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Oxidised derivatives of silybin and their antiradical and antioxidant activity

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Abstract—Carboxylic acids derived from silybin (**1**) and 2,3-dehydrosilybin (**2**) with improved water solubility were prepared by selective oxidation of parent compounds and a new inexpensive method for preparation of 2,3-dehydrosilybin from silybin was developed and optimised. The antioxidative properties of the above-mentioned compounds and of side product **3a** from oxidation of compound **1** were determined by cyclic voltammetry, free radical scavenging (DPPH, superoxide) assays, and by inhibition of in vitro generated liver microsomal lipid peroxidation. Dehydrogenation at $C_{(2)}$–$C_{(3)}$ in flavonolignans (silybin vs 2,3-dehydrosilybin; silybinic acid vs 2,3-dehydrosilybinic acid) strongly improved antioxidative properties (analogously as in flavonoids taxifolin vs quercetin). Thus, in antioxidative properties, dehydrosilybin was superior to silybin by one order, but its water solubility is too low for application in aqueous milieu. On the other hand, 2,3-dehydrosilybinic acid is a fairly soluble derivative with antilipoperoxidation and antiradical activities better than that of silybin.

1. Introduction

Flavonolignan silybin (**1**), isolated from seeds of the milk thistle (*Silybum marianum*), is an active component in a number of phytopreparations, for example, Silymarin-Forte™ (Natur-Produkt, CZ), Legalon™ (Madaus, D), widely used in human therapy for liver function improvement and as a protectant against a number of hepatotoxins (CCl_4, galactosamine, *tert*-butylhydroperoxide, phalloidine, α-amanitine).[1] Natural **1** consists of two diastereomers, silybin A (3-(*R*),5,7-trihydroxy-2-(*R*)-[3-(*R*)-(4-hydroxy-3-methoxyphenyl)-2-(*R*)-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]chroman-4-one) and silybin B (3-(*R*)-5,7-trihydroxy-2-(*R*)-[3-(*S*)-(4-hydroxy-3-methoxyphenyl)-2-(*S*)-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-chroman-4-one)[2]—in the ratio ca. 45:55. Cytoprotectivity of **1** consists in several mechanisms operating at various cell levels. Silybin acts as a radical scavenger[3] and chain breaking antilipoperoxidant.[4] Other important antioxidative effects of **1** are due to its influence on the superoxide dismutase[5] and the enzyme system associated with glutathione.[4] Recently, low density lipoprotein (LDL) antioxidant activity of **1** has been reported,[6] demonstrating thus its antiatherosclerotic effects.[7] Cell regenerating activity is associated with its ability to activate the proteosynthesis by DNA-dependent RNA-polymerase I stimulation.[8]

The bioavailability and therapeutic efficiency of silybin is rather limited by its very low water solubility (430 mg/L). The solubility was improved by the preparation of silybin 3,23-*O*-bis-hemisuccinate[9] that enabled intravenous application of silybin (Legalon-SIL) for the treatment of acute liver intoxication by mycotoxins. Glycosylation was demonstrated to be another way of improving silybin solubility.[2,10]

All commercial preparations of silybin suffer from spontaneous oxidation. The raw material for silybin preparation, for example, complex extract from seeds of

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S. marianum denoted as silymarin, contains some oxidation products, which were practically neglected in the studies of the silybin (silymarin) biological activity.[11] 2,3-Dehydrosilybin (**2**), (3,5,7-trihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-4*H*-chromen-4-one), was found as a common component in the extracts from seeds of *S. marianum* subsp. *anatolicum*[12] but according to our (unpublished) experience it is present in almost all silymarin and silybin preparations. This compound (and probably other derivatives) causes the characteristic yellow colour of silybin preparations, whereas pure silybin is practically colourless. 2,3-Dehydrosilybin (**2**) may be also one of the products of in vivo silybin oxidation during its antioxidative action.

The preparation of **2** from silybin was accomplished by H_2O_2 oxidation in $NaHCO_3$ solution[13] or alternatively by iodine oxidation in glacial acetic acid.[14] Its activity against the toxins of *Amanita phalloides* is considerably lower compared to silybin[13] and this was probably the reason why this compound was somehow ignored in further biological studies. However, in our preliminary experiments **2** was better antioxidant than silybin. This compound has positive effect on some skin diseases as, for example, psoriasis and atopical dermatitis (our unpublished results). *C*-Isoprenylated or geranylated derivates of **2** also constitute potentially effective modulators of P-glycoprotein.[15]

An important side product arising from oxidation of silybin[13] is a 'diketone' 3-[3-(4-hydroxy-3-methoxy-phenyl)-2-hydroxymethyl-2,3-dihydro-benzo[1,4]dioxin-6-yl]-1-(2,4,6-trihydroxy-phenyl)-propane-1,2-dione (**3**), which is also a plausible product of silybin oxidation in vivo and a contaminant of silybin preparations (Fig. 1). This compound can form under specific conditions an intramolecular hemiacetal (**3a**) (2,4,6-trihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-1-benzofuran-3(2*H*)-one), which was isolated and characterised in this study for the first time.

Another, so far unknown, oxidation products of both **1** and **2**, e.g., silybinic (**11**) and 2,3-dehydrosilybinic (**13**) acids are presented in this paper. It is expected that these compounds bearing easily ionisable carboxy functionality will be more hydrophilic while maintaining beneficial biological effects of their precursors (**1**, **2**). All the new compounds prepared in this study will also help to understand molecular mechanisms of antiradical and antioxidative activity of silybin and further optimise its applications (for the structures see Chart 1).

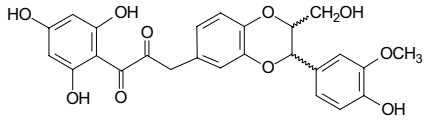
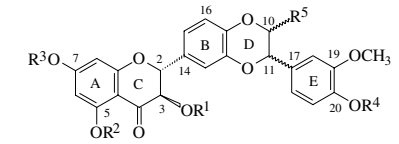
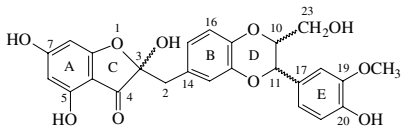


Figure 1. 'α-Diketone' **3**—degradation product of silybin in alkaline aqueous solutions.

	R^1	R^2	R^3	R^4	R^5
1	H	H	H	H	CH_2OH
4	H	H	H	H	$CH_2OTBDMS$
5	Ac	Ac	Ac	Ac	$CH_2OTBDMS$
6	Ac	Ac	Ac	Ac	CH_2OH
10	Ac	Ac	Ac	Ac	COOH
11	H	H	H	H	COOH





3a

	R^1	R^2	R^3	R^4	R^5
2	H	H	H	H	CH_2OH
7	H	H	H	H	$CH_2OTBDMS$
8	Ac	H	Ac	Ac	$CH_2OTBDMS$
9	Ac	H	Ac	Ac	CH_2OH
12	Ac	H	Ac	Ac	COOH
13	H	H	H	H	COOH

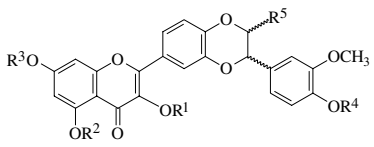


Chart 1.

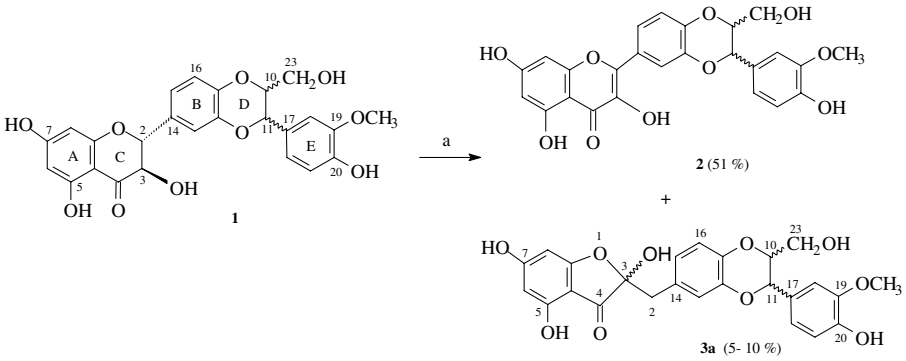
2. Results and discussion

2.1. Chemistry

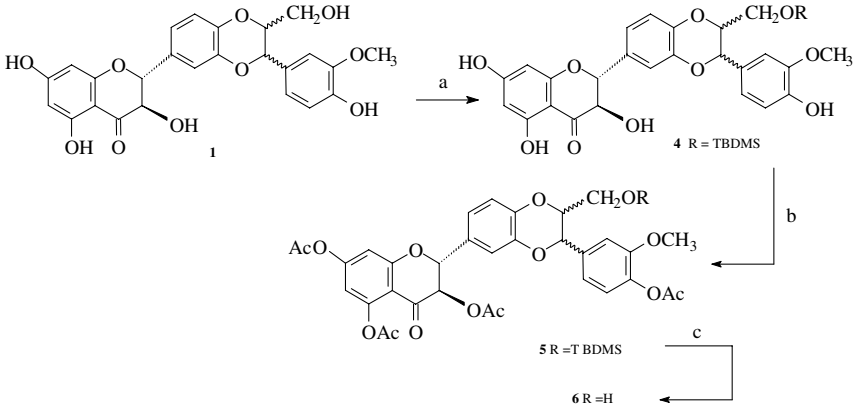
2.1.1. Preparation of 3,5,7-trihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-4*H*-chromen-4-one (2,3-dehydrosilybin, 2). Refluxing of silychristin (a flavonolignan from silymarin group containing the same benzopyran-4-on moiety as silybin) in dry pyridine (116 °C) with access to air leads to 2,3-dehydrosilychristin almost quantitatively.[16] However, silybin polymerises under the same conditions and forms hemiacetal **3a**. The decrease in the reaction temperature to 80–90 °C significantly reduced formation of these by-products (see Scheme 1). Use of pure oxygen did not give better results than the use of air.

The reaction of silybin with aqueous solution of *N*-methyl-D-glucamine in an inert atmosphere yields diketone **3**, however, under access of air also **2** is formed.[13] Weak aprotic base such as pyridine probably catalyses the formation of thermodynamically stabilised hemiacetal **3a**. Comparison of ^{1}H NMR spectrum of the compound **3a** with that of silybin (**1**) showed that the AB system of vicinal protons H-2 and H-3 was replaced by another one (in fact two, presumably stemming from silybin A and B, ratio ca. 45:55) representing geminal protons (^2J = 13.7 or 14.2 Hz) coupled to the same carbon resonating at 40.49 ppm. This methylene group is located at the benzylic position to the ring B, as evident from long-range interproton couplings to H-13 and H-15, as well as from heteronuclear couplings of these protons to C-13, C-14 and C-15. The H-2 protons are also coupled to carbons resonating at 192.7 (C-4) and 105.4 ppm (assigned to C-3). Therefore, the C-ring is five-membered and contains a hemiacetal group (**3a**).

2.1.2. Preparation of the carboxy derivatives of 1 and 2. The silylation of **1** or **2** by *tert*-butyldimethylsilyl chlo-



Scheme 1. Reagents and conditions: (a) pyridine, air, 95 °C, 100 h.



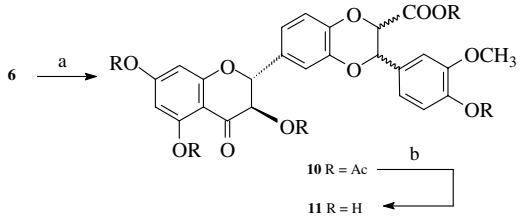
Scheme 2. Reagents and conditions: (a) TBDMSCl, pyridine, $AgNO_3$ (cat.), 40 °C, 2 h; (b) Ac_2O, pyridine, rt, 24 h; (c) $BF_3{\cdot}Et_2O$, $CHCl_3$, rt, 12 h.

ride in pyridine catalysed by $AgNO_3$[17] yielded 23-*O*-TBDMS ethers **4** or **7** (67% and 60%, respectively). The analogous alkylation of **1** by triphenylmethyl chloride (or bromide) afforded only traces of the corresponding trityl ether. The ethers **4** and **7** were acetylated by a standard Ac_2O/pyridine procedure yielding corresponding acetates **5** or **8**. The TBDMS group was removed with boron trifluoride etherate in $CHCl_3$ giving respective alcohols **6** or **9** (Scheme 2).[18] The use of Bu_4NF in THF failed in this case.

The oxidation of the alcohols **6** and **9** to the corresponding acids **10** and **12** was carried out using solution of H_5IO_6/CrO_3 in wet CH_3CN (0.75% v/v water) (Scheme 3).[19]

The NMR data of individual silybin A and B were already published in CD_3OD and CD_3COCD_3 solutions.[2,20] We have chosen DMSO-d_6 as a solvent in order to use the OH resonances for the experimental signal assignment (Fig. 2). The chemical shift differences were small in the carbon dimension so that both diastereomers mostly gave common crosspeaks. However, for some atoms different signals were observed (Tables 1 and 2) but were not assigned to the individual silybins. On the contrary, only one set of NMR signals was observed in the 2,3-dehydrosilybin series (Tables 3 and 4).

The structures of compounds **4** and **7** were confirmed by NMR. The position of TBDMS group was determined indirectly, proving the presence of hydroxyls by HMBC. Similarly, with acetyl derivatives **5**, **6**, **8** and **9**, the acetylated positions were inferred from gHMQC (relying upon the ^4J couplings[21,22] between the acetyl protons and carbons at the site of acetylation in the case of enol acetates). Because of signal doubling in the ^1H NMR spectra of **6** and **10**, the actual number of acetyl groups



Scheme 3. Reagents and conditions: (a) H_5IO_6/CrO_3, CH_3CN, 0 °C, 1.5 h; (b) K_2CO_3, MeOH, H_2O, rt, 24 h.

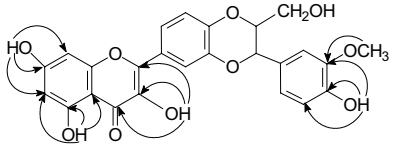


Figure 2. Diagnostic heteronuclear couplings in **2** observable by HMBC (DMSO-d_6).

Table 1. ^{1}H NMR data (399.89 MHz, 30 °C) of silybin derivatives

Proton	**4**[a]	**6**[b]	**10**[b]	**11**[a]
2	5.081 d (11.3)	5.359 d (12.1)	5.342 d (12.2)	5.066 d (11.2)
		5.368 d (12.1)	5.349 d (12.2)	5.068 d (11.2)
3	4.592 dd (11.3, 6.2)	5.682 d (12.1)	5.676 d (12.2)	4.597 d (11.2)
	4.603 dd (11.3, 6.2)	5.706 d (12.1)	5.679 d (12.2)	
6	5.199 d (2.1)	6.593 d (2.2)	6.594 d (2.2)	5.919 d (2.1)
		6.596 d (2.2)	6.596 d (2.2)	
8	5.871 d (2.1)	6.784 d (2.2)	6.785 d (2.2)	5.887 d (2.1)
	5.876 d (2.1)	6.792 d (2.2)	6.791 d (2.2)	
10	4.230 ddd (8.0, 3.7, 2.4)	4.037 ddd (8.2, 3.6, 2.4)	5.308 d (5.3)	4.975 d (4.7)
	4.240 ddd (8.0, 3.7, 2.4)	4.053 ddd (8.2, 3.6, 2.4)	5.315 d (5.3)	4.986 d (4.7)
11	4.881 d (8.0)	5.061 d (8.2)	4.836 d (5.3)	5.285 d (4.7)
		5.065 d (8.2)	4.841 d (5.3)	5.289 d (4.7)
13	7.082 d (2.0)	7.126 d (2.0)	7.098 d (2.0)	7.089 d (2.2)
	7.093 d (2.0)		7.142 d (2.0)	
15	7.016 dd (8.2, 2.0)	6.980 dd (8.2, 2.0)	6.988 dd (8.2, 2.0)	6.999 dd (8.3, 2.2)
	7.025 dd (8.2, 2.0)	6.894 dd (8.3, 2.0)	7.000 dd (8.0, 2.0)	7.005 dd (8.3, 2.2)
16	6.957 d (8.2)	7.016 d (8.2)	7.022 d (8.2)	6.938 d (8.3)
	6.962 d (8.2)	7.020 d (8.2)		6.941 d (8.3)
18	7.009 d (1.9)	7.061 d (1.7)	6.987 d (2.0)	7.007 d (2.0)
		7.075 d (1.7)	6.993 d (2.0)	
21	6.814 d (8.2)	7.100 d (8.0)	7.038 d (8.1)	6.735 d (8.1)
			7.043 d (8.1)	6.738 d (8.1)
22	6.865 dd (8.2, 1.9)	7.047 dd (8.0, 1.7)	6.953 dd (8.1, 2.0)	6.848 dd (8.1, 2.0)
		7.052 dd (8.0, 1.7)	6.969 dd (8.1, 2.0)	
23d[c]	3.521 dd (12.0, 3.7)	3.870 dd (12.6, 2.4)	—	—
			—	—
23u[c]	3.775 dd (12.0, 2.4)	3.599 dd (12.6, 3.6)	—	—
	3.777 dd (12.0, 2.4)		—	—
OMe	3.776 s	3.871 s	3.799 s	3.726 s
	3.779 s	3.879 s	3.803 s	3.729 s

Additional signals—**4**: 0.009 (3H, s, Si–Me), 0.018 (3H, s, Si–Me), 0.857 (9H, s, Me_3C), 5.786 (1H, d, J = 6.2, 3-OH), 9.138 (1H, s, 20-OH), 10.832 (1H, s, 7-OH), 11.877 (1H, s, 5-OH); **6**: 2.051 s, 2.055 s, 2.299 s, 2.331 s, 2.377 s, 2.379 s, (4 × Ac); **10**: 1.992 s, 1.998 s (3-Ac), 2.296 s (7-Ac), 2.304 s (20-Ac), 2.372 s, 2.375 s (5-Ac), 5.921 (1H, br s, COOH); **11**: 5.796 (1H, d, J = 4.4, 3-OH), 9.094 (1H, s, 20-OH), 11.138 (1H, s, 7-OH), 11.855, 11.889 (1H, s, 5-OH).

[a] In DMSO-d_6.

[b] In $CDCl_3$.

[c] d—Downfield, u—upfield.

was deduced from the number of crosspeaks between acetyls and carbonyls in HMBC. The formation of acids **10**–**13** was confirmed by the transformation of the partial structure –CH(O-)CH(O-)CH_2OH to the –CH(O-)CH(O-)COOH moiety, also corroborated by heteronuclear couplings (Fig. 3).

However, whereas both in silybin and dehydrosilybin series the value of $J_{10,11}$ is 7.8–8.2 Hz, it amounts 5.3, 4.7, 4.3 and 3.8 Hz in the acids **10**–**13**. Neolignans caffeicins A–D having a *cis*-configuration at the dioxane ring, used as models, exhibit J = 3 and 4 Hz.[23] Since the inversion of configuration at C-11 is unlikely for our reaction sequence, we explain the observed values by an equilibrium between two conformations (Fig. 4) shifted towards the right. Similar conformational equilibrium is well known with C-ring of flavans[24] or dihydroisoflavanols.[25]

2.2. Antioxidant activity

The new compounds **3a**, **11** and **13** were tested for their electron-donating potency and for their free radical scavenging ability and inhibition of lipid peroxidation in a comparison with the parent compounds **1**, **2** and structurally related flavonoids taxifolin (**14**) and quercetin (**15**) (Fig. 5, Table 5).

The reducing potency of **1**, **2**, **3a**, **11** and **13** has been evaluated using cyclic voltammetry (CV) and characterised as the anodic peak potential (E_{pa}) values determined from cyclic voltammograms (Table 5). The E_{pa} values, although obtained at the specific in vitro conditions, are considered to be able to predict radical scavenging properties and antioxidant behaviour in biological systems.[26]

In voltammograms of the parent compound **1** and of its derivatives **3a**, **11** and **13** one anodic wave was detected at peak potentials ranging from 524 to 564 mV. The E_{pa} values observed qualify these substances as rather poor antioxidants as compared, for example, with antioxidant vitamins (ascorbic acid, E_{pa} = 370 mV),[26] phenolic acids (caffeic acid, E_{pa} = 100 mV)[27] or flavonoids **14**, **15** (Table 5), and do not suggest significant change of antioxidant potency in derivatives **3a**, **11** and **13** in comparison with **1**. Only compound **2** showed two anodic waves (Fig. 6) that indicates the presence of two sites with different

Table 2. ^{13}C NMR data (100.55 MHz, 30 °C) of silybin derivatives

Carbon	**4**[a]	**6**[b]	**10**[b]	**11**[a]
2	82.53	81.03	80.85	82.53
	82.57	81.10		82.55
3	71.44	73.23	73.21	71.41
	71.51		73.26	
4	197.68	185.24	185.25	197.69
			185.27	
4a	100.50	110.63	110.55	100.44
		110.66	110.57	
5	163.33	151.48	151.33	163.31
		151.60	151.39	
6	96.10	111.19	110.99	96.14
		111.21		
7	166.85	156.39	156.34	167.00
			156.35	
8	95.05	108.92	108.98	95.12
			109.00	
8a	162.48	162.52	162.45	162.47
		162.56	162.47	
10	77.72	78.32	75.76	75.77
		78.36	75.85	
11	75.99	75.98	75.27	75.09
		76.03	75.32	
12a	143.24	143.72	142.72	142.23
	143.27	143.81		142.25
13	116.55	116.44	116.43	116.76
	116.71		116.60	116.86
14	130.11	126.48	129.09	130.45
	130.15	126.59	129.14	130.47
15	121.26	120.75	121.37	121.39
	121.47	120.99	121.41	121.47
16	116.21	117.17	117.31	116.29
	116.28	117.23	117.41	116.31
16a	143.64	144.22	142.48	142.72
	143.67	144.27	142.56	
17	127.13	134.72	133.93	127.29
			133.94	
18	111.88	111.26	111.21	111.66
	111.93	111.38	111.23	
19	147.66	151.48	151.36	147.51
	147.68			
20	147.18	140.44	140.21	146.85
			140.30	
21	115.37	123.14	123.07	115.26
		123.18		
22	120.63	119.82	119.34	119.97
		119.87	119.51	
23	62.23	61.52	170.26	169.05
			170.28	
19-OMe	55.70	56.06	55.93	55.67
		56.11	55.96	

Additional signals—**4**: −5.45, −5.30 (2 × Si–Me), 17.98 (C–Si), 25.76 (Me_3C); **6**: 20.41, 20.63, 20.91, 21.13 (4 × Ac), 167.75, 168.76, 169.05 (2C), (4 × CO); **10**: 20.27, 20.29 (3-Ac), 20.62 (20-Ac), 20.91 (5-Ac), 21.13 (7-Ac), 167.88 (7-CO), 169.03, 169.06 (20-CO), 169.21, 169.22 (5-CO), 169.24, 169.27 (3-CO).

[a] In DMSO-d_6.

[b] In $CDCl_3$.

reducing power in the molecule. The wave at higher peak potential, observed also in voltammograms of other flavonolignan derivatives, most probably reflects the electron donating ability of *o*-methoxy-phenolic moiety at the E ring, inferred from the pulse radiolysis as an exclusive target for one electron oxidation of **1**.[28] The wave at lower potential characterises stronger reducing properties of **2** associated most likely with the unsaturated hydroxy-chromanone moiety (in the ring C) of flavonolignan molecule. This observation is consistent with conclusions of structure–antioxidant activity relationship studies of flavonoids, which have demonstrated that the 3-OH group attached to the 2,3-double bond in conjugation with the 4-oxo function in the C ring is very important for effective radical scavenging.[29] This is also clearly shown by the E_{pa} values of **14** (containing a structural motif of **1**) and **15** (containing a structural motif of **2**) demonstrating stronger antioxidant activity of **2**. However, substantially stronger antioxidant capacity of quercetin (**15**) and taxifolin (**14**) compared to silybin derivatives are mostly due to the activity of catechol portion of their molecules because the intramolecular hydrogen bond in the semiquinone radical produced after the H-atom transfer to the abstracting radical (e.g., alkyl peroxyls) is much stronger than that in the parent phenol.[30] Surprisingly, the wave at lower potential was not detected in the voltammogram of acid **13** (Fig. 6), which may be explained by the presence of carboxylic moiety—see hereunder.

In fact, the poor superoxide radical scavenging capacity of silybin (**1**) was previously reported.[3] Our results have confirmed weaker ability of **1** to scavenge both chemically generated superoxide and a stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Table 5). Oxidation of C-23 of silybin (**1**) to carboxy derivative **11** further reduced radical scavenging properties. However, significant improvement of these properties was observed in both 2,3-dehydroderivatives **2** and **13**. Consistently, oxidation at C-23 led again to the scavenging activity suppression. This phenomenon was recently explained by Foti et al.[31] who demonstrated by kinetic measurements that the reaction between phenols and DPPH$^{\cdot}$ occurs in alcohols by electron transfer from the phenoxide anions to DPPH$^{\cdot}$. The presence of carboxylic acid groups in the molecules or even of adventitious acids (in the solvents) reduces the quantity of phenoxide anions and thus the reactivity toward the radical. Hemiacetal **3a** exhibited better antioxidant properties than **1** itself.

The results with radical scavenging capacity of various mixtures of **1** and **2** (Fig. 7) show that possible contamination of **1** by its oxidative product **2** strongly improve radical scavenging properties of silybin preparations. The mixtures containing ⩾50% of **2** reached the IC_{50} values corresponding to those of pure **2**. Already the presence of 10% of **2** in **1** increases the antiradical–scavenging activity more than 3 times compared to the pure silybin. This clearly demonstrates synergistic effect of both compounds. This finding is of a great importance for the formulation and optimisation of mixed preparation based on both **1** and **2**. Moreover, it helps to produce cheaper preparations of **2**, where no complete conversion is necessary and it also helps to avoid laborious purification steps for removing of traces of unreacted **1** from the final preparations. These results also demonstrate why some complex silybin-based preparations like silymarin (which is a mere mixture of flavonoids from *S. marianum* seeds with standardised

Table 3. ^{1}H NMR data (399.89 MHz, DMSO-d_6, 30 °C) of dehydrosilybin derivatives

Proton	2	7	9	12	13
6	6.194 d (2.0)	6.193 d (2.1)	6.727 d (2.0)	7.406 d (2.0)	6.265 d (2.1)
8	6.459 d (2.0)	6.454 d (2.1)	7.135 d (2.0)	7.146 d (2.0)	6.530 d (2.1)
10	4.275 ddd (7.9, 4.5, 2.5)	4.346 ddd (7.9, 3.8, 2.5)	4.413 ddd (7.8, 4.4, 2.6)	5.536 d (3.8)	5.306 d (4.3)
11	4.967 d (7.9)	4.930 d (7.9)	5.154d (7.8)	5.660 d (3.8)	5.425 d (4.3)
13	7.669 d (2.2)	7.773 d (2.2)	7.581 d (2.2)	7.619 d (2.1)	7.490 d (2.2)
15	7.756 dd (9.0, 2.2)	7.752 dd (8.3, 2.2)	7.532 dd (8.6, 2.2)	7.513 dd (8.7, 2.1)	7.437 dd (8.7, 2.2)
16	7.120 d (9.0)	7.125 dd (8.6, 0.6)	7.197 d (8.6)	7.170 d (8.7)	7.137 d (8.7)
18	7.045 d (2.0)	7.039 d (1.9)	7.284 d (1.8)	7.279 d (2.0)	7.054 d (2.2)
21	6.853 d (8.1)	6.823 d (8.1)	7.155 d (8.1)	7.086 d (8.2)	6.748 d (8.2)
22	6.891 dd (8.1, 2.0)	6.884 dd (8.1, 1.9)	7.090 dd (8.1, 1.8)	7.006 dd (8.2, 2.0)	6.848 dd (8.2, 2.2)
23	3.573 ddd (12.3, 5.0, 2.6)	3.793 dd (12.0, 2.5)	3.636 ddd (12.5, 4.8, 2.6)	—	—
	3.373 ddd (12.3, 5.1, 4.5)	3.543 dd (12.0, 3.8)	3.407 ddd (12.5, 5.8, 4.4)	—	—
19-OMe	3.792 s	3.787 s	3.802 s	3.758 s	3.740 s
3-OH	9.532 s	9.537 s	—	—	not observed
5-OH	12.404 s	12.402 s	12.130 s	12.111 s	12.116 s
7-OH	10.788 s	10.800 s	—	—	11.080 s
20-OH	9.130 s	9.159 s	—	—	9.142 s
23-OH	4.967 dd (5.1, 5.0)	—	5.095 dd (5.8, 4.8)	—	—

Additional signals—**7**: 0.009 (3H, s, Si–Me), 0.014 (3H, s, Si–Me), 0.851 (9H, s, Me_3C); **9**: 2.271 (3H, s, 20-Ac), 2.308 (3H, s, 7-Ac), 2.361 (3H, s, 3-Ac); **12**: 2.239 (3H, s, 20-Ac), 2.312 (3H, s, 7-Ac), 2.358 (3H, s, 3-Ac).

Table 4. ^{13}C NMR data (100.55 MHz, DMSO-d_6, 30 °C) of dehydrosilybin derivatives

Carbon	2	7	9	12	13
2	145.80	145.77	156.33	156.12	155.10
3	136.33	136.37	130.83	130.95	167.94
4	176.02	176.04	175.58	175.58	174.97
4a	103.12	103.14	107.98	108.00	103.62
5	160.72	160.74	160.32	160.32	161.07
6	98.28	98.29	105.50	105.52	99.24
7	164.07	164.09	156.20	156.22	164.86
8	93.58	93.59	101.98	102.03	94.42
8a	156.26	156.27	155.56	155.57	156.74
10	78.55	78.09	78.37	74.73	75.23
11	75.89	76.03	75.57	74.38	74.87
12a	143.40	143.39	143.62	142.39	142.68
13	116.21	116.30	126.82	117.12	116.88
14	123.77	123.86	121.40	122.11	130.39
15	121.27	121.41	122.27	122.65	122.20
16	116.83	116.76	117.57	117.64	117.40
16a	145.05	145.01	146.79	145.56	145.36
17	127.27	126.90	135.11	134.88	126.57
18	111.85	111.94	112.28	111.77	111.68
19	147.69	147.70	151.01	150.95	147.65
20	147.14	147.25	139.71	139.44	147.07
21	115.38	115.39	122.91	122.89	115.35
22	120.59	120.69	120.10	119.06	119.98
23	60.12	62.17	59.90	168.68	168.77
OMe	55.77	55.71	55.96	55.95	55.77

Additional signals—**7**: −5.46 (Si–Me), −5.36 (Si–Me), 17.97 (C–Si), 25.74 (CMe_3); **9**: 20.24 (3-Ac), 20.39 (20-Ac), 20.90 (7-Ac), 167.79 (3-CO), 168.34 (7-CO), 168.44 (20-CO); **12**: 20.21 (3-Ac), 20.36 (20-Ac), 20.90 (7-Ac), 167.77 (3-CO), 168.34 (7-CO), 168.40 (20-CO).

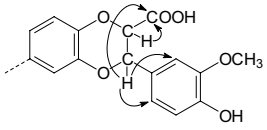


Figure 3. Diagnostic heteronuclear couplings in compounds **10**–**13**.

silybin content) give often controversial, unreproducible results,[11] which may be caused by varying content of the oxidation products of original flavonoids, namely **2**.

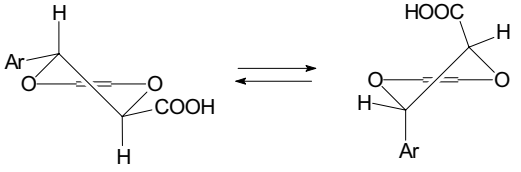


Figure 4. The equilibrium between diaxial and diequatorial substituents of the D-ring.

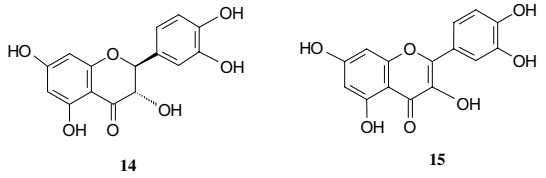


Figure 5. Flavonoids taxifolin (**14**) and quercetin (**15**).

Reaction mixtures of **1** and **2** (and its mixtures) with DPPH· were analysed by HPLC and MALDI-MS, to disclose the reaction products of the both radical scavengers. Silybin upon reaction with DPPH· gives very complex reaction mixture composed mostly of insoluble brownish precipitates, which are obviously polymers (radical-initiated polymerisation). Dehydrosilybin reacts with DPPH· faster than **1** yielding dimer (according to MS analysis) of unknown structure and some other non-identified products. All substances tested were found to be efficient inhibitors of ADP/Fe^{3+}/NADPH-induced lipid peroxidation of rat liver microsomal membranes, **2** being the most effective. Again, the presence of a carboxyl at C-10 of **11** and **13** reduces partly their inhibitory efficiency. The differences in the antioxidant activity of the flavonolignans **1**, **2**, **3a**, **11** and **13** and structurally related flavonoids **14**, **15** at the hydrophilic–hydrophobic interphase of microsomal membranes are much less pronounced than in the scavenging activities evaluated in the hydrophilic environment.

To assess the contribution of lipophilicity to the inhibitory effect on lipid peroxidation the partition co-efficients of **1**, **2**, **3a**, **11** and **13** were determined in the system 1-octanol/water at pH 7.4 of the aqueous phase (Table 6). The observed order of lipophilicity **2 > 1 > 3a > 13 > 11** is not fully related to the antilipoperoxidation effect order **2 > 13 > 1 > 3a > 11**. This result indicates also that at hydrophilic–hydrophobic interphases typical for biological systems structural determinants are important for antioxidant activity.

2.3. Conclusion

New, cheaper and faster method for synthesis of 2,3-dehydrosilybin (**2**) was developed and new carboxylic acids derived from **1** and **2** were prepared for the first time. 2,3-Dehydrosilybin is more lipophilic and less water-soluble than silybin, which predetermines it for an application in lipophilic milieu (cell membrane antioxidant, ointments). However, respective carboxylic derivative combines its considerably better antioxidative properties together with improved hydrophilicity.

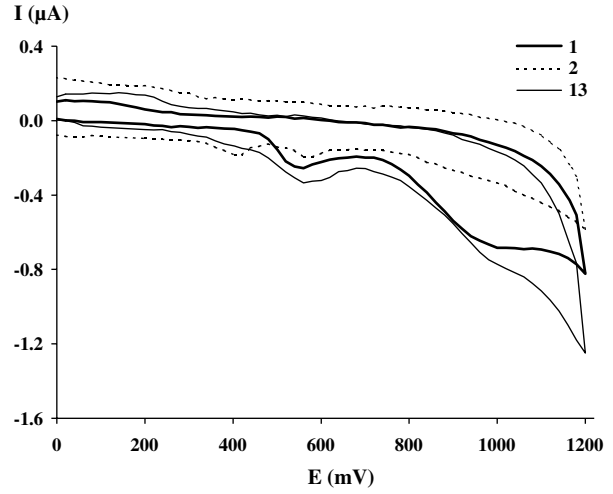


Figure 6. Cyclic voltammograms of silybin (**1**), 2,3-dehydrosilybin (**2**) and 2,3-dehydrosilybinic acid (**13**).

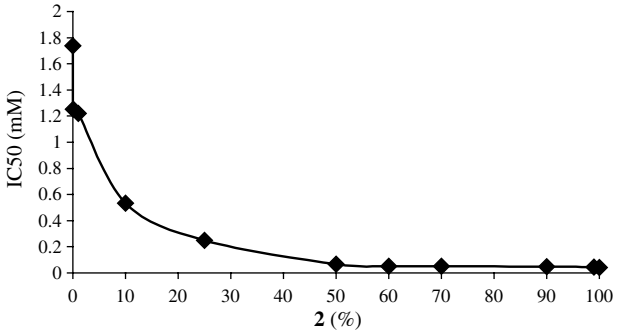


Figure 7. Radical scavenging activity of the mixtures **1** and **2**. Data are expressed as means from three measurements, SD < 0.02.

Table 5. Cyclic voltammetry peak potentials, radical scavenging data and antilipoperoxidant activity of silybin, its derivatives and structural analogues

Compound	CV[a] E_{pa} (mV)	DPPH IC_{50} (μM)	$O_2^{\cdot -}$ IC_{50} (μM)	LPx IC_{50} (μM)
Silybin (**1**)	524	1745 ± 65	55.2 ± 2.8	33.6 ± 1.2
2,3-Dehydrosilybin (**2**)	573; 397	73 ± 3	4.2 ± 0.2	3.6 ± 2.7
'Hemiacetal' **3a**	544	646 ± 42	8.2 ± 0.4	47.3 ± 6.7
Silybinic acid (**11**)	557	2645 ± 10	52.0 ± 2.6	61.5 ± 2.0
2,3-Dehydrosilybinic acid (**13**)	564	934 ± 10	15.5 ± 0.8	30.8 ± 3.1
Taxifolin (**14**)	258	21 ± 6	2.4 ± 0.1	16.2 ± 0.2
Quercetin (**15**)	174	11 ± 1	0.5 ± 0.03	4.7 ± 1.4

[a] CV—Cyclic voltammetry; E_{pa} (mV)—the anodic peak potential; DPPH—1,1-diphenyl-2-picrylhydrazyl radical scavenging; IC_{50} (μM)—the concentration of the tested compound required to reduce the absorbance of DPPH by 50%; $O_2^{\cdot -}$—superoxide scavenging—the antiradical activity of the tested compound is expressed as the concentration required to reduce the absorbance by 50% (IC_{50}); LPx—inhibition of microsomal lipid peroxidation—the activity was calculated as the concentration of the tested compound inhibiting the colour reaction with thiobarbiturate by 50% (IC_{50}).

Table 6. Octanol/water partition coefficients (log $P_{7.4}$) of silybin and its derivatives

Compound	Log $P_{7.4}$
Silybin (**1**)	2.2726
2,3-Dehydrosilybin (**2**)	3.9165
'Hemiacetal' **3a**	0.51490
Silybinic acid (**11**)	−0.88430
2,3-Dehydrosilybinic acid (**13**)	0.28510

The presence of a 2,3-double bond in the C-ring of molecule is connected with an increase in scavenging/antioxidative potency of the compounds. 2,3-Dehydrosilybin is obviously a superior silybin derivative both from the point of view of radical scavenging and antilipoperoxidant activity. Compound **2** is a 25 times better radical scavenger (DPPH·) and a 10 times better inhibitor of lipid peroxidation than **1** but it has ca. 100 times higher hydrophobicity. 2,3-Dehydrosilybinic acid (**13**) has a 100 times higher hydrophilicity compared to **2** but 10 times lower antioxidative activity than **2**, still better than **1**. Silybinic acid (**11**) has an approximately 10 times higher water solubility than **1** but only half of its antioxidative activity. Due to the absence of catechol ring flavonolignans **1**, **2**, **11**, **13** are significantly less potent radical scavengers compared to flavonoids **14**, **15**. On the other hand, antilipoperoxidant activities of **1** versus **14** and **2** versus **15** are comparable. Obviously, an interaction with the lipidic phase, which might be similar in both heterocyclic systems, plays here an important role.

3. Experimental

3.1. General methods

Reactions were monitored by TLC on Silica Gel F_{254} (Merck) and the spots were visualised by UV light and by charring with 5% H_2SO_4 in ethanol.

Optical rotation was measured on Perkin–Elmer 141 polarimeter at 24 °C.

NMR spectra were recorded on a Varian INOVA-400 spectrometer (399.89 MHz for ^{1}H, 100.55 MHz for ^{13}C) in $CDCl_3$ or DMSO-d_6 (see text) at 30 °C. Chemical shifts were referenced to the residual solvent signal (δ_H 7.265, δ_C 77.00; δ_H 2.50, δ_C 39.60). Digital resolution used justified reporting the proton and carbon chemical shifts to three and two decimal places, respectively. All 2D NMR experiments (HOM2DJ, gCOSY, TOCSY, HMQC, HMBC) were performed using standard manufacturers's software. The sequence for 1D-TOCSY experiments[32] was obtained through Varian User Library, the sequence gHMBC[33,34] was obtained from Varian Application Laboratory in Darmstadt (D).

Positive-ion electrospray ionisation (ESI) mass spectra were recorded on a double-focusing instrument Finnigan MAT 95 (Finnigan MAT, Bremen, D) with BE geometry. Samples dissolved in methanol–water (2:1, v/v) were continuously infused through a stainless capillary held at 3.3 kV into Finnigan ESI source via a linear syringe pump at a flow rate of 40 μL/min. For high-resolution experiments the instrument was tuned to a resolution of about 8000 (10% of valley definition) and the measurements were carried out by the peak-matching method using a mixture of polypropylene glycols (average Mr = 725) as an internal standard.

Analytical HPLC was carried out on a Spectra Physics analytical system (San Jose, USA) comprised of an SP 8800 ternary gradient pump, an SP 8880 autosampler and a Spectra Focus scanning UV/vis detector. A Nucleosil 100–5, C 18 AB column 250 × 4 mm (Macherey–Nagel) with a mobile phase MeOH/H_2O/CH_3-COOH = 42:58:0.01, flow rate 0.6 mL/min was used at 60 °C, UV detection at 275 nm.

3.2. Chemistry

3.2.1. 3,5,7-Trihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-4*H*-chromen-4-one (2). Silybin (**1**, 6 g, 12.44 mmol) was dissolved in dry pyridine (400 mL) to give 0.031 mol/mL and the mixture was heated to 95 °C for 100 h under stirring with access of air under $CaCl_2$ drying tube (TLC; chloroform/acetone/formic acid = 9:2:1). Reaction mixture was evaporated in vacuo and the residual pyridine was removed by co-evaporation with toluene. Remaining solid was dissolved in ethyl acetate (500 mL), filtered through a silica gel pad (30 g), which was washed with hot acetone (400 mL). The collected filtrates were evaporated to dryness and dissolved in hot EtOH. The cooled solution afforded after filtration the product **2** (3.05 g, 51%) as a yellow microcrystalline solid. Mother liquors afforded after partial evaporation to half volume the second crop of less pure product (0.74 g, 12%). The product was identified by HPLC comparing with the authentic standard and its structure was verified by NMR (Tables 3 and 4) and MS-ESI (*m*/*z*): 481 $[M+H]^+$.

3.2.2. 2,4,6-Trihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-1-benzofuran-3(2*H*)-one (3a). The mother liquor from the second crystallisation of **2** was concentrated in vacuo to the volume of 5 mL and then added dropwise to the excess (50 mL) of saturated aqueous solution of $NaHCO_3$. The precipitate was filtered off and the filtrate was extracted with ethyl acetate. The organic phase was dried (Na_2SO_4). The dry extract (1 g) was purified by flash chromatography (chloroform/acetone/formic acid 5:1:0.1), which yielded 0.12 g of title compound (**3a**) as a white amorphous solid (12%).

$[\alpha]_D$ +2.5 (*c* 0.16, acetone). ^{1}H NMR (DMSO-d_6): 2.877 and 2.933 (2H, AB, *J* = 13.7), 2.868 and 2.941 (2H, AB, *J* = 14.2), 3.287 (1H, dd, *J* = 12.0, 2.1, H-23), 3.289 (1H, dd, *J* = 12.0, 2.3, H-23), 3.474 (1H, dd, *J* = 12.0, 4.4, H-23), 3.476 (1H, dd, *J* = 12.0, 4.6, H-23), 3.764 (6H, s, 2 × OMe), 4.055 (1H, ddd, *J* = 7.7, 4.6, 2.3, H-10), 4.061 (1H, ddd, *J* = 7.7, 4.4, 2.1, H-10), 4.796 (1H, d, *J* = 7.7, H-11), 4.815 (1H, d, *J* = 7.7, H-11), 5.752 (1H, d, *J* = 1.7, H-8), 5.756 (1H, d, *J* = 1.7, H-8), 5.802

(2H, d, $J = 1.7$, 2 × H-6), 6.652 (2H, dd, $J = 8.2$, 2.0, 2 × H-15), 6.691 (2H, d, $J = 2.0$, 2 × H-13), 6.473 (2H, d, $J = 8.2$, 2 × H-16), 6.779 (2H, d, $J = 8.1$, 2 × H-21), 6.808 (1H, dd, $J = 8.1$, 1.5, H-22), 6.812 (1H, dd, $J = 8.1$, 1.7, H-22), 6.955 (1H, d, $J = 1.5$, H-18), 6.962 (1H, d, $J = 1.7$, H-18), 7.447 (2H, s, 2 × =C—OH), 9.107 (2H, s, 2 × =C—OH), 10.326 (1H, s, =C—OH), 11.864 (1H, s, =C—OH). ^{13}C NMR (DMSO-d_6): 40.49 (C-2); 55.67, 55.69 (OMe), 60.20 (C-23); 75.65, 75.76 (C-11); 77.98 (C-10), 89.87 (C-8); 95.89, 95.91 (C-6); 101.09 (C-4a); 105.32 (C-3); 111.70 (C-18); 115.36 (C-21); 115.91 (C-16); 118.73, 118.75 (C-13); 120.47, 120.49 (C-22); 123.35 (C-15); 127.17 (C-14), 127.70 (C-17); 141.96 (C-16a); 142.81 (C-12a); 146.98 (C-20); 147.65 (C-19); 158.23, 158.32 (C-5); 168.34 (C-7); 171.76 (C-8a); 192.79, 192.80, 192.81 (C-4). MS-ESI (*m*/*z*): 483 $[M+H]^+$.

3.2.3. 23-*O*-(*tert*-Butyldimethylsilyl)-silybin (2-(2-{[(*tert*-butyldimethylsilyl)oxy]methyl}-3-(4-hydroxy-3-methoxyphenyl)-2,3-dihydro-1,4-benzodioxin-6-yl)chroman-4-one) (4). Silybin (**1**, 1.50 g, 3.11 mmol) was dissolved at room temperature in dry pyridine (20 mL). *tert*-Bu(Me)$_2$SiCl (0.70 g, 4.64 mmol) and powdered $AgNO_3$ (0.20 g, 1.18 mmol) were added and the stirred mixture was heated under nitrogen at 40 °C. The reaction was quenched after 2 h with water (100 mL), the mixture was extracted with ethyl acetate (2 × 100 mL) and the organic phase was dried over Na_2SO_4. After evaporation, flash chromatography (petroleum ether/AcOEt 2:1) gave title compound (**4**) 1.24 g (67%) as a white amorphous solid.

$[\alpha]_D$ −4.33 (*c* 0.30, $CHCl_3$). MS-ESI (*m*/*z*): 597 $[M+H]^+$. For ^{1}H and ^{13}C NMR data see Tables 1 and 2.

3.2.4. 3,5,7,20-Tetra-*O*-acetylsilybin (3,5,7-triacetoxy-2-[3-(4-acetoxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]chroman-4-one) (6). Compound **4** (1.00 g, 1.675 mmol) was dissolved in the mixture of pyridine (15 mL) and acetanhydride (15 mL). The reaction mixture was stirred 24 h at room temperature. The mixture was then poured to saturated solution of $NaHCO_3$ (150 mL), extracted with ethyl acetate (2 × 100 mL), the organic phase was washed with water, dried over Na_2SO_4 and evaporated. The residue was dissolved in dry $CHCl_3$ (30 mL), $BF_3{\cdot}Et_2O$ (2.5 mL, 50% solution) was added and the mixture was stirred 12 h at room temperature. Then the mixture was poured into the water (150 mL), extracted with CH_2Cl_2 (2 × 100 mL), the organic phase was washed with water, dried (Na_2SO_4) and evaporated. Dry solid afforded after flash chromatography (petroleum ether/AcOEt 1:1) title compound (**6**) (535 mg, 49%) as a white amorphous solid.

$[\alpha]_D$ +51.56 (*c* 0.32, $CHCl_3$). MS-ESI (*m*/*z*): 651 $[M+H]^+$. For ^{1}H and ^{13}C NMR data see Tables 1 and 2.

3.2.5. 3,5,7,20-Tetra-*O*-acetylsilybinic acid (3-(4-acetoxy-3-methoxyphenyl)-6-[3,5,7-triacetoxy-4-oxochroman-2-yl]-2,3-dihydro-1,4-benzodioxine-2-carboxylic acid) (10). A stock solution of H_5IO_6/CrO_3 was prepared by dissolving H_5IO_6 (11.4 g, 50 mmol) and CrO_3 (0.023 g, 1.9 mmol/L in wet MeCN (0.75% v/v water) to make 114 mL (complete dissolution typically required 1–2 h). The H_5IO_6/CrO_3 solution (10 mL) was added to a solution of the alcohol **6** (470 mg, 0.722 mmol) in wet acetonitrile (5 mL, 0.75 v/v% water) during 30 min while maintaining the reaction temperature at 0–5 °C. The reaction was kept at 0 °C for 45 min, and then it was quenched by the addition of Na_2HPO_4 solution (0.5 g in 10 mL H_2O). The mixture was extracted with ethyl acetate (2 × 15 mL), organic layer was washed with 1:1 brine/water mixture (2 × 25 mL) then with an aqueous solution of $NaHSO_3$ (1 g in 25 mL H_2O) and finally with brine (25 mL). The organic phase was dried (Na_2SO_4). After evaporation flash chromatography (chloroform/acetone/formic acid 9:1:0.1) gave **10** (368 mg, 77%) as a white amorphous solid.

$[\alpha]_D$ + 38.18 (*c* 0.22, acetone). MS-ESI (*m*/*z*): 665 $[M+H]^+$. For ^{1}H and ^{13}C NMR data see Tables 1 and 2.

3.2.6. Silybinic acid (3-(4-hydroxy-3-methoxyphenyl)-6-(3,5,7-trihydroxy-4-oxochroman-2-yl)-2,3-dihydro-1,4-benzodioxine-2-carboxylic acid) (11). Compound **10** (0.29 g, 0.436 mmol) and K_2CO_3 (0.25 g, 1.809 mmol) were dissolved in MeOH/water solution (16.5 mL, 10:1 v/v). The reaction mixture was stirred 24 h at room temperature under argon. The reaction was quenched by an addition of formic acid (1 mL) and after 10 min of stirring it was evaporated to dryness. Dry solid afforded after flash chromatography (chloroform/acetone/formic acid 4:1:0.05) title compound (**11**) (150 mg, 70%) as a white amorphous solid.

$[\alpha]_D$ + 7.5 (*c* 0.16, MeOH). MS-ESI (*m*/*z*): 497 $[M+H]^+$. HRMS (ESI) (*m*/*z*): 519.0921 (calcd for $C_{25}H_{20}O_{11}Na$ $[M+Na]^+$ 519.0903). For ^{1}H and ^{13}C NMR data see Tables 1 and 2.

3.2.7. 2-(2-{[(*tert*-Butyldimethylsilyl)oxy]methyl}-3-(4-hydroxy-3-methoxyphenyl)-2,3-dihydro-1,4-benzodioxin-6-yl)-3,5,7-trihydroxy-4*H*-chromen-4-one (23-*O*-(*tert*-butyldimethyl-silyl)-2,3-dehydrosilybin, 7). 2,3-Dehydrosilybin (**2**, 0.980 g, 2.040 mmol) was dissolved in dry pyridine (15 mL). *tert*-Bu(Me)$_2$SiCl (0.407, 2.698 mmol) and powdered $AgNO_3$ (0.150 g mmol) were added and the stirred mixture was heated to 40 °C. The reaction was quenched after 1.5 h with water addition (100 mL), the mixture was extracted with ethyl acetate (2 × 70 mL) and the organic phase was dried (Na_2SO_4). After evaporation, flash chromatography (petroleum ether/AcOEt 2:1) gave title compound (**7**) 0.730 g (60%) as a yellow amorphous solid.

$[\alpha]_D$ 0 (*c* 0.34, acetone). MS-ESI (*m*/*z*): 595 $[M+H]^+$. For ^{1}H and ^{13}C NMR data see Tables 3 and 4.

3.2.8. 3,7-Diacetoxy-2-[3-(4-acetoxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-5-hydroxy-4*H*-chromen-4-one (3,7,20-tri-*O*-acetyl-2,3-dehydrosilybin, 9). Compound **7** (0.50 g, 0.841 mmol) was dissolved in the mixture of pyridine (10 mL) and acetanhydride (10 mL). The reaction and workup were performed analogously as for **6**. After evaporation, flash

chromatography (petroleum ether/AcOEt 1:1) gave the title compound (**9**) 0.393 g (77%) as a yellow amorphous solid. $[\alpha]_D$ +4.54 (*c* 0.44, acetone). MS (ESI) (*m*/*z*): 607 (M^++H). For ^1H and ^{13}C NMR data see Tables 3 and 4.

3.2.9. 3-(4-Acetoxy-3-methoxyphenyl)-6-[3,7-diacetoxy-5-hydroxy-4-oxo-4*H*-chromen-2-yl]-2,3-dihydro-1,4-benzodioxine-2-carboxylic acid (3,7,20-tri-*O*-acetyl-2,3-dehydrosilybinic acid, 12). Stock solution of H_5IO_6/CrO_3 (6.4 mL) was added to a solution of the compound **9** (0.30 g, 0.495 mmol) in wet acetonitrile (4 mL, 0.75% v/v water) during 30 min while maintaining the reaction temperature at 0–5 °C. The reaction and workup were performed analogously as for **10**. After evaporation, flash chromatography (chloroform/acetone/formic acid 9:1:0.1) yielded the title compound (**12**) (262 mg, 85%) as a yellow amorphous solid.

$[\alpha]_D$ +8.33 (*c* 0.42, acetone). MS (ESI) (*m*/*z*): 621 (M^++H). For ^1H and ^{13}C NMR data see Tables 3 and 4.

3.2.10. 3-(4-Hydroxy-3-methoxyphenyl)-6-(3,5,7-trihydroxy-4-oxo-4*H*-chromen-2-yl)-2,3-dihydro-1,4-benzodioxine-2-carboxylic acid (2,3-dehydrosilybinic acid, 13). Compound **12** (0.2 g, 0.322 mmol) and K_2CO_3 (0.2 g, 1.447 mmol) were dissolved in MeOH/water solution (11 mL, 10:1 v/v). The reaction mixture was stirred 24 h at room temperature under argon. The reaction was quenched by addition of formic acid (0.8 mL) and after 10 min of stirring evaporated to dryness. Dry solid afforded after flash chromatography (chloroform/acetone/formic acid 4:1:0.05) the title compound (**13**) (104 mg, 65%) as a yellow amorphous solid. $[\alpha]_D$ +2.72 (*c* 0.22, MeOH). MS-ESI (*m*/*z*): 495 $[M+H]^+$. HRMS-ESI (*m*/*z*): 517.0752 (calcd for $C_{25}H_{18}O_{11}Na$ $[M+Na]^+$: 517.0746). For ^1H and ^{13}C NMR data see Tables 3 and 4.

3.3. Cyclic voltammetry

Cyclic voltammetry measurements were performed using the Potentiostat/Galvanostat Model 273 (EG&G Princeton Applied Research, USA). A three-electrode system consisting of a glassy carbon working electrode MF2012 (Bioanalytical Systems, West Lafayette, IN, USA), a platinum-wire auxiliary electrode and a Hg/Hg_2Cl_2/saturated KCl reference electrode was used. The potentials mentioned throughout this work are referred to this electrode. The compounds (1.10^{-3} M, DMSO) were diluted to concentrations of 1.10^{-4} M in 0.1 M sodium-phosphate buffer pH 7.0. All measurements were performed at laboratory temperature and 200 mV s^{-1} scan rate in the range 0–1200 mV. The working electrode was polished with 0.05 μm grade alumina (Buehler, Lake Buffs, IL, USA) prior to each scan. The anodic (oxidation) peak potential E_{pa} was read from the anodic wave of the voltammogram.

3.4. Antiradical activity

(a) DPPH (1,1-diphenyl-2-picrylhydrazyl radical) scavenging: The absorbance change of DPPH was measured in the reaction mixture containing 0.75 mL of test compound solution (0.01–5.0 mM, MeOH) and 1.5 mL of DPPH (20 mg L^{-1}, MeOH) at 517 nm for 10 min.

(b) Superoxide scavenging: Superoxide was generated from O_2 in the presence of EDTA, $MnCl_2$, NADH and mercaptoethanol.[35] Reaction mixture containing triethanolamine–diethanolamine buffer (0.8 mL, 100 mM, pH 7.4), NADH (40 μL, 7.5 mM) EDTA–$MnCl_2$ (25 μL, 100/50 mM), test sample (0.1 mL, 0.001–1 mM) was incubated at 25 °C for 10 min. Reaction was started by the addition of mercaptoethanol (0.1 mL, 10 mM) and the decrease of absorbance at 340 nm was monitored for 20 min.

3.5. Inhibition of microsomal lipid peroxidation

Microsomes were prepared from rat liver homogenate and resuspended in 50 mM Tris–HCl buffer with 100 mM KCl and 0.1 mM EDTA (pH 7.4). Protein concentration in microsomal suspension was determined by the Lowry method. The mixture of microsomal suspension (0.5 mL, 0.5 mg protein/mL), and test compounds dissolved in DMSO (0.2 mL, 0.05–1.0 mM) were incubated in the presence ADP (0.1 mL, 20 mM), $FeCl_3$ (0.1 mL, 1.2 mM), NADPH (0.1 mL, 1.25 mM) in a shaking water bath at 37 °C for 30 min. The products of lipid peroxidation were determined by a standard reaction with thiobarbituric acid.[36] The activity was calculated as the concentration of the tested compound that inhibited the colour reaction with thiobarbiturate by 50% (IC_{50}).

3.6. Hydrophobicity

The 1-octanol/water partition co-efficients (log *P*) were determined by the shake-flask method using Britton–Robinson I buffer (0.04 M, pH 7.4, ionic strength $\mu = 0.15$ M) as an aqueous phase. The samples were dissolved in 1-octanol (5.10^{-5} M), the volumes of the partition phases were within 10–50 mL, volume ratios within 1:5–5:1. The partitions were performed with a modified rotary system (Unipan) at 25 rpm and 22 ± 2 °C for 3 h. The phases were separated by centrifugation at 3500 rpm for 30 min and sample concentration was determined spectrophotometrically at 289 nm. The partition co-efficients were determined at different volume ratios of the partition phases and calculated according to equation $P = kV_w/V_{oct}$ ($k = c_{oct}/c_w$). The final log *P* were obtained by extrapolation to the point of theoretical equi-partition ($k = 1$).[37]

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