Glycosylation of silybin

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Silybin glycosides—23-*O*-β-glucoside 2b, β-galactoside 3b, β-lactoside 4b and β-maltoside 5b—have been synthesized by different methods (Helferich glycosylation, Lewis acid catalysis). Separation of two silybin diastereoisomers in the form of acetylated monoglycosides has been achieved for the first time. These new silybin glycosides are 4–30 times more water-soluble, and their hepatoprotectivity is increased compared with that of the parent compound silybin 1.

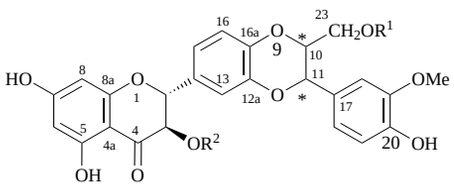
Introduction

Flavonolignan silybin **1** is an important hepatoprotective drug (Flavobion,™ Legalon™) used in therapy of liver damage of various aetiology and as a liver-protecting drug.[1] It acts as a radical scavenger, removing the reactive radicals resulting from liver detoxification of xenobiotics by monooxygenase systems[2] and stimulates the metabolism of phospholipids as anti-lipoperoxidant (probably acting on the RNA synthesis level), thereby modulating the fluidity of cell membranes of hepatocytes.[3] It is also a very effective antidote in the treatment of severe intoxications by *Amanita phalloides*.[4] This drug is used also in the treatment of some inflammatory diseases of the kidney, stomach and skin, and as a chemopreventive agent in human carcinogenesis.[1]

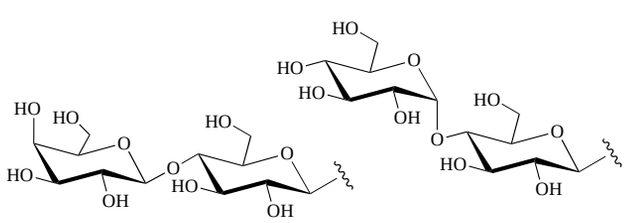
Silybin is isolated from the seeds of milk thistle *Silybum marianum*.[1] Natural silybin is an equimolar mixture of two diastereoisomers A and B having configurations 2*R*,3*R*,10*R*,11*R* and 2*R*,3*R*,10*S*,11*S*, respectively.[5] Their absolute configuration is not known. Separation of both diastereoisomers has been recently accomplished by analytical high-performance liquid chromatography (HPLC) only.[6] Preparative separation of the respective diastereoisomers and any of their derivatives has not been achieved yet.

Bioavailability and therapeutic efficiency of silybin is rather limited by its very low solubility in water (430 mg/l). The major part of the orally administered drug is not resorbed but it is excreted in bile and in faeces.[7] Solubility was improved by preparation of silybin bis-hemisuccinate[8] that enabled intravenous use of silybin (Legalon™ SIL, Madaus, Germany) for the treatment of acute liver intoxication. Another approach improving the bioavailability of silybin (not the water solubility) was the preparation of a phosphatidylcholine complex of silybin.[9] Recently, the 23-*O*-phosphate of silybin, which has better water solubility, was also prepared;[10] however, its hepatoprotective activity was inferior to that of the original drug.

Glycosylation often improves solubility of various drugs without affecting their activity. 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.[11] No natural or synthetic silybin glycoside has been reported up to now.



1 R^1 = H, R^2 = H
2a R^1 = βGlcAc$_4$, R^2 = H
2b R^1 = βGlc, R^2 = H
3a R^1 = βGalAc$_4$, R^2 = H
3b R^1 = βGal, R^2 = H
3c R^1 = R^2 = βGalAc$_4$
4a R^1 = βLacAc$_4$, R^2 = H
4b R^1 = βLac, R^2 = H
5a R^1 = βMaltAc$_4$, R^2 = H
5b R^1 = βMalt, R^2 = H



β-Lactosyl (β-Lac) β-Maltosyl (β-Malt)

Results and discussion

Silybin **1** is a rather complex target for glycosylation, bearing five OH groups of three different types. The primary alcoholic group at C-23 was chosen as the target for glycosylation because phenyl glycosides are often unstable and more acid-labile. Our aim was to prepare well defined compounds that could be used for eventual pharmacological tests. Reaction conditions were selected to give high stereo- and regio-selectivity and to avoid decomposition of starting material **1** that is rather sensitive to oxidation or radical attack.

As a first approach we tested the Koenigs–Knorr reaction.

Table 1 Optimization of silybin glycosylation

Donor (mol equiv.)[a]	Catalyst (mol equiv.)[a]	Solvent	Temp. (*T*/°C)	Time (*t*/h)	Yield[b] (%)
1.0	$BF_3{\cdot}Me_2O$ (2.0)	CH_3NO_2–CH_2Cl_2 (1:1)	−10	24	53
1.0	$BF_3{\cdot}Me_2O$ (1.5)	CH_3NO_2–CH_2Cl_2 (1:1)	20	20	46
3.0	$BF_3{\cdot}Me_2O$ (3.0)	CH_3NO_2–CH_2Cl_2 (1:1)	−10	20	54 (29)[c]
1.25	$BF_3{\cdot}Me_2O$ (1.25)	CH_3CN	−10	20	52 (54)[c]
1.25	$BF_3{\cdot}Me_2O$ (1.25)	CH_3NO_2	−10	20	58 (53)[c]
1.25	$BF_3{\cdot}Me_2O$ (1.25)	$[CH_2]_2Cl_2$	20	25	12
1.25	$BF_3{\cdot}Me_2O$ (1.25)	CH_3NO_2–$[CH_2]_2Cl_2$ (1:1)	20	25	35
3.0	$BF_3{\cdot}Me_2O$ (3.0)	CH_3NO_2–CH_2Cl_2 (1:1)	−10	20	60 (65)[c]
1.25[d]	$BF_3{\cdot}Me_2O$ (1.25)[d]	CH_3CN	−10	20	38 (43)[e]
1.25[d]	$BF_3{\cdot}Me_2O$ (1.25)[d]	CH_3NO_2	−10	20	54 (36)[e]
1.5	$BF_3{\cdot}Me_2O$ (1.25)	CH_3NO_2–$C_6H_5CH_3$ (14:11)	−10	20	70 (75)[e]
4.0	$BF_3{\cdot}Me_2O$ (2.0)	CH_3CN	−10	20	48 (45)[c]
3.0	$BF_3{\cdot}Me_2O$ (2.0)	CH_2Cl_2–CH_3CN (1:1)	20	20	84 (85)[c]
3.0[g]	$BF_3{\cdot}Me_2O$ (2.0)	CH_2Cl_2–CH_3CN (1:1)	20	22	88 (87)[e]
1.5	$BF_3{\cdot}Me_2O$ (2.0)	CH_2Cl_2–CH_3CN (1:1)	20	3	88 (88)[f]
1.5	$BF_3{\cdot}Me_2O$ (1.25)	CH_3NO_2–$C_6H_5CH_3$ (14:11)	20	3	67 (64)[f]
1.1	TMSOTf (1.0)	CH_2Cl_2	−10	24	7
1.1	TMSOTf (1.25)	CH_3NO_2–CH_2Cl_2 (1:1)	−10	20	22 (24)[c]
1.25	$SnCl_4$ (1.5)	CH_2Cl_2	−10	20	0 (2)[c]
1.25	$SnCl_4$ (1.5)	CH_2Cl_2–CH_3CN (1:1)	−10	20	12 (21)[c]

[a] 1 mol equiv. = 2.5×10^{-4} mol (penta-*O*-acetyl-β-D-glycopyranose). [b] Determined by HPLC. [c] Yield in parentheses after 4 h. [d] After 4 h another 1 mol equiv. was added. [e] Yield in brackets after 3 h. [f] Yield in brackets after 20 min. [g] Glycosyl donor − penta-*O*-acetyl-β-D-galactopyranose.

Tetraacetyl-α-bromoglucose was used as a glycosyl donor; Ag_2O served as catalyst and acid scavenger, Drierite (anhydrous $CaSO_4$) was added to remove traces of water (solvent—1,4-dioxane). A rather low yield (under 10%) of per-acetylated silybin glycoside was obtained, as confirmed by mass spectrometry (MS): both compounds gave $[M + Na]^+$ ions in positive-ion electrospray ionization mass spectroscopy (ESI MS) at *m*/*z* 835. Variation of catalysts—Ag_2CO_3 (Fetizon reagent), $AgClO_4$, $AgNO_3$—did not improve the yield and in some cases (Fetizon reagent) an oxidation of starting material was observed.

The phase-transfer glycosylation under catalysis with tetrabutylammonium hydrogen sulfate or benzyltriethylammonium bromide (tetraacetyl-α-bromoglucose as glycosyl donor, solvent $CHCl_3$/0.1 M aq. Na_3BO_4 buffer, pH 10.8; 52 °C; 4 h) gave an ~70% yield of a new product that was per-acetylated glycoside according to MS. The aglycone part, however, gave a signal 2 amu lower than compound **1**. It was later shown that silybin easily undergoes oxidative dehydrogenation yielding 2,3-dehydrosilybin. Hence, this reaction was not useful for our purposes.

Helferich glycosylation was attempted using $Hg(CN)_2$ as a catalyst and tetraacetyl-α-bromoglucose or tetraacetyl-α-bromogalactose as glycosyl donor. This reaction afforded reasonable yields of silybin glycosides (50%) and the yields were considerably improved (up to 75%) after optimization of the solvent system (nitromethane–toluene 14:11). Besides 23-*O*-β-glycosides **2a** and **3a**, a small amount of digalactoside **3c** was also isolated. This compound gave an $[M + Na]^+$ ion *m*/*z* 1097 in ESI MS. Its ^{1}H NMR spectrum contained eight OAc singlets, clearly indicating a diglycoside. The second sugar unit present in this molecule was attached to C-3 (at a secondary alcoholic group) since the corresponding carbon atom resonated 5.22 ppm downfield with respect to that in the monoglycoside **3a**. Coupling constant $J_{2,3}$ changed from 11.8 to 9.9 Hz, probably as a result of steric compression.

After multiple purification of the acylated glycosides—*vide infra*—compounds **2a** and **3a** were deacylated (Et_3N–MeOH–water 1:8:1; room temp.; overnight) and rechromatographed. The preparations, however, contained traces of Hg [16–18 mg/kg, determined by atomic absorption spectroscopy (AAS)] at levels not acceptable even for preliminary pharmacological tests. Multiple attempted purifications did not remove this contamination, probably because of weak complexation with the silybin moiety. Use of the highly toxic mercury catalyst is also unfavourable for eventual large-scale production.

Another glycosylation method using β-D-glucose penta-acetate or β-D-galactose penta-acetate as donor under catalysis by various Lewis acids was tested (Table 1). Optimum conditions using $BF_3{\cdot}Me_2O$ as a catalyst are given in the Experimental section. The best reaction time was 10–20 min. Prolongation of the reaction time gave unwanted by-products and the yields were lowered. Although it is possible to use per-acetates of both α- and β-sugars or their mixture without influencing the product stereoselectivity (giving exclusively the β-anomer) the best yields were achieved with per-acetylated β-sugars.

Per-acetylated silybin glycosides were purified by flash chromatography on silica gel (toluene–dichloromethane–MeOH 1:10:0.8). Final purification was carried out by another chromatography system (toluene–acetone–HCO_2H–CH_3CO_2H 40:10:0.7:0.7) to remove sugar impurities. During purification of the per-acylated glycosides a rather interesting phenomenon was observed: both compounds **2a** and **3a** separated into two very similar fractions.

Both fractions obtained from compound **2a** (denoted as **2a-A** and **2a-B** according to the elution order) gave $[M + Na]^+$ ions *m*/*z* 835 in positive-ion ESI MS. These ions exhibited similar

Table 2 Principal ions observed in CID mass spectra of $[M + Na]^+$ ions of silybin glycosides (see Scheme 1)

Compound	Ion type							
	$[M + Na]^+$	*a*	*b*	*c*	*d*	*e*	*f*	*g*
2a-A	835	533	487	474	533	325	413	185
3a-A	835	533	487		533		413	
2a-B	835	533	487	474	533		413	185
3a-B	835	533	487	474	533	325	413	185
2b-A	667	533	487	474	365	325	245	185
3b-A	667	533	487	474	365	325	245	185
2b-B	667	533	487	474	365	325	245	185
3b-B	667	533	487	474	365	325	245	
4b[a]	829	695	649	636	365	325	407	185
5b[a]	829	695	649		365		407	185

[a] Additional ions: *m*/*z* 533 (*m*/*z* 695 − hexose), *m*/*z* 527 (*m*/*z* 365 + hexose).

Table 3 ^{1}H NMR Data (399.95 MHz; 25 °C); chemical shifts (ppm), multiplicity and coupling constants (Hz) (italicized)

Atom	**2a-A**[a]	**3a-A**[a]	**2a-B**[a]	**3a-B**[a]	**2b-A**[b]	**3b-A**[b]	**2b-B**[b]	**3b-B**[b]	**1-A**[b]	**1-B**[b]
2	4.972 d	4.953 d	4.967 d	4.926 d	4.996 d	4.944 d	4.996 d	4.916 d	4.989 d	4.983 d
	12.0	*11.8*	*11.9*	*11.8*	*11.5*	*11.4*	*11.5*	*11.2*	*11.5*	*11.5*
3	4.505 d	4.483 d	4.513 d	4.471 d	4.525 d	4.482 d	4.534 d	4.437 d	4.517 d	4.519 d
	12.0	*11.8*	*11.9*	*11.8*	*11.5*	*11.4*	*11.5*	*11.2*	*11.5*	*11.5*
6	6.039 d	5.952 d	6.030 d	6.002 d	5.908 d	5.819 d	5.907 d	5.752 d	5.947 d	5.931 d
	2.1	*2.1*	*2.2*	*1.8*	*2.1*	*2.1*	*2.1*	*2.1*	*2.1*	*2.1*
8	5.946 d	6.028 d	5.943 d	5.916 d	5.941 d	5.848 d	5.941 d	5.776 d	5.913 d	5.898 d
	2.1	*2.1*	*2.2*	*1.8*	*2.1*	*2.1*	*2.1*	*2.1*	*2.1*	*2.1*
10	4.017 ddd	4.016 ddd	4.143 ddd	4.087 ddd	4.290 ddd	4.225 m	4.219 ddd	4.286 ddd	4.087 ddd	4.078 ddd
	8.1, 2.7, 2.2	*7.9, 2.6, 2.2*	*8.1, 5.0, 2.2*	*8.0, 5.5, 2.2*	*7.8, 4.7, 2.6*		*7.9, 3.3, 1.0*	*7.8, 4.5, 2.5*	*8.1, 4.5, 2.5*	*8.1, 4.5, 2.5*
11	4.869 d	4.863 d	4.875 d	4.799 d	5.066 d	5.156 d	5.166 d	5.071 d	4.924 d	4.934 d
	8.1	*7.9*	*8.1*	*8.0*	*7.8*	*8.0*	*7.9*	*7.8*	*8.1*	*8.1*
13	7.138 d	7.127 d	7.154 d	7.118 d	7.120 d	7.141 d	7.148 d	7.102 d	7.113 d	7.122 d
	2.0	*2.0*	*2.0*	*1.9*	*2.0*	*2.0*	*2.1*	*2.0*	*1.9*	*2.0*
15	7.077 dd	7.064 dd	7.049 dd	7.015 dd	7.060 dd	7.060 dd	7.050 dd	7.044 dd	7.059 dd	7.055 dd
	9.3, 2.0	*8.4, 2.0*	*8.3, 2.0*	*8.4, 1.9*	*8.3, 2.0*	*8.3, 2.0*	*8.3, 2.1*	*8.3, 2.0*	*8.3, 1.9*	*8.3, 2.0*
16	7.077 d	6.995 d	7.000 d	6.954 d	6.991 d	6.958 d	6.991 d	6.969 d	7.017 d	7.016 d
	8.3	*8.4*	*8.3*	*8.4*	*8.3*	*8.3*	*8.3*	*8.3*	*8.3*	*8.3*
18	6.928 m	6.923–6.941 m	6.981 d	6.865 d	7071 d	7.117 d	7.131 d	7.071 d	7.029 d	7.028 d
			1.9	*1.8*	*2.0*	*2.0*	*2.1*	*2.0*	*1.9*	*1.9*
21	6.928 m	6.923–6.941 m	6.861 d	6.900 d	6.848 d	6.856 d	6.863 d	6.837 d	6.860 d	6.858 d
			8.1	*8.1*	*8.1*	*8.2*	*8.1*	*8.2*	*8.1*	*8.1*
22	6.928 m	6.923–6.941 m	6.931 dd	6.823 dd	6.968 dd	6.994 dd	7.000 dd	6.962 dd	6.920 dd	6.921 dd
			8.1, 1.9	*8.1, 1.8*	*8.1, 2.0*	*8.2, 2.0*	*8.1, 2.1*	*8.2, 2.0*	*8.1, 1.9*	*8.1, 1.9*
23u	3.817 dd	3.827 dd	3.983 dd	3.984 dd	3.908 dd	3.897 dd	3.413 dd	3.902 dd	3.723 dd	3.725 dd
	12.2, 2.2	*12.2, 2.2*	*11.5, 2.2*	*11.4, 2.2*	*11.7, 4.7*	*n.d.*[c]	*11.3, 3.3*	*9.1, 4.5*	*12.3, 2.5*	*12.3, 2.5*
23d	3.744 dd	3.741 dd	3.497 dd	3.489 dd	3.506 dd	3.716 dd	3.340 dd	3.760 dd	3.506 dd	3.505 dd
	12.2, 2.7	*12.2, 2.6*	*11.5, 5.0*	*11.4, 5.5*	*11.7, 2.6*	*n.d.*	*11.3, 1.0*	*9.1, 2.5*	*12.3, 4.5*	*12.3, 4.5*
19-OMe	3.858 s	3.916 s	3.922 s	3.886 s	3.889 s	3.889 s	3.907 s	3.889 s	3.885 s	3.886 s

[a] $CDCl_3$. [b] CD_3OD. [c] Not determined.

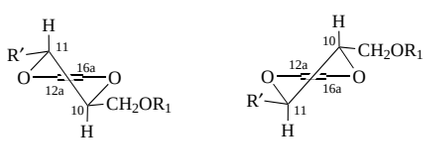


Fig. 1 Possible conformations of the dioxolane ring in the silybin derivatives

fragmentation behaviour in collisionally induced decompositions (Table 2). Their ^{1}H NMR spectra contained four acetyl singlets and the same spin systems with very similar but not identical chemical shifts and coupling constants (see Table 4). The distribution of carbon chemical shifts was also the same (see Table 5); the respective chemical shifts differed only slightly. The nature of the sugar moiety was evident from the set of characteristic vicinal coupling constants (Tables 3, 4). Judging from the value of $J_{1',2'}$ (7.8–7.9 Hz), the anomeric carbon configuration was β in all cases. The glycosylation took place at the primary alcoholic group C-23, since this carbon resonated downfield (δ_C 68.13–68.69) with respect to the free aglycone (δ_C 62.37). Coupling constant $J_{10,11}$-values ranging from 7.9 to 8.1 Hz in A- and B-type compounds (Table 3) require a nearly antiperiplanar arrangement of the involved protons, which means a different conformation (Fig. 1) for each series, the bulky substituents always occupying the pseudoequatorial positions. Therefore, separation of two derivatized silybin diastereoisomers had been achieved. Similar results were obtained with the respective galactoside **3a**.

So far the reported spectra of silybin and its derivatives have dealt with mixtures only. The absolute configuration of silybin diastereoisomers **A** and **B** is not known yet. It is noteworthy that this separation was observed only in the case of per-acetylated monoglycosides but not for any per-acylated silybin itself (Ac, Bz, *p*-BrC_6H_4CO), per-acylated diglycosides or any deacylated glycoside. Analogous separation was achieved for hydroxydihydropyranocoumarin, (±)-lomatin, which was resolved only in the glycosylated form.[12]

Preparative separation of per-acylated monoglycosides of silybin **A** + **B** enabled the first preparation of diastereoisomerically pure silybins **A** and **B**. Separation of the glycosides in their acetylated form enabled us to distinguish the NMR spectra of individual **1-A** and **1-B** diastereoisomers and to arrange their complete assignment (Tables 3–5).

Table 4 Chemical shifts (ppm) and coupling constants (Hz) (italicized) of sugar residues in silybin glycosides

Compound	H-1′	H-2′	H-3′	H-4′	H-5′	^{u}H-6′	^{d}H-6′
2a-A[a]	4.655 d	5.022 dd	5.242 dd	5.088 dd	3.709 ddd	4.101 dd	4.269 dd
	7.9	*9.7, 7.9*	*9.7, 9.3*	*10.0, 9.3*	*10.0, 4.8, 2.4*	*12.4, 2.4*	*12.4, 4.8*
2a-B[a]	4.522 d	5.043 dd	5.213 dd	5.098 dd	3.661 ddd	4.040 dd	4.204 dd
	7.8	*9.5, 7.8*	*9.5, 9.3*	*9.9, 9.3*	*9.9, 4.5, 2.5*	*12.3, 2.5*	*12.3, 2.5*
3a-A[a]	4.633 d	5.235 dd	5.046 dd	5.402 dd	3.911 ddd	4.099 dd	4.148 dd
	7.9	*1.05, 7.9*	*10.5, 3.5*	*3.5, 1.1*	*6.9, 6.6, 1.1*	*11.2, 6.9*	*11.2, 6.6*
3a-B[a]	4.503 d	5.233 dd	5.023 dd	5.371 dd	3.873 ddd	4.060 dd	4.107 dd
	7.9	*10.4, 7.9*	*10.4, 3.5*	*3.4, 1.0*	*6.8, 6.8, 1.0*	*11.2, 6.8*	*11.2, 6.8*
2b-A[b]	4.311 d	3.245 dd	3.300 dd	3.362 dd	3.260 m	3.662 dd	3.858 dd
	7.7	*9.1, 7.7*	*9.1, 8.6*	*8.6, 8.5*		*11.9, 5.6*	*11.9, 2.3*
2b-B[b]	4.181 d	3.287 dd	3.374 dd	3.268 dd	3.213 m	3.674 dd	3.287 dd
	7.8	*9.3, 7.8*	*9.3, 8.6*	*8.6, 8.6*		*12.1, 5.5*	*12.1, 2.3*
3b-A[b]	4.144 d	3.613 dd	3.477 dd	3.838 dd	3.415 dt	3.719 d	3.719 d
	7.7	*9.8, 7.7*	*9.8, 3.3*	*3.3, 1,0*	*1.0, 6.5*	*6.5*	*6.5*
3b-B[b]	4.252 d	3.578 dd	3.467 dd	3.844 dd	3.470 dt	3.726 d	3.726 d
	7.6	*9.7, 7.6*	*9.7, 3.4*	*3.4, 1.0*	*1.0, 6.1*	*6.1*	*6.1*

[a] $CDCl_3$. [b] CD_3OD. [c] Not determined. Additional signals **2a-A**: 5.874 (1 H, s, 7-OH), 11.203 (1 H, s, 5-OH); **3a-A**: 3.694 (1 H, s, 3-OH), 7.586 (1 H, s, 20-OH), 11.209 (1 H, s, 5-OH); **2a-B**: 3.578 (1 H, s, 3-OH), 11.215 (1 H, s, 5-OH); **3a-B**: 11.212 (1 H, s, 5-OH).

Table 5 ^{13}C NMR data: chemical shifts (ppm)

Atom	**2a-A**[a]	**3a-A**[a]	**2a-B**[a]	**3a-B**[a]	**2b-A**[b]	**3b-A**[b]	**2b-B**[b]	**3b-B**[b]	**1-A**[b]	**1-B**[b]
2	82.94	82.84	82.99	82.85	84.91	84.85	85.00	84.65	84.91	84.98
3	72.32	72.27	72.26	72.20	73.96	73.86	73.94	73.82	73.96	73.94
4	195.68	195.57	195.67	195.54	198.47	197.27	198.50	196.24	198.52	198.35
4a	100.71	100.55	100.70	100.46	102.04	101.05	102.06	101.25	102.10	101.95
5	163.77	163.66	163.77	163.60	164.66	164.56	164.66	164.48	164.65	164.65
6	97.06	97.00	97.06	96.99	97.77	97.86	96.71	96.77	97.70	97.83
7	163.09	162.99	163.08	162.93	169.31	165.65	169.20	165.72	169.02	169.59
8	95.94	95.92	95.91	95.92	96.72	97.86	96.71	96.77	96.67	96.82
8a	165.78	166.12	165.88	166.40	165.61	165.60	165.55	165.67	165.55	165.58
10	77.93	77.74	77.20	77.00	79.15	79.22	79.21	79.15	80.30	80.29
11	75.93	75.78	75.87	75.86	78.28	77.50	78.21	77.64	77.99	77.97
12a	144.13	144.06	143.95	143.80	145.33	145.40	145.45	145.27	145.40	145.41
13	116.74	116.08	116.39	116.35	117.99	117.82	117.86	117.87	117.92	117.84
14	129.36	129.36	129.31	129.30	131.99	132.07	131.84	132.47	131.80	131.84
15	120.81	120.82	121.22	121.14	122.39	122.53	122.55	122.33	112.41	122.50
16	117.16	117.08	117.21	117.11	118.14	117.05	118.00	118.08	118.15	118.10
16a	143.74	143.68	143.63	143.52	145.43	145.61	145.68	145.30	145.73	145.75
17	127.58	127.56	127.87	127.78	129.63	129.81	129.78	129.65	129.71	129.71
18	109.99	110.09	109.44	109.47	112.64	112.84	112.85	112.67	112.33	112.36
19	146.66	146.63	147.03	147.04	149.38	149.40	149.39	149.39	149.50	149.49
20	146.17	146.11	146.47	146.41	148.56	148.51	148.51	148.52	148.59	148.61
21	114.58	114.59	114.56	114.57	116.50	116.62	116.63	116.50	116.58	116.57
22	120.52	120.45	120.76	120.74	122.11	121.91	121.90	122.01	121.97	121.98
23	68.13	67.91	68.69	68.60	69.49	69.58	69.69	69.41	62.37	62.37
19-OMe	56.04	55.96	56.11	56.04	56.85	57.00	57.01	56.91	56.77	56.76
1′	101.58	101.93	101.16	101.55	104.95	105.83	105.29	105.55		
2′	71.38	69.00	71.39	68.95	75.52	72.85	75.40	72.94		
3′	72.57	70.65	72.83	70.67	77.70	75.22	77.54	75.19		
4′	68.47	66.99	68.30	66.98	71.95	70.57	71.78	70.49		
5′	71.76	70.60	71.86	70.65	78.31	96.91	78.29	76.98		
6′	61.91	61.73	61.84	61.13	63.06	62.67	62.88	62.69		

[a] $CDCl_3$. [b] CD_3OD. Additional signals **2a-A**: 20.56 q, 20.58 q, 20.60 q, 20.67 q, 169.55 s, 169.77 s, 170.34 s and 170.81 s; **3a-A**: 20.51 q, 20.57 q (2 C), 20.61 q, 170.12 s, 170.26 s, 170.29 s and 170.63 s; **2a-B**: 20.57 q, 20.60 q, 20.69 q, 20.76 q, 169.32 s, 169.44 s, 170.43 s and 170.92 s; **3a-B**: 20.51 q, 20.56 q (2 C), 20.82 q, 169.71 s, 170.35 s, 170.42 s and 170.71 s.

The same synthetic method was used also for the preparation of β-lactoside **4b** and β-maltoside **5b** of silybin where the per-acetyl-β-lactoside **4a** and per-acetyl-β-maltoside **5a** served as the respective glycosyl donors. No separation of diastereoisomers was achieved in these cases.

Water solubility of the new glycosides **2b**, **3b**, **4b** and **5b** compared with that of silybin **1** (0.43 g/l) was 13.0, 1.7, 3.8 and 5.6 g/l, respectively. Better solubility should increase bioavailability especially in *in vivo* tests.

Electronic spectral properties of silybin glycosides

Spectra of silybin glycosides in the UV region were strongly dependent on the presence of trace ionic impurities, due to dissociation equilibria.[13,14] Therefore reproducible results were obtained in the buffered solutions only. In acidic solutions [MeOH–trifluoroacetic acid (TFA)] three distinct transition bands were resolved (nm): 340sh ($\varepsilon_{max} \cong 3000$ dm^3 mol^{-1} cm^{-1}), 288 ($\varepsilon_{max} = 20\,000$) and 235sh ($\varepsilon_{max} = 32\,000$). Three transition bands were also observed under alkaline conditions (MeOH–KOH) (nm): 326 ($\varepsilon_{max} = 24\,000$), 290–291 ($\varepsilon_{max} = 12\,400$) and 244–247 ($\varepsilon_{max} = 18\,000$). Both types of spectra are consistent with the structure of silybin and its derivatives at C-23.[15] As expected, the electronic spectra of silybins and their glycosides are, within experimental error, identical.

As the structure of silybin diastereoisomers contains three isolated chromophores connected by means of four asymmetric carbons, circular dichroism effects ($\varepsilon_L - \varepsilon_R$) were expected. Respective Cotton effects are summarized in Table 6. The band

Table 6 CD spectra of silybin and its glycosides ($\Delta\varepsilon$)

	330 nm	295 nm	255 nm	235 nm
Acidic conditions				
Silybin **A**	+15	−58	+7 (260 nm)	+48
Silybin **B**	+12	−48	+7	−18 (245 nm)
2b-A	+	−	+	+
2b-B	+20	−70	+10	−19 (238 nm)
Alkaline conditions				
Silybin **A**	−6	−24 (297 nm)		+40 (230 nm)
Silybin **B**	−4	−24 (297 nm)	+8 (245 nm)	−5
2b-A	−	−		+
2b-B	−6	−41 (297 nm)	+12 (245 nm)	−8

at the longest wavelength was assigned to the n–π^* transition of the acetophenone moiety. The opposite signs of the Cotton effects in acidic and alkaline media indicate, however, that the formation of salts is accompanied by both different electronic structures and conformations of the molecule and cannot be used unambiguously for the determination of the absolute chirality of 3-hydroxyflavanones at C-2 and C-3; *cf.* ref. 16 (2*R*,3*R* chirality stems from biomimetic synthesis of silybin[5]). Significant differences in the Cotton effects were found for the spectral band at 240 nm under both acidic and alkaline conditions. Owing to the overlap of the transitions and the dominating character of the acetophenone chromophore, the chirality at C-10 and C-11 cannot be assigned unambiguously without some additional proof, *e.g.* crystal-structure determination of some isolated diastereoisomer derivative.

Small amounts of pure silybin **A** and **B** for CD spectra measurements were obtained by acid hydrolysis of respective glucosides **2b-A** and **2b-B** and their purity (over 99%) was determined by HPLC and by optical-rotation measurements (silybin **A** + **B** − natural) $[a]_D^{23}$ +11.38† (*c* 0.23, acetone), silybin A $[a]_D^{23}$ +6.09 (*c* 0.23, acetone), silybin B $[a]_D^{23}$ +18.28 (*c* 0.29, acetone).

NMR and mass spectroscopy of silybin glycosides

All investigated compounds exhibited intense $[M + Na]^+$ peaks in ESI MS. Supplementary information was obtained from collisionally induced decompositions (CID), observed in daughter scans of these ions. However, this fragmentation pattern is different from that found under electron impact because of different ionization mechanisms. Besides trivial losses of CH_3 and CH_2OH, leading to *m*/*z* 652 and 636 ions (Fig. 2), there are some diagnostically useful ions observed in this experiment (Table 2). The mass shift of some ions encountered with deprotected glycosides **2b-A**, **2b-B**, *etc.* confirms this interpretation. In particular, the ions *d* and *f* confirm the glycosidation at C-23.

Carbon chemical shifts of the corresponding atoms in the **A**- and **B**-type compounds are very close (largest differences are 0.5–0.7 ppm, Table 5). That is enough to enable us to observe two signals when measuring the mixture under sufficient digital resolution but makes the identification based on ^{13}C NMR spectra alone impossible. Also, some proton chemical shifts (H-10, H-11, H-23, H-6, H-8) are distinctly different so that the resonances do not overlap (Table 3). However, the safest criterion for **A**/**B** isomer differentiation seems to be the size of H-2 couplings to the CH_2O protons, which are slightly different in both series, most probably reflecting different rotamer populations. These differences are smaller with deprotected glycosides and vanish in free silybinins.

The assignment reported in Tables 3–5 was derived by concerted application of 2D NMR techniques. Several isolated spin systems were picked up by 2D homonuclear chemical-shift correlation spectroscopy (COSY). Two ABC systems of the same type (1,2,4-trisubstituted benzene ring) due to H-13, H-15, H-16 and H-18, H-21, H-22 were differentiated using nuclear Overhauser enhancement (NOE) between H-18 and the methoxy group, and long-range couplings of these aromatic protons to H-11 or H-2 (Fig. 3). The benzylic coupling of H-2 with H-13 and H-15 distinguished this proton from H-3. The signal of H-6 was broadened by an additional coupling to 5-OH that makes its differentiation from H-8 possible. Completed proton assignment was transferred to carbons using ^1H–^{13}C heteronuclear-shift correlation (HETCOR). Quaternary carbons were then assigned by a long-range HETCOR variant (optimized for the detection of geminal and vicinal couplings) and with recourse to fine splitting observed in proton-coupled ^{13}C NMR spectra (Fig. 4). Closely related compounds were then assigned by analogy.

Biological effects of new compounds

Cytoprotective effects of compounds **2b** and **3b** were studied in isolated rat hepatocytes intoxicated by CCl_4 (10 mM) under conditions described previously.[17,18] Reduction in the lactate dehydrogenase (LDH) leakage was 10% with silybin **1**, 20% with compound **2b** and 23% with compound **3b**. Similar results were obtained in hepatocytes intoxicated with *tert*-butyl hydroperoxide and allyl alcohol.[19] These results clearly justify further studies *in vitro* and *in vivo* with the new compounds.

Experimental

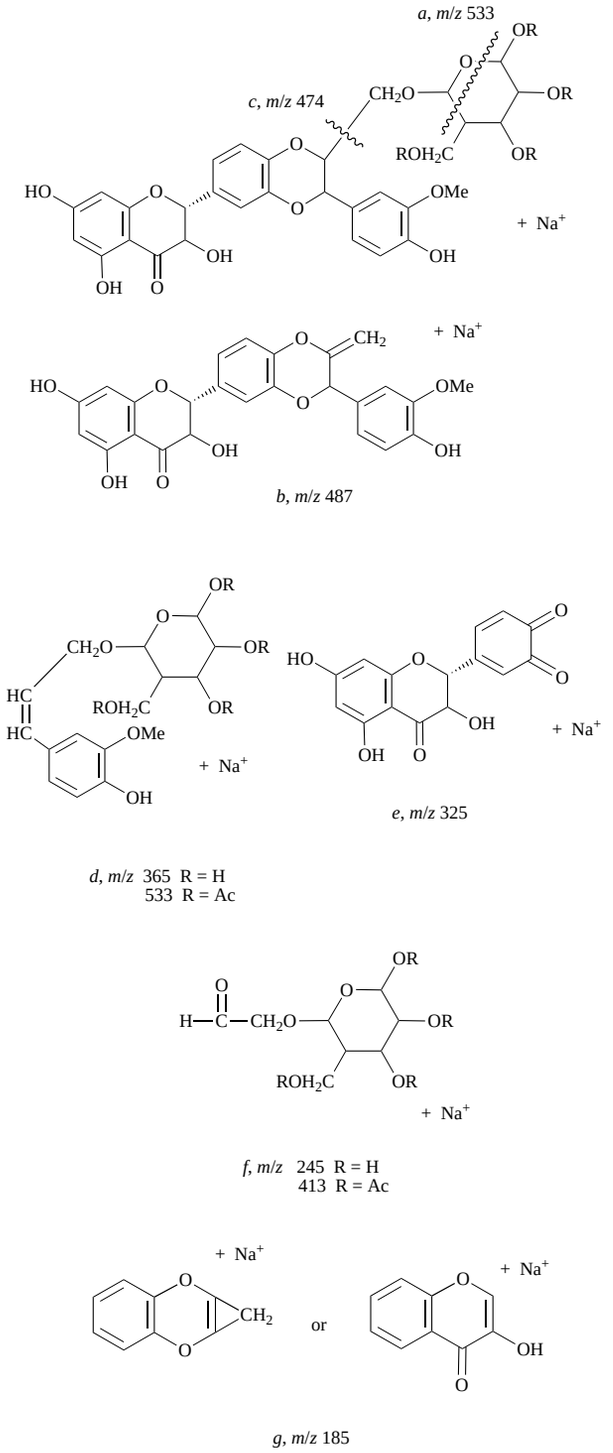
Silybin **1** was kindly donated by Galena Pharmaceuticals Ltd. (Opava, Czech Republic).

^1H and ^{13}C NMR spectra were measured on a Varian VXR-400 spectrometer (399.95 and 100.58 MHz, respectively) for solution in CD_3OD at 25 °C. Residual solvent signal (δ_H 3.33, δ_C 49.3) served as an internal reference. Chemical shifts are given in the δ-scale; digital resolution was 0.0002 and 0.006 ppm, respectively. *J*-Values are given in Hz. Carbon signal multiplicity was determined by an Attached Proton Test (APT) experiment. Manufacturer's software was used for 2D NMR (COSY, ROESY, HOM2DJ, HETCOR).

All positive-ion ESI mass spectra were recorded on a double-focusing instrument Finnigan MAT 95 (Finnigan MAT, Bremen, Germany) of BE geometry (magnetic sector preceding the electrostatic one) equipped with the Finnigan ESI source. Samples dissolved in 50% aq. methanol were continuously infused through a stainless steel capillary held at 3.8 kV into the electrospray ion source *via* linear syringe pump at a rate of 30 μl min^{-1}. A mixture of polypropylene glycols (M_r = 425) was used to calibrate the *m*/*z* scale of the mass spectrometer.

Products of CIDs in the first field-free region of the instrument were analysed by daughter-ion-linked scan (B/E constant) using the manufacturer's software. The collision gas (He) pressure was adjusted for 50% attenuation of the primary ion beam, with collision-cell voltage maintained at the ground potential.

† Specific optical rotations $[a]_D$ are reported in units of 10^{-1} deg cm^2 g^{-1}.



Scheme 1 Proposed structures of fragment ions observed in CID mass spectra of $[M + Na]^+$ ions of silybin glycosides (all ions are adducts with Na^+)

The mass range was scanned at a rate of 100 amu/40 s and the conventional resolution was adjusted to 2000 (10% valley definition).

UV and CD spectra were measured on Varian DMS 300 and Jobin Yvon Mark 5 spectrometers, respectively, in the spectral region 200–400 nm for solutions in methanol, 0.5% TFA in methanol, and 0.5% KOH in methanol. The concentrations of the compounds were 25 and 50 μM for UV and CD measurements, respectively.

General method for glycosylation of silybin (Helferich method)

Silybin **1** was dried prior to glycosylation by azeotropic distillation with toluene to remove crystal-bound water. Compound **1** (5.3 g, 11 mmol) was dissolved in a total of 450 cm^3 of a 14:11

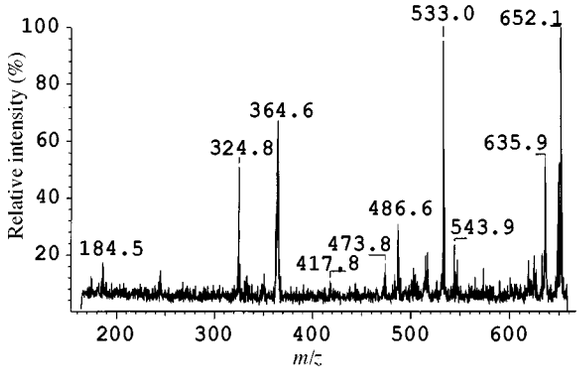


Fig. 2 CID MS of $[M + Na^+]$ ion of **2b-A**

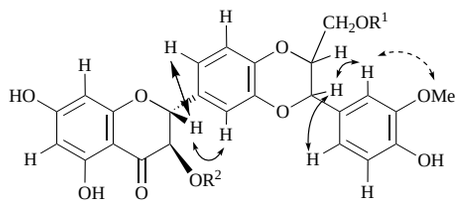


Fig. 3 Diagnostic long-range H-couplings and NOE (dashed)

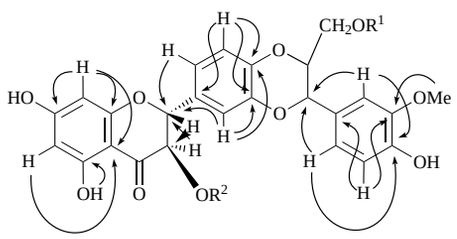


Fig. 4 Diagnostic long-range H,C-couplings

(v/v) mixture of nitromethane–toluene and ~10–20 cm^3 was distilled off using the Distler apparatus to remove traces of water. The mixture was stirred at 60 °C under nitrogen and $Hg(CN)_2$ (2.8 g, 11 mmol) was added. When all catalyst was dissolved, a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (5.2 g, 12.5 mmol) in a minimal amount of the solvent mixture was added and stirring of the mixture at 60 °C was continued for 20 h. Then another portion of tetraacetyl-α-bromogalactose (2.1 g, 5 mmol) and $Hg(CN)_2$ (0.76 g, 3 mmol) was added. The addition (1 g and 0.25 g, respectively) was repeated after another 20 h and the reaction was terminated after a total of 50 h. The mixture was evaporated to one-fifth of its original volume, cooled, and the bulk of precipitated $Hg(CN)_2$ was filtered off. The rest of the mercury was removed by extraction with 3% aq. KI (3 × 100 cm^3). The organic phase was dried over Na_2SO_4. The crude mixture of per-acetylated silybin glycosides was separated from any unchanged silybin and glycosyl donor by flash chromatography on silica gel (toluene–dichloromethane–MeOH 1:10:0.8). Final purification and separation of the per-acetylglycosides of two silybin diastereoisomers was achieved by medium-pressure liquid chromatography on a pre-packed Lobar column (silica gel, size C) (Merck, Darmstad, Germany) by the solvent mixture toluene–HCO_2H–CH_3CO_2H (40:0.7:0.7; 9.9 ml min^{-1}) with a linear acetone gradient 12–20% (v/v). Before evaporation, pooled fractions were neutralized by successive washings with water, saturated aq. $NaHCO_3$ and brine. The yield of crude product **3a** was 8.3 g (92%). Final purification afforded 0.88 g of isomer **3a-A** (having a lower retention time), 0.53 g of isomer **3a-B** and 5.0 g of the mixture **A** + **B** (total yield 71%) and a small amount of diglycoside **3c** (0.2 g). Deacetylation was performed with the mixture Et_3N–MeOH–water (1:8:1) (35 °C; 30 h) and, after evaporation, silybin glycosides were again purified by flash chromatography on silica gel (dichloromethane–

MeOH–water 8:2:0.5) to afford pure isomers **3b-A** (0.44 g, 63%) and **3b-B** (0.27 g, 65%).

Optimization of silybin glycosylation under Lewis acid catalysis

Dry silybin **1** (241 mg, 0.5 mmol) and 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose were dissolved in 15 ml of the respective solvent (for reactant amounts and solvents see entries in Table 1) under vigorous stirring (N_2) and the catalyst was quickly added. When the reaction was complete the mixture was poured into ice-cold, saturated aq. $NaHCO_3$ and twice extracted with 15 ml of chloroform, evaporated, and the residual mixture deacetylated by a mixture of Et_3N–MeOH–water (1:8:1) (35 °C; overnight). Yield of the products was determined by HPLC under the following conditions: SP 8800 ternary gradient pump (Spectra Physics), SP 8880 autosampler, Spectra Focus scanning UV–VIS detector, column 250 × 4 mm, Nucleosil 100-5 C_{18} AB (Macherey Nagel, Germany). Isocratic elution was used; MeOH–water–CH_3CO_2H (42:58:0.1 v/v), flow rate 0.5 ml min^{-1}; scan 210–360 nm, integration was performed at 285 nm (Fig. 5). For the results see Table 1.

General method for glycosylation of silybin (optimized method)

Dry silybin **1** (4.82 g, 10 mmol) and per-acetylated β-glycosyl donor (5.85 g; 15 mmol) were dissolved in 200 ml CH_2Cl_2–MeCN (1:1, v/v) and at room temperature under N_2, $BF_3{\cdot}OMe_2$ (1.83 ml, 20 mmol) was quickly added to the vigorously stirred solution. When the reaction was complete the mixture was poured into ice-cold, saturated aq. $NaHCO_3$ and twice extracted with 150 ml of dichloromethane. Extracts were pooled and dried over Na_2SO_4, evaporated, and chromatographed as in the Helferich reaction. For the respective spectra see Tables 2–6. Instead of elementary analyses, mass spectroscopy was used for new compound characterization as silybin glycosides tend to form non-stoichiometric hydrates and/or solvates.

Silybin 23-*O*-β-D-glucopyranoside 2b. Reaction of silybin **1** (4.82 g, 10 mmol) and 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (5.85 g, 15 mmol) was accomplished as given in the optimized general method. Final purification afforded 1.5 g of isomer **2a-A**, 1.2 g of isomer **2a-B** and 3.2 g of a mixture of **2a A** + **B** (total yield 73%). Deprotection followed by column chromatography afforded separate glycosides **2b** (65%). For NMR, MS and CD data see Tables 2–6.

Silybin 23-*O*-β-D-galactopyranoside 3b. Reaction of silybin **1** (4.82 g, 10 mmol) and 1,2,3,4,6-penta-*O*-β-D-galactopyranose (5.85 g, 15 mmol) was accomplished as given in the optimized general method. Final purification afforded 1.3 g of isomer **3a-A** (having a lower retention time), 1.4 g of isomer **3a-B** and 3.1 g of a mixture **3a A** + **B** (total yield 71%). Deprotection followed by column chromatography afforded separate deprotected glycosides **3b** in a total yield of 65%. For NMR, MS and CD data see Tables 2–6.

Silybin 23-*O*-β-lactoside 4b. Dry silybin **1** (3.5 g, 7.3 mmol) and per-acetyl-β-lactose (7.4 g, 11 mmol) were dissolved in 400 ml of CH_2Cl_2–CH_3CN (1:1, v/v). $BF_3{\cdot}OMe_2$ (6.7 ml, 73 mmol) was quickly added to the vigorously stirred mixture under nitrogen at room temperature. After 19 h the reaction mixture was poured into ice-cold, saturated aq. $NaHCO_3$ and then twice extracted with 150 ml of CH_2Cl_2. Extracts were pooled, dried over Na_2SO_4 and evaporated. Deprotection of the residue, followed by column chromatography (silica gel; EtOAc–MeOH–water 77:13:8, v/v), afforded lactoside **4b** (1.3 g, 22%); δ_H(400 MHz; CD_3OD) (distinct signals only) 3.882 (3 H, s, OCH_3), 3.900 (3 H, s, OCH_3), 4.221 (1 H, d, *J* 7.8), 4.282 (1 H, d, *J* 7.6), 4.339 (1 H, d, *J* 7.8) and 4.381 (1 H, d, *J* 7.6) [all anomeric H-1, β-configurational], 4.521 (1 H, d, *J* 11.5, H-3), 4.531 (1 H, d, *J* 11.5, H-3), 4.990 (2 H, d, *J* 11.5, 2 × H-2), 5.043 (1 H, d, *J* 7.8, H-11A), 5.151 (1 H, d, *J* 8.0, H-11B), 5.910 (1 H, d, *J* 2.0, H-6), 5.912 (1 H, d, *J* 2.0, H-6), 5.944 (2 H, d, *J* 2.0, 2 × H-8), 6.848 (1 H, d, *J* 8.1), 6.863 (1 H, d, *J* 8.2), 6.956 (1 H, dd, *J* 8.1 and 2.0), 6.970 (1 H, d, *J* 8.3), 6.986 (1 H, d, *J* 8.3), 6.990 (1 H, dd, *J* 8.1 and 2.0), 7.045 (1 H, dd, *J* 8.3 and 2.0), 7.055 (1 H, dd, *J* 8.3 and 2.0), 7.115 (1 H, d, *J* 2.0), 7.127 (1 H, d, *J* 2.0) and 7.135 (1 H, d, *J* 2.0); δ_C(400 MHz; CD_3OD) 56.87 q and 56.99 q (OCH_3), 62.00 t and 62.22 t (C-6′), 62.79 t (C-6″), 69.76 t (C-23), 70.59 d (C-4″), 72.82 d (C-2″ and 3′), 73.92 d (C-3), 75.02 d and 75.07 d (C-3″), 76.57 d, 76.68 d and 76.78 d (C-5″), 77.33 d (C-5′), 77.49 d and 77.67 d (C-11), 79.07 d and 79.19 d (C-10), 80.71 d and 80.98 d (C-4′), 84.86 d and 84.95 d (C-2), 96.72 d (C-6), 97.75 d (C-8), 102.05 s (C-4a), 104.71 d and 105.06 d (C-1″), 105.32 d and 105.36 d (C-1′), 112.60 d and 112.79 d (C-18), 116.51 d and 116.62 d (C-21), 117.66 d and 117.87 d (C-13), 117.99 d and 118.16 d (C-16), 121.89 d and 122.15 d (C-22), 122.42 d and 122.56 d (C-15), 129.57 s and 129.73 s (C-17), 131.80 s and 131.95 s (C-14), 145.29 s and 145.37 s (C-12a), 145.41 s and 145.63 s (C-16a), 148.47 s and 148.53 s (C-20), 149.37 s (C-19), 164.63 s (C-5), 165.51 s and 165.56 s (C-8a), 169.14 s and 169.19 s (C-7) and 198.48 s (C-4). For MS data see Table 2.

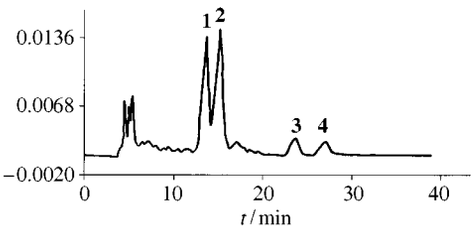


Fig. 5 HPLC of silybin β-glucosides (reaction mixture): column 250 × 4 mm, Nucleosil 100-5 C_{18} AB (Macherey Nagel, Germany) mobile phase MeOH–water–CH_3CO_2H = 42:58:0.1 (v/v), flow rate 0.5 ml min^{-1}; detection 280 nm. 1, **2b-B**; 2, **2b-A**; 3, silybin **1** B; 4; silybin **1** A

Silybin 23-*O*-β-maltoside 5b. Dry silybin **1** (3.5 g, 7.3 mmol) and per-acetylated maltose (7.4 g, 11 mmol) were dissolved in 400 ml of CH_2Cl_2–CH_3CN (1:1, v/v). $BF_3{\cdot}OMe_2$ (6.7 ml, 73 mmol) was quickly added to the vigorously stirred solution under nitrogen at room temperature. After 19 h the reaction was worked up as in the previous case to yield deprotected maltoxide **5b** (1.2 g, 20%); δ_H(400 MHz; CD_3OD) (distinct signals only) 3.883 (3 H, s, OCH_3), 3.901 (3 H, s, OCH_3), 3.466 (1 H, dd, *J* 9.7 and 3.8, H-2″), 4.204 (1 H, d, *J* 7.8), 4.323 (1 H, d, *J* 7.8), 4.523 (1 H, d, *J* 11.5, H-3), 4.532 (1 H, d, *J* 11.5, H-3), 4.992 (2 H, d, *J* 11.5, 2 × H-2), 5.040 (1 H, d, *J* 7.8, H-11A), 5.152 (1 H, d, *J* 8.0, H-11B), 5.181 (1 H, d, *J* 3.8, H-2″), 5.912 (1 H, d, *J* 2.1, H-6), 5.915 (1 H, d, *J* 2.1, H-6), 5.946 (2 H, d, *J* 2.1, 2 × H-8), 6.848 (1 H, d, *J* 8.2), 6.862 (1 H, d, *J* 8.1), 6.957 (1 H, dd, *J* 8.2 and 1.9), 6.968 (1 H, d, *J* 8.4), 6.985 (1 H, d, *J* 8.2), 7.047 (1 H, dd, *J* 8.1 and 1.9), 7.055 (1 H, dd, *J* 8.4 and 2.0), 7.114 (1 H, d, *J* 2.0), 7.125 (1 H, d, *J* 2.0) and 7.135 (1 H, d, *J* 1.9); δ_C(400 MHz, CD_3OD) 56.87 q and 57.01 q (OCH_3), 62.25 t and 62.41 t (C-6′), 63.02 t (C-6″), 69.57 t and 69.74 t (C-23), 71.79 d (C-4″), 73.93 d (C-3), 74.41 d, 74.96 d and 75.03 d (C-3″), 76.57 d, 76.68 d and 76.78 d (C-5″), 75.34 d, 76.81 d, 76.94 d, 77.50 d, 77.67 d, 77.97 d, 79.06 d and 79.18 d (C-10), 81.34 d and 81.45 d (C-4′), 84.87 d and 84.95 d (C-2), 96.68 d (C-6), 97.72 d (C-8), 102.10 s (C-4a), 103.63 d (C-1″), 104.83 d and 105.15 d (C-1′), 112.59 d and 112.81 d (C-18), 116.52 d and 116.63 d (C-21), 117.81 d and 117.88 d (C-13), 118.00 d and 118.16 d (C-16), 121.88 d and 122.16 d (C-22), 122.41 d and 122.56 d (C-15), 129.58 s and 129.73 s (C-17), 131.79 s and 131.93 s (C-14), 145.29 s and 145.38 s (C-12a), 145.42 s and 145.64 s (C-16a), 148.47 s and 148.54 s (C-20), 149.38 s (C-19), 164.63 s (C-5), 165.56 s (C-8a), 169.04 s (C-7) and 198.52 s (C-4). For MS data see Table 2.

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