed at the Shimadzu Prominence System (Shimadzu, Kyoto, JP) consisting of a DGU-20A mobile phase degasser, two LC-20AD solvent delivery units, a SIL-20AC cooling auto sampler, a CTO-10AS column oven and SPD-M20A diode array detector.

Chromatographic data were collected and processed using Shimadzu Solution software at a rate of 40 Hz and detector time constant of 0.025 seconds. The Chromolith Performance RP-18e monolithic column (100 × 3 mm i.d., Merck, DE) coupled with a guard column (5 × 4.6 mm) (Merck, DE) was used. Mobile phase A: $CH_3CN/CH_3OH/H_2O/HCO_2H$ (2/37/61/0.1; v/v/v/v), mobile phase B: $CH_3CN/H_2O/HCO_2H$ (80/20/0.1) and mobile phase C: CH_3CN/HCO_2H (99.1/0.1; v/v) were employed in the analyses. Gradient G1 (**2**): 0 - 2 min, 100% m.p. A; 2 - 10 min, 0 – 60% C; 10 - 10.5 min, 60% B; 10.5 - 11 min, 60 - 0% B; 11 – 12 min, 0 % C. The flow rate was 1.2 mL/min at 25 °C. Gradient G2 (**3a SCH**, **4a SD**): 0 - 4 min, 7 – 40% m.p. B; 4 - 6 min, 40% B; 6 - 7 min, 40 – 7% C; 7 - 8 min, 7% B. The flow rate was 1.5 mL/min at 25 °C. The PDA data were acquired in the 200-450 nm range and the 254 nm (**DHS**), 285 nm (SCHS, SDS) signal was extracted, respectively.

4.1.4. Preparative HPLC

Preparative HPLC were performed on 20×300 mm Asahipak GS-310 20F (Shodex, USA). The system was consisted of a Shimadzu LC-8A pump, a SPD-20A dual wavelength detector, a FRC-10A fraction and CBM-20A controller connected to PC. Separations were performed with methanol at 25°C, flow rate was 5 mL/min, and wavelengths used were 254 and 369 nm.

4.2. Aryl Sulfotransferase from Desulfitobacterium hafninense *(AST DH)*

Expression of the AST DH enzyme was performed as described by van der Horst et al. 2012 [14] with following amendments. The plasmid containing AST gene (kindly provided by dr. van der Horst, Univ. of Amsterdam, NL), was re-transformed into various *E. coli* strains: BL21, BL21(DE3) Gold, and BL21 (DE3)plyS. Bacterial cultures were grown in LB medium with kanamycin and respective second antibiotics (according to the *E. coli* strain) for the better selection and maintenance of plasmids. The purity of the enzyme was confirmed by 12 % SDS–PAGE analysis [15], showing a single band of 70 kDa (figure not shown). The crude lysate of the most active clones *(E. coli* BL21(DE3)Gold) was used in enzymatic reactions. Due to the enzyme stability and no sulfatase activity the purification of the protein was not necessary. The AST was always prepared fresh, standard enzymatic assays were performed at 30 ˚C. Activity of AST was expressed as released *p*-nitrophenol at 400 nm (ε_{400} = 0.2488

$cm^2.\mu mol^{-1}$). One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 mmol *p*-nitrophenol per minute. Activities were corrected for non-enzymatic *p*-nitrophenol release. The specific activity of the recombinant AST from *D. hafniense* was 28 500 $U.mg^{-1}$.

4.3. Aryl Sulfotransferase from Rat Liver (AST IV)

AstIV from rat liver enzyme was expressed as described in our previous work [10] with following modifications. To support the enzyme folding and to enhance the activity of AstIV the co-transformation with the chaperon G7 (GroEL/ GroES) was applied for *E. coli* strains (BL21(DE3)Gold**,** BL21plyS and Origami (DE3). However, no significant improvement of the enzyme activity and stability was reached. Freshly prepared whole cells, prepared according to [10] were employed in the biotransformation experiments. Standard enzymatic assays were performed at 22 ˚C and the activity of AST was expressed as *p*-nitrophenol released at 400 nm (ε_{400} = 0.2488 $cm^2.\mu mol^{-1}$). One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 mmol *p*-nitrophenol per minute. Activities were corrected for non-enzymatic release of *p*-nitrophenol.

4.4. 2,3-Dehydrosilybin Sulphation by Aryl sulfotransferase from Desulfitobacterium hafniense

Dehydrosilybin (DH, 30 mg, 0.0625 mmol) was dissolved in 1 mL of acetone. 10 mL of 100 mM Tris–glycine buffer (pH 9), *p*-nitrophenyl sulfate (25 mg 0.086 mmol Sigma Aldrich) and 1 mL of AST (2170 $U.\ mL^{-1}$) were added to the substrate solution and the mixture was incubated at 30 °C. The reaction progress was monitored by HPLC, after 4 hours the conversion was typically over 50 %.

Reaction mixture was halved by evaporation *in vacuo* so that all organic solvents were removed. pH was adjusted to 7.5-7.7 and *p*-nitrophenol (*p*-NP) and residual starting material were removed by extraction (3 × 50 mL EtOAc). The aqueous phase (15 mL) containing the sulfated product was evaporated, the residue was dissolved in 2 mL of 80 % methanol and loaded onto a Sephadex LH-20 column (30 g dry weight, 3 cm i.d.) packed and equilibrated with 80 % methanol. The fractions were analyzed by TLC (EtOAc / MeOH / HCO_2H, 4:1:0.01, v/v) and the fractions containing the respective product were collected and evaporated *in vacuo* at 45 °C.

2,3-Dehydrosilybin 20-*O*-sulfate (18) was obtained as yellowish solid (9 mg, 25.8%). Structure was determined by HRMS *m/z* calc. for $C_{25}H_{19}O_{13}S$ 559.05518, found 559.05454 and ^{13}C, ^{1}H NMR (For ^{1}H, ^{13}C NMR and MS data – see Figure S1 and Table S1, Supplementary data. Complete assignment of all NMR signals was achieved by use of a combination of gHCOSY, gHSQC, gHMBC. Structure of 2,3-dehydrosilybin 7,20-*O*-disulfate (**19**, 10 mg, 25.1 %) was determined by HRMS (*m/z* calc. for $C_{25}H_{19}O_{16}S_2$ 639.01200, found 639, 01115) and ^{13}C, ^{1}H NMR (For ^{1}H, ^{13}C NMR and MSdata – see Figure S2 and Table S2,Supplementary data;

4.5. Silychristin Sulphation by Aryl sulfotransferase from Desulfitobacterium hafniense

Silychristin (**3**, 150 mg, 0.3112 mmol, prepared as described previously [5], was sulfated using the same procedure as for 2,3-dehydrosilybin. silychristin 19-*O*-sulfate (**20**) was obtained as a yellowish solid (100 mg, 57,1 %). Its structure was determined by HRMS (*m/z* calc. for $C_{25}H_{21}O_{13}S$ 561.07083, found 561.07092) and by ^{13}C, ^{1}H NMR (see Figure S3 and Table S3, Supplementary data).

4.6. Silydianin Sulphation by Aryl sulfotransferase from Desulfitobacterium hafniense

Silydianin (**4**, 150 mg, 0.3112 mmol, prepared as described previously [5] was sulfated using the same procedure as for 2,3-dehydrosilybin. Silydianin 20-*O*-sulfate (**21**) was obtained as a yellowish solid (101 mg, 58,3%). Its structure was determined by HRMS (*m/z* calcd. for $C_{25}H_{21}O_{13}S$ 561.07083, found 561.07141) and by ^{13}C, ^{1}H NMR (see Figure S4 and Table S4, Supplementary data).

4.7. General Procedure B: Sulphation by Arylsulfotransferase from Rat Liver

Silychristin (**3**, 50 mg, 0.104 mmol) and *p*-nitrophenyl sulfate (35 mg, 0.136 mmol) were dissolved in 3 mL DMSO and 2 grams whole cells (wet based) expressing aryl sulfotransferase from rat liver (prepared as described in chapter 2.2) resuspended in potassium phosphate buffer (pH 7.5, 17 mL) was added. The reaction mixture was incubated at rotary shaker at 37 °C for 24 hours then the cells were centrifuged (30 min, 5000 rpm, 5404 R Eppendorf, DE) and a new portion of the fresh cells (2 grams, wet based, resuspended in K-phosphate buffer) were added (15 mL, tree-times in 3 days The reaction was monitored by HPLC and after 72 hours the product was purified by preparative HPLC

employing Asahipak GS-310 20F (Shodex, USA) column with isocratic elution with 100 % methanol. The fraction containing the putative sulfated product(s) was evaporated, dissolved in 1 mL of 80% methanol and loaded onto a Sephadex LH-20 column (15 g dry weight, 12 mm i.d.) packed and eluted with 80 % methanol. Fraction containing putative sulfated product was evaporated, dissolved in 1 mL of 80% methanol and loaded onto a Sephadex LH-20 column (15 g dry weight, 12 mm i.d.) packed and eluted with 80 % methanol. No sulphated product was detected.

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Bioavailability of silymarin flavonolignans: drug formulations and biotransformation

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Abstract Over the past years, great advances have been made on the development of novel delivery systems for bioactive natural compounds, in parallel to their structural modification via chemical, chemoenzymatic and enzymatic methodologies. These approaches give rise to novel formulations and derivatives that often display advantages over the parental molecule, such as enhanced bioavailability and pharmacological activity, due to improved dissolution and stability. Silymarin components suffer from poor solubility in water and lipid media and their resorption in the intestine is rather limited. Moreover, silybin undergoes intensive Phase II metabolism and is rapidly excreted in bile and urine, leading to low therapeutic efficacy. This work aims to present the current status of available silymarin formulations, and to highlight successful efforts for the biotransformation of its constituent flavonolignans towards the synthesis of novel derivatives. Herein, various pharmaceutical formulations that aim at the bioavailability improvement of these fascinating phytochemicals, i.e., liposomes, phytosomes, self-microemulsifying drug delivery systems, solid dispersions systems, dripping pills, nanosuspensions, floating tablets, and micronization, are reviewed. Silybin (semi)synthetic derivatives prepared by chemical or enzymatic methods, such as fatty acid conjugates, silybin bishemisuccinate, silybin glycosides, silybin sulfates, silybinic acid, and 2,3-dehydrosilybin, are also discussed in detail. Additionally, this work attempts to direct the attention towards the pharmacological implications of optically pure silybin A and silybin B and their biotransformation reactions, both Phase I and II, in relation to bioavailability.

Keywords Silymarin · Silybin · Silibinin · Bioavailability · Formulations · Biotransformation

Abbreviations

CYP	Cytochrome P450
DPPH	2,2-Diphenyl-1-picrylhydrazyl
GI	Gastrointestinal

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GST	Glutathione S-transferase
MDA	Malonaldehyde
PEG	Polyethylene glycol
Pgp	P–glycoprotein
ROS	Reactive oxygen species
SEDDS	Self emulsifying drug delivery system
SMEDDS	Self-micro emulsifying drug delivery system
UDP	Uridine diphosphate

Introduction

In the last decades, silymarin and silybin have been in the spotlight due to multiple beneficial biological activities. Silymarin functions as antioxidant that stabilizes cell membranes, stimulates detoxification pathways, regenerates the liver tissue, inhibits the growth and exerts direct cytotoxic activity toward various cancer cell lines, and enhances the efficacy of some chemotherapy agents (Post-White et al. 2007; Gažák et al. 2010b). However, the in vivo effectiveness of silymarin is governed by the bioavailability and the achieved therapeutic concentrations of the flavonolignans that reach the organs of interest (Manach et al. 2005; Silberberg et al. 2006). The components of silymarin are poorly soluble in water, and studies using both humans and laboratory animals have shown only ng mL^{-1} concentrations in plasma after oral administration of powdered extracts (Barzaghi et al. 1990; Schandalik et al. 1992). Silybin is barely absorbed orally and cannot be dissolved directly for parenteral preparations, due to its poor solubility in aqueous and lipid media (Song et al. 2008). Even though the oral route of administration is preferred, limited drug absorption resulting in poor bioavailability is dominant amongst the potential problems that can be encountered (Vasconcelos et al. 2007). When an active agent is orally delivered, it must first dissolve in gastric and/or intestinal fluids in order to permeate the membranes of the GI tract to reach systemic circulation. For that reason, the pharmaceutical community focuses on enhancing the therapeutic efficacy of active agents by increasing the solubility of poorly water-soluble drugs and the permeability of poorly permeable drugs (Dhirendra et al. 2009; Javed et al. 2011).

The purpose of this review is to discuss ways to improve the dissolution and bioavailability of silymarin or silybin and to stimulate the interest for new potent clinical applications of these attractive natural compounds.

Historical background

Milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)] is a hardy and often invasive plant indigenous to the Mediterranean region whose achenes, often referred to as seeds, have been valued for their medicinal properties (Gažák et al. 2007; Kroll et al. 2007). The extract of the seeds has been used in European folk medicine for centuries as a natural remedy for treating hepatitis and cirrhosis, and to protect liver from toxic substances (Flora et al. 1998; Lee et al. 2006). The oldest reference belongs to Pedanius Dioscorides (ca. 40–90 AD), the most renown pharmacologist and botanist of ancient Greece, who first described the plant in his "De Materia Medica" (ca 70 AD) and suggested that a tea of the seeds can cure "those that be bitten of serpents". Dioscorides used the term "silybon" to describe thistle-like plants, while Pliny the Elder (23–79 AD), a Roman naturalist, called it "silybum" and noted that the plant juice mixed with honey is excellent for "carrying off bile" (Foster 1996; Flora et al. 1998; Lee et al. 2006). In 1596, John Gerard (1545–1612), one of the best known English herbalists of his time, advocated the use of milk thistle for the therapy of melancholy, a disease that was in Middle Ages related to the liver, and also called "black bile" (Mulrow et al. 2000). In the sixteenth century, another well-known English herbalist, Nicolas Culpepper (1616–1654), suggested milk thistle for obstructions of the liver and the spleen, breaking and expelling stones, treating jaundice and plague infections, and cleansing the blood (Flora et al. 1998). In the United States, milk thistle gained popularity in the nineteenth century due to the Eclectics movement, an officially recognized school of medical herbalists, who used milk thistle for congestion of the liver, spleen, and kidneys and for varicose veins and menstrual difficulty (Foster 1996). The first attempts to isolate the active components of the seeds appeared in 1958, and almost a decade later, a research by Wagner at the University of Munich successfully led to the isolation of silymarin, which was believed to be a single compound. With improved chemical separation methods, it was later revealed that

silymarin was not a single component but a complex of flavonolignans (Foster 1996; Abascal and Yarnell 2003).

Silymarin and silybin preparation

Typically, the seeds of *S. marianum* are partially defatted by pressing, which lowers the fat content from approximately 25 to 8 %. Then the seeds are extracted with acetone (alternatively ethanol, methanol or ethyl acetate). Acetone extract is partially evaporated and the remaining fat is removed by hexane extraction. Crude silymarin (complex) precipitates after further evaporation. Pure silybin (Fig. 1a) is prepared by dissolving silymarin in absolute ethanol followed by addition of about 10 % water. Crude silybin, which precipitates can be further purified by recrystallization from ethanol (personal communication with Dr. L. Cvak, TEVA Pharmaceuticals, Opava, CZ).

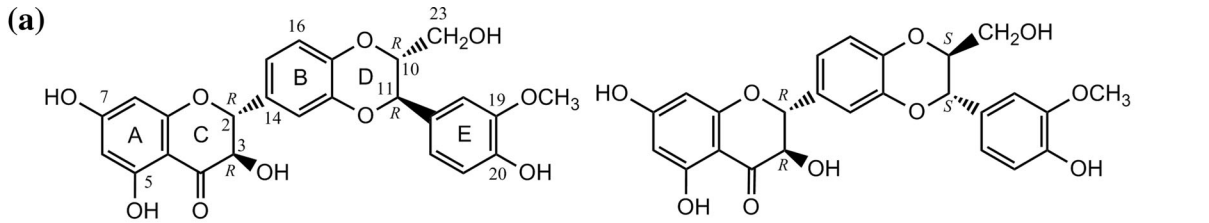
Silymarin is a complex of at least 7 flavonolignans and 1 flavonoid (taxifolin) (Fig. 1b) (Kroll et al. 2007). The most prevalent (50–70 %) and biologically active compounds in silymarin are the two diastereomers of silybin (CAS No. 22888-70-6; in pharmacopoeias the name *silibinin* is used): silybin 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-(hydroxy-methyl)-1,4 benzodioxin-6-yl)-2,3-dihydro-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one (typically **1b** is slightly prevalent in most preparations).

Biological activities of *S. marianum* components

Silymarin and silybin exert antioxidant activity and support redox homeostasis in several in vitro and in vivo models. It has been shown that administration of silymarin increases the activities of anti-oxidant enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S transferase (GST) together with a decrease in the levels of malondialdehyde (MDA), a marker for lipid peroxidation, in erythrocytes exposed to H_2O_2 (Kiruthiga et al. 2007). While Zhao et al. (2000) showed that silymarin inhibits the MDA formation by epidermal microsomes in a dose-dependent manner, and also inhibits 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and benzoyl peroxide (BPO)-induced lipid peroxidation in mouse skin epidermis, demonstrating strong in vivo anti-oxidant activity. The molecular mechanisms of the antioxidant activity of silymarin flavonolignans, as well as the responsible functional groups, have been described (Trouillas et al. 2008; Gažák et al. 2009). In depth knowledge of the role of the respective hydroxyl groups and moieties of the silybin molecule allowed the rational identification of suitable sites for silybin derivatization without losing the biological activity of the resulting conjugates (Gažák et al. 2010a).

In last decades silymarin and silybin were widely examinated as hepatoprotective agents (Miguez et al. 1994; Flora et al. 1998; Parés et al. 1998; Lieber 2000; Horváth et al. 2001; Saller et al. 2001; Wellington and Jarvis 2001; Mayer et al. 2005; Pradhan and Girish 2006; Loguercio and Festi 2011; Křen and Walterová 2005). Silymarin anti-oxidative, anti-fibrotic, anti-inflammatory, membrane stabilizing, immunomodulatory and liver regenerating properties play an important role in experimental liver diseases (Post-White et al. 2007). Silybin has been reported to stimulate RNA polymerase I, and consequently ribosomal RNA synthesis and proteosynthesis, both in the liver and in cultured hepatocytes (Plíšková et al. 2005).

Furthermore, silymarin has been extensively studied, both in vitro and in vivo, for its chemopreventive potential against various cancers (Post-White et al. 2007; Ramasamy and Agarwal 2008). Silymarin exerts anticancer properties by multiple molecular mechanisms that inhibit all stages of carcinogenesis. Its anti-invasive and anti-metastatic effects render silymarin a promising prophylactic and therapeutic agent in the treatment of more advanced and aggressive forms of cancer as an adjunct to established therapies, to prevent or reduce chemotherapy consequences as well as radiotherapy-induced toxicity (Agarwal et al. 2006; Ramasamy and Agarwal 2008). Studies have shown that silymarin and silybin down-regulate the signaling of the epidermal growth factor receptor (EGFR) by inhibiting expression and secretion of growth factors, leading to the subsequent impairment of downstream mitogenic events causing anti-cancer effect in tumor cell lines (Deep and Agarwal 2007). The mini review of Ramasamy and



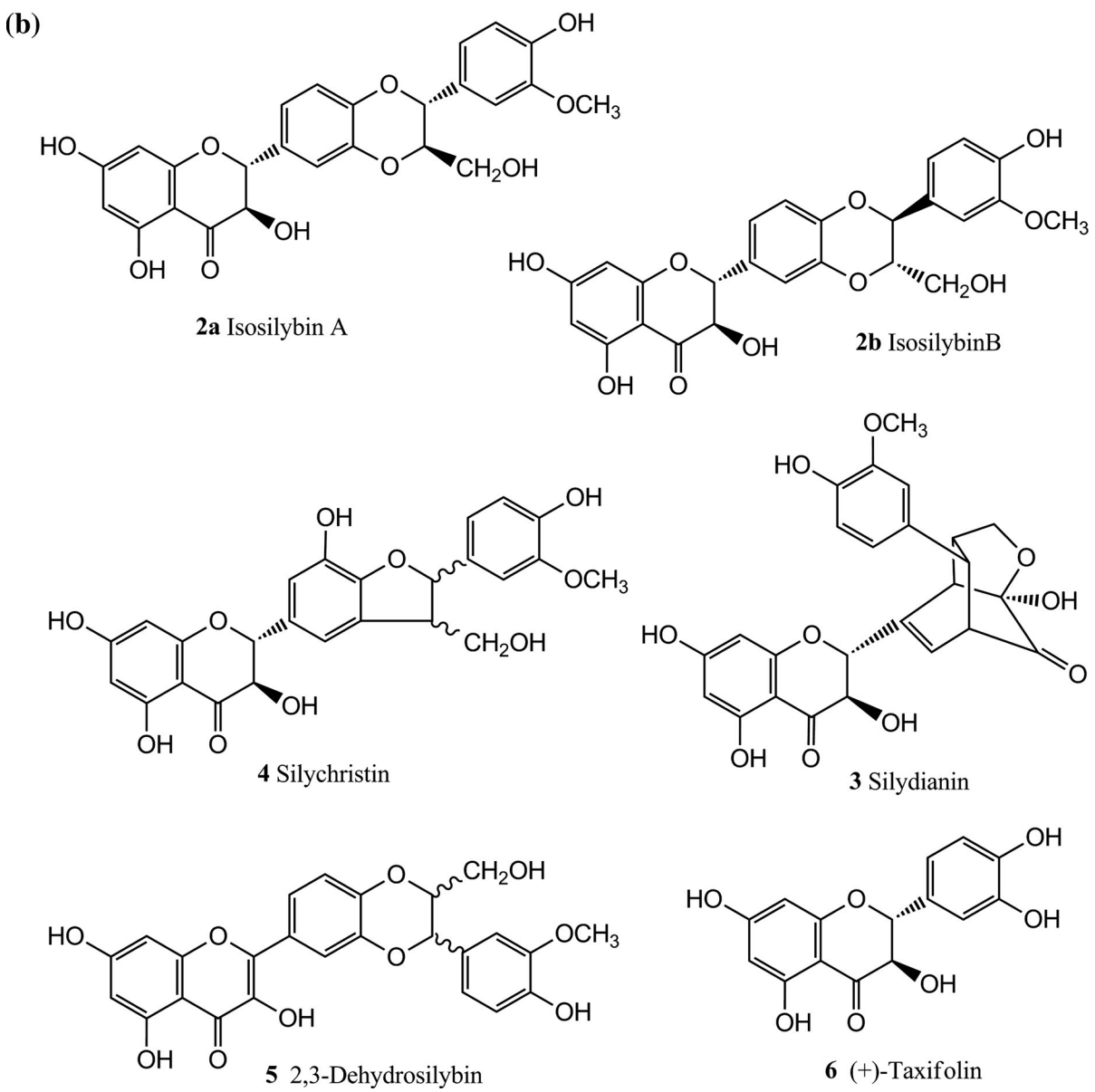


Fig. 1 **a** Structures of silybin A and silybin B. **b** Structures of the major silybin congeners and the flavanonol taxifolin

Agarwal (2008) summarizes in an excellent way multi-targeted chemopreventive and interventive mechanisms of silymarin and silybin in various in vitro and in vivo cancer models.

Aspects of the optical purity of silybin and other flavonolignans from the silymarin complex have been largely neglected. When silybin is used for applications other than as an antioxidant in an isotropic environment its stereochemistry plays an extremely important role, and the respective biological activities also need to be studied using optically pure compounds (Šimánek et al. 2000). New data on the pharmacological activity of pure silybin A and B appeared recently and clearly demonstrated the different biological activities of the two silybin diastereomers. Plíšková et al. (2005) have presented the proof that silybin B interacts with an estrogenic receptor, whereas diastereomer A is inactive. Later, a research on the activity of pure silybin A and B, isosilybin A and B, and other silymarin components on human prostate carcinoma demonstrated that isosilybin B is the most effective in suppressing the topoisomerase IIR gene promoter in DU145 cells (Davis-Searles et al. 2005). Silybin B was the most active in the G1 cell cycle accumulation of DU145 cells. However, a major problem hampering studies with optically pure silybin is the extremely complicated separation of its diastereomers.

Despite having exceptional biological properties in vitro, silymarin flavonolignans, like many polyphenols, exhibit lower or no in vivo activity, likely due to several factors. Firstly, polyphenols are multiplering molecules, too large to be absorbed by simple diffusion, whilst they are not absorbed actively as occurs with some vitamins and minerals (Manach et al. 2004; Kidd and Head 2005). Secondly, flavonolignans display poor solubility in aqueous and lipid media that severely hampers their ability to pass across the lipid-rich outer membranes of the enterocytes of the small intestine (Kosina et al. 2002; Kidd and Head 2005). Besides that, silybin and its congeners are rather quickly metabolized mainly by the Phase II biotransformation enzymes and they are therefore, rather quickly excreted (Wu and Tsai 2007). These properties are the major limiting factors for molecules to pass the biological membranes, and to be absorbed systematically after oral or topical administration. In addition, some components of plant extracts are destroyed in gastric environment when taken orally (Bhattacharya 2009).

Metabolic biotransformations of silybin and its congeners

Silybin and its congeners contained in silymarin are largely metabolized by both Phase I and Phase II biotransformation enzymes in mammals. Their metabolism is strictly dependent on the stereochemistry of the respective flavonolignans. Profound knowledge of the pharmacokinecs and pharmacodynamics of the metabolic biotransformations is of the utmost importance for assessing various silymarin and silybin formulations.

Biotransformations of silybin by Phase I enzymes

Studies conducted the last decade clearly show an interaction between silybin and only a limited number of CYPs, in addition, researchers mainly focused on CYP1A1/2, CYP2C8/9, CYP2D6, CYP2E1, and CYP3A4 (Sridar et al. 2004; Brantley et al. 2010). This is important for possible drug–drug interactions, moreover supported by albeit few in vivo studies. *O*-Demethylation of silybin mediated by CYP2C8 is the only Phase I biotransformation known up to date. The production of demethylsilybin (*nor*-silybin, **14**) was confirmed by MS (Jančová et al. 2007) or/and LC–MS (Gunaratna and Zhang 2003), and these methods were sufficient for analysis of this product (single OMe group in the molecule; Fig. 2). A number of other hydroxylated metabolites of silybin were identified by Gunaratna and Zhang (2003), however, the site of hydroxylation was not determined by NMR and the exact structure of these metabolites remained unknown.

Biotransformations of silybin by Phase II enzymes

In general, polyphenolic substances are a facile target for conjugation reactions, and these are actually main

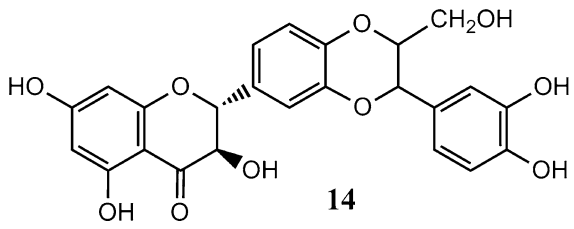


Fig. 2 Structure of one of the major silybin metabolites of Phase I—*nor*-silybin (Jančová et al. 2007; Gunaratna and Zhang 2003)

biotransformation pathways resulting in their excretion from the human body. As we mentioned above, silybin undergoes rapid conjugation, which is strictly stereoselective, e.g. silybin A (**1a**) and silybin B (**1b**) have different metabolic profiles. Characteristically, the peak plasma levels of total and unconjugated silybin reach ca. 76 and 8.5 μg ml^{-1} respectively (per os dose 500 mg kg^{-1} in rats) within 30–40 min after administration (Wu et al. 2007). Miranda et al. (2008) investigated the biliary excretion of silymarin flavonolignans (silybin A and B, isosilybin A and B, silychristin and silydianin), glucuronide and sulphate conjugates in perfused rat livers. Sulfonation was the major metabolic route for silybin B (78 %), isosilybin A (84 %) and isosilybin B (85 %). On the contrary, glucuronidation was the primary pathway for silydianin (81 %) and silychristin (ca 60 %) conjugation. Silybin A (**1a**) was sulphonated and glucuronidated to about the same degree (overall to a lower extent than **1b**). The synthesis of optically pure silybin and isosilybin sulfates was very recently achieved using aryl-sulfate sulfotransferase (EC 2.8.2.22) from *Desulfitobacterium hafniens* and a cheap sulfate donor, i.e., *p*-nitrophenyl sulfate. The used enzyme provided nearly quantitative regiospecific conversion of the flavonolignans into silybin A-20-*O*-sulfate, silybin B-20-*O*-sulfate, isosilybin A-20-*O*-sulfate and isosilybin B-20-*O*-sulfate, giving now the possibility to employ authentic standards in the metabolic studies of both silybins and isosilybins (Marhol et al. 2013). Besides, it was demonstrated that aryl sulfotransferase AstIV from rat liver (expressed in *Escherichia coli*) produces 20-*O*-silybin B sulfate from silybin B, whereas silybin A is completely resistant to the sulfation reaction (Purchartová et al. 2013). This is in a good agreement with the previous observations demonstrating that silybin B undergoes rather quick conjugation reaction, sulfonation being the major one, whereas metabolism of silybin A is considerably slower (Miranda et al. 2008).

Křen et al. (2000) prepared the 7-*O*-β-D-glucuronide (**15**), 20-*O*-β-D-glucuronide (**16**), and 5-*O*-β-D-glucuronide (**17**) of optically pure silybin A [*n.b. according to the current nomenclature this was actually silybin B* (**1b**)] by enzymatic synthesis using ovine liver microsomes (Fig. 3). These conjugates were formed in vitro in the proportion **15** (62.5 %), **16** (27 %), **17** (2.5 %), which reflects approximate regiopreference of UDP-glucuronosyltransferases (UGT) towards silybin B molecule. This and the work of Han et al. (2004) are probably the only studies so far describing the full spectral and therefore structural evidence (^{1}H, ^{13}C NMR and MS) of the silybin metabolites. They established (Křen et al. 2000) that silybin 20-*β*-D-glucuronide (**16**) is a major silybin B conjugate in humans, while the C-7 regioisomer (**15**) is

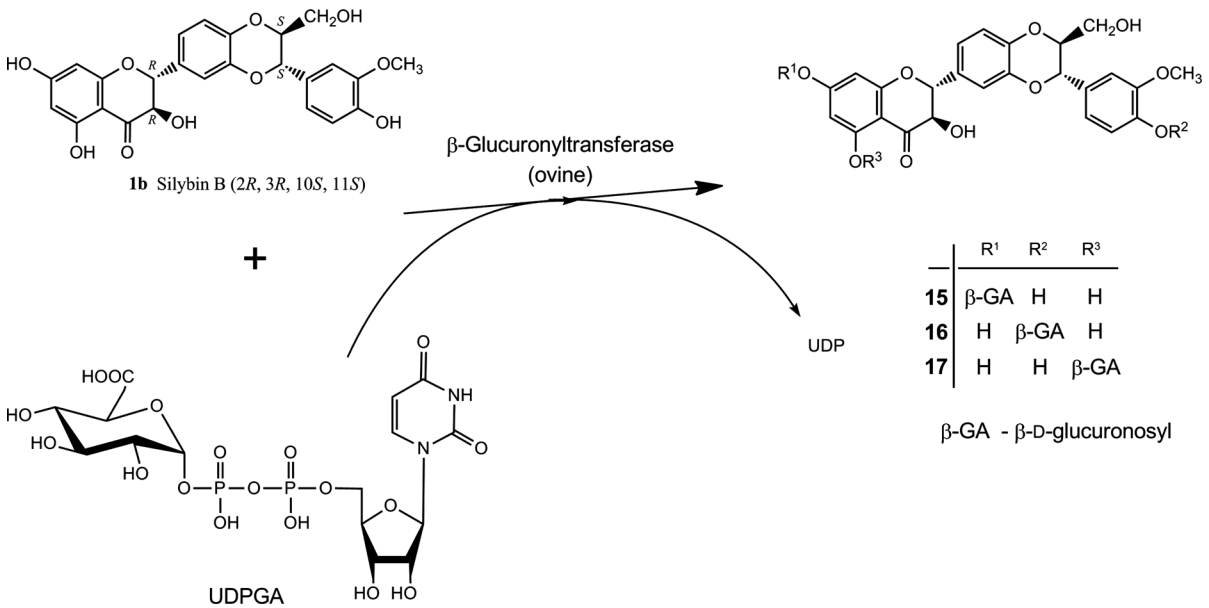


Fig. 3 Enzymatic preparation of silybin B *β*-glucuronides (Křen et al. 2000)

also formed, but in a lower proportion. They also tested the radical scavenging activity of both conjugates and proved that 7-*O*-silybin-β-D-glucuronide is a better antioxidant than nonconjugated silybin.

Han et al. (2004) identified four major metabolites (using bovine liver microsomes) of silybin; two for silybin A and two for silybin B. Silybin B is extensively metabolized, yielding 20-*O*-*β*-D-glucuronide (**16**) as a major metabolite and a slightly lower amount of the 7-*O*-*β*-D-glucuronide (**15**) of silybin B. Silybin A is glucuronidated similarly at both the 7 and 20 position, however the major product is 7-*O*-*β*-D-glucuronide and the 20-isomer is produced at a slightly lower rate. When comparing conjugation rates, silybin B is metabolized by the bovine microsomes 2–3 times more rapidly than silybin A. There were also differences in the respective silybin A and B metabolism when both diastereomers were incubated individually or in a diastereomeric mixture with the microsomes. This means that both diastereomers influence the respective metabolism of each other.

The solubility of silybin in water is ca. 0.43 mg mL^{-1}, but it strongly depends on pH. As a compound that is phenolic in nature, it dissolves well with increasing pH (phenolic OH ionization). However, alkaline solutions of all silymarin flavonolignans tend to oxidize (or disproportionate) forming strongly yellow 2,3-dehydroderivatives (Gažák et al. 2013b). The solubility of silymarin in other solvents of pharmacological relevance is as follows: transcutol, 350 mg mL^{-1}; ethanol, 225 mg mL^{-1}; polysorbate 20, 131 mg mL^{-1}; and glyceryl monooleate, 33 mg mL^{-1} (Woo et al. 2007). This may be also one of the reasons for the high tolerability of extremely high doses of silybin, since a major proportion of the silybin remains unresorbed (50–80 %) in the intestines.

Silymarin and silybin formulations

Liposomes

Liposomes are microparticulate lipoidal vesicles that have been used as carriers for the improved delivery of various drugs, such as chemotherapeutic agents, imaging agents, antigens, genetic materials, and immunomodulators. Conventional liposomes are typically composed of only phospholipids (neutral and/or negatively charged) and/or cholesterol (Verma and Garg 2001). Liposomes can also be used as solubilizers of poorly water soluble drugs for intravenous administration. In such conditions, they may behave in a comparable way to solvent formulations and do not influence the pharmacokinetics and pharmacodynamics of the drug (Fahr et al. 2005).

Maheshwari and coworkers, encaged silymarin into liposomal structures to investigate hepatoprotective activity against carbon tetrachloride induced hepatotoxicity and gastroprotective activity in mice. They prepared small unilamellar liposmal vesicles in various formulation and different process variables (lipid composition, impact of charge imparting agent, nature of hydration medium) were optimized to improve the efficacy of the drug entrapment. The results obtained from the in vivo studies, indicated the improved performance of silymarin in liposomes at a level of 55.6 % hepatoprotection in comparison to 33.08 % of the plain drug. Liposomal silymarin and plain liposomes also showed significant antiulcer activity when compared with plain silymarin and controls (Maheshwari et al. 2003).

Aiming at improving the poor bioavailability of silymarin from oral products, another research group prepared silymarin hybrid liposomes for buccal administration, and investigated in vivo the hepatoprotective efficiency against CCl_4-induced oxidative stress in albino rats. Silymarin loaded hybrid liposomes were composed of lecithin, cholesterol, stearyl amine and Tween 20 in molar ratio of 9:1:1:0.5. The degree of protection was measured using biochemical parameters like serum glutamic oxalacetate transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT). The introduced silymarin hybrid liposomes produced a significant decrease in both transaminase levels in comparison with orally administered silymarin suspension, and the improvement was also confirmed histopathologically (El-Samaligy et al. 2006).

Phytosomes

Phytosomes are forms of herbal formulations that are better absorbed compared with conventional herbal extracts. They are prepared by incorporating the standardized plant extract or the bioactive phytoconstituents into phospholipids, mainly phosphatidylcholine (PC), to produce lipid compatible molecular complexes (Bhattacharya 2009; Suryawanshi 2011).

PC acts not only as a passive "carrier", but additionally as bioactive nutrient with documented clinical efficacy for liver disease, including alcoholic hepatic steatosis, drug-induced liver damage, and hepatitis. Phytosomes differ structurally from liposomes. With the phytosome process, the phosphatidylcholine and the individual components form a 1:1 or a 2:1 complex depending on the substance, whereas the liposome is an aggregate of many phospholipid molecules that can enclose active phytomolecules without bonding to them (Bombardelli 1994; Kidd and Head 2005).

Phytosomal silybin is more rapidly absorbed than silymarin. It is also absorbed at least four times more completely than silymarin, reaching rapidly the liver and appearing in the bile within a few hours. While silymarin must be taken at doses of approximately 420 mg daily to achieve benefit, phytosomal silybin (Siliphos) can produce benefit at intakes as low as 120 mg daily, but can be safely administered at doses of 240–360 mg (Kidd and Head 2005). In an in vivo experimental model, Gallo et al. (2003) investigated the growth inhibitory effects of silymarin phytosome IdB 1016 as a single agent against human ovarian cancer, as well as the plasma and tumour bioavailability of silybin after repeated administration of IdB 1016. Female nude mice bearing human ovarian cancer xenografts (A2780) received daily 450 mg kg^{-1} IdB 1016 by oral gavage. This treatment with a total of 20 administrations produced a tumour weight inhibition (TWI) of 78 % and a $\log_{10}$ Cell Kill (LCK) of 1.1, while free silybin levels were determined to be 7 ± 5 μg mL^{-1} and 183 ± 86 ng per g tissue in the plasma and tumour samples, respectively. In conclusion, they noted that IdB 1016 can be a good candidate with clinical potential in the management of recurrent ovarian cancer.

Similarly, Yanyu et al. (2006) studied the physicochemical properties of silymarin phytosome and compared its pharmacokinetic profile and bioavailability after oral administration with silybin-*N*-methylglucamine in rats. They observed that the bioavailability of silybin was increased remarkably after oral administration of silybin–phospholipid complex compared with silybin-*N*-methylglucamine, and this was attributed to an impressive improvement of the lipophilic property of silybin-phospholipid complex and improvement of the biological effect of silybin.

Self-microemulsifying drug delivery system (SMEDDS)

Self-emulsifying drug delivery systems (SEDDS) are isotropic mixtures of triglyceride oils, surfactants, solvents and co-solvents/surfactants that are used to enhance the bioavailability of hydrophobic drugs (Pouton 1997; Gursoy and Benita 2004; Tang et al. 2008). They can be prepared either in a liquid form or by solidification of liquid/semisolid self-emulsifying (SE) ingredients into powders (Tang et al. 2008). When such a formulation is released into the lumen of the gut it forms a fine emulsion and the active compound remains in solution. By this way, the dissolution step, which often limits the rate of absorption of hydrophobic drugs from the crystalline state, is circumvented (Pouton 1997; Wu et al. 2006).

Wu et al. (2006) prepared silymarin SMEDDS formulations composed of silymarin, ethyl linoleate, Tween 80, and ethyl alcohol. The bioavailability of orally administered silymarin SMEDDS in rabbits, the effect of microemulsion particle size, and release characteristics on absorption were included in their study. Relative bioavailability of silymarin SMEDDS was dramatically enhanced ca. 1.88- and 48.82-fold than that of silymarin PEG 400 solution and suspension, respectively.

In order to improve the dissolution rate of silymarin, Woo et al. (2007) also formulated SMEDDS. The optimum SMEDDS consisted of 15 % silymarin, 10 % glyceryl monooleate as the oil phase, a mixture of polysorbate 20/HCO-50 (1:1) as the surfactant and transcutol as the co-surfactant, with a surfactant/cosurfactant ratio of 1. They observed that after oral administration to rats, the % release of silybin from the SMEDDS after 6 h was 2.5 times higher, and the bioavailability of the drug was 3.6 times higher than the reference capsule.

Finally, another research group developed silymarin SMEDDS with enhanced oral absorption. In their case, the optimal formulation with the best self-microemulsifying and solubilization ability consisted of 10 % ethyl linoleate, 30 % Cremophor EL, and 60 % ethyl alcohol. They observed that the oral absorption of silymarin SMEDDS in fasted dogs was improved about 2.2-fold, and considerably faster, than that from the commercial silymarin preparation hard capsule [Legalon(R)] (Li et al. 2010).

Solid dispersion systems

Solid dispersions systems are one of the most promising strategies for enhancing the dissolution rate and hence the bioavailability of various hydrophobic drugs. These solid preparations consist usually of a hydrophilic matrix and a hydrophobic drug. The matrix can be crystalline or amorphous, while the active compound can be dispersed molecularly, in amorphous particles (clusters) or in crystalline ones (Dhirendra et al. 2009). By reducing drug particle size to the absolute minimum, and hence improving drug wettability, bioavailability may be significantly improved (Vasconcelos et al. 2007). Although these preparations seem to be a competent technique their commercial use remained limited, mainly due to manufacturing difficulties, stability problems, difficulties in dosage form development, poor reproducibility of physicochemical properties and lack of feasibility for scale-up of manufacturing processes (Sethia and Squillante 2003; Sun et al. 2008).

Solid dispersions of silymarin were prepared by Li and Hu (2004) applying the fusion method with the intention of improving the dissolution properties of silymarin. Their results showed that the dissolution rate of silymarin was significantly improved when formulated in solid dispersions with polyethylene glycol 6000 (PEG 6000) compared with the original drug. They observed that the dissolution rate of original silymarin was very low, only 8 % of flavonoids was released within 30 min, and less than 40 % of silymarin released even after 12 h, while for the silymarin solid dispersions about 50 % of flavonoids in silymarin were released within the first 1 h, and the flavonoids released nearly completely after 2 h.

The study of Qiu et al. (2005) describes a new preparation of solid dispersions in the form of "dripping pills" designed to enhance solubility. Dripping pills (3–4 mm diameter), containing 5 mg of silymarin each, were prepared by the traditional fusion method using a mixture of silymarin and PEG 6000 at a 1:4 ratio. The dissolution rate of the silymarin dripping pill was found to be considerably higher (by a factor of 7.5–11) compared with three other silymarin preparations (Yiganling Film-Coating Tablet, Yiganling Sugar-Coating Tablet, and Legalon Capsule).

Nanosuspensions

Although there are various methods that can be employed for the formulation of water-insoluble drugs, no general approach exists that can be applied to all drugs. Nanosuspensions seem to be a promising technology to overcome difficulties when delivering poorly water- and lipid-soluble drugs, due to its simplicity and the advantages over other strategies. Nanosuspensions can be defined as colloidal dispersions of nano-sized drug particles that are produced by a suitable method and stabilized by a suitable stabilizer (Patravale et al. 2004).

Silybin nanosuspensions were prepared very recently by Wang et al. (2010) and the effect of the new formulation on silybin permeation across the Caco-2 cell monolayer was investigated. They observed that nanosuspensions of silybin had a significant effect on drug transport across the Caco-2 cell monolayer and the enhanced dissolution velocity was attributed to the increased permeability. This in vivo pharmacokinetic study further confirmed the in vitro results, and demonstrated that oral administration of silybin nanosuspensions significantly increased its bioavailability compared to silybin coarse powder. Moreover, they found that nanosuspensions with smaller particle size, exhibited greater potential to increase oral bioavailability, while for intravenous infusion the lower pressure produced silybin nanosuspensions appeared to maintain a more sustained drug release profile.

Floating tablets

Drugs that have narrow absorption window in the GI tract will have poor absorption. For these drugs, gastroretentive drug delivery systems offer the advantage in prolonging the gastric emptying time. Swellable, floating, and sustained release tablets are developed by using a combination of hydrophilic polymer (hydroxypropyl methylcellulose), swelling agents (crospovidone, sodium starch glycolate, and croscarmelose sodium) and effervescent substance (sodium bicarbonate). These formulations are evaluated for percentage swelling, in vitro drug release, floating lag time, total duration of floating, and mean residence time (MRT) in the stomach (Arza et al. 2009).

Garg and Das Gupta (2009) designed floating tablets of silymarin with improved bioavailability by prolonging its gastric residence time. The aim of their study was to evaluate gastroretentive floating effervescent and noneffervescent silymarin tablets with increased drug efficacy and stability in the stomach. They examined various materials like hydroxypropyl methylcellulose, psyllium husk, swelling agent as crospovidone and microcrystalline cellulose and gas generating agent like sodium bicarbonate and citric acid. Floating non effervescent tablets were prepared by polypropylene foam powder and different matrix forming polymers like HPMC K 4M, Carbopol 934P, xanthan gum and sodium alginate. They performed in vitro drug release studies and they found that the drug release pattern could be efficiently adjusted by altering formulation parameters such as concentration of swelling agent and β-cyclodextrin, type of matrix forming polymers and blend of matrix forming polymers. They concluded that the developed silymarin floating tablets could be used in clinical applications for prolonged drug release for at least 24 h, thus improving the bioavailability and patient compliance.

Micronization

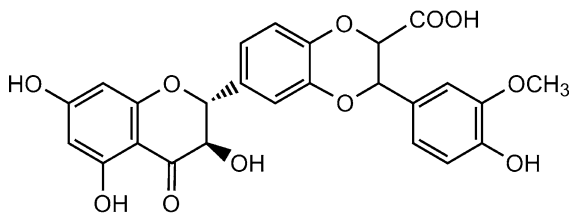
As already mentioned, the bioavailability of a sparingly soluble drug is often limited by the rate of dissolution of the drug substance. Drugs in a micronized form can also be employed to maximize their bioavailability. Recently, micronized spherical and rod-shaped silybin particles were successfully prepared using sodium dodecyl sulfate (SDS) by applying the emulsion solvent diffusion method. The dissolution tests conducted shown that both types of micronized silybin particles displayed considerably enhanced dissolution rate when compared with commercial silybin powder (Zhang et al. 2009).

Silybin synthetic derivatives

Hydrophilic derivatives

Biological availability of pharmaceuticals can be enhanced via introduction of hydrophilic moieties in the molecule and the therapeutic action can be prolonged via introduction of protecting groups. Side effects of drugs may be reduced and drug stability may be increased by modification of the parent molecule (Giri et al. 2001). In 1985, the introduction of Legalon-SIL (silybin 3,23-*O*-bis-hemisuccinate), enabled intravenous application of silybin (Madaus GmbH, DE) for the treatment of acute liver intoxication of death cap fungus poisoning. Another water-soluble semisynthetic derivative, 23-*O*-phosphate of silybin, displayed better water solubility; however, its hepatoprotective activity was inferior to that of the original drug. The preparation of silybin glycosides was demonstrated to be another way of improving the water solubility of the parental molecule (Křen et al. 1997; Kubisch et al. 2001; Kosina et al. 2002).

Glycosylation often improves solubility of various drugs without affecting their activity, while respective glycosides may act as pro-drugs. Moreover, β-galactosylation can improve the drug targeting to the liver cells known to contain high concentrations of β-galactoside receptors (Křen et al. 1997). Křen et al. (2009) synthesized silybin glycosides, 23-*O*-β-glucoside, β-galactoside, β-lactoside and β-maltoside. Phenolic glycosides are often unstable and more acid labile therefore primary alcoholic group at C-23 was chosen as the target for glycosylation. Finally, optimized method using peracetylated glycosyl donors under catalysis of $BF_3.Et_2O$ enabled selective glycosylation without the need of protection/deprotection of silybin. Moreover, peracetylated silybin glycosides enabled separation of both silybin diastereomers. These silybin glycosides were 4–30 times more water soluble and their hepatoprotectivity was increased compared with the parent compound silybin. The glycosides are potent antioxidants and free radical scavengers in several in vitro models. Increase in the water solubility due to glycosylation potentiated the free radical scavenging properties of silybin namely in the hydrophylic compartments. In the cellular models used, silybin glycosides displayed slightly better cytoprotective activity than silybin. Since



7 Silybinic acid

Fig. 4 Structure of silybinic acid

glycosylation reduced silybin toxicity in primary hepatocyte cultures, silybin glycosides appear to be promising for long term studies (Kosina et al. 2002).

Gažák et al. (2004) synthesized silybinic acid (**7**) with improved water solubility (Fig. 4), by selective oxidation of the parental molecule at C-23, and investigated their antioxidant and cytoprotective activities. It was expected that these compounds bearing easily ionisable carboxy functionality would be more hydrophilic while maintaining the beneficial biological effects of silybin. However, modifications of silybin molecule leading to an increase of its water solubility usually lead to an impairment of its antioxidant (antiradical) activity in the lipophilic milieu (Gažák et al. 2010b).

Lipophilic derivatives

Lipophilization of the silybin molecule by its conjugation to fatty acids cannot only improve its antioxidant but also its anti-influenza virus activities. The aim of the study conducted by Gažák et al. (2010b) was to develop methods for selective acylation of silybin with long-chain monocarboxylic acids and compare the antioxidant and anti-influenza virus activities of the novel derivatives to silybin. An additional aim of this work was to determine the effect of the acyl chain length on the biological properties of the synthesized silybin lipoderivatives. They presented two selective acylation methods for silybin esterification with monocarboxylic acids, yielding a series of silybin 7-*O*- and 23-*O*-acyl-derivatives of different acyl chain lengths. They demonstrated that the length of the acyl chain is decisive for lipid peroxidation inhibitory activity, with the derivatives bearing short acyl chains (C4–C8) being inferior inhibitors than the parent silybin and longer acyl derivatives (C12 and mainly C16) better inhibitors. Esterification of silybin by the short-chain acids probably improves its interaction with biomembranes compared with silybin itself; however, it simultaneously reduces its mobility and hinders its ability for self-stabilization by dimerization. Long-chain fatty acid esters could probably restore this ability due to higher lipophilicity and better mobility within membranes; thus leading to higher inhibitory activity in both types of silybin substitutions. The results of experiments focusing on the inhibition of the lipid peroxidation of microsomes induced by tertbutylhydroperoxide proved that the length of the acyl chain of the corresponding ester has a decisive role in this activity. However, the site of silybin acylation was found to be less significant in achieving a high inhibitory activity (Gažák et al. 2010b).

New silybin derivatives, with different patterns of substituents such as aliphatic and aromatic groups at the position C-23, were prepared by Huang et al. (2009) with the purpose of changing the chain length and modulating the electron density of the lead structure in anticipation for better activities. Twelve 23-esterified silybin derivatives with different patterns of substituents such as aromatic and aliphatic groups were designed and synthesized. New silybin derivatives exhibited improved inhibitory effects against rat liver homogenate lipid peroxidation compared with silybin and displayed their protective properties on DNA cleavage in a dose-dependent manner.

An oxidized form of silybin 2,3-dehydrosilybin (**5**) (Fig. 1b), which represents a minor component in silymarin (Bandopadhyay et al. 1972), has significantly better anticancer and antioxidant activity than silybin (Huber et al. 2008; Thongphasuk et al. 2008, 2009; Gažák et al. 2004.; Zatloukalová et al. 2011). Moreover, some *C*-prenylated (Maitrejean et al. 2000) and certain *O*-alkylated (Džubák et al. 2006) derivatives of 2,3-dehydrosilybin constitute effective modulators of P-glycoprotein (Pgp). Pgp-like transporter in *Leishmania* spp. was also found to be inhibited by 8-prenyl-dehydrosilybin (Pérez-Victoria et al. 2001) that led to the parasite sensitization towards daunomycin. 2,3-Dehydrosilybin itself was also an effective inhibitor of chloroquine-sensitive and -resistant *Plasmodium* strains (Monbrison et al. 2006).

2,3-Dehydrosilybin is also shown to be a potent protectant against H_2O_2-induced oxidative stress in human keratinocytes and mouse fibroblasts (Svobodová et al. 2006), and to suppress UVA-caused oxidative stress and may be used in the treatment of UVA-induced skin damage (Svobodová et al. 2007). Besides the skin, 2,3-dehydrosilybin decreases ROS formation also in the isolated rat heart mitochondria, and causes the increase in respiration and the decrease in the membrane potential of these organelles (Gabrielová et al. 2010).

Silybin and 2,3-dehydrosilybin were identified as strong inhibitors of glucose uptake in multiple cell lines, including adipocytes and Chinese hamster ovarian (CHO) cells, by direct competitive inhibition

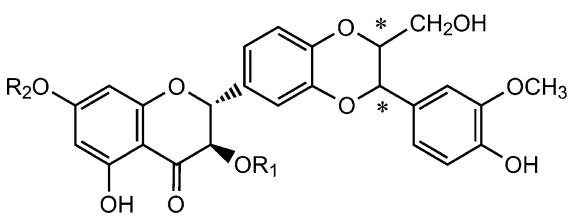
of GLUT4-mediated transport (Zhan et al. 2011). The reduction in glucose availability caused by silybin and more effectively by 2,3-dehydrosilybin treatment represents a new possible explanation for the anti-cancer effects of these flavonolignans.

Silybin biotransformation

Biotransformations may be used to carry out several reactions (e.g. regio- and stereoselective hydroxylation, oxido-reduction, hydrogenation, glycosylation, methylation, acylation, amino-acylation) of a wide range of substrates using as catalysts plant, animal or microbial cells or purified enzymes at non extreme pH values and temperatures (Sasson 1991; Ishihara et al. 2003). The production of totally new drugs, and the modification of already existing ones, is usually extremely complex and expensive when chemical synthesis is employed. For that reason, biotransformations have enormous potential for the production of more drastic or less toxic compounds, and the selective conversion of compounds to useful derivatives which are difficult to produce synthetically (Giri et al. 2001; Kim et al. 2006).

Microbial transformations

Chemical synthetic approaches to obtain new silybin derivatives would engage impractical complicated protection/deprotection procedures (Křen et al. 1997). Silybin biotransformation by microorganisms may offer prospects to obtain novel metabolites with higher water solubility, improved bioavailability and therapeutic efficiency. Kim et al. (2006) using *Trichoderma koningii* KCTC 6042 prepared silybin glucopyranosides from optically pure silybins (Fig. 5) reaching yields that ranged from 5 to 18 % with the preference for the C-3 position. Substrate and culture controls demonstrated that the metabolites were formed as a result of enzymatic activity, but not as a consequence of degradation or non-metabolic changes. Differences in this silybin A and silybin B biotransformation were not observed.



8 R_1 = β-Glc, R_2 = H Silybin 3-*O*-β-D-glucopyranoside
9 R_1 = H, R_2 = β-Glc Silybin 7-*O*-β-D-glucopyranoside

Fig. 5 Structures of silybin glucopyranosides obtained by *Trichoderma koningii* biotransformation of silybin (Kim et al. 2006)

Abourashed et al. (2012), performed recently bioconversion of silybin (mixture A and B) using a series of fungal cultures. The screening afforded four strains producing new metabolites (Fig. 6); i.e., *Beauveria bassiana* oxidized silybin to 8-hydroxysilybin (**10**), *Cunninghamella blakesleana* yielded silybin-7-sulfate (**11**) and 2,3-dehydrosilybin-7-sulfate (**12**) and *Cunninghamella* sp. afforded 2,3-dehydrosilybin-3-*β*-*O*-glucopyranoside (**13**). The antioxidant DPPH radical scavenging activity of all the new compounds that could be Phase I and Phase II metabolites of silybin was evaluated. They observed that metabolite **10** was tenfold better radical scavenger than silybin, whereas sulfate **11** was threefold inferior than silybin. Sulfate **12** was threefold better scavenger than silybin but this is caused presumably by the 2,3-enolic moiety as 2,3-dehydrosilybin is up to 25-fold better radical scavenger than silybin (Gažák et al. 2004).

Cultured plant cells

Glucosylation of silybin at the phenolic OH group at C-7 has been achieved using a plant cell culture of *Papaver somniferum* (Křen et al. 1998), and this is probably the first study using optically pure silybin. Another enzymatic extension of silybin-23-*β*-glucosides using cyclodextrin glucanotransferase afforded oligomaltosides of silybin with very good water solubility (Kubisch et al. 2001), while the regioselective formation of silybin-23-*O*-β-D-glucoside by glucosylation of silybin with cultured plant cells of *Eucalyptus periniana* was reported very recently (Shimoda et al. 2012).

Lipases

Lipases proved to be effective not only for the preparation of acylated silybin derivatives with novel biological properties but also for the selective deacylation of the molecule to obtain selectively protected

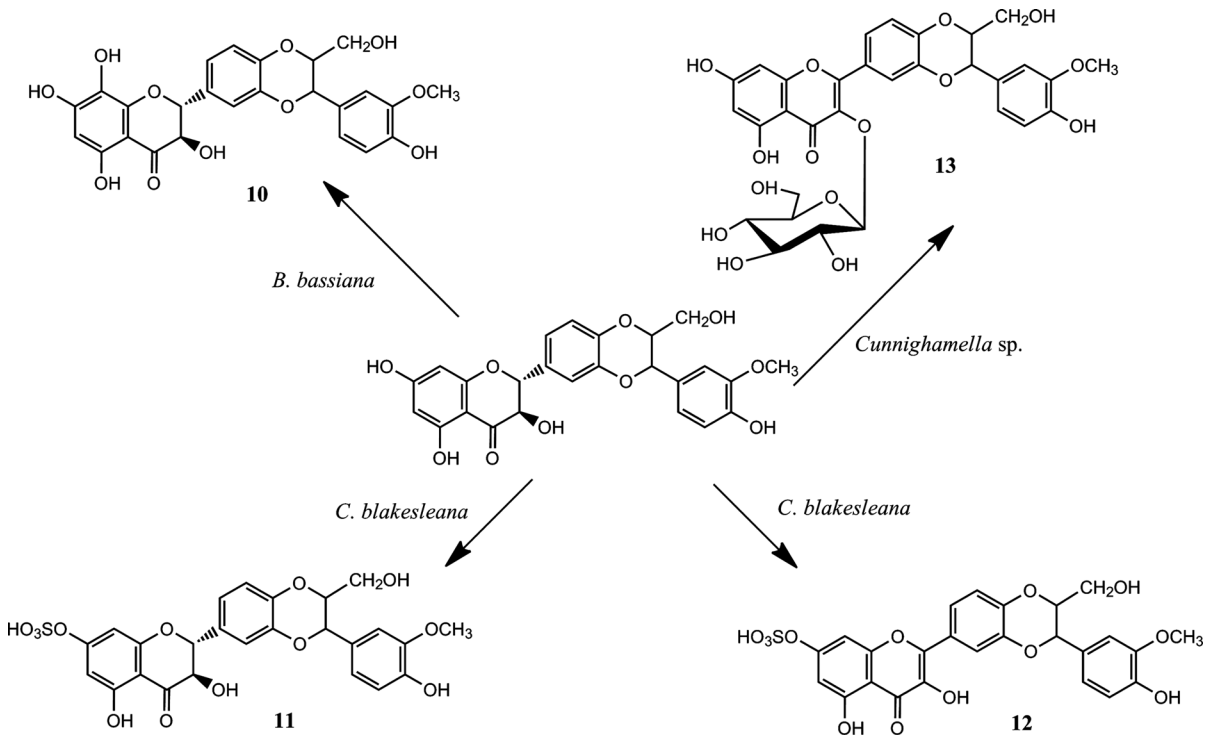


Fig. 6 Silybin Phase I and Phase II microbial metabolites of silybin (Abourashed et al. 2012)

silybin. Silybin bears 5 various OH group, therefore protection/deprotection strategy is of utmost importance in most of the modifications of this molecule. Peracetylated silybin A and B was selectively deacetylated using a large panel (24) of lipases (Purchartová et al. 2011). Lipase AK (*Pseudomonas* sp., Amano) proved to be efficient in selective deacetylation (methyltertbutyl ether:BuOH 9:1 v/v) of silybin pentaacetate yielding thus 3,5,20,23-tetra-*O*-acetyl-silybin A and B and 3,20,23-tri-*O*-acetyl-silybin A and B. The formation of both silybin acetates is kinetically controlled enabling the direct preparation of each derivative in sufficient purity.

Candida antarctica lipase B (Novozym®435) was used in a study for the preparation of novel acylated silybin derivatives using free fatty acids or their esters, in organic solvents and imidazolium-based ionic liquids comprising of either BF_4^- or PF_6^- anions. It was found that the synthesis of the silybin esters depended both on the reaction medium and the acyl donor used. The anti-proliferative effect of the enzymatically synthesized derivatives, 23-*O*-butyryl-silybin and 23-*O*-lauryl silybin, was also investigated using K562 human leukemia cells, and was found that they induced a concentration-dependent cell growth inhibition, though with different degrees of efficiency. The new lipophilic silybin analogues retained partly the tumor growth inhibitory activity of the parental compound, and the length of the acyl donor seemed not to dictate this activity in an important extend (Theodosiou et al. 2009).

Acylated derivatives of silybin with dicarboxylic acids (hexanedioic, dodecanedioic and hexadecanedioic acid) were also prepared using Novozym®435 in various organic solvents. In this study, both silybin monoesters and diesters were identified as well as the oxidized form 2,3-dehydrosilybin. Higher conversion yields were obtained using acetone or acetonitrile as reaction medium while the enzymatic process depended on the chain length of the acyl donor. The anti-proliferative effect of the derivatives, as well as the ability to modulate the secretion of vascular endothelial growth factor (VEGF) were estimated using K562 human leukemia cells, and it was found that the biological function of silybin was not only retained but in some cases (silybin monoester

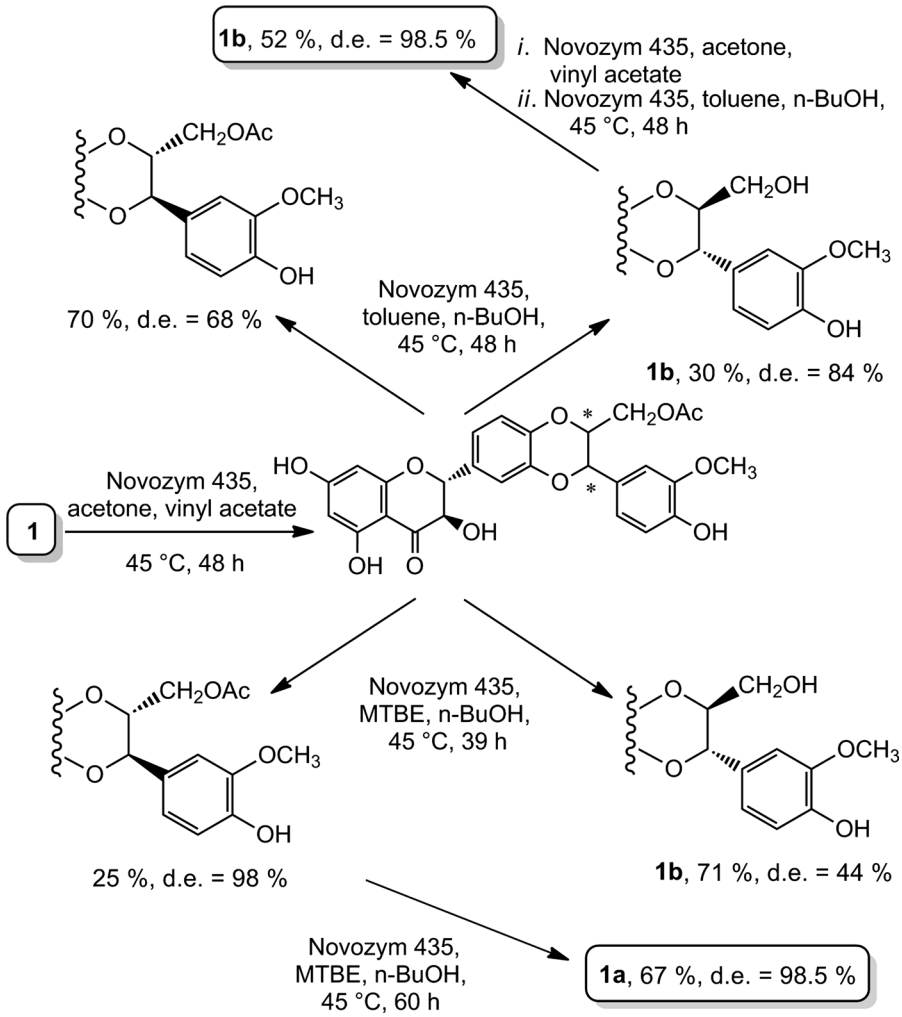


Fig. 7 Separation of silybin A and B using selective acylation/deacylation with Novozym®435 (Gažák et al. 2010a)

with hexadecanedioic acid) was more effective, indicating that they might possess improved anti-angiogenic and anti-tumor properties (Theodosiou et al. 2011).

Lipases and proteases in diastereomeric discrimination of silybin A and B

Lipases and proteases proved to be highly efficient in the chiral separation of a large number of racemates (Carrea and Riva 2000). In addition, enzymatic acylations of flavonoids have been used for various compounds and systems (Chebil et al. 2006; Xanthakis et al. 2010). Silybin as flavonolignan possesses rather specific features that distinguish it from most other flavonoids, e.g. the presence of a primary alcoholic group enabling a variety of derivatizations. Křen and coworkers applied this methodology for the preparative separation of silybins A and B in sufficient amounts and with high diastereomeric purity. A panel of 26 lipases and proteases has been, therefore, tested both for selective 23-*O*-acylation and deacylation of diastereomeric mixture of respective silybin derivatives (Monti et al. 2010). Only a few enzymes were able to transacetylate silybin (acetyl donor vinyl acetate) or cleave silybin-23-*O*-acetate (*n*BuOH) probably due to lipase inhibition by flavonoids (Sharp et al. 2007). Novozym®435 and lipase from *Candida rugosa* proved to be suitable for chiral discrimination of both silybin diastereomers. Novozym®435 proved to be the most suitable enzyme by means of variation and combination of various reaction conditions and solvents; thus it was chosen for the design of a high-yielding preparatory procedure for the separation of

silybin A (**1a**) and B (**1b**) in multigram quantities (Fig. 7) (Gažák et al. 2010a). This enzyme can be used also for a preparative separation of other silybin congeners, such as isosilybin A and B (Gažák et al. 2013a).

Conclusions

The primary objective of this review article has been to present available methodologies, ranging from various formulations to synthetic or enzymatic structural modifications, which can be applied for the improvement of silymarin and silybin bioavailability, and concomitantly therapeutic efficacy. All these approaches attempt to confront the reduced silymarin (as well as silybin) bioavailability that is mainly due to (1) low water solubility, (2) intensive Phase II metabolism, (3) rapid excretion in bile and urine, and (4) low permeability across intestinal epithelia cells. Herein, the high necessity of using optically pure diastereomers of silymarin (silybin A, silybin B, isosilybin A, isosilybin B) in various pharmacological implications is stressed, since they display different biological properties. Only in this way we can draw concrete conclusions and take the maximum therapeutic benefit of these compounds.

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Biotransformation of Silybin and its Congeners

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Abstract: Silybin and its congeners belong to a group of flavonolignans with strong biological activities. These compounds are potentially applicable in human medicine, e. g. due to their cytoprotective activity. As a part of herbal preparations available on the open market, they face the risk of potential negative drug-drug interactions. This review aims to evaluate current knowledge on the metabolism of these compounds by biotransformation enzymes, interactions with other drugs, their pharmacokinetics, and bioavailability. While silybin and its derivatives interact with cytochrome P450s, only metabolism of silybin by cytochrome P450 2C8 poses a risk of adverse effects. The main biotransformation route of silybin and derivatives was identified as conjugation, which is sterospecific in case of silybin. Studies of the metabolism, pharmacokinetics, potentional drug – drug interactions and increasing bioavailability of these flavonolignans play an important facet of possible therapeutical use of these compounds. The goal of our review is to aid future developments in the area of silybin research.

Keywords: Silybin, silibinin, silymarin, biotransformation, diastereoisomers, sulfonation, glucuronidation, cytochrome P450.

1. INTRODUCTION

The flavonolignan silybin (CAS No. 22888-70-6; in pharmacological literature also denoted as *silibinin*) and its congeners such as 2,3-dehydrosilybin, isosilybin, silydianin and silychristin are important natural compounds obtained from silymarin, an extract from the seeds of *Silybum marianum* (L.) Gaertn. (*Carduus marianus* L., Asteraceae; milk thistle). While both silybin and silymarin are fully characterized, the former a defined chemical substance the latter a complex mixture of flavonolignans containing approximately 30% of silybin, the terms silybin and silymarin are often muddled [1, 2]. In the last decades, silymarin and silybin have been in the public eye mainly because of marketing which claims multiple beneficial biological activities. Originally known for its anti-phalloidin activity [3], and usable for the treatment of various liver disorders (for a review, see [4]), silybin and its congeners, frequently as constituents of milk thistle extract, were later identified as antioxidants with cytoprotective effects [5, 6] and also possible hypocholesterolemic drugs [7, 8]. One of the first reports on tumor inhibition by silymarin components in 1994 by the Agarwal group [9] triggered broad interest in the detailed study of these activities. More recently, silybin derivatives prepared by chemical and enzymatic methods garnered attention thanks to their anticancerogenic activity [10, 11]. The cytoprotective activity of silybin is thought to be mainly mediated by its antioxidative and radical-scavenging properties but new biological effects based on interaction with a specific receptor were discovered, e.g. estrogenic, antiangiogenic, drug transporter modulation (P-glycoprotein; P-gp), and acting on DNA-expression via nuclear factor-κB (NF-κB) suppression (as reviewed in [12]). Silybin may be applied in the treatment of other diseases besides the liver and gastro-intestinal disorders, e. g., pancreatic problems and balancing glycaemia, kidney and lung diseases, treatment of various disorders of the prostate including adenocarcinoma, and, moreover, in dermatology and cosmetics. Administration of silymarin (or silybin) proved to be often beneficial for cancer patients – either by releasing negative side effects of chemotherapy or by potentiation of anticancer treatment [12].

The molecular mechanism of silybin action is not completely clear; partly because it must be studied with scarcely available (optically) pure compounds and partly due to its rather pleiotropic activity. There are speculations that the broad activities of silybin may be explained by its non-specific interaction with ATP binding sites or with estrogenic receptors. Only antioxidant and antiradical mechanisms were investigated and described in detail [13, 14] 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. The oxidation of silybin to 2,3-dehydrosilybin creates a new strong antiradical center- the enolic C-3 OH that has strong antiradical activity [14], even at low pH.

Silybin and its congeners undergo complex biotransformation processes and interact with both phase I. and II. enzymes. Due to the complex nature of the silybin molecule, which has five OH groups and four stereogenic centers, its biotransformation and interactions with other (macro)-molecules are very complex. Silybin, isosilybin, silychristin and isosilychristin occur in silymarin as pairs of diastereomers, denoted A and B (Figs. **1** and **2**). Stereochemistry of silybin is of the utmost importance, as it is now clear that the stereomers silybin A (**1a**, SilA) and silybin B (**1b**, SilB) are metabolised in completely different ways, both in terms of pharmacokinetics [15, 16] and pharmacodynamics, yielding different metabolites [17].

The topic of silybin has been attracting increasing attention, reflected in a high number of papers published recently (over 200 papers per year on average; e.g., 277 papers in 2012 (ISI - Web of Science)). The emerging role of silybin adjuvant cancer therapy or even a potential therapy on its own motivated us to conduct this review on metabolic transformations of silybin and its congeners present in silymarin complex.

2. DIASTEREOMERS OF SILYBIN AND THEIR BIOLOGICAL ACTIVITY

Silybin exists in nature as an approximately equimolar mixture of two diastereoisomers, silybin A (SilA, **1a**) − (2*R*,3*R*)-2-[(2*R*,3*R*)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-

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benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one − and silybin B (SilB, **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**). Typically SilB is slightly prevalent in most preparations. Taking this fact into account is of utmost importance in any biological study or any interaction with an anisotropic system; the existence of two silybin diastereomers may not be neglected in metabolic studies either. It has been clearly demonstrated that SilA and SilB have different metabolic profiles in the pioneering work by Weyhenmeyer *et al.* [15] - at that time authors referred to the silybin diastereomers as Isomer 1 and 2 without knowing their absolute configuration. We surmise that these isomers were SilA and SilB, respectively, as inferred from the order of elution of the respective peaks in HPLC profile *cf.* [16, 18, 19].

Silybin and other major flavonolignans contained in silymarin, i.e isosilybin, silychristin, and silydianin, are formed by the oxidative (radical) coupling of coniferyl alcohol to the catechol moiety of taxifolin. This reaction is not stereospecific [20] and thus all respective flavonolignans occur in the silymarin complex in diastereomeric pairs, always in the *trans*- configuration, but in various proportions.

However, until recently both SilA and SilB were technically inseparable, except *via* preparative HPLC that gave milligram amounts of each.

Most biological tests were performed with natural silybin, which is a natural mixture of SilA and SilB, except for a minor number of studies [17, 21-23]. In many instances, however, the silymarin complex was used in experiments. The following examples illustrate the importance of the use of optically pure stereoisomers of silybin. In a study of human prostate cancer, it was shown that the tested substances - SilA and SilB, isosilybin A and B, and other components of silymarin - are effective at suppressing the expression of topoisomerase II α gene in DU145 cells (androgen-independent prostate cancer cell line). Isosilybin B and SilB were the most active in the G1 phase of the cell cycle of DU145 cells [21]. Moreover, it was found that SilB interacts with the estrogenic receptor while SilA is inactive [22]. Recently, we demonstrated that SilB (**1b**) and some of its galloyl derivatives have strong antiangiogenic activity, whereas SilA (**1a**) is almost inactive [23]. These and also other studies demonstrate that the stereomeric structures of silybin and isosilybin have a profound influence on their biological activities. Therefore any proper biological study must be done with pure stereomers.

High demand for optically pure silybins stimulated research in this field. Upscaling the analytical HPLC methods enabled minor amounts of the optically pure compounds to be obtained. The first successful preparatory method yielding optically pure silybins based on the chromatographic separation of silybin glycosides was published as early as 1997 [24]. At that time the absolute configuration of the respective silybins was not known, the pure silybins were denoted A and B. However, Lee *et al.* [18] and Kim *et al.* [25] used repeated HPLC separation and determined the absolute configuration of silybins and isosilybins, but unfortunately used the opposite notation to the previous study [24] - as inferred from the optical rotations of SilA and SilB. Therefore, in the literature before 2003, the nomenclature for SilA and SilB is reversed. Optical rotation ($[\alpha]_D^t$) is the easiest way to assign the absolute configuration of both compounds: natural silybin (a mixture of A and B in the ratio ca 1:1) has $[\alpha]_D^{23}$ = +11.4 (c = 0.29, acetone) [24], silybin A has a higher $[\alpha]_D^{23}$ = +20 (c = 0.21, acetone) than the mixture, and silybin B has a lower $[\alpha]_D^{23}$ = - 1.07 (c = 0.21, acetone) [18].

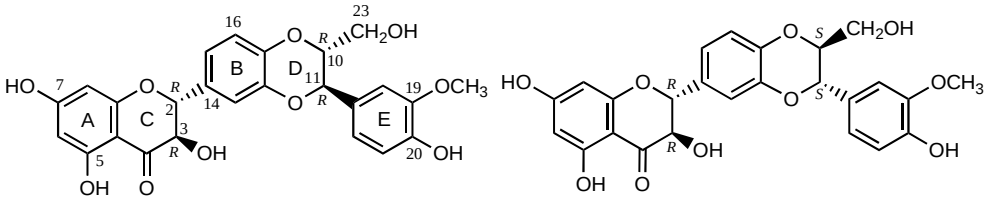
Later, a chemoenzymatic method for the preparatory separation of SilA and SilB using β-galactosidases was published [19] and this provided preparatory amounts of SilA and SilB on a large scale, however in unsatisfactory purity (under 95%). Therefore, a new and very efficient method for separating silybin diastereomers based on the use of lipases was developed. This method employs the selective alcoholysis of 23-*O*-acetylsilybins with Novozym 435 (lipase B from *Candida antarctica*) [26]). The method was then scaled up yielding multigram amouts of SilA and SilB in 99+ % purity [27]. These methods were the first to enable the large-scale use of optically pure silybins in animal tests and in pharmacological preparations including synthetic procedures.

3. METABOLIC BIOTRANSFORMATIONS OF SILYBIN AND ITS CONGENERS

Investigations into biotransformations of silybin and its congeners encompass experiments conducted *in vitro*, usually employing individual substances, as well as *in vivo*, often in the form of a milk thistle extract. Risks in extrapolating the *in vitro* findings to the *in vivo* situation or clinical applications are hidden mainly in the uncertain concentration of multiple constituents in the target tissue, e.g. hepatic. Potential risks identified for specific substance at a well defined concentration range maybe irrelevant *in vivo* because the *in vitro* experimental design does not account for multiple issues including but not limited to the composition of milk thistle extract, bioavailability of individual constituents and tissue distribution. Therefore *in vitro* experiments only point out potential risks that need to be evaluated *in vivo*. Challenges of *in vitro* methodologies used for evaluation of herbal products in general are discussed in a recent review [28].

3.1. Interaction with Phase I. Biotransformation Enzymes

The metabolic detoxification of any compound entering the human body includes various mechanisms one of which involves a broad class of monooxygenases known as cytochrome P450s (CYPs). These enzymes are often the first line of defense against xenobiotics and are consequently labeled phase I. biotransformation enzymes. Only a few are considered crucial for drug metabolism forming a group of CYPs involved in negative side effects of drugs or unwelcome drug-drug interactions. Of these major CYPs many are expressed in hepatocytes, with a smaller number found in the intestine as well as other extrahepatic tissues [29]. They comprise,



1a Silybin A (2*R*, 3*R*, 10*R*, 11*R*) **1b** Silybin B (2*R*, 3*R*, 10*S*, 11*S*)

Fig. (1). Structures of silybin **A** and **B**.

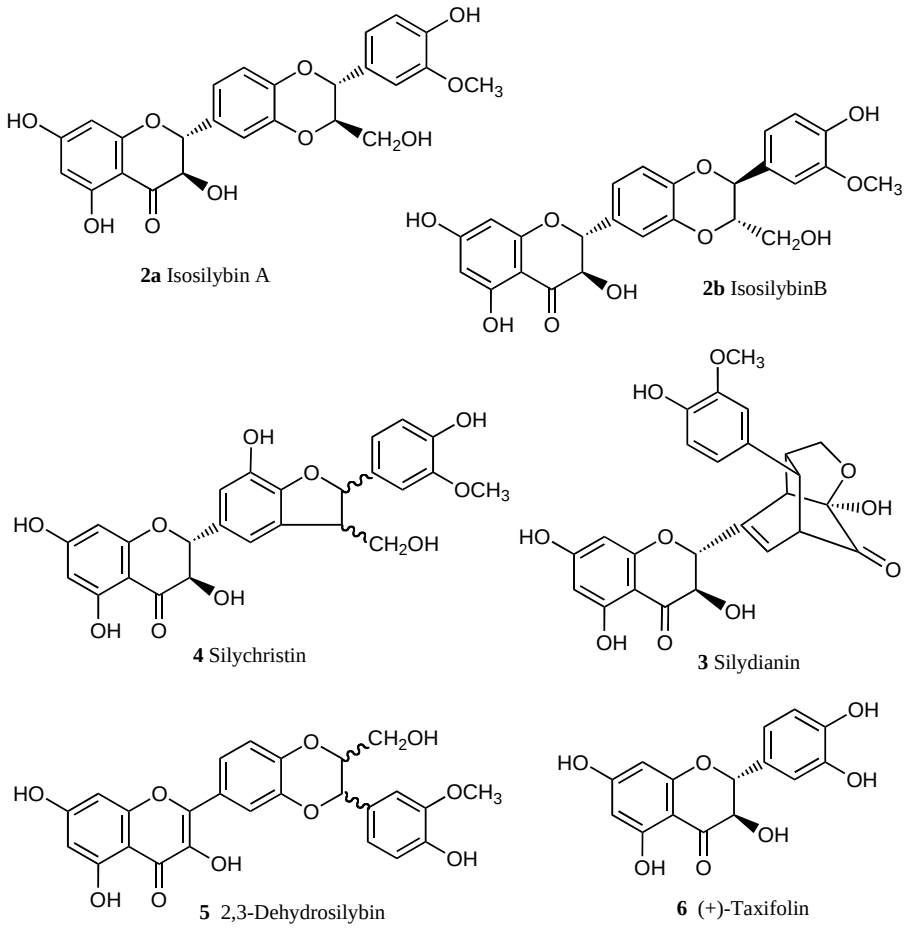


Fig. (2). Structures of the major components of silymarin, *i. e*. silybin congeners.

in particular, CYP1A1/2, CYP2C8/9, CYP2D6, CYP2E1, and CYP3A4 enzymes. CYPs are able to biotransform silybin and its congeners, despite multiple hydroxyl groups already present in the molecule (Fig. **1**).

Studies on the interactions of silybin and its congeners with CYPs have only been done in the last two decades, ever since supplements containing milk thistle extract became a food supplement for patients with hepatic disorders. But potential use in adjuvant cancer therapy calls for attention of possible drug-drug interactions.

Recombinant CYP1A1 activity is inhibited by silybin and even more potently by dehydrosilybin [30]. In contrast, *in vitro* investigations showed negligible effect on CYP1A2 catalytic activity by silybin or silymarin [31-33]. Lack of effect was confirmed *in vivo*, suggesting that milk thistle supplementation poses negligible risk of herb-drug interactions [34]. Silymarin reduced the induction in CYP1A2 expression induced by pyrogallol and rifampicin co-treatment, which reduced hepatotoxic activity of these compounds [35]. On the other hand, no effect of silybin and its glycosides on CYP1A2 expression was observed in human hepatocytes [36]; thus, the hepatoprotective effect of silybin seems to be based on its antioxidant activity.

Interestingly, the influence of silybin and its congeners on the activity and expression of CYP2C8 is mostly ignored in publications. Therefore discovering CYP2C8 as the enzyme responsible for the *O*-demethylation of silybin in 2007 was rather surprising [33] and stipulated the issue of a comprehensive study on this topic [37].

It was shown that silybin or silymarin may inhibit CYP2C9 activity *in vitro* [31, 32, 38], which is clinically relevant in relation to (*S*)-warfarin metabolism and poses a threat of negative side-effects [39]. Metronidazole, an antibacterial (anaerobic), antiprotozoal and amoebicide drug is metabolized to hydroxymetronidazole. The biotransformation of metronidazole is related to CYP2C9. Interaction of silymarin with **metronidazole** (Flagyl) was studied in detail [40]. Silymarin pretreatment increased the clearance of metronidazole and the metabolite, hydroxymetronidazole by 29.51% and 31.90%, respectively, with a decrease in half-life, C-max and AUC (0-48). Urinary excretions of metronidazole and its metabolites were decreased after 48 h.

Important anticancer drug is **cisplatin**, it interacts with CYP2C9 and CYP2D6. Cisplatinum-induced nephrotoxicity was significantly reduced by silymarin in rats [41]. Silybin increased cisplatinum cytotoxicity in ovarian cancer cells and increased the cytotoxicity of doxorubicin in MCF-7 doxorubicin-resistant breast-cancer cells [42]. When combining silybin with cisplatin, a dose-dependent significant increase in cisplatinum activity was observed in *in vivo* experiments [43]. The co-administration of both drugs potentiated the anticancer activity. Interestingly, mice receiving the combination recovered earlier in terms of body weight loss than cisplatin-treated mice. The data suggest that silybin or silymarin may modulate CYP-mediated metabolism of the co-administered drug while maintaining their cytoprotective activity.

Indeed, **doxorubicin**, one of the most widely used cancerostatics, is metabolized by CYP2C8 and CYP3A4. The patients suffer from cardiotoxicity which is unrelated to the mechanisms of doxorubicin cancerostatic activity. It was demonstrated that silymarin and the respective flavonolignans display a dose-dependent cytoprotection against doxorubicin [44]; of the pure flavonolignans

tested - silybin, dehydrosilybin, silychristin and silydianin - the last one produced the best effect without compromising doxorubicin activity.

The inhibition effects of silybin or silymarin on *in vitro* transformations mediated by CYP2D6 have K_i in the low [31, 45] to high micromolar range [32]. The *in vivo* effect, however, is claimed to be negligible because the active concentration values found exceeded those present in plasma. This was also confirmed *in vivo* [34].

CYP2E1, responsible for the metabolism of small molecules such as CCl_4 or acetaminophen, was considered as a probable target of inhibition by silybin and its congeners However, Miguez *et al*. [46] showed that silymarin's hepatoprotective activity is not associated with modulating reactions with CYP2E1 and this conclusion was confirmed by other studies [31, 32, 45]. Only a high concentration of silybin was shown to affect CYP2E1 when the metabolism of chlorzoxazone was followed [32], and similar results were obtained when *p*-nitrophenol metabolism was monitored [45][40]. In summary, these studies offer evidence that silybin and its congeners are not metabolized by CYP2E1. On the other hand, silybin may interact with the cytochrome directly according to *in vitro* model [47]. However, the same group of authors admitted that CYP 2E1 protein expression was not significantly reduced in *in vivo* model, in both female and male tumor-bearing mice. Moreover, they reported that dietary silybin did not reduce the activity of CYP2E1 induced by diethylnitrosamin or by tumor progression. In addition, no differences between sexes were observed and no aggravating effect of ethanol was displayed [48].

CYP3A4 is considered the most important known CYP thanks to its involvement in the metabolism of most drugs. Evidence was found on CYP3A4 inhibition *in vitro* [31, 32, 38, 45, 49, 50] and silybin was shown to be a mechanism-based inactivator of CYP3A4 [38]. Follow-up systematic study demonstrated mechanism-based inhibition of recombinant CYP3A4 by the semipurified extract of silybin and its constitutents SilA and SilB [51]. *In vivo* experiments proved the assumption that silybin may not efficiently inhibit CYP3A4 in humans due to its low concentrations.

Silymarin does not significantly influence the pharmacokinetics of **irinotecan**, a campthotecin derivative metabolized by CYP3A4, in cancer patients [52]. Similarly, the influence of silymarin on the pharmacokinetics of **indinavir,** an HIV protease inhibitor used as an antiviral drug and a known CYP3A4 substrate, was investigated in healthy volunteers [53, 54]. 175 mg of silybum seed extract (equivalent to 153 mg silymarin) administered three times a day for 3 weeks caused a 9% and 25% reduction in the indinavir AUC and mean trough level, respectively [53]. It was concluded that the interaction was minimal and silymarin does not interfere with indinavir therapy of AIDS. Silybin had little effect on CYP3A4 *in vitro* [54]. Evaluation of four herbal supplements one of which was milk thistle found no influence on midazolam, a probe substrate of CYP3A, in healthy human volunteers [34].

Recent studies in rats found significant effects of silybin on loratadine [55], paclitaxel [56], and tamoxifen bioavailability [57] or pharmacokinetics, identifying the interaction with CYP3A4 as the culprit.

There was no effect noted on the induction or down-regulation of CYP3A4 by silybin-related compounds in primary human hepatocytes [36]. However, recent study that employed LS180 colon adenocarcinoma cells identified silybin and isosilybin as strong inhibitors of PXR-mediated CYP3A4 induction. Computational predicted binding affinity of silybin and isosilybin to PXR was comparable to hyperforin, a known PXR activator [58].

In sum, it appears that silybin interacts with only a low number of CYPs (Table **1**) and the *in vivo* data suggest adverse interactions of silybin with drugs metabolized by CYP2C8/9. We list select drugs that are in wide clinical use and may display drug-drug interaction when co-administered with silymarin or its individual flavonolignans (Table **2**). Our conclusion is in agreement with the overview of *in vivo* studies of possible drug-drug interactions of milk thistle extract [59]. The main reason is likely *O*-demethylation of silybin mediated by CYP2C8, the only phase I. biotransformation known to date [33]. The production of demethylsilybin (*nor*-silybin) was confirmed by MS methods (See Fig. **3**). This metabolite was also verified by Gunaratna *et al*. [60] who performed an LC-MS study. Other hydroxylated metabolites of silybin were also identified but without the identification of exact metabolite structures [60]. Although studies in laboratory animals may suggest potential drug-drug interactions *in vivo* human studies are needed for verification. For example the suggestion of potential interactions made in case of CYP3A4 [55-57] may not be a high risk because of human *in vivo* study [34]. Table **2** lists drugs with high-risk drug-drug interaction when co-administered with silybin or its congeners.

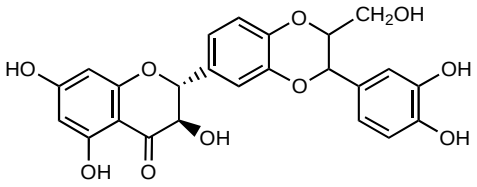


Fig. (3). Structure of the major silybin metabolite - *nor*-silybin.

3.2. Interaction with Phase II. Biotransformation Enzymes

Polyphenolic substances are an easy target for conjugation reactions, and these are indeed the major biotransformation pathway leading to their excretion from the human body. Generally, UDP-glucuronosyl transferases (UGT), sulfotransferases (SULT), *N*-acetyl transferases (NAT), glutathione *S*-transferases (GST), thiopurin *S*-methyl transferases (TPMT) and *O*-methyl transferases (COMT) are enzymes playing fundamental roles in phase II. conjugation reactions in the metabolism of xenobiotics [61]. Silybin undergoes rapid conjugation, which is strictly stereoselective, e.g. SilA and SilB have different conjugation profiles. Typically, the peak plasma levels of total and unconjugated silybin reach ca 76 μg/ml and 8.5 μg/ml respectively (*per os* dose 500 mg/kg, rats) [62], within 30-40 min after administration. This clearly demonstrates a high rate of silybin conjugation. Numerous studies in rodents as well as in humans showed that the biliary excretion of sulfate and glucuronate conjugates of silybin is the major route for silybin elimination [63]. Animal studies, therefore, indicate the same general route of phase II. biotransformation of silybin and its congeners, e.g. glucuronidation, but the particular (iso)enzymes involved in the process are not necessarily the same as human equivalents. Results obtained with the use of recombinant enzymes may help in pointing out a possible interaction with silybin and its congeners, however, expression of a particular enzyme *in vivo* may be too low to be relevant stressing again the need for human *in vivo* studies.

3.2.1. Silybin Sulfonation

Sulfonation converts xenobiotics into more water-soluble and less toxic metabolites, which are then excreted into bile or urine [64]. Sulfonation is also important in activation or inactivation of a number of xeno- and eubiotics. Sulfated conjugates are often incorrectly referred to as "sulfates" because the transfer of a sulfonyl group to a hydroxyl acceptor generates a sulfate ester [65]. Sulfonation processes are catalysed by members of the cytosolic sulfotransferase (SULT) superfamily consisting of at least ten functional (human) genes [66, 67]. Sulfotransferases are found in various tissues including the liver, kidney, brain, adrenals, gut and platelets [64]. The availability of co-substrate/donor 3′-phosphoadenosine-

Table 1. Interaction of silybin and its congeners with cytochromes P450. References are given as upper index.

Cytochrome P450	Interaction *in vitro*	Interaction *in vivo*
1A1	inhibition of recombinant enzyme [31]	-
1A2	negligible effects in microsomes [32,33]	negligible effects in humans[34]
2C8	substrate in microsomes [33]	-
2C9	inhibition in microsomes [32,33,37] inhibition of recombinant enzyme [37,38]	suspected inhibition in rats[57]
2D6	inhibition in microsomes [31,32,45]	negligible effects in humans[34]
2E1	inhibition in microsomes [32,41,45]	negligible effects in humans[34]
3A4	inhibition of recombinant enzyme [51] inhibition in microsomes [31,32,38,45] in isolated rat hepatocytes [49] and in human hepatocytes [50]	negligible effects in humans[34]

Table 2. Select drugs in clinical use that may display drug-drug interaction with silybin.

Drug	indication	Phase I biotransformation
(S)-warfarin	indirect anticoagulant agent	CYP2C9
metronidazole	antibacterial	CYP2C9
cisplatin	anticancer	CYP2C9, CYP2D6
doxorubicin	anticancer	CYP2C8, CYP3A4
paclitaxel	anticancer	CYP2C8
diclofenac	non-steroidal antiinflammatory drug	CYP2C8
fluvastatin	hypolipidemic (statin)	CYP2C8
DMZ	antibacterial	CYP2C9

5´-phosphosulfate (PAPS) is of utmost importance for effective sulfate conjugation [68-70].

Notably, almost all recent studies of silybin conjugation were so far only done with a silybin mixture or with crude silymarin, although some authors attempted to analyse the metabolites of SilA and SilB separately. Miranda *et al.* studied the biliary excretion of silymarin flavonolignans, SilA and SilB, isosilybin A&B, silychristin, and silydianin, their glucuronide and sulphate conjugates in perfused rat livers. They found sulfonation to be the major metabolic pathway for SilB (78%) and for isosilybin A (84%) and isosilybin B (85%). On the other hand, the primary route for silydianin conjugation is glucuronidation (80.5%) and this also holds true to a lesser extent for silychristin (ca 60%). SilA is sulphonated and glucuronidated to about the same degree, overall to a lower extent than SilB. Therefore, it is obvious that all flavonolignans present in silymarin, including their diastereomers, are metabolized by phase II. biotransformation [63].

Hoh *et al*. [71] studied silybin distribution and metabolism in patients with colorectal carcinoma. Three different silybin monosulfates were identified in plasma based on HPLC-MS profiles. However, due to multiple diastereoisomers of silybin derivatives and without authentic reference samples they could not assign regioisomers to the multiple peaks seen in the single ion. This again demonstrates the need to work with optically pure silybins.

Recently, Purchartová *et al*. applied recombinant aryl sulfotransferase IV (AstIV) from rat liver for preparation of silybin sulfates. The data demonstrate that sulfation reaction of silybin is strictly stereoselective. Moreover, the study employed for the first time optically pure silybins for the sulfation reactions by a mammalian enzyme. As a result, SilB was sulfated yielding 20-*O*-silybin B sulfate, whereas SilA was completely resistant to the sulfation reaction. The report included an indirect evidence that SilA is not an inhibitor of AstIV. When the natural mixture of silybin diastereomers was used for the sulfation, only SilB was sulfated in the position C-20 while Sil A was not sulfated [72].

Marhol *et al*. obtained conjugates of SilA and SilB and isosilybin A and B using AstIV from *Desulfitobacterium hafniense*. This bacterial enzyme was able to catalyze sulfation of both silybins, although the reaction with the SilB was slower and not stereospecific. It is remarkable that in this study the sulfation position occured at C-20 in both silybins and isosilybins. We can speculate that the conjugation reaction in the case of silybin could be dictated by the structure of the molecule itself and not by the enzyme regardless of source organism [73].

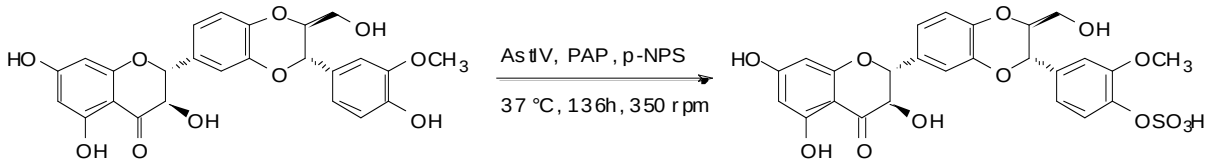
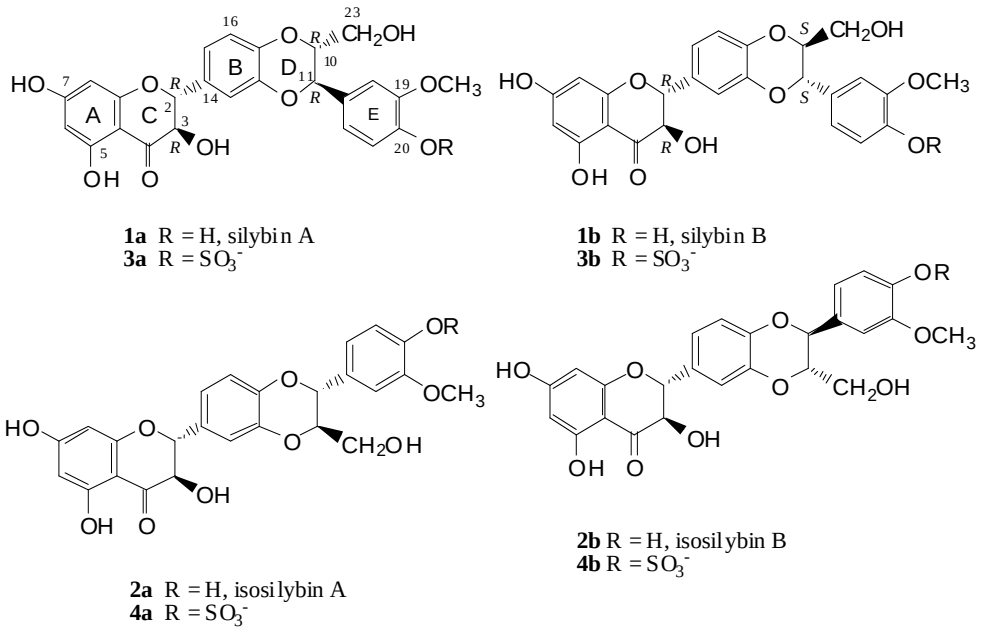


Fig. (4). Enzymatic preparation of 20-*O*-silybin sulfate B *via* mammalian aryl sulfotransferase IV [72].



1a R = H, silybin A
3a R = SO_3^-

1b R = H, silybin B
3b R = SO_3^-

2a R = H, isosilybin A
4a R = SO_3^-

2b R = H, isosilybin B
4b R = SO_3^-

Fig. (5). Silybin, isosilybin and respective sulfates prepared using aryl sulfotransferase from *Desulfitobacterium hafniense.* [73].

While sulfatase driven sulfation yields 20-*O*-silybin B sulfate, chemical synthesis yields sulfation at other positions. Interestingly, these sulfated derivatives display biological activity. Zarelli *et al.* reported chemical synthesis of 23-*O*-silybin sulfate [74], Abourashed *et al* used fungus *Cunninghamella blakesleana* to obtain 7-*O*-silybin sulfate [75]. Both groups prepared silybin sulfates from the natural mixture of SilA and SilB, both derivatives were tested for their antioxidant activity. Agarwal *et al.* prepared silybin 7,23-disulfates from both optically pure SilA and SilB by employing chemical synthesis and report anti-cancer efficacy of this derivative [76].

3.2.2. Silybin Glucuronidation

Glucuronidation is one of the three main conjugative reactions of flavonoids [77, 78]. It is catalysed by UDP-glucuronosyl transferases (UGTs), the microsomal enzymes encoded by a multigene family in humans. UGTs catalyse conjugation of nucleophile substrates such as aliphatic alcohols, phenols, carboxylic acids, thiols or amines with UDP-glucuronic acid (UDP-GA) [79]. Besides xenobiotics, the UGTs metabolize eubiotics such as bilirubin, steroid hormones, and thyroid hormones [80, 81]. These enzymes are expressed in liver [82] and gastrointestinal cells [82-85].

Křen *et al.* [17] prepared the 7-*O*-β-D-glucuronide (**7**), 20-*O*-β-D-glucuronide (**8**), and 5-*O*-β-D-glucuronide (**9**) of optically pure SilB by enzymatic synthesis using ovine liver microsomes (Fig. **6**). These conjugates were formed (*in vitro*) in the proportion **7** (62.5%), **8** (27%), **9** (2.5%), thereby reflecting the approximate regio-preference of UGT for SilB molecule. This and Han's paper [16] are probably the only studies so far describing the full spectral, and therefore structural evidence (^{1}H, ^{13}C NMR and MS) of the silybin metabolites. Křen *et al.* [17] established that silybin 20-β-D-glucuronide is a major SilB conjugate in humans, while the C-7 regioisomer is also formed, but in a lower proportion.

It was shown that SilB yields 20-*O*-β-D-glucuronide as a major metabolite and a slightly lower amount of the 7-*O*-β-D-glucuronide of SilB, contrary to SilA. SilB is metabolised by the bovine microsomes ca 2-3× quicker than SilA and in mixture, both diastereomers influence the respective metabolism of each other [16]. Jančová *et al.* [82] tested silybin metabolite formation *in vitro* by incubating with a human liver microsomal fraction, then with primary cultures of human hepatocytes and with 12 recombinant isoenzymes of UGTs. Only three recombinant UGTs, UGT1A4, UGT2B4 and UGT2B17, do not participate in silybin glucuronidation processes. Chen *et al.* also [78] investigated the glucuronidation of various flavonoids by recombinant UGT1A3 and UGT1A9. Hoh *et al.* identified four silybin monoglucuronides and two diglucuronides (HPLC-MS) in patients with colorectal carcinoma after the administration of Silipide (Indena) containing SilA and SilB [71]. Summary of phase II biotransformation of silybin and its congeners is given in Table **3**.

3.2.3. Silybin as an Inhibitor of Conjugative Enzymes

Flavonoids inhibit several groups of biotransformation enzymes and silybin is no exception [32, 38]. Unfortunately, there are no direct studies on the silybin inhibition of SULT. Several studies show that silymarin, and its main constituent silybin, inhibit glutathione *S*-transferase [86, 87]. On the other hand, Zhao *et al.* [88] showed that orally administered silybin in mice results in a significant induction of glutathione *S*-transferase (GST) and quinone reductase (QR) [88]. Latest studies of Marhol *et al.* and Purchartová

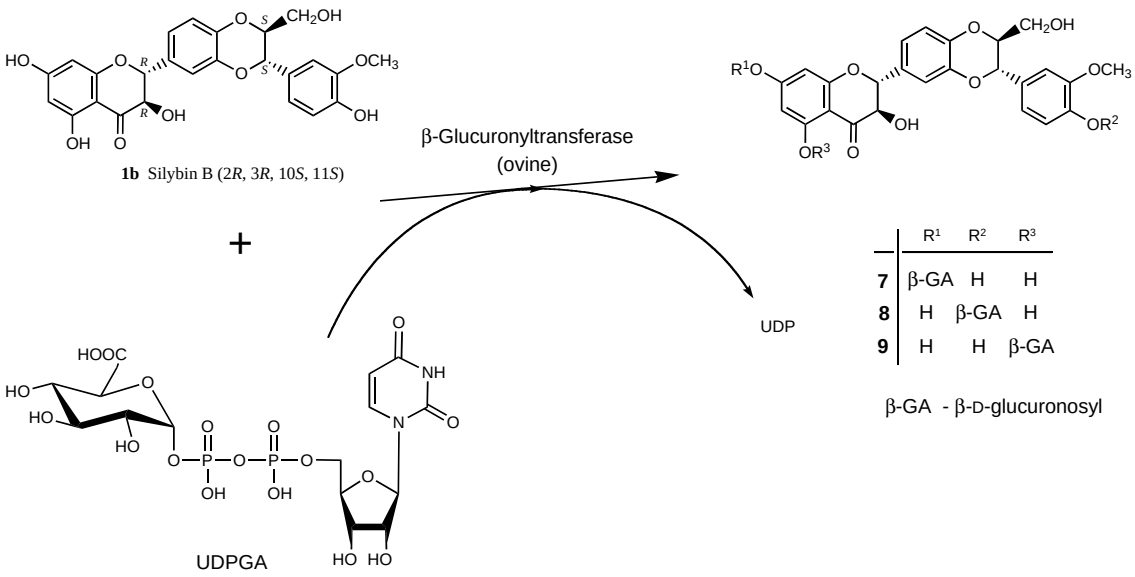


Fig. (6). Enzymatic preparation of silybin B β-glucuronides [17].

Table 3. Biotransformation of silybin and its congeners by phase II enzymes. References are shown as upper index. Preferred substrates or positions for each conjugation reaction are given in bold. Silybin 20-β-D-glucuronide is a major SilB conjugate in humans [17].

flavonolignan	Position of conjugation	Enzyme/enzymatic system/organ	*In vivo* evidence
sulfation			
Sil A Sil B **isosilybin A** **isosilybin B** silydianin silychristin	C-20	Perfused rat liver [63] Rat recombinant AstIV [72] AstIV from *Desulfitobacterium hafniense* [73]	flavonolignan sulfates detected in humans [71] and in mice [88]
Glucuronidation			
Sil A **Sil B** isosilybin A isosilybin B silydianin silychristin	C-5 C-7 **C-20**	Ovine liver microsomes [17] bovine liver microsomes [16] human liver microsomes [82] human hepatocytes [82] human recombinant glucuronidases [78,82]	flavonolignan glucuronides detected in humans [17,71], in rats [99] and in mice [88]

et al. report preparation of silybin or isosilybin sulfates by employing bacterial or mammalian aryl sulfotransferases shown no possible inhibition of these enzymes [72, 73].

Sridar *et al*. showed that silybin is a potential inhibitor of recombinant UGTs, namely 1A1, 1A6, 1A9, 2B7 and 2B15 with IC_{50} values of 1.4 μM, 28 μM, 20 μM, 92 μM, and 75 μM, respectively. Moreover, they demonstrated quite selective inhibition of UGT1A1 by silybin [38]. Unfortunately only a mixture of SilA and SilB was used in this study, we may expect that the inhibition of the respective conjugation enzymes by individual silybin diastereomers will differ.

D´Andrea *et al*. confirmed the previous results that silybin (silymarin) inhibits UGT1A1 and 1A6 [89]. Importantly, silybin glucuronides prepared as a crude mixture of glucuronides by microsomes plus UDP-GA exerted rather strong competitive inhibition of UGT1A with K_i = 14 ug/ml for *p*-nitrophenyl glucuronidation. This value is in the range of the physiological concentration that can be reached in the tissues upon silybin administration. Therefore, this fact must be considered in terms of possible drug-drug interactions. [89].

Flaig *et al*. indirectly confirmed inhibition of UGT1A1 in humans during a phase I. trial of high doses of silybin administered as silybin-phytosome complex with phosphatidylcholine (PC) [90]. Volunteers receiving doses higher than 10 g of phytosome/day, equivalent to ca 3 g of silybin, developed in some cases asymptomatic hyperbilirubinemia that improved with the cessation of treat-

ment resembling Gilbert's syndrome caused by a decreased functionality of UGT1A1, a common genetic disorder that manifests itself in asymptomatic hyperbilirubinemia. This effect should be considered when combining high doses of silybin with some cancerostatics, e.g., irinotecan that could develop toxicity effects [52].

Silymarin also influences deconjugating enzymes, e.g. those active in the colon, which can participate in the enterohepatic circulation of xenobiotics. Silybin and two at the time unidentified silymarin components, presumably silychristin and silydianin, act as specific inhibitors of intestinal bacteria β-glucuronidase (e.g. *E. coli* HB101) [91]. This inhibition, 53% inhibition by 0.8 mg/ml silybin, can improve hepatoprotection upon xenobiotic metabolism, with the toxic metabolites secreted *via* hepatobiliar routes as glucuronides by preventing their enterohepatic circulation.

3.2.4. Biological Activity of Silybin Conjugates

Majority of silymarin conjugates are excreted by the hepatobiliar route into the intestinal system, where they can act locally or enter enterohepatic circulation. The plasma and tissue concentration of free, unconjugated silybin is generally ca five to ten fold lower than that of conjugated silybin, depending on dose and time. It is reasonable to suppose that at least some silybin conjugates share pharmacologic activity with the parent molecule.

Generally, conjugated metabolites are devoid of pharmacological activities, except for a few but important over-the-counter examples: e.g. morphine-6-glucuronide has ca three-fold better analgesic activity, lacks the unwanted side effect of morphine (nausea), and as a result it is used as a morphine alternative [92, 93]. Another example are retinoid glucuronates that have a higher biological activity and lower toxicity than the parent vitamins [94]. Minoxidil sulfate, a vasodilatans for treatment of alopecia, has a faster vasodilatation activity than the unconjugated drug via acting on the norepinephrine contraction of the smooth muscles of the arteries [95]. As a result, the study of the pharmacological activities of silybin is a great challenge, as it can produce new drugs with good bioavailability having new, targeted activities. This is especially important for silybin sulfates which could be prepared by relatively simple chemical synthesis in multigram amounts.

There have been limited attempts to prepare silybin metabolites to test their biological activity. For example silybin glucuronides were prepared using liver microsomes in sufficient purity and quantities to enable their structural determination and some basic biological tests [16, 17]. This method is quite feasible, but the amount of product is limited by the high price of UDP-glucuronate used as the glucuronosyl donor. Purification of the respective conjugates is not trivial and usually involves preparative HPLC. Radical scavenging activity of two silybin glucuronides (C-20 and C-7) was tested and demonstrated that 7-*O*-silybin-β-D-glucuronide is a better antioxidant than nonconjugated silybin [17]. Han *et al*. identified [16] four major metabolites of silybin - two for SilA and two for SilB using bovine liver microsomes.

23-*O*-silybin sulfate prepared by chemical synthesis [74], and 7-*O*-silybin sulfate obtained *via* biotransformation using fungus *Cunninghamella blakesleana* [75], were tested for their antioxidant activity by DPPH radical scavenging assay. In both cases were silybin sulfates prepared from the diastereomeric mixture of SilA and SilB. 23-*O*-Silybin sulfate showed antiradical activity very similar to silybin [74], whereas silybin sulfation at C-7 led to a substantial reduction in the radical scavenging activity [75]. Recently, Agarwal *et al*. prepared silybin 7,23-disulfates from optically pure SilA and SilB and natural silybin. Only 7,23-silybin disulfate from natural silybin, however, was applied in futher biological tests[76]. This compound and other derivatives of silybin, neither sulfates nor glucuronides, were examinated for their anticancer efficiency using human bladder cancer HTB9, colon cancer HCT116 and prostate carcinoma PC3 cells. Silybin 7,23-disulfate did not display any significant improvement in inhibition of cancer cell growth in both HTB9 and HCT116 cells. Moreover, the disulfate appeared to be a slightly worse growth inhibitor than silybin. On the other hand, the very same disulfate seemed to be better inhibitor of cancer cells growth than silybin in case of PC3 cells [76].

4. SILYBIN PHARMACOKINETICS

Silybin and all its silymarin congeners suffer from low water solubility, which strongly dependent on pH. At alkaline pH, the solubility dramatically increases. Under physiological conditions, the solubility of silybin in an aqueous milieu is ca 0.5 g/l, but this depends on the presence of other components (e.g. aminoacids, metals). High doses of silybin are not well absorbed and this is also one of the possible reasons for silybin's excellent safety record for peroral administration, even at extremely high doses (up to 7 g silybin/d) [90]. Therefore, the unabsorbed silybin/silymarin, which is eventually excreted *via* feces, must be also considered for its local effects in the intestines. The resorption of silybin can be strongly improved, e. g. by complexing silybin with phosphatidylcholine (Phytosome, Silipid, Silyphos, IdB1016), or for intravenous administration in cases of mushroom poisoning as the semisynthetic preparation silybin bishemisuccinate (Legalon-SIL), which is highly water soluble.

We have to consider, however, that preparations of marketed silymarin contain highly variable amounts of constituent flavonolignans carrying a high probability of different pharmacokinetic parameters obtained with various extracts. Additional layer of uncertainty comes with general extrapolation of *in vitro* studies to the *in vivo* situation as discussed in detail by Markowitz and Zhu [28]. The use of experimental animals aids the assessment of pharmacokinetics of silybin and its congeners but human *in vivo* studies will be necessary. Pharmacokinetics is strongly influenced by the concentration or dose of the silybin preparation. Kroll *et al*. [2] correctly warn that the concentration of silymarin constituents should be given as separate concentrations of the individual chemicals, e.g. 50 μM silybin as actually 25 μM SilA and 25 μM SilB.

In his pioneering pharmacokinetic work, Weyhenmeyer *et al*. [15] clearly demonstrated that SilA and SilB have different metabolic profiles[15]. At that time authors referred to Isomer 1 and 2, which were plausibly SilA and SilB, respectively. A single dose of silymarin was administered to 6 healthy males (Legalon 140, containing 51 mg silybin). They showed that the bioavailability and peak plasma concentration (free) of Isomer 1 (SilA) were 2-3-fold greater than that of Isomer 2 (SilB). The data are in agreement with faster conjugation of SilB.

Recent evaluation of free silymarin flavonolignans pharmacokinetics in healthy human volunteers confirmed the stereoselectivity of flavonolignan disposition. Moreover, rapid absorption and elimination of six major silymarin flavonolignans was demonstrated [96].

Interesting pharmacokinetic data were obtained by comparing silybin/silymarin administration to patients with cholecystectomy [97] and with extrahepatic biliary obstruction [98]. In the first study [97], both silymarin and silipidewere used and dosed at 120 mg total silybin. Peak concentrations of silybin reached ca 116 μg/mL (240 μM) within an hour in the bile after silipide, a preparation of silymarin similar to Phytosome, and 29 μg/mL (60 μM) after silymarin administration. Biliary concentrations were 250 to 1000 times higher than plasma concentrations. The bile also contained considerable amounts of isosilybin and very low concentrations of silydianin and silychristin. Silybin recovered in the bile in both conjugated and free form within 48 h accounted for 11% of the dose after silipide and for 3% of the dose after silymarin, which indicates better silybin availability from silipide than from silymarin. The second study [98] on patients with cholestasis secondary to biliary obstruction demonstrated that silybin, equivalent to 120 mg dose of silybin, reached a peak in plasma after 3-4 h and remained at rather high values (ca 400 ng/ml). On average, the total

silybin was more than 40-fold greater than the AUC for free silybin. These data suggest that extrahepatic biliary obstruction is associated with a lower clearance of conjugated silybin, due to impaired excretion of the conjugate in bile.

Wu *et al*. demonstrated that silybin has dose-related pharmacokinetics when administered in doses of 10 to 50 mg/kg. They worked with normal and cirrhotic rat liver, where dimethylnitrosamin (DMN) induced liver cirrhosis, and suggested that the phase II. conjugative reaction of silybin is blocked by treatment with DMN. They also reported that silybin excretion is partially inhibited by P-glycoprotein [99].

Interaction of silybin and its congeners with organic anion-transporting polypeptides (OATP) is an important facet of pharmacokinetics of OATP substrates. Hepatic and extrahepatic OATPs mediate transport of wide variety of drugs including antibiotics, anticancer drugs and statins. Köck *et al*. evaluated inhibition of overexpressed OATPs by silymarin and its individual constituents [100]. Although inhibition of OATPs by individual silymarin flavonolignans was demonstrated, the authors concluded that silymarin-drug interactions in hepatic uptake are low risk and will very much depend on actual concentrations of flavonolignans at the site of drug transport.

Zhao and Agarwal determined the *in vivo* distribution and conjugate formation of systemically administered silybin in the pancreas, stomach, lung, skin, liver and prostate in mice [88]. They also studied the effect of orally administrated silybin on phase II. biotransformation enzyme activity in the small intestine, skin, lung, liver and stomach. The study shows that silybin is quickly absorbed after oral application and spreads into various tissues. Maximum concentrations of free silybin were reached after half an hour in the lung, liver, stomach and pancreas, and after one hour in the prostate and skin. Silybin conjugates were found in the lung and stomach after 30 minutes and after one hour in the liver, pancreas, prostate and skin. The levels of both free and conjugated silybin decreased in an exponential fashion with an elimination half-life of 57-127 min for free and 45-94 min for conjugated silybin after appearing in tissues. Studies examining the effect of silybin on phase II. biotransformation enzymes showed a moderate to highly significant increase in both glutathione reductase and glutathione S-transferase activities in the small intestine, liver, skin, lung, and stomach [88]. This study did not show the ratio of silybin sulfates or glucuronides, did not identify the type of silybin conjugates formed, and did not explore the stereoselectivity of silybin pharmacokinetics.

Silybin in its conjugated form is quickly exported, presumably actively *via* P-gp exporters, into the bile thus producing a bile concentration ca 100-fold higher than that in plasma [62]. There is also a secondary peak of silybin conjugates in plasma after a single oral dose of 600 mg silymarin in humans indicating enterohepatic circulation [101]. Animal studies therefore point out the same general routes of clearance of silybin and its congeners. The similarity may be related to similar phase II biotransformation. However, therapeutic application in humans with potential disbalance in drug clearance, must be carefully considered.

5. SILYBIN BIOAVAILABILITY

The bioavailability of silybin and/or silymarin is an important issue, due to the relatively low solubility of this drug both in water and in oleinaceous vehicles [102]. This may be also one of the reasons for the high tolerability of extremely high doses of silybin, since a major proportion of the silybin remains unabsorbed (50-80%) in the intestines. Bioavailability may be strongly improved by various pharmacological procedures, e.g. by complexing silybin (1:2, w/w) with phosphatidylcholine (PC), or by intravenous administration in cases of mushroom poisoning as the semisynthetic preparation silybin bishemisuccinate, which is highly water-soluble.

The solubility of silybin in water is ca 400 mg/L, but it strongly depends on pH - as a compound that is phenolic in nature, it dissolves well with increasing pH because of phenolic OH ionization. However, alkaline solutions of all silymarin flavonolignans tend to oxidize quickly by oxygen from the air, forming strongly yellow 2,3-dehydroderivatives. The solubility of silymarin in other solvents of pharmacological relevance is as follows: transcutol, 350.1 mg/mL; ethanol, 225.2 mg/mL; polysorbate 20, 131.3 mg/mL; and glyceryl monooleate, 33.2 mg/mL [103].

In conclusion, there are four major reasons for restricted silymarin, and consequently silybin, bioavailability: *i*) low water solubility, *ii*) phase II biotransformation, *iii*) rapid excretion in bile and urine, and *iv*) low permeability across intestinal epithelial cells.

The availability of silymarin is addressed by numerous approaches, some of them are available on the market. Theodosiou *et al*. reviewed recent drug formulation approaches used in improving bioavailability of silymarin and its constituents [104]. Here in this review we are only highliting the most usable strategies. A very effective method is silybin micronization using a special milling that forms particles on the micrometer scale (2-5 μM) or even nanonization (5-300 nm), which strongly improves resorption and is now used in a number of commercial preparations e.g. for dermal delivery of silymarin [105]. A large number of other methods have been developed and are now being tested for commercial application: Liposomes can improve solubility and target silymarin better to liver tissues [106]; complexation with cyclodextrin has been used in many other drugs with poor solubility - this has also been tested with silymarin as well and a considerable increase in bioavailability was reported [107].

Besides these pharmacological methods, chemical methods for improving silybin solubility and availability have also been developed. We synthesized a series of silybin glycosides that proved to be considerably more water soluble than the parental silybin, e.g. silybin-23-*O*-glucopyranoside (30-fold), silybin-23-*O*-galactopyranoside (four-fold) [24]. Extending the glycosidic moiety by attaching more α-Glc residues using enzymatic methods further improved their solubility [108]. Another attempt to improve silybin solubility was the synthesis of its phosphate [109]. Although the solubility was improved, antioxidant activity proved to be inferior to that of silybin. The synthesis of long fatty acid esters of silybin aimed at improving its solubility in a lipidic milieu and targeting of the esters to membrane compartments [110]. Resulting compounds eventually also proved to have antiviral activity. All of the above synthetic prodrugs still remain to be tested *in vivo* before they can enter the application sphere. The tests will undoubtedly use experimental animals but the results are not readily applicable to humans, for example due to different intestinal microbiome that may influence the bioavailability.

6. CONCLUSION

Key points of the review are summarized in Table **4**. The metabolism of silybin and its congeners is dominantly linked to the phase II. biotransformation, although their interaction with cytochrome P450s cannot be completely discounted. CYP2C8-mediated biotransformation is identified as the only potential drug-drug interaction risk. While CYP biotransformation appears to be non-selective, conjugation reactions are stereoselective, with SilB the preferred substrate. Because silybin and its congeners undergo mainly glucuronidation, adverse drug interactions may manifest in case of silymarin/silybin co-administration with other drugs. Biological activity of conjugated silybins varies depending on cell type and may be stronger or weaker than the parent flavonolignan. Therefore conjugated derivatives of silybin and its congeners need to be evaluated if considered for adjuvant or combination therapy.

In order to develop more soluble and thus better bioavailable silybin derivatives its biotransformation routes as well as stereochemistry must be taken into account. These factors have a pro-

Table 4. Summary of review key points.

Key point	*In vivo*/clinical relevance
Metabolism of silybin by CYP2C8	Risk of drug-drug interactions with CYP2C8/9 substrates
Stereoselective phase II biotransformation	Variable biological activity of extracts containing unknown proportion of flavonolignan stereoisomers
Limited bioavailability	Limited therapeutic effect, but negligible toxicity

found influence on silybin pharmacokinetics and therapeutic effects. Thanks to its newly discovered biological activities, silybin and its derivatives are prominent compounds with a great potential in pharmacology. Thus, understanding the mechanisms behind its interactions, metabolism and bioavailability is a fundamental issue.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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LIST OF ABBREVIATIONS

AstIV	=	Aryl sulfotransferase IV
ATP	=	Adenosine triphosphate
AUC	=	Area under the curve
CCl_4	=	Tetrachlomethane
C-max	=	Maximum (peak) concentration
COMT	=	*O*-methyl transferase
CYPs	=	cytochrome P450s
DMZ	=	4-methyl-*N*-methyl-*N*-(2-phenyl-2*H*-pyrazol-3-yl)benzenesulfonamide
DPPH	=	2,2-diphenyl-1-picrylhydrazyl radical scavenging activity
E. coli HB101	=	*Escherichia coli* hybrid K12 × B strain bacterium containing the *rec*A13 mutation
GST	=	Glutathione *S*-transferase
HCT116	=	Colon cancer cells
HPLC	=	High-performance liquid chromatography
HTB9	=	Human bladder cancer cells
DMN	=	Dimethylnitrosamin
DU145	=	Androgen-independent prostate cancer cell line
Ki	=	Dissociation constant
LC-MS	=	Liquid chromatography–mass spectrometry
MCF-7	=	Doxorubicin-resistant breast-cancer cells
NAT	=	*N*-acetyl transferase
NF-κB	=	Nuclear factor-kappaB
OATP	=	Organic anion transport polypeptide
PAPS	=	3´-phosphoadenosine-5´-phosphosulfate
PC	=	Phosphatidylcholine
PC3	=	Prostate carcinoma cells
P-gp	=	P-glycoprotein
QR	=	Quinone reductase
SilA	=	Silybin A (**1a**)
SilB	=	Silybin B (**1b**)
SULT	=	Sulfotransferase
TPMT	=	Thiopurin *S*-methyl transferase
UDP-GA	=	UDP-glucuronic acid
UGT	=	UDP-glucuronosyl transferase

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