Mechanism of the Antioxidant Action of Silybin and 2,3-Dehydrosilybin Flavonolignans: A Joint Experimental and Theoretical Study

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Flavonolignans from silymarin, the standardized plant extract obtained from thistle, exhibit various antioxidant activities, which correlate with the other biological and therapeutic properties of that extract. To highlight the mode of action of flavonolignans as free radical scavengers and antioxidants, 10 flavonolignans, selectively methylated at different positions, were tested in vitro for their capacity to scavenge radicals (DPPH and superoxide) and to inhibit the lipid peroxidation induced on microsome membranes. The results are rationalized on the basis of (i) the oxidation potentials experimentally obtained by cyclic voltammetry and (ii) the theoretical redox properties obtained by quantum-chemical calculations (using a polarizable continuum model (PCM)−density functional theory (DFT) approach) of the ionization potentials and the O−H bond dissociation enthalpies (BDEs) of each OH group of the 10 compounds. We clearly establish the importance of the 3-OH and 20-OH groups as H donors, in the presence of the 2,3 double bond and the catechol moiety in the E-ring, respectively. For silybin derivatives (i.e., in the absence of the 2,3 double bond), secondary mechanisms (i.e., electron transfer (ET) mechanism and adduct formation with radicals) could become more important (or predominant) as the active sites for H atom transfer (HAT) mechanism are much less effective (high BDEs).

Introduction

Silymarin, the standardized plant extract obtained from seeds of the milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)],[1,2] is used as a hepatoprotective drug (Flavobion, Legalon)[3,4] and has also demonstrated beneficial effects in the prevention and the treatment of various liver diseases.[5] The major component of this extract is the flavonolignan silybin (Figure 1), but silymarin also contains other flavonolignans including silydianin, silychristin, isosilybin, dehydrosilybin, various flavonoids including taxifolin and quercetin, and other unidentified polyphenolic compounds (10−30%).[3] Silybin naturally occurs (in plants as well as in silymarin) as an equimolar mixture of two diastereoisomers: silybin A (3,5,7-trihydroxy-2-[3-(*R*)-(4-hydroxy-3-methoxyphenyl)-2-(*R*)-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]chroman-4-one) and silybin B (3,5,7-trihydroxy-2-[3-(*S*)-(4-hydroxy-3-methoxyphenyl)-2-(*S*)-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]chroman-4-one).[6]

Flavonolignans from silymarin exhibit free radical scavenging properties[7] and protective effects on the oxidation of lipid membranes[1,4] and low-density lipoprotein (LDL).[8] This antioxidant effect is often correlated with the other biological activities of those compounds, such as modulation of cell-signaling pathways involved in cell growth, differentiation, and apoptosis.[6] Recent studies demonstrated that both silybin and silymarin are effective against photoinduced and chemical-induced skin damage in vitro and in vivo.[9] We also demonstrated that silybin B exhibits an estrogenic activity, whereas silybin A is inactive.[10]

While the structure−antioxidant activity relationship of flavonoids has been extensively investigated and has clearly been established,[11,12] the mechanism of action of silybin and its derivatives as radical scavengers and antioxidants has not been systematically studied and it remains unclear. Only one study reported on the role of the hydroxyl group at C-20.[13]

The aim of the present study is to investigate the role of the different OH groups in the antioxidant activity of silybin (i.e., compound **1** in Figure 1, which is characterized by the absence of the 2,3 double bond) and 2,3-dehydrosilybin (i.e., compound **2** in Figure 1, which is characterized by the presence of the 2,3 double bond). Seven derivatives of those two compounds were synthesized by selective monomethylations of the different OH groups (Figure 1). Nor-dehydrosilybin (compound **3**) was obtained by hydroxylation of the 19-OCH_3 group of **2**. All the 10 compounds presented in Figure 1 were tested in vitro for their capacity to scavenge the stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl), the superoxide radical, and the lipid peroxidation induced on microsome membranes. The results are

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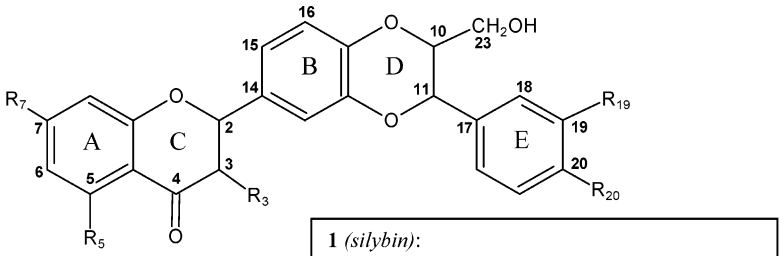
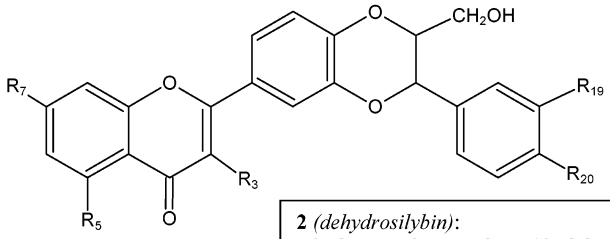
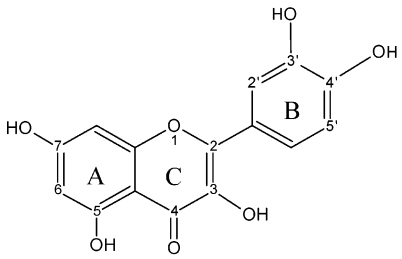


1 *(silybin)*:
R3=OH R5=OH R7=OH R19=OCH_3 R20=OH
1a *(3-O-*Me-*silybin)*:
R3=OCH_3 R5=OH R7=OH R19=OCH_3 R20=OH
1b *(7-O-*Me-*silybin)*:
R3=OH R5=OH R7=OCH_3 R19=OCH_3 R20=OH
1c *(20-O-*Me-*silybin)*:
R3=OH R5=OH R7=OH R19=OCH_3 R20= OCH_3

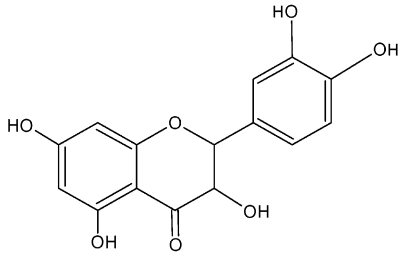


2 *(dehydrosilybin)*:
R3=OH R5=OH R7=OH R19=OCH_3 R20=OH
2a *(3-O-*Me-*dehydrosilybin)*:
R3=OCH_3 R5=OH R7=OH R19=OCH_3 R20=OH
2b *(5-O-*Me-*dehydrosilybin)*:
R3=OH R5= OCH_3 R7=OH R19=OCH_3 R20=OH
2c *(7-O-*Me-*dehydrosilybin)*:
R3=OH R5=OH R7=OCH_3 R19=OCH_3 R20=OH
2d *(20-O-*Me-*dehydrosilybin)*:
R3=OH R5=OH R7=OH R19=OCH_3 R20= OCH_3

3 (19-*Nor-dehydrosilybin)*:
R3=OH R5=OH R7=OH R19=OH R20=OH



Quercetin



Taxifolin

Figure 1. Chemical structure of silybin (**1**), dehydrosilybin (**2**), their monomethylated derivatives, nor-dehydrosilybin (**3**), quercetin, and taxifolin.

rationalized on the basis of (i) the oxidation potentials experimentally obtained by cyclic voltammetry and (ii) the theoretical redox properties obtained by quantum-chemical calculations.

The redox reactivity of phenolic compounds (ArOH) can follow two different chemical pathways,

(i) H atom transfer (HAT) mechanism:

$$\mathrm{ArOH} + \mathrm{R}^{\bullet} \rightarrow \mathrm{ArO}^{\bullet} + \mathrm{RH} \quad (1)$$

(ii) electron transfer (ET) mechanism:

$$\mathrm{ArOH} + \mathrm{R}^{\bullet} \rightarrow \mathrm{ArOH}^{+\bullet} + \mathrm{R}^{-} \rightarrow \mathrm{ArO}^{\bullet} + \mathrm{RH} \quad (2)$$

$R^{\bullet}$ is any radical involved in oxidative stress (including $^{\bullet}OH$, $O_2^{\bullet-}$, $ROO^{\bullet}$) and for example the model-radical DPPH.

Reaction 1 corresponds to the homolytic dissociation of an O−H bond. This reaction can occur on each OH group of the phenolic compound (the flavonolignan, for example) depending on the bond dissociation enthalpy (BDE) of the OH group (i.e., $\mathrm{BDE} = H_{298\mathrm{K}}(\mathrm{ArO}^{\bullet}) + H_{298\mathrm{K}}(\mathrm{H}^{\bullet}) - H_{298\mathrm{K}}(\mathrm{ArOH})$) and on the enthalpy difference ΔH_1 of reaction 1. The BDE is an intrinsic thermodynamical parameter of a given OH group in a phenolic compound, while ΔH_1 depends on the radical reacting with the flavonolignan. The lower the BDE, the easier the O−H bond breaking and the more important its role in the antioxidant reactivity. To be active on a given radical $R^{\bullet}$, the OH group must exhibit a negative ΔH_1, so that reaction 1 is exothermic. The second mechanism (reaction 2) is governed by the electron-transfer capacity, in other words, the ionization potential ($\mathrm{IP} = E(\mathrm{ArOH}^{\bullet+}) - E(\mathrm{ArOH})$) or the enthalpy difference of the first step of reaction 2 (i.e., $\Delta H_2 = [H(\mathrm{ArOH}^{\bullet+}) + H(\mathrm{R}^{-})] - [H(\mathrm{ArOH}) + H(\mathrm{R}^{\bullet})]$). The second step of this reaction is the heterolytic O−H bond dissociation, which is strongly exothermic for phenolic compounds.[14]

The conformational features of the flavonolignans and the corresponding radicals (ArO$^{\bullet}$) obtained after H abstraction, the ionization potentials, and the O−H BDEs of each OH group of the 10 compounds are obtained by using density functional theory (DFT) calculations. The solvent effects are taken into account by using a polarizable continuum model (PCM) approach. From the correlation between the experimental data and those calculations, the role of each OH group and the contribution of the two mechanisms (HAT or ET) are discussed.

Experimental and Computational Methods

Chemicals. Compound **1** (as a mixture of both diastereoisomers silybin A and B) was kindly donated by Galena-Teva Co. (Opava, Czech Republic). Compound **2** and monomethylated derivatives of **1** (**1b** and **1c**) and **2** (**2a**, **2c**, **2d**) as well as **3** were prepared as we previously described.[15,16] For the preparation of the new compounds **1a** and **2b**, and the corresponding structural data, see Supporting Information. Because of problems in its synthesis, the 5-*O*-methylsilybin has not been obtained, and as a consequence, neither experimental nor theoretical data will be presented here for this derivative. Quercetin and taxifolin (Figure 1) were purchased from Pliva-Lachema (Czech Republic) and ROTH (Germany), respectively. All other chemicals were provided by Sigma-Aldrich.

Identification. All the compounds were identified by NMR and mass spectrometry. NMR (HOM2DJ, COSY, TOCSY, HMQC, HMBC) 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 at 303 K. The sequence for 1D-TOCSY was obtained through Varian User Library, and the HMQC sequence was obtained from Varian Application Laboratory in Darmstadt. Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing instrument Finnigan MAT 95 (Finnigan MAT, Bremen, FRG) 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 the Finnigan ESI source, via a linear syringe pump at a flow rate of 40 μL/min.

Free Radical Scavenging Activity. *(a) DPPH Scavenging.* The DPPH scavenging activity was measured as the decrease in the DPPH characteristic UV absorption peak (517 nm) induced by the presence of the tested compound. This was measured in a reaction mixture containing 0.05 mL of a methanol solution of the compound (final concentration ranging from 0.001 to 5 mM) and 0.1 mL of DPPH solution (0.5 μM, MeOH), 10 min after preparation. IC_{50} values (compound concentration that scavenges 50% of the DPPH free radicals) were graphically obtained from the dose−response curves.

(b) Superoxide Scavenging. Superoxide was generated from molecular oxygen in the presence of EDTA, $MnCl_2$, and mercaptoethanol and evaluated spectrophotometrically as the decrease in absorbance of NADPH which is oxidized during the reaction.[17] The reaction mixture containing a triethanolamine−diethanolamine buffer (0.2 mL, 100 mM, pH 7.4), NADPH (10 μL, 7.5 mM), EDTA/$MnCl_2$ (10 μL, 100 mM/50 mM), and test sample/DMSO (from 1 μM to 5 mM in 5 μL DMSO) was incubated 10 min at 25 °C. The reaction was started with the addition of mercaptoethanol (25 μL, 10 mM), and the absorbance (340 nm) decrease was monitored 15 min after. IC_{50} are only measured for **1**, **2**, **3**, quercetin, and taxifolin.

Inhibition of Microsomal Lipid Peroxidation. Microsomes were prepared from rat liver homogenates and resuspended in a 50 mM Tris-HCl buffer with 100 mM KCl and 0.1 mM EDTA (pH 7.4). The protein concentration in the microsomal suspension was determined by the Lowry method. This suspension (0.49 mL, 1.0 mg protein/mL) was then mixed with the tested compounds (from 0.51 to 5.1 mM in 0.01 mL of DMSO) and incubated for 10 min at 37 °C. *tert*-Butyl hydroperoxide (0.01 mL, 2.5%, DMSO) was then added, and the mixture was incubated for 30 min at 37 °C. Controls for each experiment were performed with the same volumes of DMSO (i.e., instead of both tested compounds and *tert*-butyl hydroperoxide). The products of lipid peroxidation were observed by a standard reaction with thiobarbituric acid (TBA): (i) addition of 0.5 mL of TCA (2.86%) and 0.25 mL of TBA (1%), (ii) heating (90 °C, 30 min), (iii) cooling down, (iv) separation by centrifuge (10 min, 10 000 rpm, 4 °C), and (v) absorption measurement at 535 nm. The anti-lipoperoxidant activity is expressed as the concentration of the tested compound that inhibits the color reaction with thiobarbiturate by 50% (Table 1).

TABLE 1: Antioxidant Activities of Silybin and Dehydrosilybin Derivatives, Quercetin, and Taxifolin in Terms of DPPH and Superoxide Scavenging, Lipid Peroxidation Inhibition, and Half-Wave Anodic Potentials ($E_{a/2}$)

	IC_{50} (μM)			
compound	DPPH	superoxide	LPx	$E_{a/2}$ (mV)
1	1745 ± 65	55.20 ± 2.76	73.92 ± 2.11	542
1a	2541 ± 620		33.69 ± 0.46	572
1b	5750 ± 226		25.87 ± 0.39	531
1c	3546 ± 62		89.73 ± 1.97	-
2	54 ± 1	4.20 ± 0.21	15.11 ± 0.17	410; 600
2a	680 ± 19		24.64 ± 1.23	578
2b	292 ± 10		93.30 ± 5.22	443; 560
2c	66 ± 1		26.54 ± 2.12	467; 613
2d	60 ± 6		13.41 ± 0.71	403; 634
3	24 ± 8	0.49 ± 0.04	0.89 ± 0.01	266; 420
quercetin	11 ± 1	0.54 ± 0.01	4.73 ± 1.39	189
taxifolin	21 ± 6	2.42 ± 0.12	16.22 ± 0.16	273

Cyclic Voltammetry. Cyclic voltammetry measurements were performed using a potentiostat/galvanostat model 273 (EG&G Princeton Applied Research). The three-electrode system used is formed by a glassy carbon working electrode MF2012 (Bioanalytical Systems, West Lafayette, IN), a platinum-wire auxiliary electrode, and a Ag/AgCl reference electrode. The potential values quoted in this work are given with reference to the latter electrode. The compounds (10^{-2} M, DMSO) were diluted to concentrations of 10^{-4} M in 0.1 M sodium phosphate buffer (pH 7.0). All measurements were performed at room temperature and using a 200 mV/s scan rate in the range 0−1200 mV. The working electrode was polished with 0.05 μm grade alumina (Buehler, Lake Buffs, IL) prior to each scan. The half-wave anodic (oxidation) potentials $E_{a/2}$ were read from the detected anodic wave of the voltammograms.

Calculations. O−H BDEs were calculated as the difference in total enthalpy between the corresponding phenoxy radical (ArO$^{\bullet}$) formed after H abstraction and the flavonolignan (ArOH), according to the following reaction ArOH → ArO$^{\bullet}$ + H$^{\bullet}$.

Several approaches can be used to calculate such properties. Semiempirical[18,19] and more recently DFT[20−28] calculations have been successfully used for evaluating O−H BDEs for phenolic compounds smaller than flavonolignans (e.g., flavonoids and diarylheptanoids). Because it takes into account electron correlation at a lower computational cost compared to the post-Hartree−Fock methods, DFT appears as the most effective method for BDE estimation of small phenolic compounds in regard to the experimental data. The extension of the use of DFT to larger systems, such as flavonoids, only appeared very recently. Within the DFT formalism, it is well-known that the

functional and the basis set significantly influence the accuracy of the results. The combination of the B3P86 functional with a large basis set (6-311+G(d,p)) leads to excellent results for the BDE estimation of phenol in the gas phase compared to the experimental data ($BDE_{theoretical}$ = 86.9 kcal/mol vs $BDE_{experimental} = 87.0 \pm 1.5$ kcal/mol). Compared to the widely used B3LYP functional, B3P86 calculations show an improvement in the BDE values by about 4 kcal/mol for phenol and catechol.[28] On that basis, here we extend the use of the DFT/B3P86 approach to flavonolignans.

Because the correct description of electron delocalization is very important in the flavonolignan derivatives, especially for explaining their antioxidant behavior, we used the 6-311+G(d,p) basis set. This is quite large for a molecular system of such size; this choice is motivated by the fact that this basis set gives (i) reliable BDE estimation in the gas phase and (ii) a better description of electronic interaction far from the nucleus (i.e., electron delocalization) due to the joint use of polarization functions (d, p) and diffuse functions (+). The full B3P86/6-311+G(d,p) scheme (i.e., energy calculations and geometry optimization) is used for compounds **1**−**3**. We then tested for those three compounds the B3P86/6-311+G(d,p)//B3P86/6-31G(d) scheme. The reduction in the basis set used for the geometry optimization does not influence significantly the final energies, and the difference in BDE between the two approaches is lower than 0.5 kcal/mol for those three systems. $ArO^{\bullet}$ radicals were evaluated by using an unrestricted scheme (U) in order to take spin polarization into account, which is required in such DFT calculations.

Because of the computational cost for such systems, corrections related to zero point energy (ZPE) as well as translational, rotational, and vibrational energies were extrapolated from those obtained for quercetin and taxifolin at the (U)B3P86/6-311+G(d,p) level.[28] In this case, BDEs are calculated at 298 K using the sum of electronic and thermal enthalpies, which is the total electronic energy + ZPE + $E_{vibration}$ + $E_{rotation}$ + $E_{translation}$ + RT. For quercetin, the corrections on the BDEs are 8.4, 7.9, 7.9, 9.1, and 8.6 kcal/mol for the 3-OH, 3′-OH, 4′-OH, 5-OH, and 7-OH groups, respectively; for taxifolin they are 9.3, 7.9, 7.9, 8.9, and 9.0 kcal/mol for the 3-OH, 3′-OH, 4′-OH, 5-OH and 7-OH groups, respectively. We thus used those corrections for the dehydrosilybin and silybin derivatives to the corresponding OH groups.[29] Let us notice that these corrections are significant and can be of importance when comparing the calculated BDEs with experimental values and with other reference antioxidants.

Geometry optimization of each $ArO^{\bullet}$ radical was performed starting from the optimized structure of the parent molecule, after the H atom was removed from the 3, 5, 7, 19, or 20 position.

Calculations were carried out without and with taking into account the solvent effects. Guerra and co-workers studied the effect of explicit water molecules on phenol.[30] They concluded that solvent molecules could be arranged in cages (of at least six water molecules) in the vicinity of the OH group of phenol. Such calculations for each OH group of the 10 flavonolignan compounds would have been untractable. Thus, we used a PCM (polarizable continuum model) approach, as implemented in Gaussian 03,[31−33] which considers that the molecule under study is embedded in a polarizable dielectric representing the solvent, with no explicit solvent molecules included. The polarizable dielectric medium is described by the dielectric constant of the solvent (ϵ = 78.39 for water). Concerning the correction to enthalpy at 298 K, the same procedure than in the gas phase has been used (i.e., extrapolation from the corrections obtained for quercetin and taxifolin at the PCM-(U)B3P86/6-311+G(d,p) level). In this case the corrections on the BDEs are (i) for quercetin 7.0, 7.7, 7.4, 6.8, and 6.8 kcal/mol for the 3-OH, 3′-OH, 4′-OH, 5-OH, and 7-OH groups, respectively, and (ii) for taxifolin 9.1, 8.4, 8.4, 7.4, and 7.4 kcal/mol for the 3-OH, 3′-OH, 4′-OH, 5-OH, and 7-OH groups, respectively.

Therefore, the results reported in Tables 2 and 3 for the 10 compounds correspond to (U)B3P86/6-311+G(d,p)//(U)B3P86/6-31G(d) calculations of BDEs and enthalpy differences at 298 K, with or without the influence of the PCM solvent. All calculations were carried out with the Gaussian03 software.[34]

TABLE 2: BDEs (kcal/mol) at 298 K for Each OH Group of the Ten Silybin and Dehydrosilybin Derivatives, Quercetin, and Taxifolin in the Gas Phase (a) and with the Influence of the PCM Solvent (b)[a]

	3-OH	5-OH	7-OH	19-OH	20-OH	3′-OH	4′-OH
(a) BDE in the gas phase							
1	106.8	99.1	92.3		84.8		
1a		106.1	91.6		84.8		
1b	106.7	98.6			84.7		
1c	106.8	99.1	92.2				
2	83.4	97.6	89.2		85.0		
2a		100.8	89.8		85.0		
2b	85.3		86.0		84.8		
2c	83.2	96.9			85.0		
2d	83.4	97.6	89.1				
3				76.6	76.6		
quercetin	83.7	99.3	88.6			77.0	74.6
taxifolin	106.8	99.7	92.1			76.1	76.1
(b) BDE with the influence of the PCM solvent							
1	105.7	93.0	94.6		85.9		
1a		103.6	104.2		85.6		
1b	105.5	92.9			85.6		
1c	105.6	93.0	94.5				
2	80.2	91.4	90.7		86.3		
2a		94.1	92.7		86.4		
2b	80.6		89.6		86.2		
2c	80.0	90.9			86.3		
2d	80.1	91.5	90.8				
3				81.1	81.0		
quercetin	80.0	92.1	90.3			82.4	79.7
taxifolin	105.9	93.4	94.8			80.9	80.7

[a] The values for quercetin and taxifolin are from ref 28.

TABLE 3: Ionization Potential (IP, eV) and Energy Difference (ΔH_2, kcal/mol) of Reaction 2 (i.e., Electron Transfer from the Molecule to DPPH or to Peroxy Radicals)[a]

	IP (eV)		DPPH ΔH_2 (kcal/mol)		ROO$^{\bullet}$ ΔH_2 (kcal/mol)	
compd	gas phase	solvent	gas phase	solvent	gas phase	solvent
1	7.88	6.39	90.9	24.9	145.4	37.6
1a	7.86		90.5		145.1	
1b	7.81		89.4		144.0	
1c	7.74	6.36	87.7	24.3	142.3	36.9
2	7.61	6.14	84.8	19.2	139.4	31.8
2a	7.57	6.25	83.8	21.7	138.4	34.4
2b	7.34	6.05	78.6	17.1	133.1	29.8
2c	7.52	6.13	82.6	19.1	137.2	31.8
2d	7.54	6.13	83.2	19.1	137.7	31.8
3	7.65	6.13	85.6	19.1	140.2	31.8

[a] See ref 39 for additional computational details on DPPH and ROO$^{\bullet}$.

Results

Antioxidant Activity. The antioxidant capacity (DPPH and superoxide radical scavenging and lipid peroxidation inhibition)

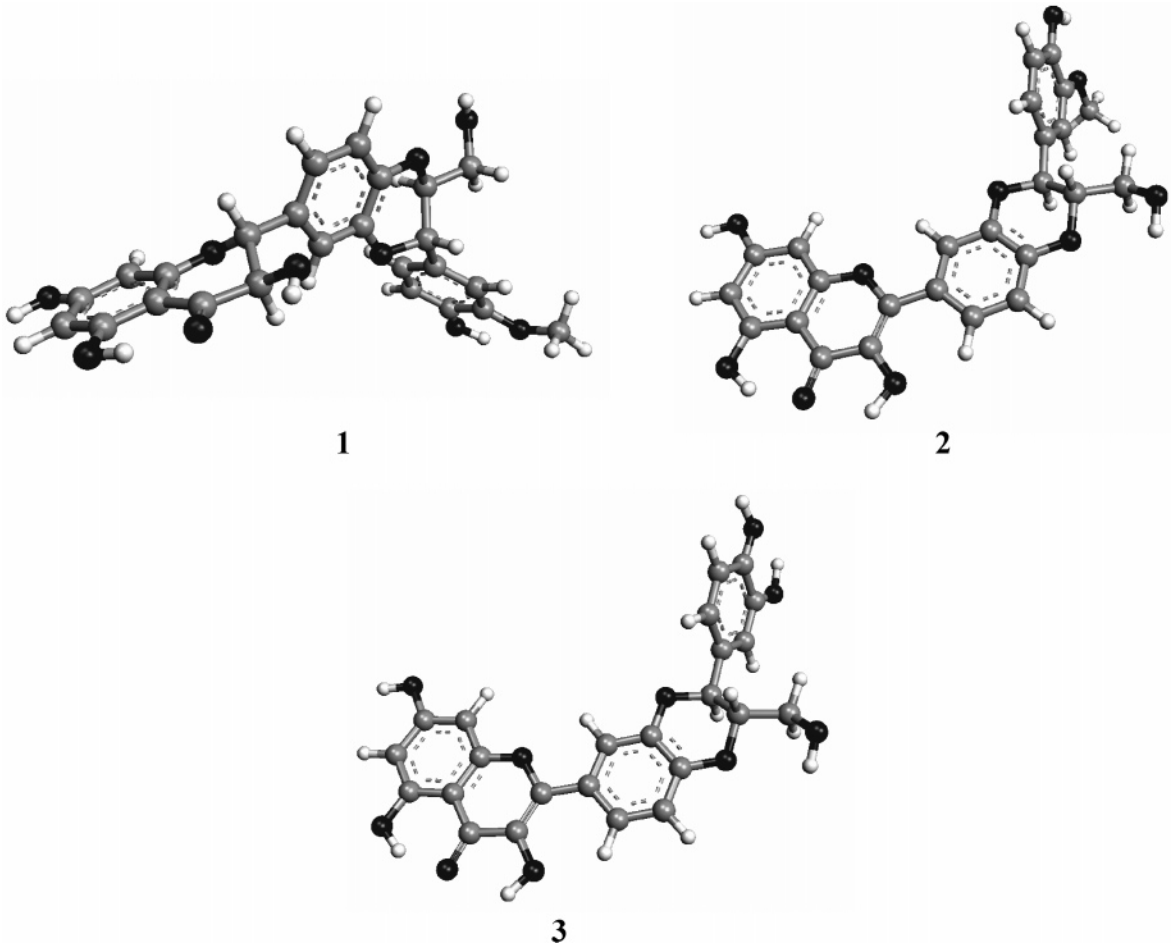


Figure 2. Conformations of **1**, **2**, and **3**.

is reported (Table 1) for all the silybin and dehydrosilybin derivatives studied in this paper, along with quercetin and taxifolin, two well-known flavonoids. Quercetin is a reference phenolic antioxidant, and taxifolin is its dihydroflavonol counterpart (Figure 1). The difference between those two compounds comes from the presence of the 2,3-double bond in quercetin, which is important to explain the activity of the 3-OH group.[28]

As previously proposed,[13] here we confirm that silybin (compound **1**) exhibits only low radical scavenging capacity compared to the other phenolic compounds (quercetin, for example). The corresponding methylated compounds (**1a**, **1b**, and **1c**) also exhibit poor activity. It must be noted that due to low water solubility of the methylated derivatives and their subsequent precipitation in the assay mixture, IC_{50} values for the superoxide scavenging action are not available (Table 1).

The free radical scavenging capacity of flavonolignans with the 2,3-double bond (**2** and the corresponding methylated derivatives, **2a** to **2d**) is much higher than for the silybin derivatives (**1**, **1a**, **1b**, and **1c**), which do not possess this double bond. The selective methylations of **2** give some valuable indications to understand the role of the various OH groups. Concerning the DPPH scavenging capacity, the following hierarchy is obtained: **2** $\sim$ **2c** = **2d** > **2b** > **2a** (Table 1). This indicates that 3-OH is the most important group involved in this activity. The 5-OH group also seems to influence this activity while the other two groups (7- and 20-OH) appear to give only a small contribution.

Compound **3** exhibits a much higher antioxidant activity compared to the other compounds, with a DPPH and superoxide scavenging capacity very close to that of quercetin. This compound is a better inhibitor of lipid peroxidation than quercetin, probably because it possesses the same redox capacity (approximately same DPPH scavenging) and it is more lipophilic.

Let us also note that no linear relationship is found between the IC_{50}s of DPPH scavenging and the IC_{50}s of lipid peroxidation inhibition. First, the mode of action for DPPH and $ROO^{\bullet}$ scavenging could be different (due to the different redox properties of those two free radicals). Second, DPPH can undergo different types of reaction including adduct formation, while lipid peroxidation is strongly influenced by the lipophilicity of the compounds, which could distort the experimental results on the pure scavenging (or inhibition) capacity. Therefore, the correlation between those two tests is not necessarily good.

Conformational Analysis. Let us analyze the conformational behavior of these compounds. To our knowledge, no quantum-chemical studies have been performed for these compounds. Only one study reported on a force-field investigation of silybin, and ligand binding to erbB1-Shc, in prostate cancer DU145 cells.[35] It is thus important to examine the conformational characteristics of these compounds and their influence on the electron delocalization, since the electron delocalization in the parent molecule (ArOH) and in the phenoxy radicals ($ArO^{\bullet}$) is one major electronic feature correlated with a high antioxidant capacity.

A conformational analysis has been performed following the two torsion angles Phi = C3−C2−C14−C15 and Psi = C10−C11−C17−C18 (Figure 1). For compound **2**, we find that the flavonoid moiety (A, B, and C rings) is planar (Figure 2),

allowing π electron delocalization; this is confirmed by the shape of the highest occupied molecular orbital (HOMO), which is delocalized over this moiety. For compound **1**, the planarity is lost and Phi is around 74° (Figure 2). This strong distortion from plane is obviously due to the loss in the 2,3-double bond and subsequently the loss in π electron delocalization. The HOMO of **1** is localized on the E-ring, and the HOMO-1 (which is very close in energy to the HOMO) is centered on the B-ring. The torsion angle Psi is 106° for **1** and **2** to minimize the steric hindrance between the hydrogen atom on the C18 position and the substituent on the C10 position.

The conformational analysis also highlights the importance of H-bonding in the E-ring. A stabilizing effect is observed by H-bonding between the H atom of the hydroxy group and the O atom of the methoxy group. This feature has previously been observed in curcumin[27] and chalcones.[14]

Methylations only weakly influence the conformations except for the 3-OH group. In this case methylation leads to a change in the torsion angle Phi to reduce the steric hindrance with the B-ring. For silybin derivatives, the angle changes from 74° (in **1**) to 94° (in **1a**) in the gas phase and from −111° (in **1**) to −104° (in **1a**) with the influence of the PCM solvent. For the dehydrosilybin derivatives, methylation of the 3-OH group induces a twist from planarity, from 180° (in **2**) to 160° (in **2a**) in the gas phase as well as in the solvent. However, this loss of planarity does not significantly influence the HOMO distribution. Methylation of the other groups does not significantly change the molecular conformations except for the OCH_3 group itself: to decrease the interaction with the 4-keto group, the 5-OCH_3 group (in **2b**) is oriented in the opposite direction, which is not the case in **2**, for which the 5-OH group exhibits a H bonding interaction with the 4-keto group. The same behavior is observed for methylation of position 20 (i.e., twisting of the 20-OCH_3 group in **1c** or **2d** compared to the 20-OH group in **1** or **2**).

HAT Mechanism and BDE Calculations. In order to estimate the contribution of the HAT mechanism in silybin derivatives, the BDEs were calculated for each OH group of the 10 compounds and are reported in Table 2. Concerning **1** and the corresponding methylated derivatives, the lowest BDE is obtained for the 20-OH group (around 85 kcal/mol in the gas phase). Let us note that in Table 2 we only quoted BDEs for the silybin A isomer (2R3R10R11R) of compound **1**. The diastereoisomer counterpart silybin B (2R3R10S11S) possesses almost the same theoretical BDE values (i.e., differences lower than 0.2 kcal/mol, in the gas phase). For **2** and other derivatives, the lowest BDEs are those of the 3- and 20-OH groups (around 83 and 85 kcal/mol, respectively). The 3-OH BDE of **1** is high (around 107 kcal/mol), indicating that this group does not participate in the HAT mechanism. Figure 3a shows the calculated spin density of the radical obtained after H abstraction from the 3-OH group. The more delocalized the spin density in the radical, the easier the radical is formed and the lower the BDE. For the 3-OH group of **1**, it is strongly localized on the O atom from where the H atom is removed, while it is delocalized on the 2,3 bond for compound **2** (Figure 3a), which is in agreement with the high and low BDE, respectively. The H-transfer from the 5- and 7-OH groups seems to be less effective (high BDE), indicating that those groups only weakly participate in the HAT mechanism.

The O−H BDEs in the E-ring of compound **3** is around 77 kcal/mol in the gas phase, indicating the strong capacity of those groups to transfer H atoms. This is a special characteristic of the catechol moiety, as already observed for quercetin, taxifolin, and other flavonoids having the same feature (see Table 1 and ref 24). In this case the spin density is delocalized over the E-ring (Figure 3b), indicating efficient stabilization of the formed radical.

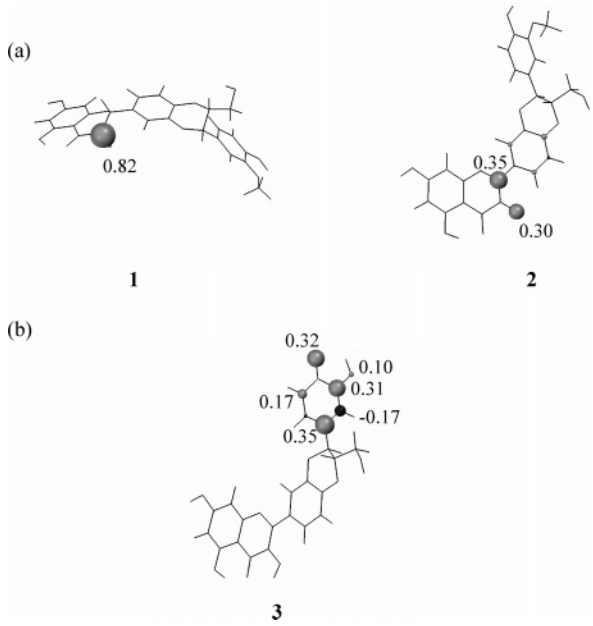


Figure 3. Spin density distribution of the 3-OH radical for compounds **1** and **2** (a) and the 20-OH radical for compound **3** (b). Only the sites with values higher than 0.10 are shown.

The primary effect of methylation is obviously to block the methylated OH group, but this could also influence the reactivity of the other OH groups. As we can see in Table 2, methylation of the 5-OH group (i.e., compound **2b**) leads to an increase in BDE for the 3-OH group by about 2 kcal/mol. This is due to the loss of the H-bond between the 5-OH and 4-oxo groups, which allows an increase in the interaction between the 3-OH and 4-oxo groups. Indeed, the distance between the oxygen atom of the 4-oxo group and the H atom of the 3-OH group decreases from 2.07 Å (in **2**) to 1.92 Å (in **2b**). This result is in very good agreement with the antioxidant tests (i.e., increase in DPPH IC_{50} values from 54 to 292 and increase in lipid peroxidation IC_{50} from 15.1 to 93.3, from compound **2** to **2b**).

As shown in Table 2b, the presence of the solvent (PCM calculations) significantly changes the BDEs. This change is different for the 3-OH and the 5-OH groups compared to the 7-OH group and the OH groups of the E-ring. For example, the BDE is decreased by ∼2.7 kcal/mol for the 3-OH group whereas it is increased by ∼1.3 and 4.4 kcal/mol for 20-OH in **2** and **3**, respectively. Such a result has previously been reported for flavonoid compounds[14] and is attributed to a difference in interaction between the solvent and the different parts of the molecule. As a result, the 3-OH BDE (∼80.1 kcal/mol) of **2** and other derivatives is much lower than for the other groups and very close to the BDEs in the E-ring of **3**, which clearly points to the important role of the 3-OH group in flavonolignan reactivity.

ET Mechanism. In order to estimate the contribution of the ET mechanism of flavonolignans, we calculated the IP (as the energy required to remove an electron from the ground state of the molecule) for all the 10 compounds. As shown in Table 3, the lowest IP is obtained for compounds having the 2,3-double bond (**2**, **2a**, **2b**, **2c**, **2d**, and **3**), whereas the other ones (**1**, **1a**, **1b**, and **1c**) exhibit relatively high IPs; the difference in energy is ∼0.30 eV (i.e., ∼7 kcal/mol) in the gas phase and 0.25 eV

(i.e., ∼6 kcal/mol), with the influence of the solvent. The presence or absence of the 2,3-double bond appears to be the most important chemical parameter that influences the IP values. Except for that chemical feature, the difference in IP among the different methylated flavonolignans compounds is weak, especially for the methylation of the 7-OH group and the groups of the E-ring, indicating that those substitutions do not strongly influence the intrinsic capacity to remove an electron from the molecule. The methylation of the 3-OH group (compound **2a**) induces a reduction in π electron delocalization (due to the loss of planarity), thus inducing a slight but significant increase in IP compared to **2**. In contrast, the methylation of the 5-OH group (in compound **2b**) probably reenforces the delocalization effect between the 3-OH group and the 4-oxo group (due to a stronger interaction between the two groups), inducing a lower IP.

The enthalpy difference ΔH_2 of the first step of reaction 2 (ET mechanism) has been calculated in the case of DPPH and ROO$^\bullet$ radicals and is reported in Table 3. Considering the role of the solvent strongly influences the results. Such a strong effect, observed in Table 3 between the gas phase and the solvent calculations, has previously been found with chalcones[14] and definitively leads to the conclusion that the solvent must be taken into account in such calculations, in order to obtain a good description of the redox properties of polyphenolic compounds, especially when charged species are involved. Nonetheless, even when taking into account the solvent effects, ΔH_2 is still relatively high and positive (i.e., higher than ∼+17 kcal/mol). This value (plus the energy barrier estimated to be ∼6 kcal/mol) indicates that the ET mechanism is a thermodynamically unfavorable event for those compounds.

Cyclic Voltammetry. The redox capacity has also been evaluated using cyclic voltammetry and characterized by the half-wave anodic (oxidation) potential ($E_{a/2}$) values. The $E_{a/2}$ values can be used to predict the radical scavenging properties and the antioxidant behavior of molecules in biological systems.[36] Generally, compounds having an $E_{a/2}$ value lower than 200 mV can be considered as valuable antioxidants. Compound **1** and the corresponding derivatives exhibit one peak ranging from 542 to 572 mV. Compound **2** and derivatives exhibit two anodic waves, suggesting the presence of two sites in the molecule with different reducing power. The higher $E_{a/2}$ values correspond to those observed for flavonolignans without the 2,3-double bond, and the lower values (410−467 mV) reflect the stronger reducing capacity of flavonolignans with the double bond. A new peak at ∼270 mV appears for compound **3**, demonstrating the major role of the catechol moiety in the redox properties of this compound.

The most significant change due to methylation is observed for compound **2a** (3-OH methylation of **2**) (Figure 4). In this case, the lowest peak ($E_{a/2}$ ∼ 410 mV) is lost, giving a voltammogram very close to that of compound **1**. Methylation of the 20-OH group leads to a significant decrease in the first peak for compound **1**; the peak is still present for compound **2** (Table 1), but its intensity is decreased on the voltammogram. Those results point to the importance of the 3-OH and 20-OH groups in the redox properties of silybin derivatives.

Discussion

Our approach in this work is to support the experimental results by the theoretical calculations of chemical parameters related to the redox capacity, in order to establish some general trends (distinguishing two subclasses, presence or absence of the 2,3-double bond) and to understand the mode of action of flavonolignans.

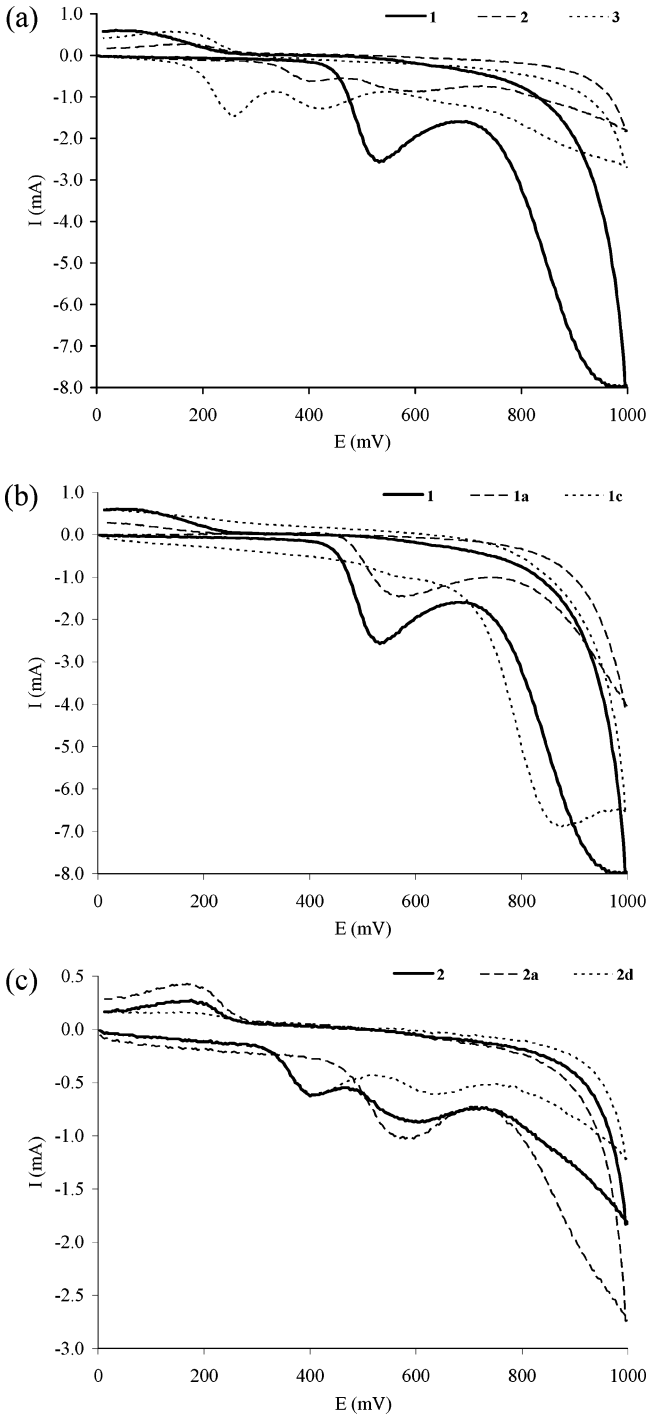


Figure 4. Cyclic voltamogramms for compounds **1**, **2**, and **3** (a) and the methylation effect on silybin **1** (b) and dehydrosilybin **2** (c).

Dehydrosilybin Derivatives (2, 2a, 2b, 2c, 2d, and 3). Concerning compound **2** and the corresponding derivatives (including **3**), the following hierarchy is obtained for the DPPH test: **3** > **2** ∼ **2c** = **2d** > **2b** > **2a**. The low activity of **2a** (3-OH methylation) shows the role of the 3-OH group. For the superoxide scavenging test, compound **3** is also more active than **2**. The hierarchy is almost the same for the lipid peroxidation inhibition, except for **2a** and **2b**. For this test, the difference in activity between compounds is relatively small, which most probably reflects the occurrence of solubility problems rather than a different mode of action.

The loss of the oxidation peak at 410 mV, followed by a slight increase in the ionization potential (Tables 1 and 3), for compound **2a** (methylation of 3-OH) could at first glance

indicate a good correlation with the decrease in the antioxidant property. However, the corresponding ΔH_2 values (enthalpy difference for the ET mechanism reported in Table 3) for the DPPH and ROO$^\bullet$ radicals are too high to consider the ET mechanism as the major process. Indeed, the ET mechanism gives endothermic reactions with DPPH as well as with peroxy radicals (i.e., ~+20 and +35 kcal/mol, respectively). A small increase in ΔH_2 is observed from **2** to **2a** (i.e., ~2.5 and 2.8 kcal/mol for DPPH and ROO$^\bullet$, respectively), which is followed by a strong decrease in the antioxidant activity. However, the decrease in ΔH_2 by about 2.1 (DPPH) and 2.5 kcal/mol (ROO$^\bullet$) from **2** to **2b** is concomitant with a decrease in the antioxidant activity. Thus, the parameters governing the ET mechanism do not correlate with the antioxidant activity, indicating that in solution this process is unfavorable. The preliminary observation made for compound **2a** (i.e., IP increase and lower antioxidant activity compared to **2**) may suggest that the ET mechanism participates as a secondary process. On the contrary, the calculated BDEs are in very good agreement with the experimental trends. The BDEs of the OH groups can be compared to the BDEs of DPPH−**H** and ROO−**H**, which have been experimentally estimated to be 80[37] and 88 kcal/mol.[38] To be active for the HAT mechanism, the OH group of the phenolic compound must exhibit a BDE lower than those values, so that reaction 1 is exothermic. From the theoretical calculations (Tables 2 and 3),[39] it appears that the HAT mechanism is thermodynamically favorable for the 3-OH group of the flavonolignans (i.e., $\Delta H_1 = +2.0$ and -7.6 kcal/mol for DPPH and ROO$^\bullet$, respectively). This calculation does not take into account the energy barrier but clearly gives the best thermodynamic balance. The lower activity of **2b** (5-OH methylation) is attributed to the loss in H bond between the 5-OH and the 4-oxo groups, which leads to a slight increase of the 3-OH BDE.

For compound **3**, the 3-OH group is still active, but a second effective redox site is present in the E-ring. Indeed, it is clear that in the presence of the catechol moiety (19-OH, 20-OH groups) the redox behavior is close to that of the 3-OH group (Table 2b). The O−H bond dissociation capacity of the 20-OH group of compound **3** leads to a favorable H transfer to the radicals (i.e., $\Delta H_1 = +2.9$ and -6.6 kcal/mol for DPPH and ROO$^\bullet$, respectively), while the ET mechanism appears strongly endothermic (i.e., $\Delta H_2 = +19.1$ and $+31.8$ kcal/mol for DPPH and ROO$^\bullet$, respectively). Moreover, for compounds **2** and **3**, the ΔH_2 values are identical whereas the antioxidant capacity is much higher for compound **3**. Thus, the E-ring of **3** most probably acts as a H atom donor rather than an electron donor, even though the ET mechanism could act as a minor process, depending on the radical. Let us note that the presence of two effective groups (i.e., the 3-OH and 20-OH groups with low BDEs in **3**), rather than one (i.e., the 3-OH group in **2**), leads to a 2-fold increase in the DPPH activity. Considering the DPPH test as a good indicator of the redox capacity of effective antioxidants, it seems that in the case of **3** HAT from 3-OH and 20-OH are clearly the predominant modes of action.

Silybin Derivatives (1, 1a, 1b, and 1c). The DPPH scavenging activities of compound **1** and its methyl ethers are much lower compared to **2** and its methyl derivatives (including the nor-derivative **3**), except compound **2a**. The superoxide scavenging activity of **1** is much lower than **2** and **3**, the three compounds for which IC_{50}s have been successfully determined (Table 1). Even though the antioxidant activities in the three tests are relatively low (and thus more sensitive to experimental fluctuations), let us give the hierarchy observed for **1** and its derivatives: **1** ~ **1a** > **1c** > **1b** (for DPPH) and **1a** ~ **1b** > **1** > **1c** (for lipid peroxidation).

From those results it appears that only the 7-OH and 20-OH groups contribute to the DPPH scavenging activity of **1**. One can suggest that 7-OH reacts with DPPH by adduct formation and by this way contributes to the overall DPPH activity. Concerning lipid peroxidation inhibition, methylation increases the activity, which is attributed to the better lipophilic properties of the methylated compounds (due to the presence of an additional OCH_3 group which is less polar than the OH group). Interestingly, this is what we observe for **1a** (3-OH methylation) and **1b** (7-OH methylation) compared to **1**, but not for **1c** (20-OH methylation). On the contrary, the 20-OH methylation (from **1** to **1c**) slightly decreases the lipid peroxidation inhibition. In that case, the beneficial effect of the increase in lipophilicity is probably masked by a loss in the redox property of the E-ring, as observed in the DPPH test (lower activity of **1c** compared to **1**).

The role of the 20-OH, 19-OCH_3 moiety (E ring of silybin **1**) has previously been discussed for silybin[13] and theoretically explained for curcumin[27] and chalcones.[14] In those studies, the HAT mechanism is involved to explain the radical scavenging capacity of this moiety. Here we must note that the role of this moiety in the antioxidant capacity is relatively low compared to that of 3-OH in **2** and 20-OH in **3**: no changes in the antioxidant capacities (Table 1) are observed after the 20-OH methylation (from **2** to **2d**).

Comparison with Flavonoids. It is interesting to compare the experimental structure−activity relationship as well as the theoretical calculations obtained for flavonolignans to those of flavonoids. The recent literature reported BDE calculations at the DFT level on different compounds including quercetin and taxifolin.[23−25] The role of the B-ring has been pointed out, especially in the presence of the catechol moiety (quercetin and taxifolin). The role of the 3-OH group was also discussed and confirmed in the presence of the 2,3-double bond.[28]

Here we note that flavonoids and flavonolignans exhibit the same behavior for the 3-OH group. The BDE is ~80 kcal/mol for compound **2** and quercetin, whereas it is higher than 105 kcal/mol for compound **1** and taxifolin.[40] Similar to quercetin,[28] the low value obtained for the 3-OH BDE in compound **2** (i.e., in the presence of the 2,3 double bond) comes from the capacity to delocalize the spin density in the phenoxy radical ArO$^\bullet$ formed after H abstraction (Figure 3). On the contrary, for taxifolin as well as for **1**, the spin density of the corresponding ArO$^\bullet$ radical is strongly localized on the O atom (Figure 3). Thus, the lack in π electron delocalization in **1** makes the H abstraction from the 3-OH group a very unfavorable thermodynamic event.

The E-ring of silybin derivatives exhibits a behavior similar to the B-ring in flavonoids. The BDEs are relatively low, especially in the case of compound **3**. Again, these low BDEs are attributed in part to the strong capacity for radical delocalization in the phenoxy radical ArO$^\bullet$ (Figure 3), which is in agreement with the possibility of quinone formation for flavonoids.[41]

The redox behavior of flavonolignans can then be divided into two distinguishable parts: the one attributed to the flavonoid moiety and the one attributed to the E-ring. In solution, the total redox activity of flavonolignans is probably just an addition of both contributions. This additive effect must be attributed to the weak electronic coupling between the two parts of the flavonolignan molecules.

Conclusions

In this paper, the selective methylation of the different compounds is performed to highlight the mode of action of flavonolignans as free radical scavengers and antioxidants. Here we clearly establish the importance of the 3-OH group in dehydrosilybin (**2**) and nor-dehydrosilybin (**3**) (i.e., in the presence of the 2,3 double bond) as an H donor. For compound **3**, the HAT mechanism is also likely to occur in the E-ring, in which the 20-OH group could easily transfer H atom to free radicals. For those two compounds, the ET mechanism and the formation of adducts with DPPH radicals probably act as secondary modes of action.

For silybin (**1**), these secondary mechanisms could become more important (or predominant) as the active sites for HAT mechanism are much less effective (they show high BDEs). In this case, the 20-OH group could act as H-donor, especially to $ROO^{\bullet}$ radicals, but the ET mechanism could give a significant contribution and the 7-OH group could undergo adduct formation with DPPH.

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Supporting Information Available: Detailed procedures and ^{1}H and ^{13}C NMR data for the monomethylated derivative synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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(29) In order to support our choice to extrapolate the temperature correction from the results obtained on quercetin and taxifolin, we also performed the frequency calculation at the B3P86/6-31G(d) level, with a scale factor of 0.9759. Nonetheless, we obtained the following correction for (i) dehydrosilybin 6.6, 7.2, 7.0, and 6.3 kcal/mol for 3-OH, 5-OH, 7-OH, and 20-OH, respectively, and (ii) silybin 7.6, 7.4, 7.3, and 6.6 kcal/mol. Those corrections follow the same trend compared to our extrapolation methodology but the corrections are shifted by about 1−2 kcal/mol. We also performed the frequency calculation for quercetin at the B3P86/6-31G-(d) level, using a scale factor of 0.9759, and we obtained 6.6, 7.2, 7.0, and 6.2 kcal/mol for 3-OH, 5-OH, 7-OH, and the B-ring, respectively. We thus concluded that the scaled-B3P86/6-31G(d) level gives less accurate results for the temperature correction. We finally used the extrapolated scheme (i.e., thermal corrections obtained at the B3P86/6−311+G(d,p) for quercetin and taxifolin and extrapolated to all the flavonolignan derivatives).

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