HETEROCYCLES, Vol. 54, No. 2, pp. 901-915, Received, 12th July, 2000

CHEMOENZYMATIC PREPARATION OF OLIGOGLYCOSIDES OF SILYBIN, THE FLAVONOLIGNAN FROM *Silybum marianum*

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Abstract - Starting from 23-*O*-b-D-glucopyranosyl silybin (**2**) and 23-*O*-b-D-galactopyranosyl silybin (**3**), a-oligoglucosides of silybin were synthesized employing cyclodextrin glucanotransferase from *Bacillus stearothermophillus* (E.C. 2.4.1.19). Oligoglycosides of silybin were tested for the LDL antioxidant activity *in vitro*.

Flavonolignan silybin (**1**) (Figure 1), isolated from seeds of the milk thistle (*Silybum marianum*), is an active component in a number of phytopreparations, e.g., Flavobion™, Legalon™, widely used in human therapy for improving liver function.[1] Cytoprotective activity of **1** consists in several mechanisms operating at various cell levels. Silybin acts as a radical scavenger (antilipoperoxidant), removing the reactive toxic radicals resulting from oxidative detoxification of xenobiotics by liver monooxygenase systems (P-450 and others).[2] Cell regenerating activity is associated with its ability to activate the proteosynthesis by DNA-dependent RNA-polymerase I stimulation.[3] Cytoprotective activity of **1** is believed to be based on its antioxidant properties.[4a] Silybin is considered to act as a chain-breaking antioxidant by scavenging free radicals which induce lipid peroxidation.[4b] Other important antioxidative effects of **1** are due to its influence on enzyme system associated with glutathione[4a] and superoxide dismutase.[4c] Antioxidant activity of **1** in different models is widely documented.[1a,2,4] Recently, low

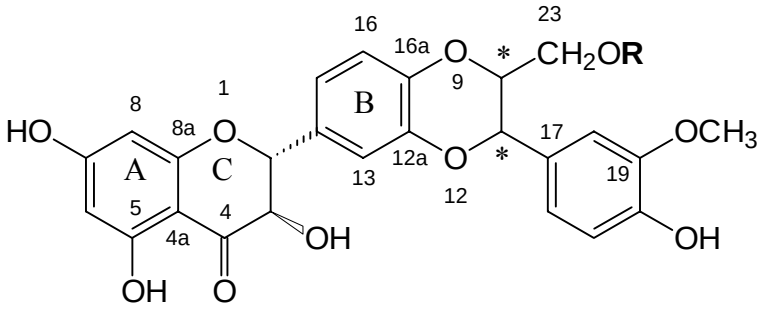
density lipoprotein (LDL) antioxidant activity of **1** has been reported.[5] Oxidatively modified LDL is supposed to play very important role in atherosclerosis initiation and propagation.[6]

Bioavailability and therapeutic efficiency of **1** are rather limited by its very low solubility in water (430 mg/L). Solubility was improved by preparation of silybin 3,23-*O*-bishemisuccinate[7] that enabled intravenous application of silybin (Legalon-SIL™, Madaus, D) for the treatment of acute liver intoxication. Another approach improving the bioavailability of silybin (not water solubility) was the preparation of a silybin-phosphatidylcholine complex (IdB 1016, Indena, I).[8]

Series of silybin 23-*O*-b-D-glycosides[9] was prepared by chemical glycosylation, silybin 7-*O*-β-D-glucoside was obtained by biotransformation of **1** using plant cell culture.[10] New glycosides exhibited good solubility in water,[9] higher cytoprotectivity, and lower cytotoxicity[11] in comparison with parent compound (**1**).

Introduction of the a- or b-glucosyl moieties usually lead to a substantial increase of solubility. a-D-Glucopyranosyl (a-D-Glc*p*) moiety seems to be more suitable than the b-D-Glc*p* moiety because of easier cleavage by liver glycosidases, and possibly also better resorption from small intestine mediated by transmembrane a-glucosidase (sucrase).[12] We have, therefore, decided to prepare silybin glycosides bearing one or more a-glucopyranosyl units.

Natural silybin is an equimolar mixture of two diastereoisomers having absolute configurations 2*R*, 3*R*, 10*R*, 11*R* and 2*R*, 3*R*, 10*S*, 11*S*, respectively (Figure 1).[13] To date, separation of both diastereoisomers has been achieved by analytical HPLC only.[14]



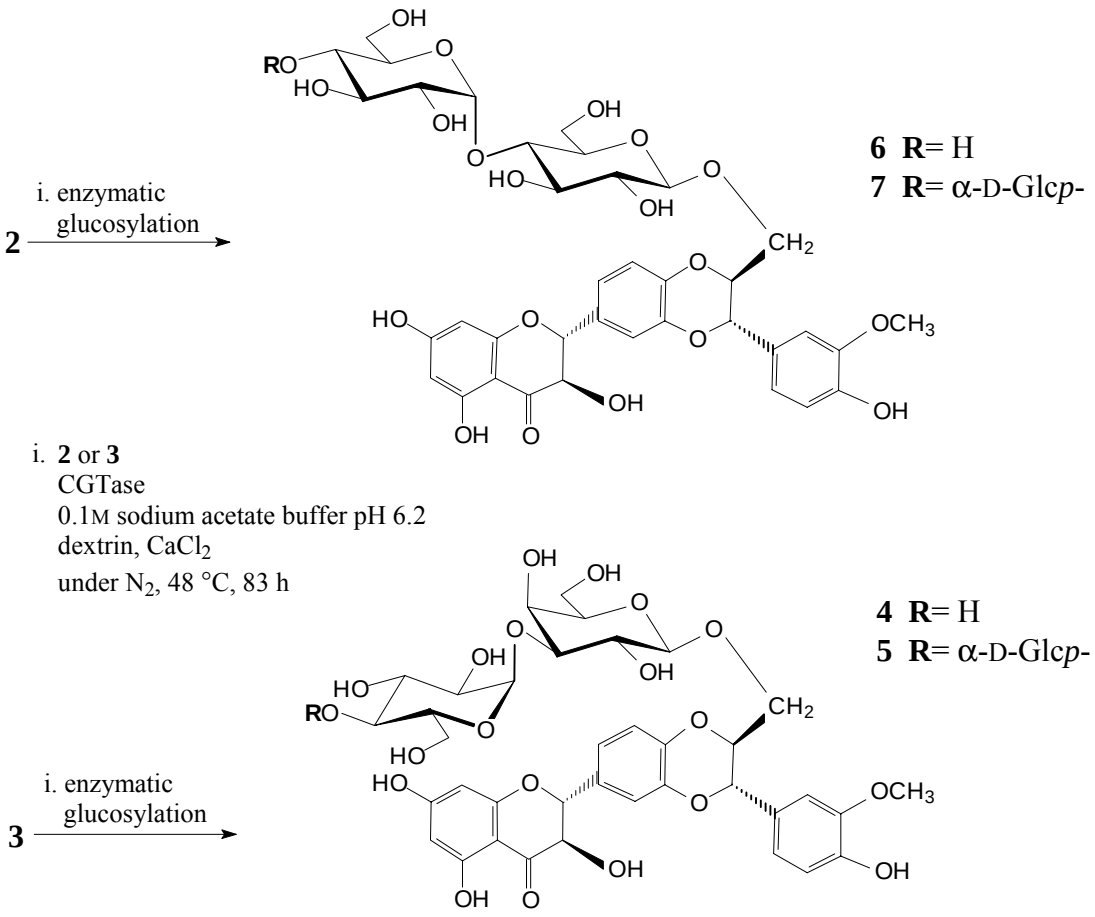
1 **R** = H
2 **R** = β-D-glucopyranosyl
3 **R** = β-D-galactopyranosyl

Figure 1

Here we report on enzymatic synthesis of the complex silybin glycosides employing CGTase from *Bacillus stearothermophillus.* Antioxidant activities of the compounds were tested *in vitro* using a model of copper-induced oxidation of LDL.[5c,15a]

RESULTS AND DISCUSSION

CGTase (cyclodextrin glucanotransferase, E.C. 2.4.1.19) from *Bacillus stearothermophillus* was chosen to be suitable tool for the preparation of silybin glycosides. Primarily, this enzyme catalyzes synthesis of cyclodextrins from starch.[16] It is also able to catalyze the transfer of a-glucosyl unit to various acceptors, mainly glycosides, extending the sugar chain by one or more a-D-Glc*p* moieties. The acceptor specificity of CGTase is rather broad. In the glycosides with the terminal D-Glc*p* moiety, the transfer is rather regioselective with the preference for 4-OH group so that the second Glc unit is usually attached by an a(1→4) bond. In other glycosides the regioselectivity is rather poor.[16]



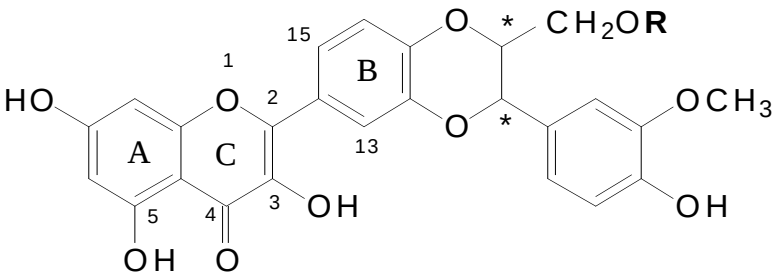
Scheme 1 Glycosides of silybin prepared by enzymatic extension

23-*O*-b-D-Glucopyranosyl silybin (**2**) or 23-*O*-b-D-galactopyranosyl silybin (**3**)[9] (Figure 1) served as the glycosyl acceptors for enzymatic Glc*p* unit transfer (Scheme 1). We have used - mostly for easier spectral characterization - diastereomerically pure silybin glycosides that were prepared *via* peracetylated glycosides.[9] Dextrin was used as a glucosyl donor, $CaCl_2$ was used to protect the enzyme against thermal denaturation.[16b] Transglycosylation was catalyzed by CGTase from *Bacillus stearothermophillus.* Silybin molecule is very sensitive to the oxidation by molecular oxygen, especially in the alkaline medium[17] (antioxidant properties represent major mechanism of its hepatoprotective activity). Therefore, the reaction mixture was kept under nitrogen atmosphere.

Table 1 HR ESI-MS data of prepared glycosides

Glycoside	Elemental composition	$[M+Na]^+_{calcd.}$	$[M+Na]^+_{found}$
4	$C_{37}H_{42}O_{20}Na$	829.2167	829.2167
4a	$C_{37}H_{40}O_{20}Na$	827.2011	827.2011
5	$C_{43}H_{52}O_{25}Na$	991.2695	991.2703
5a	$C_{43}H_{50}O_{25}Na$	989.2539	989.2535
6	$C_{37}H_{42}O_{20}Na$	829.2167	829.2163
6a	$C_{37}H_{40}O_{20}Na$	827.2011	827.2009
7	$C_{43}H_{52}O_{25}Na$	991.2695	991.2689
7a	$C_{43}H_{50}O_{25}Na$	989.2539	989.2533

High resolution MS spectrometry of the products (**4**, **6** and **5**, **7**) (Table 1) indicated the presence of two or three glycosyl units in the molecule, respectively. A comparison of proton and carbon chemical shifts (Tables 2-5) of the aglycone in new glycosides (**4**, **5**, **6** and **7**) with the starting glycosides (**2** and **3**) did not show any significant differences what implies the substitution in sugar part of the molecule. The anomeric configuration of all newly formed glycosidic bonds is a as shown from the sugar coupling constants $J_{1,2}$ (3.7 - 3.9 Hz). The attachment of the monosaccharide unit was determined on the basis of the downfield shift of the substituted carbon (C-23, C-3', C-4', C-4''). Heteronuclear couplings between atoms of different rings (H-23→C-1',H-1'→C-23,H-1''→C-x',H-x'→C-1'',H-1'''→C-x'',H-x''→C-1''') in HMBC experiments clearly indicated the type of this attachment. Different configuration (Gal *versus* Glc), anomeric configuration (a- *versus* b-) and couplings H-1'→C-23, H-23→C-1' were used for the discrimination of the first monosaccharide unit (attached to the aglycone).



4a R = Glc*p*-α-(1→3)-Gal*p*-β-
5a R = Glc*p*-α-(1→4)-Glc*p*-α-(1→3)-Gal*p*-β -
6a R = Glc*p*-α-(1→4)-Glc*p*-β-
7a R = Glc*p*-α-(1→4)-Glc*p*-α-(1→4)-Glc*p*-β-

Figure 2

Table 2 ^{1}H NMR Data (399.91 MHz, CD_3OD, 30 °C); aglycone part: chemical shifts [ppm], multiplicity and coupling constants [Hz] (italicized)

Proton	**4**	**4a**	**5**	**5a**
2	4.987 d; *11.5*	-	5.004 d; *11.5*	-
3	4.561 d; *11.5*	-	4.533 d; *11.5*	-
6	5.934 d; *2.1*	6.170 m	5.943 d; *2.1*	6.181 d; *2.0*
8	5.904 d; *2.1*	6.358 m	5.915 d; *2.1*	6.381 d; *2.0*
10	4.262 ddd; *7.7, 4.3, 2.6*	4.287 ddd; *7.9, 4.3, 4.1*	4.279 ddd; *7.7, 4.3, 2.5*	4.318 ddd; *7.9, 3.9, 2.8*
11	5.074 d; *7.7*	5.081 d; *7.9*	5.094 d; *7.7*	5.110 d; *7.9*
13	7.115 d; *2.0*	7.785 d; *2.0*	7.121 d; *2.1*	7.819 d; *2.1*
15	7.051 dd; *8.4, 2.0*	7.731 dd; *8.5, 2.0*	7.060 dd; *8.3, 2.1*	7.766 dd; *8.5, 2.1*
16	6.977 d; *8.4*	7.001 d; *8.5*	6.986 d; *8.3*	7.040 d; *8.5*
18	7.075 d; *2.0*	7.101 d; *1.8*	7.084 d; *2.0*	7.110 d; *1.9*
21	6.846 d; *8.1*	6.866 d; *8.1*	6.850 d; *8.2*	6.869 d; *8.2*
22	6.964 dd; *8.1, 2.0*	6.987 dd; *8.1, 1.8*	6.976 dd; *8.2, 2.0*	6.998 dd; *8.2, 1.9*
23u[1]	3.789 dd; *12.3, 2.6*	3.905 dd; *11.7, 4.1*	3.890 dd; *12.3, 2.0*	3.900 dd; *11.7, 1.9*
23d[1]	3.899 dd; *12.3, 4.3*	3.812 dd; *11.7, 4.3*	3.800 dd; *12.3, 4.3*	3.830 dd; *11.7, 1.9*
19-OMe	3.895 s	3.920 s	3.904 s	3.923 s

Table 3 ^{1}H NMR data (399.91 MHz, CD_3OD, 30 °C); sugar part: chemical shifts [ppm], multiplicity and coupling constants [Hz] (italicized)

Proton	**4**	**4a**	**5**	**5a**
1'	4.324 d; *7.4*	4.337 d; *7.4*	4.331 d; *7.5*	4.344 d; *7.4*
2'	3.706 dd; *9.7, 7.4*	3.718 dd; *9.7, 7.4*	3.68 m	3.709 dd; *9.7, 7.4*
3'	3.649 dd; *9.7, 3.1*	3.664 dd; *9.7, 3.0*	3.64 m	3.66 m
4'	4.057 dd; *3.1, 0.8*	4.067 dd; *3.0, 1.0*	4.056 dd; *2.9, 1.0*	4.063 dd; *2.8, 0.9*
5'	3.482 dt; *0.8, 6.1*	3.496 dt; *1.0, 6.0*	3.487 dt; *1.0, 6.1*	3.496dt; *0.9, 6.1*
6'	3.755 d (2H); *6.1*	3.764 d (2H); *6.0*	3.756 d (2H); *6.1*	3.762 d (2H); *6.1*
1"	5.006 d; *3.7*	5.012 d; *3.8*	5.003 d; *3.8*	5.007 d; *3.9*
2"	3.457 dd; *9.7, 3.7*	3.458 dd; *9.8, 3.8*	3.508 dd; *9.8, 3.8*	3.509 dd; *9.8, 3.9*
3"	3.735 dd; *9.7, 9.0*	3.763 dd; *9.8, 8.9*	3.994 dd; *9.8, 9.0*	3.996 dd; *9.7, 9.0*
4"	3.367 dd; *10.0, 9.0*	3.364 dd; *10.1, 8.9*	3.585 dd; *9.9, 9.0*	3.584 dd; *10.0, 9.0*
5"	4.019 ddd; *10.0, 5.0, 2.4*	4.020 ddd; *10.1, 5.0, 2.4*	4.132 ddd; *9.9, 3.5, 2.6*	4.134 ddd *10.0, 5.0, 2.4*
6"u[a]	3.698 dd; *11.9, 5.0*	3.665 dd; *11.8, 5.0*	3.82 m	3.82 m
6"d[a]	3.752 dd; *11.9, 2.4*	3.810 dd; *11.8, 2.4*	3.85 m	3.84 m
1"'	-	-	5.184 d; *3.8*	5.178 d; *3.8*
2"'	-	-	3.461 dd; *9.7, 3.8*	3.454 dd; *9.7, 3.8*
3"'	-	-	3.623 dd; *9.7, 8.8*	3.62 m
4"'	-	-	3.280 dd; *9.8, 8.8*	3.272 dd; *9.6, 9.0*
5"'	-	-	3.69 m	3.69 m
6"'u[a]	-	-	3.67 m	3.66 m
6"'d[a]	-	-	3.84 m	3.83 m

[a] u – upfield; d - downfield

Even though the reaction was kept under nitrogen atmosphere, the yellow by-products with R_f very similar to the respective glycosides (**4**, **5**, **6** and **7**) were formed, too. Molecular MS of these by-products were 2 amu lower than those of corresponding desired glycosides (Table 1). NMR spectra of these by-products did not contain typical AB system of protons H-2, H-3. Protons of the ring A were markedly

shifted downfield. The presence of two carbon signals of the sp^2-type instead of two oxymethine signals suggested the formation of a tetrasubstituted double bond. One sp^2-type carbon resonated in proton-coupled ^{13}C NMR spectra as a singlet (no hydrogen atom in its vicinity). Upfield shift of the carbon C-4

Table 4 ^{13}C NMR data (100.57 MHz, CD_3OD, 30 °C); aglycone part: chemical shifts [ppm]

Carbon	**4**	**4a**	**5**	**5a**	**6**	**6a**
2	84.90	147.10	84.94	147.12	84.88	146.87
3	73.96	138.00	73.51	137.99	73.59	137.96
4	198.44	177.59	198.47	177.60	198.12	177.47
4a	102.03	104.72	102.07	104.65	102.73	104.45
5	165.54	162.68	165.61	162.73	165.63	162.65
6	97.81	99.87	97.80	99.96	98.06	100.13
7	169.34	166.55	169.34	166.89	170.54	167.46
8	96.77	95.02	96.76	95.07	97.06	95.19
8a	164.64	158.52	164.68	158.63	164.64	158.60
10	79.21	79.58	79.26	79.61	78.01	79.59
11	77.66	77.67	77.69	77.71	77.68	77.68
12a	145.32	145.27	145.36	145.32	145.35	145.25
13	117.97	117.96	117.96	117.98	117.96	117.92
14	131.98	126.07	132.00	126.11	n.o.[a]	126.11
15	122.39	122.77	122.42	122.80	122.40	122.73
16	118.16	118.22	118.16	118.26	118.13	118.20
16a	145.46	146.61	145.50	146.65	145.75	146.51
17	129.65	129.48	129.69	129.49	129.64	129.44
18	112.73	112.80	112.79	112.81	112.61	112.65
19	149.39	149.43	149.41	149.44	149.45	149.42
20	148.49	148.58	148.58	148.60	148.59	148.61
21	116.52	116.57	166.55	116.57	116.50	116.54
22	122.01	122.08	122.04	122.08	122.17	122.20
23	69.44	69.37	69.44	69.35	69.42	69.41
19-OMe	56.94	57.00	59.96	56.99	56.86	56.90

[a] not observed

(177 ppm instead of 198 ppm) implies extended conjugation. The same effect explains the downfield shift of the protons of the ring A. The structures of $\Delta^{2,3}$-derivatives (**4a**, **5a**, **6a** and **7a**) were derived from these spectral observations (Figure 2). The comparison of the carbon chemical shifts with flavone quercetin, containing the same substructure was very satisfactory.

Table 5 ^{13}C NMR data (100.57 MHz, CD_3OD, 30 °C); sugar part: chemical shifts [ppm]

Carbon	4	4a	5	5a	6
1'	105.54	105.55	105.56	105.55	101.74
2'	71.30	71.33	71.27	71.28	73.96
3'	80.55	80.57	80.61	80.60	75.18
4'	66.96	66.98	66.98	66.98	81.49
5'	76.57	76.61	76.60	76.60	72.68
6'	62.65	62.68	62.67	62.67	62.50
1"	97.90	97.92	97.80	97.79	103.16
2"	73.81	73.83	73.51	73.51	74.57
3"	75.31	75.33	75.12	75.11	75.08
4"	71.91	71.94	81.75	81.73	71.81
5"	73.78	73.80	72.47	72.46	75.42
6"	62.80	62.82	62.22	62.21	63.04
1'"	-	-	103.08	103.07	-
2'"	-	-	74.59	74.58	-
3'"	-	-	75.46	75.44	-
4'"	-	-	71.90	71.88	-
5'"	-	-	75.00	74.99	-
6'"	-	-	63.11	63.07	-

This oxidative derivatives of silybin are, to our knowledge, the first isolated and structurally characterized oxidative products of silybin. These findings are of utmost importance not only for pharmacology and studies of silybin biotransformation but also for the stability and quality information of various formulations containing silybin. Pure silybin is faintly yellow compound, however, most of commercial preparations containing silybin are intensively yellow indicating the possible presence of the

above $\Delta^{2,3}$ oxidative products. $\Delta^{2,3}$-Silybin has been characterized also by UV spectroscopy and compared with silybin (Table 6) as we assume that these data are important for differential UV spectrometry and HPLC analysis of various silybin preparations. UV spectra of silybin and its 23-*O*-glycosides are virtually identical,[9] therefore, we have measured electronic spectra of $\Delta^{2,3}$-silybin that was liberated from its glycosides by mild acidic hydrolysis.[9] To obtain reproducible results (dissociation equilibria of the phenolic groups)[9] the spectra were recorded in buffered solutions (MeOH/HCl or MeOH/MeONa).

Table 6 UV spectra of silybin and 2,3-dehydrosilybin

	Silybin		$\Delta^{2,3}$-Silybin	
	λ_{max} [nm]	ε_{max} [L.mol^{-1}.cm^{-1}]	λ_{max} [nm]	ε_{max} [L.mol^{-1}.cm^{-1}]
MeOH/HCl	288.0	26 300	254.4	18 500
			308.8	8 700
			367.8	16 700
MeOH/MeONa	211.0	76 600	258.0	22 100
	250.9	19 000	277.6	21 000
	290.4	13 400	306.8	11 200
	326.9	31 700	401.2	12 800

With compounds (**6**) and (**7**), the configuration and position of all newly formed glycosidic bonds were a-(1→4). This arrangement is in the agreement with the known pattern of CGTase acting on *gluco*-structures. Analogous regioselectivity was observed in the oxidized by-products (**6a**) and (**7a**).[16]

The new glycosidic bond between galactose and glucose moieties in compounds (**4**) and (**5**) was a(1→3). This difference is obviously given by axial 4'-OH group of the galactoside (**3**) that is not accessible to the a-glucose transfer. Analogous selectivity was observed in corresponding oxidized by-products (**4a**) and (**5a**).[16] The transfer of the a-glucose moiety upon **3** is the yield-limited step in the whole reaction cascade; therefore, the yields of oligoglucosides resulting from **3** are generally lower.

Mixtures of silybin oligoglycosides were separated by gel chromatography on Sephadex LH-20 with the mobile phase MeOH-H_2O 4:1 (v/v). Regardless of their molecular weight, all oxidized silybin glycosides (**4a**, **5a** and **6a**, **7a**) eluted later than the respective silybin glycosides (**4**, **5** and **6**, **7**). This separation pattern implies large difference in solvation caused by dehydrogenation and conformational changes.

Even though the separation pattern does not correspond with generally accepted concept of exclusion chromatography (e.g., separation based on the molecular masses) this effect enabled clear separation of oxidized silybin glycosides from the parent compounds.

Glycosides (**4**, **5**, **4a** and **5a**) were completely spectrally characterized by MS and NMR (see Tables 1-5). With compounds (**6**, **7**, **6a** and **7a**) not all NMR signals were assigned, however, there is a sufficient spectral support for the given structures. When b-Glc*p* unit serves as an acceptor for the glucosylation, all newly formed glycosidic bonds are a(1→4).

Aside from the products described also mixtures of higher oligoglycosides (homologues) of silybin resulting from **2** and **3** were isolated but not spectrally characterized.

As it was shown previously, silybin possesses the LDL antioxidant activity *in vitro*.[5b,c] However, *in vivo* the LDL antioxidant effect of silybin was observed only when administered to the rats as a dietary supplement of more bioavailable forms as silybin galactoside or silipide.[15b] The aim of this study was to define the influence of different glycosylation on the LDL antioxidant activity of silybin *in vitro*.

Table 7 Antioxidant effect of silybin glycosides on copper-induced oxidation of LDL expressed as percentual prolongation of the lag time

Compound[a]	Prolongation of the lag time [%]
silybin (**1**)	22.49
silybin 23-*O*-b-D-glucopyranoside (**2**)[b]	5.93
silybin 23-*O*-b-D-galactopyranoside (**3**)[b]	12.69
4	8.38
5	6.63
6	2.21
7	9.08
silybin 23-*O*-b-D-lactoside[b]	11.84

[a] mixture of diastereoisomers A and B (in aglycon part);[b] prepared previously[9]

Table 7 summarizes our findings. Silybin added in LDL (*in vitro*) caused the prolongation of lag time, a parameter accepted to express the improved resistance of LDL to oxidation by copper ions.[15a] Glycosylation of silybin led to the suppression of antioxidant effect of silybin since all glycosides were

less effective to prolong lag time than silybin. Better antioxidant effect of glycosylated silybin than that of silybin *in vitro*[15b] may be associated with its better bioavailability.

EXPERIMENTAL

General methods.- Reactions were monitored by TLC on silica gel F_{254} plates (Merck) using the solvent system EtOAc-MeOH-H_2O 77:13:10 (v/v). The spots were visualized by UV light and by charring with 10 % H_2SO_4 in ethanol. Solid phase extraction was performed using XAD-4 Amberlite (BDH Chemicals Ltd., England) resin column (150×40 mm). After extensive washing with water, retained silybin glycosides were eluted with methanol and acetone. Size exclusion chromatography was performed on Sephadex LH-20 (column 950×25 mm) with MeOH-H_2O 4:1 (v/v) as a mobile phase. Optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22 °C in MeOH.

^1H and ^{13}C NMR spectra were measured on a Varian INOVA-400 spectrometer (399.91 and 100.57 MHz, respectively) in CD_3OD at 30 °C. Residual solvent signal (d_H 3.33, d_C 49.3) served as an internal reference. Chemical shifts are given in the d scale, *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, HOM2DJ, ROESY, HMQC, HMBC).

Positive ion electrospray ionization spectra (ESI-MS) were recorded on a double-focusing instrument Finnigan MAT 95 (Finnigan MAT, Bremen, FRG) with BE geometry. Samples dissolved in MeOH-H_2O 2:1 (v/v) were continuously infused through a stainless capillary held at 3.3 kV into Finnigan ESI source *via* linear syringe pump at a flow rate of 40 μL/min. For high-resolution experiments the instrument was tuned to a resolution of about 8 000 (10% valley definition) and the measurements were carried out by the peak-matching method using a mixture of polypropylene glycols (average M_r = 725) as an internal standard.

LDL antioxidant activities of new silybin glycosides were measured using well established methods.[5c,15a]

Typical procedure of the preparation of silybin oligoglycosides from 23-*O*-b-D-glucopyranosylsilybin (2). Glycoside (**2**) (207 mg, 0.32 mmol) was dissolved in 0.1M sodium acetate buffer (pH 6.2, 7 mL), and dextrin (690 mg, PINE-DEX No. 1, DE 8, Matsutani Chemicals Kogyo Co. Ltd., Japan) and 5% $CaCl_2$ (170 μL) were added. pH value of the resulting solution was checked and adjusted to pH 6.2 with 0.1M CH_3COOH. CGTase (150 μL, 223 U) from *Bacillus stearothermophillus* (E.C. 2.4.1.19) (kind gift of Hayashibara Co., Okayama, Japan) was added and the solution was bubbled

by nitrogen and kept under its atmosphere at 46 °C. After 51 h another portion of dextrin (100 mg) was added. After 83 h the reaction was stopped by boiling (10 min). Reaction mixture was diluted with 40 mL of distilled water and loaded on the XAD-4 Amberlite resin column equilibrated with water. After extensive washing with water retained silybin glycosides were eluted with methanol (300 mL) and finally with acetone (100 mL) affording mixture of silybin glycosides (458 mg). Resulting mixture (400 mg) was fractionated by gel chromatography on Sephadex LH-20 affording glycosides (**6**, **6a**, **7** and **7a**).

D-Glucopyranosyl-a-(1→4)-D-glucopyranosyl-23-*O*-b-silybin (**6**). 20 mg, amorphous yellowish solid, $[a]_D^{23}$ +0.2º (*c* 1.18, methanol), TLC: R_f 0.34; ^{1}H NMR: 3.895 (3 H, s, OMe), 4.335 (d, *J* 7.8 Hz), 4.373 (d, *J* 7.7 Hz), 4.523 (d, *J* 11.4 Hz, H-3), 4.992 (d, *J* 11.4 Hz, H-2), 5.072 (d, *J* 7.9 Hz, H-11), 5.081 (d, *J* 7.9 Hz, H-11), 5.187 (d, *J* 4.0 Hz), 5.213 (d, *J* 3.9 Hz), 5.888 (d, *J* 2.1 Hz), 5.916 (d, *J* 2.1 Hz), 6.851 (d, *J* 8.2 Hz), 6.975 (dd, *J* 8.2, 2.1 Hz), 7.216 (d, *J* 8.4 Hz), 7.002 (d, *J* 2.1 Hz), 7.063 (dd, *J* 8.4, 1.9 Hz), 7.066 (dd, *J* 8.4, 1.9 Hz), 7.120 (d, *J* 1.9 Hz); ^{13}C NMR: 56.86 q, 62.50 t, 63.04 t, 69.42 t, 71.81 d, 71.91 d, 72.68 d, 73.59 d, 73.96 d, 74.18 d, 74.57 d, 75.00 d, 75.03 d, 75.18 d, 75.25 d, 75.42 d, 77.71 d, 77.88 d, 78.01 d, 81.49 d, 81.79 d, 84.88 d, 97.06 d, 98.06 d, 101.64 d, 101.74 d, 102.73 s, 102.94 d, 103.12 d, 112.61 d, 116.50 d, 117.96 d, 118.13 d, 122.17 d, 122.40 d, 129.64 s, 132.11 s, 145.35 s, 145.75 s, 148.59 s, 149.45 s, 164.64 s, 165.63 s, 170.54 s, 198.12 s.

D-Glucopyranosyl-a-(1→4)-D-glucopyranosyl-23-*O*-b-D^{2,3}-silybin (**6a**). 13 mg, amorphous yellowish solid, $[a]_D^{23}$ +0.4° (*c* 1.81, methanol), TLC: R_f 0.35; ^{1}H NMR: 3.910 (3 H, s, OMe), 4.305 (m), 4.337 (d, *J* 7.8 Hz), 4.383 (d, *J* 7.7 Hz), 5.184 (d, *J* 3.9 Hz), 5.186 (d, *J* 3.9 Hz), 6.159 (br s), 6.346 (br s), arom. protons – overlap; ^{13}C NMR: 56.90 q, 62.44 t, 62.81 t, 63.05 t, 69.41 t, 69.50 t, 71.68 d, 71.80 d, 71.88 d, 74.17 d, 74.23 d, 74.33 d, 74.46 d, 75.07 d, 75.10 d, 75.37 d, 75.45 d, 76.99 d, 77.68 d, 77.85 d, 78.01 d, 79.46 d, 79.59 d, 81.47 d, 87.22 d, 95.19 d, 100.13 d, 101.92 d, 103.15 d, 104.45 s, 104.87 d, 104.98 d, 112.65 d, 116.53 d, 117.92 d, 118.20 d, 122.20 d, 122.73 d, 126.11 s, 129.44 s, 137.96 s, 145.25 s, 146.51 s, 146.87 s, 148.61 s, 149.42 s, 158.60 s, 162.65 s, 167.46 s, 177.47 s.

D-Glucopyranosyl-a-(1→4)-D-glucopyranosyl-a-(1→4)-D-glucopyranosyl-23-*O*-b-silybin (**7**). 14 mg, amorphous yellowish solid, $[a]_D^{23}$ +0.4° (*c* 1.27, methanol), TLC: R_f 0.21.

D-Glucopyranosyl-a-(1→4)-D-glucopyranosyl-a-(1→4)-D-glucopyranosyl-23-*O*-b-D^{2,3} silybin (**7a**). 10 mg, amorphous yellowish solid, $[a]_D^{23}$ +0.3° (*c* 0.91, methanol), TLC: R_f 0.22; ^{1}H NMR: 3.914 (3 H, s, OMe), 4.352 (d, *J* 7.4 Hz), 4.383 (d, *J* 7.5 Hz), 5.108 (d, *J* 7.9 Hz, H-11), 5.169 (d, *J* 3.7 Hz), 5.211 (d, *J* 3.9 Hz), 6.202 (d, *J* 2.1 Hz), 6.413 (d, *J* 2.1 Hz), 6.873 (d, *J* 8.2 Hz), 6.912 (d, *J* 8.2 Hz), 7.000 (dd, *J* 8.2, 2.0 Hz), 7.054 (d, *J* 8.7 Hz), 7.103 (d, *J* 8.7 Hz), 7.118 (d, *J* 1.8 Hz), 7.787 (dd, *J* 8.7, 2.1 Hz), 7.836 (d, *J* 2.1 Hz).

HR ESI-MS of the glycosides (**6**, **6a**, **7** and **7a**) - see Table 1.

Typical procedure of the preparation of silybin oligoglycosides from 23-*O*-b-D-galactopyranosylsilybin (**3**). Glycoside (**3**) (300 mg, 0.47 mmol) was dissolved in 0.1M sodium acetate buffer (pH 6.2, 10 mL), and dextrin (1000 mg, PINE-DEX No. 1, DE 8, Matsutani Chemicals Kogyo Co.

Ltd., Japan) and 5% $CaCl_2$ (250 μL) were added. pH value of the resulting solution was checked and adjusted to pH 6.2 with 0.1M CH_3COOH.

CGTase (250 μL, 371 U) from *B. stearothermophillus* was added and the solution was sparged by nitrogen and kept under its atmosphere at 46 °C. After 50 h another portions of dextrin (100 mg) and CGTase (100 μL, 148 U) were added. After 89 h the reaction was stopped by boiling (10 min). Reaction mixture was diluted with 40 mL of dist. water and loaded on the XAD-4 resin column equilibrated with water. After extensive washing with water retained silybin glycosides were eluted with methanol (300 mL) and finally with acetone (100 mL) affording mixture of silybin glycosides (500 mg). The mixture (500 mg) was consequently fractionated by gel chromatography on Sephadex LH-20 affording glycosides (**4**, **4a**, **5** and **5a**). For HR ESI-MS and NMR data see Tables 1-5.

D-Glucopyranosyl-a-(1→3)-D-galactopyranosyl-23-*O*-b-silybin (**4**). 37 mg, amorphous yellowish solid, $[a]_D^{23}$ +0.3° (*c* 3.40, methanol), TLC: R_f 0.49.

D-Glucopyranosyl-a-(1→3)-D-galactopyranosyl-23-*O*-b-D^{2,3}-silybin (**4a**). 26 mg, amorphous yellowish solid, $[a]_D^{23}$ +0.1° (*c* 2.36, methanol), TLC: R_f 0.50.

D-Glucopyranosyl-a-(1→4)-D-glucopyranosyl-a-(1→3)-D-galactopyranosyl-23-*O*-b-silybin (**5**). 20 mg, amorphous yellowish solid, $[a]_D^{23}$ +0.2° (*c* 1.81, methanol), TLC: R_f 0.34.

D-Glucopyranosyl-a-(1→4)-D-glucopyranosyl-a-(1→3)-D-galactopyranosyl-23-*O*-b-D^{2,3}-silybin (**5a**). 16 mg, amorphous yellowish solid, $[a]_D^{23}$ +0.5° (*c* 1.45, methanol), TLC: R_f 0.35.

HR ESI-MS of the glycosides(**4**, **4a**, **5** and **5a**) - see Table 1, for ^1H and ^{13}C spectra see Tables 2-5.

ACKNOWLEDGEMENTS

This work was supported by the grants No. 303/98/0414 and 303/99/1382 from the Grant Agency of the Czech Republic, No. PP-Z1/13/96 from The Ministry of Industry and Commerce, and the grant No. LB98233 from The Ministry of Education of the Czech Republic.

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