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Silychristin: Skeletal Alterations and Biological Activities

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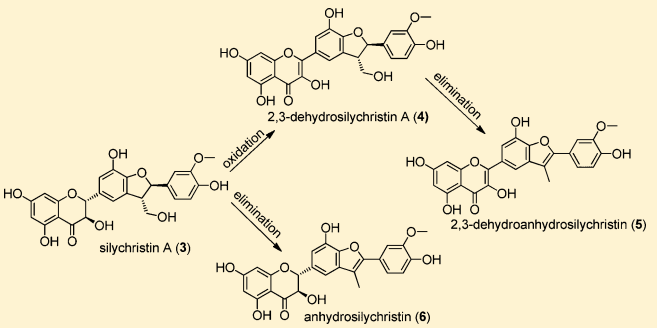
Supporting Information

ABSTRACT: Silychristin is the second most abundant flavonolignan (after silybin) present in the fruits of *Silybum marianum*. A group of compounds containing silychristin (**3**) and its derivatives such as 2,3-dehydrosilychristin (**4**), 2,3-dehydroanhydrosilychristin (**5**), anhydrosilychristin (**6**), silyhermin (**7**), and isosilychristin (**8**) were studied. Physicochemical data of these compounds acquired at high resolution were compared. The absolute configuration of silyhermin (**7**) was proposed to be identical to silychristin A (**3a**) in ring D (10*R*,11*S*). The preparation of 2,3-dehydrosilychristin (**4**) was optimized. The Folin−Ciocalteau reduction and DPPH and ABTS radical scavenging assays revealed silychristin and its analogues to be powerful antioxidants, which were found to be more potent than silybin and 2,3-dehydrosilybin. Compounds **4**−**6** exhibited inhibition of microsomal lipoperoxidation (IC_{50} 4−6 μM). Moreover, compounds **4**−**8** were found to be almost noncytotoxic for 10 human cell lines of different histogenetic origins. On the basis of these results, compounds **3**−**6** are likely responsible for most of the antioxidant properties of silymarin attributed traditionally to silybin (silibinin).



Silymarin is a crude extract of the fruits (*cypselae*) of milk thistle (*Silybum marianum* L. Gaertn., Asteraceae), which is used in a plethora of nutraceutical and dietary supplement preparations due to a purported chemo- and hepatoprotective action. Depending on the plant cultivar and extraction method used, silymarin contains more than 10 structurally closely related flavonolignans, which are all biogenetic congeners. The most abundant flavonolignan and the easiest to isolate from silymarin is silybin (**1**, silibinin),[1] typically used as a natural mixture of diastereomers silybin A (**1a**) and silybin B (**1b**) in a ratio of ca. 1:1 (the numbers denote here natural mixtures of diastereomers of silybin (**1**) and silychristin (**3**) and the numbers with letters denote individual diastereomers). Silybin (**1**) has been thoroughly researched in the past, and there exists a strong, however unspoken, bias of ascribing all the potentially beneficial properties of silymarin to silybin (**1**). Recently, the silybin oxidative derivative and minor silymarin constituent 2,3-dehydrosilybin (**2**) have attracted research attention due to their biological activities.[2] On the other hand, the second most abundant flavonolignan in silymarin, silychristin (**3**), has infrequently been isolated, and little is known about its biological activities.[3]

Prior to the medieval period, application of milk thistle was restricted to a culinary use where mostly the leaves were eaten as a salad or were boiled. The first reference to its medical properties is in the nine books of *Physica* by Hildegard von Bingen (written between 1150 and 1160 A.D. in Disibodenberg monastery, at present in Germany), and since then, milk thistle has been mentioned in all major herbals up to the modern times. The herbal of Pietro A. Mattioli (German edition, Prague, 1563) contains a possible reference of the usage of the alcoholic seed extract against liver problems ("*Das Wasser von Blettern gebrandt vnd ein zimlichen Trunck darvon gethan bringt gute hilff wider das Seitenstechen. Besser ist es aber so man ein halb quentlen deß zerstoffenen Samens darzu thut.*"). Adam Lonitzer in his *Kräuterbuch* (1557, Frankfurt am Main) in a text similar to that of Mattioli mentions the use against "side pain" (probably liver or gall bladder problems) as well. The most specific reference in his book mentions usage against "inflamed liver", "*Tüchlein in dem Wasser genetzt und übergelegt ist es gut zu der*

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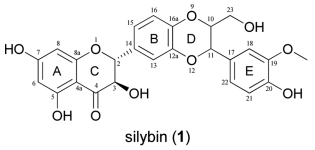
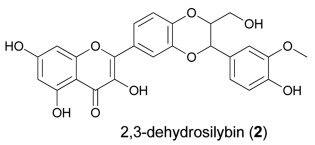
entzündeten Leber für Ohnmacht und Schwachheit". In this context, it is important to note that only silychristin (**2**) and silydianin[4] are readily soluble in protic solvents (such as ethanol), and the remaining flavonolignans, such as silybin (**1**), precipitate from EtOH. This means that most of beneficial effects described in these ancient sources might be ascribed to silychristin (**3**) and silydianin, as discussed recently.[2]

In the present work, fundamental properties and biological activities of silychristin (**3**) and several of its so far unknown or poorly studied structural analogues such as 2,3-dehydrosilychristin (**4**), 2,3-dehydroanhydrosilychristin (**5**), anhydrosilychristin (**6**), silyhermin (**7**, from the white flowering variety of *S. marianum*), and isosilychristin (**8**, from wild *S. marianum* from Austria) were studied using standard methods in a panel of structurally related compounds. Scattered data from existing older literature[5−8] are often not comparable due to variability of the methods used and also questionable quality (purity) of the substances tested.

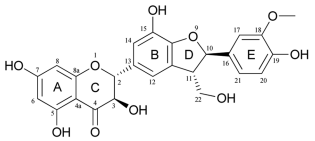
Similar to silybin, natural silychristin from *S. marianum* consists of two diastereomers: silychristin A (**3a**) and silychristin B (**3b**).[8] Silychristin A (**3a**) is prevalent in the natural material with a diastereomeric ratio of approximately 95:5 (**3a**/**3b**), depending on the source. In the present investigation, natural silychristin (**3**) (actually **3a** in a purity of ca. 95%) was used for chemical modifications. Pure silychristin B (**3b**), due to its paucity, was used only for the determination of its physicochemical and spectroscopic properties. Silybin (**1**) and 2,3-dehydrosilybin (**2**) were included in the panel of compounds tested as control substances.



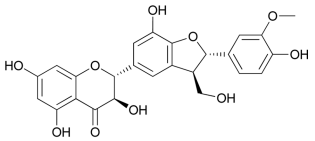
silybin (**1**)



2,3-dehydrosilybin (**2**)



silychristin A (**3a**)



silychristin B (**3b**)

RESULTS AND DISCUSSION

Most isolation methods for silychristin rely upon RP-HPLC. However, separation on this stationary phase, despite using various mobile phases, always leads to coelution of silychristin B (**3b**) with silydianin.[9] There exists no practical method for the separation of silychristin B (**3b**) and silydianin using silica gel chromatography. A novel method based on LH-20 gel filtration,[9] which allows preparative separation of silychristin (**3**) from silydianin and other flavonolignans, has recently been developed, although it is unable to separate diastereomers **3a** and **3b** from each other. A diastereomeric mixture of **3a** and **3b** devoid of silydianin can be then separated by preparative HPLC chromatography on silica gel (Labiospher PSI 100 10 μm, Labio a.s., Prague, Czech Republic) to yield optically pure silychristin A (**3a**) and silychristin B (**3b**). The complexity of the isolation and very low content of silychristin B (**3b**) in the natural material is an obvious reason that it has not been described until 2005, 29 years after the discovery of silychristin (**3**).[4]

2,3-Dehydrosilychristin (**4**) was first prepared in 1982[6] by the aerial oxidation of silychristin (**3**), and it has been virtually ignored ever since. Although the initial author reported a high yield, in this study it never exceeded 14% (with total conversion of the starting material). The low yields are probably caused by the formation of insoluble polymers, which deposit as a thin transparent layer on the walls of the reaction vessel. Originally, the oxidation was carried out in hot pyridine under an oxygen atmosphere typically for 14 days.[2,6] During optimization of this reaction various combinations of bases and solvents were tested (i.e., DMF, DMSO, acetone, ethanol, and methanol as solvents and DMAP, triethylamine, CH_3COOK, and K_2CO_3 as bases). A new efficient method has been developed and optimized, allowing ca. 20× shorter reaction time and double yields. No formation of insoluble polymers was observed using this optimized method. Oxidation of silychristin was performed in DMSO with addition of triethylamine at 60 °C under an O_2 atmosphere overnight, giving **4** in 27% yield.

It has been known since 1978[5] that the primary alcoholic group of silychristin readily eliminates water to give **6** under acidic conditions. The previous authors have coined the term "anhydrosilychristin" for this product, which was not investigated in their study,[5] and its preparation was not repeated until now. Anhydrosilychristin (**6**) was prepared by the treatment of silychristin with HCl in hot EtOH (Scheme 1). The reaction proceeded fast (full conversion in 1 h), and although the yield was low (24%), no soluble byproducts were observed. While small amounts of **6** were isolated during the separation of silychristin from natural silymarin, it remained unclear if it is present in the natural source or whether it is an artifact of the isolation procedure. Elimination of the primary alcoholic group of **4** (the same method as for **6**) resulted in the formation of **5**, an achiral flavonolignan and so far undescribed compound, identified as 2,3-dehydroanhydrosilychristin (**5**).

Two natural compounds, silyhermin (**7**) and isosilychristin (**8**), isolated from a white flowering variety of the milk thistle and from wild samples collected in Austria, respectively, were also included due to their close structural analogy to silychristin (**3**), to complete the test compound panel.

A polyvinyl column, ASAHIPAK GS-301 20F (Shodex, USA), suitable for the purification of complex mixtures of flavonoids, was used for the separation of reaction mixtures and for the isolation of **7** and **8**. Silyhermin (**7**), a 3-deoxy analogue of silychristin A (**3a**), was obtained by direct extraction of the crushed fruits of a white flowering variety of *S. marianum* with acetone−water (95:5). The crude extract was concentrated and defatted with hexane.[10] An extract containing **7** as a major compound was chromatographed on ASAHIPAK GS-301 20F in methanol to obtain pure **7**, which was identified by NMR and HRMS (together with the results of chiroptical methods available in Table S56 and Figures S54, S55, and S57−S62, Supporting Information).

The proposed structure of **7** was corroborated using the electronic circular dichroism (ECD) spectrum. In a previous paper describing silyhermin (**7**),[10] the absolute configurations of the stereocenters on the D-ring were not determined, mainly due to the lack of a suitable analogous compound with known absolute configuration. The UV/vis absorption and ECD spectra of silychristin A (**3a**) were found to be almost identical with those of silyhermin (**7**), with the ECD spectrum of silychristin B (**3b**) in the UV region found to be different from the ECD of silyhermin (**7**, Table 1, Figure S62, Supporting Information). Therefore, it may be inferred that the absolute

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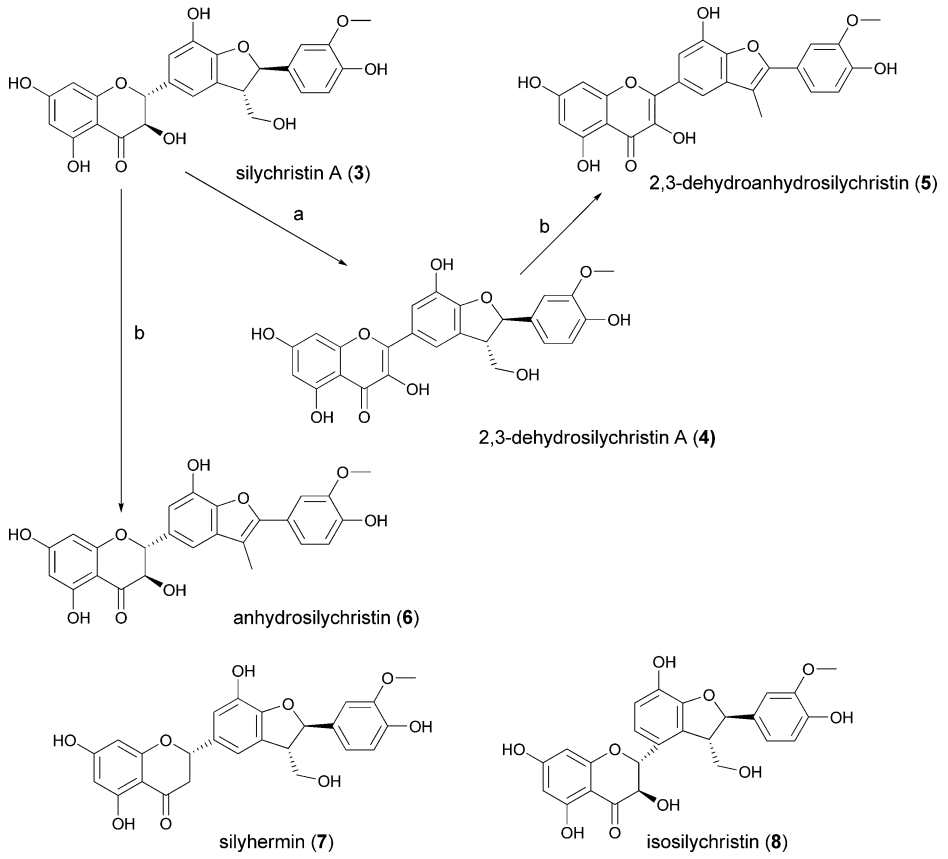
Scheme 1. Silychristin Derivatives and Analogues[a]



[a]Reagents and conditions: (a) O_2, DMSO, triethylamine, 60 °C, overnight; (b) HCl, EtOH, 70 °C, overnight.

Table 1. ECD Spectra of Silychristins (2a, 2b) and Silyhermin (7)[a]

compound	208 nm	216 nm	223 nm	244 nm	261 nm	295 nm	330 nm
silychristin A (**3a**)	21	5.4	11.8	+	+	−9.4	3.1
silychristin B (**3b**)	45 (203 nm)	−14.7	26.3	−8.35	4.6	−28.3	6.3
silyhermin (**7**)	13.5	5.1	9.3	+	+	−7.1	2.6

[a]For full spectra see Figures S11, S21, S62, and S73, Supporting Information.

Table 2. Radical Scavenging and Inhibition of Lipoperoxidation by Silychristin (3) and Its Structural Analogues and Derivatives

	radical scavenging assay				
compound	FCR [GAE][a]	DPPH[b] (IC_{50} [μM])	ABTS [TE][c]	Lpx[d] (IC_{50} [μM])	log*P*[e]
silybin (**1**)	0.92 ± 0.03[f]	553 ± 13.4[f]	0.47 ± 0.04[f]	67.0 ± 3.75[f]	−0.63
2,3-dehydrosilybin (**2**)	1.29 ± 0.07[f,g]	9.32 ± 0.22[f]	0.56 ± 0.02[f,j]	13.0 ± 0.5[f,l]	−0.54
silychristin (**3**)	1.49 ± 0.06	18.97 ± 0.68[i]	1.09 ± 0.06[k]	10.3 ± 0.6	−0.39
2,3-dehydrosilychristin (**4**)	3.06 ± 0.09	4.88 ± 0.38	1.79 ± 0.12	5.45 ± 0.09	−1.01
2,3-dehydroanhydrosilychristin (**5**)	2.13 ± 0.06	3.56 ± 0.18	1.43 ± 0.08	6.43 ± 0.17	−0.24
anhydrosilychristin (**6**)	1.74 ± 0.06[h]	17.94 ± 0.53[i]	0.99 ± 0.06[k]	4.12 ± 0.12	−0.74
silyhermin (**7**)	1.24 ± 0.01[g]	42.99 ± 2.05	0.77 ± 0.06	12.6 ± 0.4[m]	−0.37
isosilychristin (**8**)	1.74 ± 0.04[h]	53.11 ± 2.88	0.65 ± 0.06[j]	36.7 ± 0.9	−0.47

[a]Folin−Ciocalteau reduction (gallic acid equivalents, GAE). Values in the same column marked with the same letter are not significantly different ($p < 0.05$). [b]1,1-Diphenyl-2-picrylhydrazyl scavenging. [c][2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation scavenging (trolox equivalents, TE). [d]Inhibition of *tert*-butyl hydroperoxide-induced peroxidation of rat liver microsomes. [e]Partition coefficient. [f]Published previously.[2]

configuration of silyhermin (**7**) is identical to silychristin A (**3a**) in ring D (10*R*,11*S*) (see also Figure S73, Supporting Information).

Isosilychristin[11] (**8**) was isolated from wild purple flowering *S. marianum* plants growing in Austria by direct extraction of the seeds with acetone. Here, the material contained at least five different compounds, from which only isosilychristin (**8**) could be isolated in pure form by chromatography on ASAHIPAK GS-301 20F in methanol.

All radical scavenging and antioxidant assays were performed as at least three independent experiments in triplicate. The antioxidant potential of silychristin (**3**) and its derivatives and

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analogues was evaluated as their ability to reduce the Folin–Ciocalteau reagent (FCR), to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) cation ($ABTS^{+\bullet}$) radicals, and to inhibit lipid peroxidation induced in rat liver microsomes by *tert*-butyl hydroperoxide.

The FCR assay was originally developed as a total phenol analysis, but it also indicates the overall reducing capacity of the sample.[12] The most active compound in this assay was 2,3-dehydrosilychristin (**4**; 3.06 gallic acid equivalents, GAE) followed by 2,3-dehydroanhydrosilychristin (**5**; 2.13 GAE). Anhydrosilychristin (**6**) and isosilychristin (**8**) displayed similar activity (1.74 GAE), while silychristin (**3**) was slightly less potent (1.49 GAE). Silyhermin (**7**) was the least potent (1.24 GAE) reducing agent in the panel studied, but still better than silybin (**1**) and comparable to 2,3-dehydrosilybin (**2**; Table 2). A similar hierarchy was obtained for DPPH scavenging, expressed as IC_{50} values, which were inversely proportional to the radical-scavenging capacity: compound **5** (IC_{50} 3.6 μM) > **4** > **3** ∼ **6** ≫ **7** > **8** (IC_{50} 53 μM) ≫ **1** (IC_{50} 553 μM), with 2,3-dehydroanhydrosilychristin being the most active scavenger (Table 2). For $ABTS^{+\bullet}$ scavenging, the hierarchy was only slightly different, with the most active compound being 2,3-dehydrosilychristin (**4**; 1.79 trolox equivalents, TE), followed by **5** > **3** ∼ **6** > **7** > **8** (0.65 TE) ∼ **2** > **1** (Table 2). In all assays, the 2,3-dehydro derivatives **4** and **5** proved to be more active than their parent molecules and more active than 2,3-dehydrosilybin (**2**), which, under the same experimental conditions, displayed activity equivalent to 1.3 GAE, IC_{50} 9.3 μM, and 0.56 TE, respectively.[2] The antioxidant activity of anhydrosilychristin (**6**) was similar to silychristin (**2**), while silyhermin (**7**) and isosilychristin (**8**) were considerably less potent. Nevertheless, all silychristin derivatives and analogues were considerably more potent than silybin (**1**), in having 0.9 GAE, DPPH IC_{50} 553 μM, and 0.47 TE.[2]

In the case of lipid peroxidation inhibition, the most active compound was anhydrosilychristin (**6**; IC_{50} 4.1 μM), and then the inhibitory potential decreased as follows: **4** (IC_{50} 5.5 μM) > **5** > **3** > **2** ∼ **7** ≫ **8** (IC_{50} 37 μM) ≫ **1** (IC_{50} 67 μM). The most striking difference compared to other assays is the remarkably efficient inhibition by anhydrosilychristin (**6**), which was greater than both the 2,3-dehydro derivatives. This could be related to an ability to efficiently interact with membrane lipids.[2] Therefore, the lipophilicity of all compounds under study was determined and expressed as log*P*. However, no clear relationship between log*P* and antilipoperoxidant activity could be established.

Overall, the data for silychristin were in accordance with previous results.[2] In contrast, 2,3-dehydrosilychristin used in the present study was considerably more active. Stereochemical attributes play no role in these assays, as demonstrated in previous studies[13] and also due to the fact that all assays are performed in isotropic systems.

Cytotoxic properties of prepared compounds were assessed against a panel of 10 human cell lines of different tissue origin and representing different models of human disease. The panel included breast, prostate, and hepatocellular carcinoma cell lines, leukemia cell lines, and normal cells (Table S74, Supporting Information). Cell viability was assayed by an ATPlite (PerkinElmer, Waltham, MA, USA) chemoluminiscence assay after 48 h of incubation. In this assay, the level of cellular ATP is taken as the measure of cell viability. In contrast to the control substances, represented by campthotecin, silybins A and B (**1a** and **1b**), and 2,3-dehydrosilybin (**2**), all silychristin derivatives showed very little cytotoxicity within the concentration range tested (330 pM to 100 μM). Silychristin (**3**) and isosilychristin (**8**) were not cytotoxic for any of the tested cell lines. As expected, some activity was observed with 2,3-dehydro compounds **4** and **5**, but lower than that displayed by 2,3-dehydrosilybin (**2**). Leukemic cell lines were found to be the most sensitive, which might be connected with their proliferation potential (Table S74, Supporting Information).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a Rudolph Autopol polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA) in MeOH at room temperature. Electronic circular dichroism spectra were recorded in a JASCO-815 (JASCO Analytical Instruments, Easton, MD, USA) spectrometer with the following experimental setup: spectral interval from 200 to 600 nm, scanning speed 5 nm/min, time response 16 s, 1 mm quartz cell, sample concentration 0.3 mmol/L (**2a** and **5**), 0.13 mmol/L (**2b**), 0.82 mmol/L (**4**), 0.15 mmol/L (**6**, **7**), and 0.27 mmol/L (**8**), all dissolved in MeOH. NMR spectra were recorded in DMSO-d_6 (30 °C) using a Bruker AVANCE 600 NMR spectrometer (600 MHz for ^{1}H, 151 MHz for ^{13}C) with the residual solvent peak as the internal standard. Mass spectra were measured using an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source. The mobile phase consisted of CH_3OH–H_2O (4:1), flow rate 30 mL/min, and the samples were injected using a 2 μL loop. The mass spectra of negatively charged ions were internally calibrated using deprotonated palmitic acid as lock mass. Data were acquired and processed using Xcalibur software (Thermo Fisher Scientific).

The preparative HPLC (Shimadzu, Kyoto, Japan) system consisted of a LC-8A high-pressure pump with an SPD-20A dual-wavelength detector (with preparative cell), FRC-10A, and fraction collector. The system was connected to a PC using command module CBM-20A and controlled by a LabSolution 1.24 SP1 software suite. All preparative HPLC separations were performed using an ASAHIPAK GS-310 20F column (Shodex, Munich, Germany), with MeOH as mobile phase, flow rate 5 mL/min, and detection at 254 and 369 nm. All analytical HPLC analyses were performed with the Shimadzu Prominence System (Shimadzu) consisting of a DGU-20A mobile phase degasser, two LC-20AD solvent delivery units, a SIL-20AC cooling autosampler, a CTO-10AS column oven, and an SPD-M20A diode array detector. Chromatographic data were collected and processed using Shimadzu Solution software at a rate of 40 Hz and detector time constant of 0.025 s. The Chromolith Performance RP-18e monolithic column (100 × 3 mm i.d., Merck, Darmstadt, Germany) coupled with a guard column (5 × 4.6 mm, Merck) was used. Mobile phase A [CH_3OH–HCO_2H (100:0.1)] and phase B [CH_3OH–H_2O–HCO_2H (10:90:0.1)] were employed in the analyses; gradient: 0–9 min 10–60% A; 9–12 min 60% A, 12–14 min 60–10% A. The flow rate was 1.2 mL/min at 25 °C. The PDA data were acquired in the 200–450 nm range, and 285 nm signals were extracted.

Plant Material. Silymarin was purchased from Liaoning Senrong Pharmaceutical Co. (Panjin, People's Republic of China, batch no. 120501). Silychristin was isolated from *S. marianum* (chemovariety MIREL, plant material samples deposited at Central Institute for Supervising and Testing in Agriculture, Brno, Czech Republic (http://eagri.cz/, sample number MED 11272). The material for isolation of silyhermin (**7**) was the white flowering variety of *S. marianum* (chemovariety AIDA, plant material samples deposited at Central Institute for Supervising and Testing in Agriculture, Brno, Czech Republic (http://eagri.cz/), sample number MED 22367). The material for isolation of isosilychristin (**8**) was wild purple flowering *S. marianum* collected in Austria (GPS coordinates: 46°44′25.2″ N 15°50′01.6″ E, plant material sample was deposited at Dr. Martin Buchta, Stolařská 601/4, CZ-747 14 Ludgeřovice, Czech Republic).

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Extraction and Isolation. Silybin was isolated from silymarin by suspending silymarin in MeOH and filtration. 2,3-Dehydrosilybin was prepared as described before.[14]

*Silychristin (**3**).* **3** (natural mixture of diastereomers A and B; total content of **3** is more than 96%) was isolated from *S. marianum* using LH-20 column chromatography as published previously[9] as a pale yellow amorphous solid, and it was used for further reactions and separations.

*Silychristin A (**3a**) and Silychristin B (**3b**).* A 1.0 g aliquot of natural silychristin (**3**) was injected onto an HPLC column (25 × 250 mm, silica gel, 10 μm particle size) in 10 injections (3 mL total volume injected in acetone, mobile phase $CHCl_3$–acetone–HCO_2H, 9:1:0.01) to obtain silychristin A (**3a**, 332 mg, purity 95.2%) and silychristin B, which was reinjected twice to obtain pure compound (**3b**, 13 mg, purity >98%).

3a: white, amorphous solid; $[\alpha]_D$ +6.1 (*c* 0.014, MeOH), lit.[7] +112 (*c* 0.3, MeOH, 26 °C); UV (MeOH) λ_{max} 205, 230, 238, 288 nm; ^{1}H, ^{13}C NMR, MS, ECD, and UV data, see Supporting Information (Table S5, Figures S3, S4, S6–S11); ESIMS *m/z* 481 (100%) $[M - H]^-$, 451 (1), 385 (1), 309 (3), 293 (9), 249 (5), 216 (2); (−)-HRESIMS *m/z* 481.1131 (calcd for $C_{25}H_{21}O_{10}$ 479.1140).

3b: white, amorphous solid; $[\alpha]_D$ −10.0 (*c* 0.0063, MeOH), lit.[8] +47 (*c* 0.03, MeOH); UV (MeOH) λ_{max} 204, 231, 239, 288 nm; ^{1}H, ^{13}C NMR, MS, ECD, and UV data see Supporting Information (Table S15, Figures S13, S14, S16–S21); ESIMS *m/z* 481 (100%) $[M - H]^-$, 451 (2), 375 (4), 337 (1), 309 (14), 293 (49); (−)-HRESIMS *m/z* 481.1131 (calcd for $C_{25}H_{21}O_{10}$ 479.1140).

*Silyhermin (**7**).* A crude extract containing **7** was obtained by extraction of the crushed fruits of *S. marianum* with acetone–H_2O (95:5). The crude extract was concentrated and defatted by extraction with hexane, and the solvents were evaporated. A red-brown residue containing about 15% (HPLC) of **7** was separated on preparative HPLC (see General Experimental Procedures). Then 908 mg of the mixture was injected in three portions (1 mL total volume), with **7** eluting at 31 min. The recovery was 83%. The combined fractions were evaporated and lyophilized from *t*-BuOH to obtain **7** (90 mg, purity >95%). The structure of **7** was inferred from its spectroscopic data, which were in accordance with previously characterized 7-pentaacetate.[10] White lyophilizate; $[\alpha]_D$ +23.5 (*c* 0.0068, MeOH); UV (MeOH) λ_{max} 203, 230, 287 nm; ^{1}H, ^{13}C NMR, MS, ECD, and UV data, see Supporting Information (Table S56, Figures S54, S55, S57–S62); ESIMS *m/z* 465.1 $[M - H]^-$ (100), 435.1 (15), 293.2 (13); (−)-HRESIMS *m/z* 465.1183 (calcd for $C_{25}H_{21}O_9$ 465.1191).

*Isosilychristin (**8**).* A crude extract containing **8** was obtained by extraction of the crushed fruits of *S. marianum* with acetone–H_2O (95:5). The crude extract was concentrated and defatted with hexane, and solvents were evaporated. A red-brown residue containing about 50% (HPLC) of **8** was separated by preparative HPLC. Then, 835 mg of the mixture was injected in four portions, with **8** eluting at 21 min. Recovery was 70%. The combined fractions were evaporated and lyophilized from *t*-BuOH to obtain **8** (213 mg, purity over 95%), which was identified by comparison with literature data.[11] White lyophilizate; $[\alpha]_D$ +212.0 (*c* 0.012, MeOH), lit.[11] +245 (*c* 0.1, pyridine); UV (MeOH) λ_{max} 203, 230, 288 nm; ^{1}H, ^{13}C NMR, MS, ECD, and UV data, see Supporting Information (Table S66, Figures S64, S65, S67–S72); ESIMS *m/z* 481.1 $[M - H]^-$ (100); (−)-HRESIMS *m/z* 483.1284 (calcd for $C_{25}H_{23}O_{10}$ 483.1286).

Synthetic Procedures. *2,3-Dehydrosilychristin (**4**).* A new optimized procedure was used. (For optimization conditions, see Supporting Information Table S23.) To a solution of silychristin (**3**, 100 mg, 0.21 mmol in 5 mL of DMSO) was added 40 μL of triethylamine, and the mixture was stirred at 60 °C under an O_2 atmosphere overnight. Then, the mixture was diluted with water and extracted with ethyl acetate. The organic phase was dried over Na_2SO_4 and concentrated in vacuo. The residue was separated by preparative HPLC (see General Experimental Procedures) and lyophilized (*t*-BuOH) to obtain the desired compound (27 mg, 27%). Yellow lyophilizate; $[\alpha]_D$ +72.1 (*c* 0.039, MeOH), lit.[6] +71.2 (solvent and *c* not given); UV (MeOH) λ_{max} 230, 256, 373 nm; ^{1}H, ^{13}C NMR, MS, ECD, and UV data, see Supporting Information (Table S26, Figures S24, S25, S26–S32); ESIMS *m/z* 479.1 (100%) $[M - H]^-$, 375.2 (7), 309.2 (15), 301.0 (39), 293.2 (84); (−)-HRESIMS *m/z* 497.0976 (calcd for $C_{25}H_{19}O_{10}$ 479.0984).

*2,3-Dehydroanhydrosilychristin (**5**).* To a solution of 2,3-dehydrosilychristin (**4**, 200 mg, 0.42 mmol) in EtOH–H_2O (15 mL, 9:1) was added concentrated HCl (5 mL), and the mixture was stirred at 80 °C for 1 h in a sealed glass vessel. The mixture was cooled and filtered through a pad of silica gel, which was then washed with EtOH, and the combined filtrates were evaporated to yield compound **5** (80 mg, 41%) as a brown, amorphous solid, which was then lyophilized from *t*-BuOH. Reddish-brown lyophilizate; UV (MeOH) λ_{max} 267, 303, 367, 425 nm; ^{1}H, ^{13}C NMR, MS, and UV data, see Supporting Information (Table S36, Figures S34, S35, S37–S42); ESIMS *m/z* 461.1 $[M - H]^-$ (100%), 301.0 (7); (−)-HRESIMS *m/z* 461.0868 (calcd for $C_{25}H_{17}O_9$ 461.0878).

*Anhydrosilychristin (**6**).* An improved procedure[5] was used. To a solution of silychristin (**3**, 100 mg, 0.21 mmol) in EtOH (5 mL, 90%) was added concentrated HCl (2.5 mL), and the mixture was stirred at 70 °C overnight. The mixture was then evaporated, re-evaporated twice with water, and purified by preparative HPLC to obtain **6** (23 mg, 24%), which was lyophilized from *t*-BuOH, yielding a pale yellow lyophilizate: $[\alpha]_D$ −4.3 (*c* 0.0067, MeOH); UV (MeOH) λ_{max} 204, 290, 317 nm; ^{1}H, ^{13}C NMR, MS, ECD, and UV data, see Supporting Information (Table S46, Figures S44, S45, S47–S52); ESIMS *m/z* 463.1 $[M - H]^-$ (88), 411.2 (2), 375.2 (9), 337.1 (3), 309.2 (19), 293.2 (100); (−)-HRESIMS *m/z* 463.1025 (calcd for $C_{25}H_{19}O_9$ 463.1035).

Antiradical and Antioxidant Assays. The compounds were dissolved in DMSO; stock solutions (100 mM) were stored at −20 °C and before each assay further diluted with the respective solvent. DMSO content in the reaction mixtures was always kept below 0.1%.

Folin–Ciocalteau Reduction Assay. Folin–Ciocalteau reduction capacity was determined as described previously,[15] with some modifications. Briefly, 5 μL of samples (1 mM) or standard (gallic acid, 0.25–4 mM) in phosphate-buffered saline (PBS, pH 7.4) was mixed with 100 μL of Folin–Ciocalteau reagent (Merck) diluted 10-fold with distilled water. After 5 min at room temperature, 100 μL of Na_2CO_3 solution (75 g/L) was added, and the reaction mixture was further incubated for 90 min at room temperature. The absorbance was measured at 725 nm using a Tecan Sunrise plate reader (Tecan Group Ltd., Männedorf, Switzerland), and the reducing capacity was expressed as gallic acid equivalents.

DPPH Assay. The antiradical activity was evaluated using the DPPH (Sigma-Aldrich) radical as previously described[16,17] with minor modifications. A 15 μL aliquot of each test substance (final concentration 0–150 μM in MeOH) was mixed with 285 μL of methanolic DPPH solution (final concentration 20 μM). After 30 min, the absorbance was read at 517 nm. The percentage inhibition was calculated using the negative control, and IC_{50} values were obtained from the inhibition curves.

$ABTS^{+\bullet}$ Scavenging. The capacity to scavenge $ABTS^{+\bullet}$ was evaluated using an antioxidant assay kit (CS0790, Sigma-Aldrich), according to the manufacturer's instructions. Briefly, 10 μL of the sample (final concentration 100 μM), blank, or standard in an assay buffer (pH 7.48) was mixed with 20 μL of myoglobin (3.5 mg/L of the assay buffer), and 150 μL of a working solution containing 0.2 mM ABTS and 2.45 mM H_2O_2 in a phosphate-citrate buffer, pH 5, was added. The reaction mixture (pH 5.09) was incubated at room temperature for ≥5 min, and then 100 μL of a stop solution was added. Absorbance was measured at 405 nm, and the scavenging activity was expressed as trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalents (TE) from the trolox calibration curve.

Measurement of log*P*. The hydrophobicity of the compounds was determined by the measurement of partition coefficient *P* in a mixture of two immiscible phases, octan-1-ol and 6.6 mM phosphate buffer (pH 7.4), to simulate physiological conditions. Before use, octan-1-ol was stirred with the buffer for 16 h at 25 °C to achieve saturation of both phases, which were then separated. Stock solutions (0.5 mM) of the compounds tested were prepared in octan-1-ol; 300

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μL of the stock solutions was mixed with 300 μL of the phosphate buffer in microcentrifuge tubes (1.5 mL) and stirred (750 rpm) for 2 h at 25 °C in triplicate. Phases were separated, and the solute concentration in each phase was determined in a micro UV cuvette (Brand, Wertheim, Germany) using a UV−vis spectrometer (UV-mini-1240, Shimadzu, Prague, Czech Republic) at 285 nm. Log*P* was calculated as follows: $\log P_{oct/buffer} = \log([A]_{octan\text{-}1\text{-}ol}/[A]_{buffer})$.

Inhibition of Microsomal Lipid Peroxidation. Pooled microsomes from male rat livers (M9066, Sigma-Aldrich) were washed 5× using centrifugation (13 500 rpm, 5 min, 4 °C) and PBS to remove sucrose and diluted to 0.625 mg protein/mL with PBS before use. Then, 400 μL of the diluted microsomal suspension was mixed with 50 μL of each test compound (final concentration, 0−40 μM in PBS) and *tert*-butyl hydroperoxide (50 μL in PBS; final concentration 1 mM), and the mixture was incubated at 37 °C for 60 min. The products of lipid peroxidation were determined as thiobarbituric acid reactive substances (TBARS) after addition of 700 μL of trichloroacetic acid (26 mM) with thiobarbituric acid (918 mM), with the mixture heated (90 °C; 15 min), cooled, centrifuged (5 min; 13 500 rpm; 4 °C), and the absorbance of the supernatant measured at 535 nm. The activity was calculated as the concentration of the tested compound that inhibited the color reaction with thiobarbiturate (without the tested compound) by 50% (IC_{50}).

Cell Lines. Cellular evaluation was carried out with a panel of cell lines of diverse tissue origin: RPE-1, human normal immortalized cells from pigmented epithelium in retina (ATCC CRL-4000); BJ, human primary fibroblasts (ATCC CRL-2522); hPPMC, human primary prostate mesenchymal cells derived from biopsies of patients diagnosed with benign prostate hyperplasia; Hep G2, human hepatocellular carcinoma; K562, human chronic myelogenous leukemia (ATCC CCL-243); HL-60, human acute myeloid leukemia (ATCC CCL); MDA-MB-231, human breast adenocarcinoma (MDA-MB-231); PC-3, human prostate adenocarcinoma (ATCC CRL-1435); MCF7, human breast adenocarcinoma (HTB-22); Cal-51, human breast adenocarcinoma.

Cell Culture. HepG2 cells were provided by Promega (Madison, WI, USA) within an internal collaboration, and Cal51 cells were a kind gift from Dr. Lumír Krejčí (Masaryk University, Brno, Czech Republic). All other cell lines were purchased from the American Type Culture Collection (ATCC) in the last several years. Cells were propagated in a monolayer in the cell culture medium supplemented with 10% fetal bovine serum, 2 mM glutaMAX (Thermo Fisher Scientific), 1 mM nonessential amino acids, and penicillin/streptomycin (Thermo Fisher Scientific) and incubated in a 5% CO_2-humidified atmosphere at 37 °C. DMEM was used as a culture medium for RPE-1, Hep G2, BJ, and MDA-MB-231 cells, F-12K for PC3, and IMDM for K562 and HL-60. BJ cells were propagated in the medium supplemented with 10 ng/mL of bFGF.

Cell Viability Assay. Cells were propagated in the cell growth medium to the amount needed for the experiment. Cells were harvested, counted, diluted in the growth medium to the final concentration of 0.2×10^6/mL, and dispensed with the liquid dispenser Multidrop (Thermo Fisher Scientific), to the cell culture treated, in white solid 1536-well plates (Corning Inc., Corning, NY, USA) at 1000 cells/well in 5 μL of total medium volume. Compounds to be tested were diluted in DMSO and transferred to the cells using an Echo 520 acoustic dispenser (Labcyte, Sunnyvale, CA, USA) integrated in the fully automated robotic HTS station Cell::Explorer (PerkinElmer). Compounds were tested at 10 different concentration points in the wide concentration range from 10 nM to 100 μM, in triplicate. Cell viability was determined after 48 h of incubation with test compounds using ATPlite (PerkinElmer) reagent according to the manufacturer's protocol. Luciferase signal was measured on an Envision multimode plate reader (PerkinElmer). Data were collected and processed with an in-house-developed LIMS system ScreenX. The data were normalized, and IC_{50} values were calculated using a nonlinear regression function (dose−response, variable slope).

Statistical Analysis. Data on the free radical scavenging capacity and inhibition of lipoperoxidation were analyzed with one-way ANOVA, and post hoc comparisons among pairs of means were performed using the Scheffe test and least-squares difference test, all using the statistical package Statext ver. 2.1 (STATEXT LLC, Wayne, NJ, USA). Differences were considered statistically significant when $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00750.

^{1}H and ^{13}C NMR data, MS, ECD, and UV spectra of all new compounds, and HPLC chromatograms of all new final products, synthesis of **4**, its optimization, and cell profiling with human cell lines (PDF)

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Notes

The authors declare no competing financial interest.

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