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Laccase-mediated dimerization of the flavonolignan silybin

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Abstract

Flavonolignan silybin (**1**) present in the seeds of the milk thistle (*Silybum marianum*) is widely used in human therapy of liver dysfunctions and as a hepatoprotectant thanks to its dual function: it acts as a highly effective radical scavenger (antilipoperoxidant) and also as an antioxidant. Molecular mechanisms of antiradical action of **1** and even functional groups responsible for this activity are not well known so far. Silybin forms during in vitro reaction with stable radicals (e.g., DPPH) or with enzyme laccase (*Trametes pubescens*) complex mixtures of oligomeric and polymeric products whose structural analysis is virtually impossible. Methylation of 7-OH in **1** yields under laccase-mediated oxidation C–O and C–C dimers in the ratio ca. 1:2.5. Using this approach, the hydroxyl groups responsible for antiradical activity of silybin (20-OH group) were determined and the molecular mechanism of the E-ring antiradical activity was explained.

Keywords: Silybin; Silymarin; Dimer formation; Antiradical mechanism; Laccase (*Trametes pubescens*)

1. Introduction

Flavonolignan silybin (**1**) is a major biologically active component of an extract from the seeds of *Silybum marianum* (milk thistle) known as silymarin. Besides this compound, silymarin contains silybin congeners silydianin, silychristin, isosilybin, 2,3-dehydrosilybin, flavonoids taxifolin, and quercetin (Fig. 1), and other unidentified polymeric phenolic compounds (10–30%) [1].

Silybin and silymarin are active constituents of numerous phytopreparations used in the prevention and the treatment of various liver diseases and as a protectant against a number of hepatotoxins and mycotoxins [2]. Natural silybin (**1**) consists of two diastereomers – preparatively inseparable – in nearly equimolar ratio (Fig. 1) [3,4]. Cytoprotectivity of **1** is based on several mechanisms operating at various cell levels. Silybin acts mainly as an effective radical scavenger (antilipoperoxidant) [5], and also as an antioxidant [6–8].

Recently, silybin (and/or silymarin) has received attention due to its alternative beneficial activities that are not directly related to its hepatoprotective and/or antioxidant (radical scavenging) properties [1,2,9]. These include mostly anticancer and chemopreventive actions, as well as hypocholesterolemic [10,11], cardioprotective [12,13], neuroactive, and neuroprotective activities [14].

Although pharmacology of silybin has been intensively studied (ca. 400 papers published on silybin in the last 5 years—ISI), the molecular mechanisms of the antiradical and antioxidative action of the silybin molecule have not been systematically investigated and are not quite clear so far. There exists only a single study devoted to the molecular mechanism of silybin antioxidant action, stressing the essential role of hydroxyl at C-20 of silybin [15].

However, neither a clear mechanism of the antiradical action nor the products formed during the radical oxidation of **1** have been described yet. The main reason of these failures consists in the fact that, during in vitro reactions with stable radicals (e.g., *N*,*N*-diphenyl-*N*′-picrylhydrazyl—DPPH), silybin tends to form complex mixtures of oligomeric and polymeric products [16], whose structural analysis is virtually impossible. This is probably caused by the presence of more than one reactive site in its molecule, and consequently, by the parallel reactions of these functionalities. We reasoned that the use of selectively substituted (e.g., methylated) derivatives could lead to the deceleration of the polymerization process due to selective blocking of one active site and, as a consequence, to the possibility of isolating the intermediate dimers formed in the first step of the radical (or one-electron oxidation) reaction. The application of

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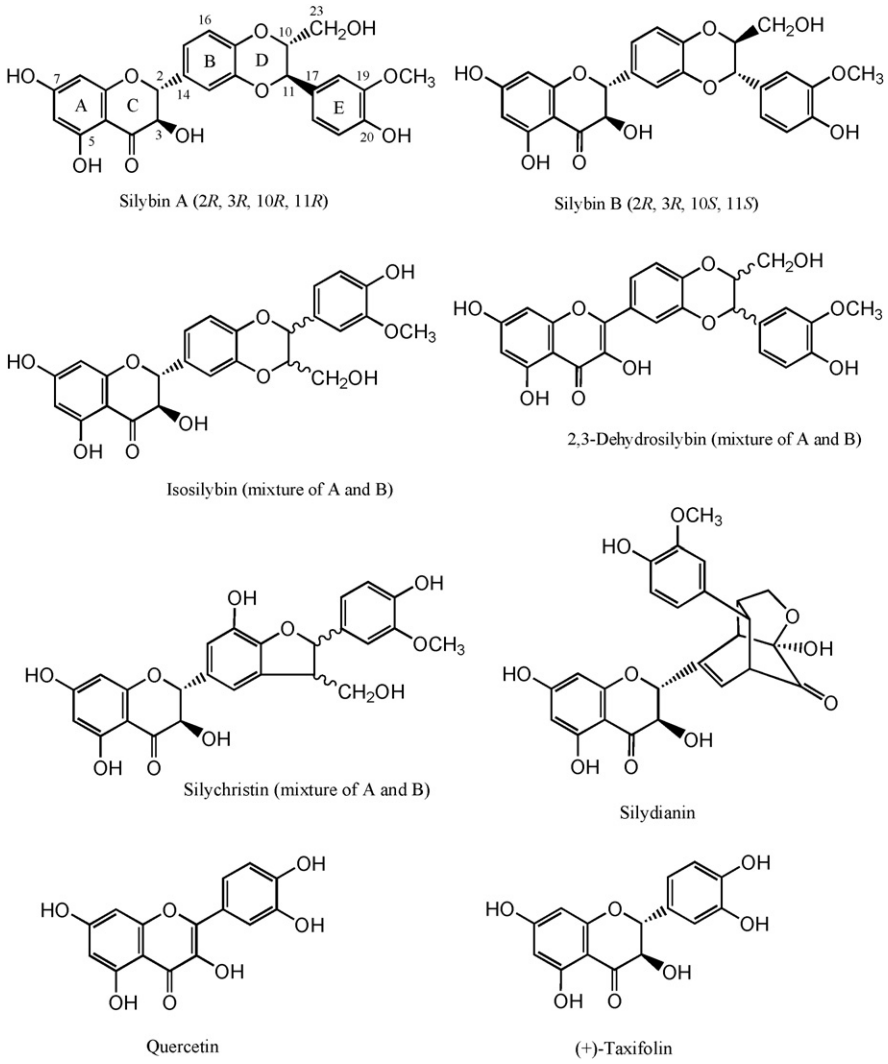

Fig. 1. Flavonoids from *Silybum marianum*.

laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2, [17]) for this purpose offers another possibility for the deceleration of the polymerization since the one-electron oxidation of phenols by laccases are usually substantially slower than the similar reaction with radical species (e.g., DPPH). The effectivity of this approach was confirmed recently by the successful dimerization of the phytoalexin resveratrol [18], of the steroid hormone β-estradiol [19], flavonoid (+)-catechin [20], and of the antibiotic totarol [21].

2. Experimental

2.1. General methods

NMR spectra were recorded on a Varian INOVA-400 spectrometer (399.89 MHz for ^{1}H, 100.55 MHz for ^{13}C) in DMSO-d_6 at 30 °C. Chemical shifts were referenced to the residual solvent signal (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 manufacturer's software. The sequence for 1D-TOCSY experiments was obtained through Varian User Library, the sequence gHMQC was obtained from Varian Application Laboratory in Darmstadt (DE).

Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing instrument Finnigan MAT 95 (Finnigan MAT, Bremen, DE) 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.

2.2. Chemicals

Silybin (mixture of both diastereomers A and B, ca. 1:1) was kindly donated by Dr. L. Cvak (TEVA Galena Co., Opava, CZ). The laccase from *Trametes pubescens* (TL) was provided by Prof. D. Haltrich (University of Natural Resources and Applied Life Sciences, Vienna, AT). Monomethylated deriva-

Table 1
^{1}H NMR data (399.87 MHz, DMSO-d_6, 30 °C) of dimers derived from **2**

H	**4**	**5**	
		North	South
2	5.124 d (11.4)	5.136 d (11.4)	
	5.120 d (11.4)	5.124 d (11.3)	
3	4.660 d (11.4)	4.654 m	
	4.647 d (11.4)		
6	6.108 d (2.3)	6.117 d (2.1)	6.107 d (2.1)
	6.106 d (2.3)	6.109 d (2.1)	
8	6.089 d (2.3)	6.096 d (2.1)	6.090 d (2.1)
	6.086 d (2.3)	6.085 d (2.1)	
10	4.225 ddd (7.9,4.6,2.5)	4.416 m	4.208 m
	4.213 ddd (7.9,4.6,2.5)		
11	4.918 d (7.9)	4.900 d (7.8)	5.006 d (7.8)
13	7.106 d (1.9)	7.114 d (2.0)	7.096 d (2.0)
	7.099 d (1.9)	7.105 d (1.9)	
15	7.011 dd (8.3,1.9)	7.038 dd (8.2,2.0)	
		7.032 dd (8.2,1.9)	
16	6.966 d (8.3)	6.988 d (8.2)	
		6.993 d (8.2)	
18	6.974 d (1.8)	6.624 d (1.9)	7.199 d (1.9)
		6.615 d (1.8)	7.192 d (1.9)
21	–	–	6.688 d (8.2)
			6.683 d (8.2)
22	6.894 d (1.8)	6.933 d (1.9)	6.961 dd (8.2,1.9)
	6.887 d (1.8)	6.929 d (1.8)	6.959 dd (8.2,1.9)
23d	3.580 dd (12.5,2.5)	3.559 dm (12.3)	3.582 dm (12.3)
23u	3.423 dd (12.5,4.6)	3.366 dm (12.3)	
7-OMe[a]	3.779 s	3.786 s	
		3.784 s	
19-OMe[a]	3.816 s	3.844 s	
		3.842 s	
3-OH	5.847 m		
5-OH	12.445 s	11.863 s	
20-OH	8.171 s	9.002 s	

[a] 3H.

tives of silybin (7-*O*- and 20-*O*-methylsilybin) were prepared as described previously [22]. All other chemicals were purchased from Sigma–Aldrich.

2.3. General procedure for the laccase oxidation

Monomethyl silybin (200 mg, 0.403 mmol) was dissolved in ethyl acetate (50 mL), while the laccase (100 U) was dissolved in 45 mL of 20 mM acetate buffer, pH 4.5. The biphasic system was stirred at room temperature for 24 h. Two phases were separated, the organic solvent was evaporated and the crude residue was purified by flash chromatography.

2.4. Methylsilybin dimers *4* and *5*

7-*O*-Methylsilybin (**2**, 200 mg, 0.403 mmol) was reacted according to the general procedure. After work up and flash chromatography (chloroform/acetone/formic acid 8:2:0.1) C-21–C-21′-dimer **4** (50 mg, 25%) and C-20-*O*–C-21′-dimer **5** (18 mg, 9%) were obtained as brownish amorphous solids.

Positive ESI-MS (*m*/*z*): 991 $[M+H]^+$ for both compounds (**4** and **5**). For ^{1}H and ^{13}C NMR data see Tables 1 and 2.

3. Results and discussion

3.1. Reaction of silybin and its monomethyl derivatives with laccase

The synthesis of some selectively methylated derivatives of **1** was recently reported by us [22]. Selective methylation of the 7-OH position of **1**, yielding compound **2**, was achieved using MeI in boiling acetone in the presence of K_2CO_3. The application of slight excess (2–2.5 equiv.) of NaH in DMF yielded 20-*O*-methylsilybin (**3**) providing 1 equiv. of MeI was used (Fig. 2).

The formation of dimeric intermediates in the course of radical-polymerization reactions was observed several times. Actually, the alleged dimer of silybin was mentioned for the first time in 1975, when it was found that it possesses ca. 10 times higher antiphalloidine activity than silybin itself [23–25]. Unfortunately, neither the structure of this dimer nor the method of its preparation was disclosed (only MS indicating the mass of a dimer—962 amu).

The reaction of nonderivatized silybin (**1**) with laccase (*T. pubescens*) led to fast oligo- and polymerization, similarly to what was obtained by reacting **1** with DPPH. On the contrary, the attempted oxidation of 20-*O*-methylsilybin (**3**) with this

Table 2
^{13}C NMR data (100.55 MHz, DMSO-d_6, 30 °C) of dimers derived from **2**

C	**4**	**5**	
		North	South
2	82.73	82.66	
	82.70		
3	71.57	71.58	
	71.50	71.48	
4	198.31	198.31	
	198.28		
4a	101.39	101.39	
5	163.01	163.03	
6	94.95	94.95	
7	167.59	167.60	
8	93.85	93.87	
8a	162.43	162.40	
	162.41		
10	78.19	78.01	
11	76.10	75.60	
12a	143.35	143.1	
13	116.62	116.65	
		116.57	
14	129.87	129.90	129.97
	129.83		
15	121.28	121.45	121.20
	121.14	121.27	
16	116.31	116.46	
	116.28	116.38	
16a	143.74	143.6	
	143.71		
17	125.53	127.02	131.54
	125.48		
18	110.00	112.3	112.3
	109.91		
19	148.39	149.18	149.50
20	146.42	138.4	146.55
	146.36		
21	126.24	143.2	116.6
22	122.80	107.3	120.2
	122.77		
23	60.33	60.12	
7-OMe	55.96	55.97	
19-OMe	56.03	55.84	56.20

Note: Chemical shifts given in one decimal place are HMQC and HMBC readouts.

enzyme did not proceed at all. Interestingly, 7-*O*-methylsilybin (**2**) was oxidized by the laccase to give the dimeric products **4** and **5** (Figs. 3 and 4) in a ca. 2.5:1 ratio. These dimers still possessed some antiradical activity as, using an excess of enzyme or longer reaction times, they further underwent oligo- and polymerization.

Although compound **3** did not react with laccase to give dimeric product(s), its oxidation (at its 7-OH) to the corresponding radical cannot be excluded. According to our observation, compound **3** still possesses a slight antiradical activity (ca. 30% of the parent silybin activity, as confirmed using the DPPH scavenging assay: protection at 50 μM of the tested compound against DPPH – expressed as mean values from three independent measurements – was 2.3% for **3**, 5.9% for **2**, and 7.5% for silybin (**1**) [26]) and possibly participates in silybin radical polymerization. In order to confirm this hypothesis, further study of the role of the 7-OH of **1** in its antioxidant mechanism is required. However, as a matter of fact, the differences between the products obtained from the laccase-catalyzed oxidation of **1** and **2** (polymerization vs. dimer formation) strongly suggest a significant contribution of the 7-OH to the overall antiradical activity of silybin.

3.2. Structure characterization

The $[M+H]^+$ ions of compounds **4** and **5** had *m*/*z* values of 991 in positive ESI-MS, corresponding to dimeric structures. The ^{1}H and ^{13}C NMR data for both compounds are reported in Tables 1 and 2.

C–C dimer **4**. Seven signals of the aromatic C–H type in the ^{13}C NMR and the HMQC spectra of this compound instead of the eight present in the parent compound indicated that the dimerization took place at the expense of two aromatic protons. All the hydroxyl protons at C-3, C-5, and C-20 were retained (see Table 1). There were only two protons (H-18, H-22) left on the E-ring with mutual *meta*-coupling (J = 1.9 Hz). In the HMBC spectrum, the crosspeak between H-22 and C-21 due to a weak ^2J coupling to C-21 and to a strong ^3J coupling to C-21′ further supported the C-21–C-21′ linkage (Fig. 5).

C–O dimer **5**. Both the ^{1}H and the ^{13}C NMR spectra indicated that **5** was an unsymmetrical molecule. Two OH signals at 11.836 and 9.002 ppm with intensity ratio 2:1 (due to the 5-OH and 20-OH, respectively) suggested a C–O dimerization using the latter OH. Two different spin systems were found for the E-ring protons of the northern and southern half of the molecule: an ABC and an AB system, respectively. The carbon chemical shift of the "northern" C-21 was 143.2 ppm, in accord with an aromatic C–O bond. All these data are in accordance with the proposed structure **5**.

As the dimerization started from a mixture of two diastereoisomers of silybin (with absolute configurations 2*R*, 3*R*, 10*S*, 11*S* and 2*R*, 3*R*, 10*R*, 11*R*—their chromatographic separation is not possible without further derivatization and the use

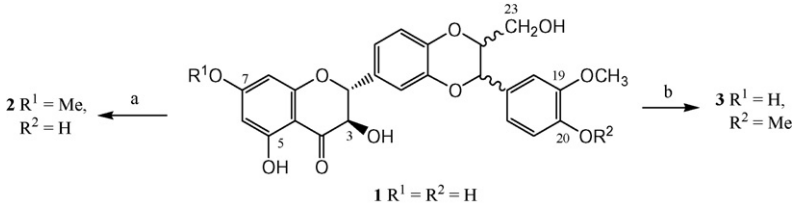

Fig. 2. Preparation of the monomethyl derivatives **2** and **3**. (a) MeI (1.5 equiv.), K_2CO_3, acetone, reflux, 3.5 h, 53%; (b) MeI (1.2 equiv.), NaH (3 equiv.), DMF, rt, 2 h, 54%.

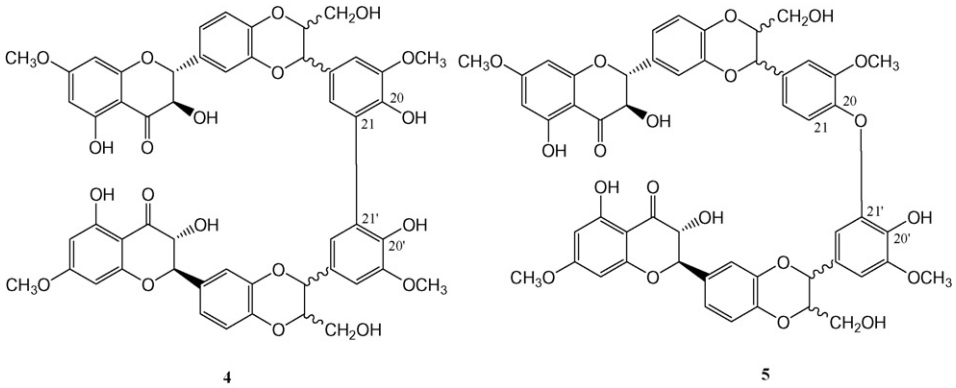

Fig. 3. Structures of the dimeric products **4** and **5**.

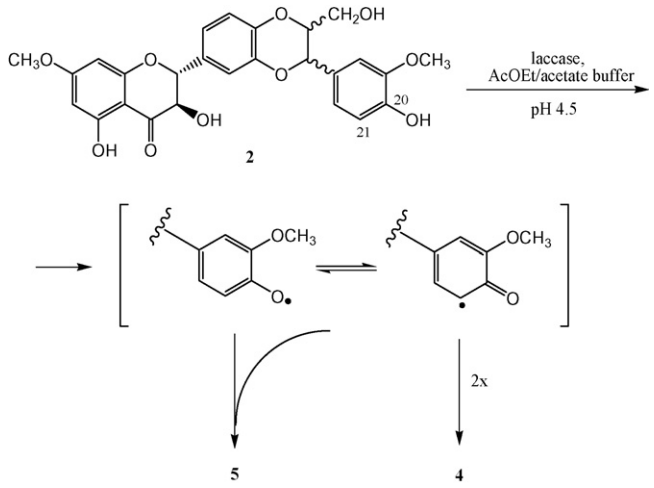

Fig. 4. Mechanism of the laccase-catalyzed oxidation of **2** to give the dimers **4** and **5**.

of preparative HPLC for this purpose gives negligible quantities of pure diastereoisomers only) four optically active compounds of each dimer could be expected. Due to the symmetry of the dimer **4**, two sets of signals were observed in ^{1}H and ^{13}C NMR spectra. The asymmetrical dimer **5** gave analogously four sets of signals in ^{1}H NMR spectrum but only three sets in ^{13}C NMR spectrum; however, a lot of signals of the same position were superimposed (both in ^{1}H and ^{13}C NMR) giving often only single signal for all four species of the dimer present. Nevertheless, according to the distinguishable NMR signals all possible isomers of each respective dimers **4** and **5** were formed in nearly equimolar ratio. This is probably caused by the fact that radical reactions (e.g., silybin radical dimerization) are characterized by low or no stereoselectivity at all.

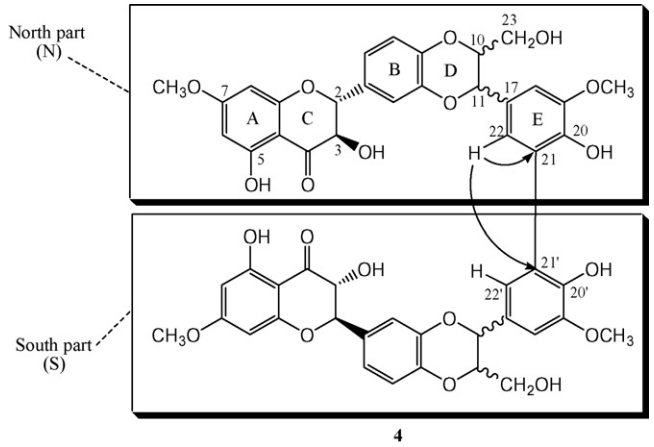

Fig. 5. The key HMBC-interactions of the dimer **4**.

4. Conclusions

To the best of our knowledge, this is the first report on the successful preparation and structure identification of silybin dimers. We confirmed, exploiting the laccase-catalyzed radical-oxidation of selectively protected derivatives of silybin, previous assumption [15] that the most important silybin moiety for its radical-scavenging activity is the phenolic 20-OH. Moreover, based on the structures of respective dimers, we were able for the first time to determine the mechanism of their formation and to explain the mechanism of silybin ring E antioxidant (antiradical) action on the molecular level (Fig. 4).

Furthermore, contrary to the previous hypothesis [15], we have found that the 7-OH in **1** still possesses a slight antiradical activity and probably contributes to the overall radical scavenging ability of silybin.

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