Molecular Structure and Stereochemistry of Silybin A, Silybin B, Isosilybin A, and Isosilybin B, Isolated from *Silybum marianum* (Milk Thistle)

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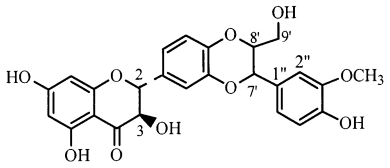
Two pairs of diastereoisomeric flavonolignans, silybin A, silybin B, isosilybin A, and isosilybin B, were successfully separated from *Silybum marianum* by sequential silica gel column chromatography, preparative reversed-phase HPLC, and recrystallization. Complete stereochemical assignments at C-2, C-3, C-7′, and C-8′ of these flavonolignans have been achieved. On the basis of X-ray crystallographic analysis and optical rotation data, coupled with comprehensive ^{1}H and ^{13}C NMR spectral data interpretation including COSY, HMQC, and HMBC, the stereochemistry of these diastereoisomers was determined unambiguously as silybin A (**4**), 2*R*, 3*R*, 7′*R*, 8′*R*; silybin B (**5**), 2*R*, 3*R*, 7′*S*, 8′*S*; isosilybin A (**6**), 2*R*, 3*R*, 7′*R*, 8′*R*; and isosilybin B (**7**), 2*R*, 3*R*, 7′*S*, 8′*S*.

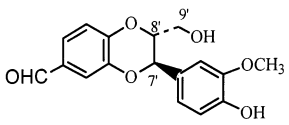
Silymarin is a mixture of flavonolignans extracted from the seeds of milk thistle, *Silybum marianum* (L.) Gaertn. (Asteraceae) and is used traditionally as a heptoprotective agent. Previous studies have revealed that silymarin is composed of silybin, isosilybin, silychristin, silydianin, and other phenolic compounds.[1] The common feature of these structural isomers is a flavonolignan skeleton (C_{25} $H_{22}O_{10}$, mol wt 482). Silymarin has been marketed as dietary supplement in the United States under a variety of brand names. In general, these crude extracts contain about 80 wt % of flavonolignans.

The structure of silybin (**1**) was first established in 1975 by Pelter and Hänsel using a degradative method.[2] The synthesis of dehydrosilybin pentamethyl ether was also achieved by these authors.[3] The structure was further confirmed by biomimetic synthesis by Merlini et al.[4,5] However, the synthesized product was a mixture of regioisomers, silybin and isosilybin (57:43, HPLC). The regioisomers were separated by initial crystallization from methanol−water (9:1) followed by a secondary crystallization from EtOAc. Arnone et al.[6] from the same research group reported that silybin is a diasteroisomeric mixture in a ca. 1:1 ratio, based on the observation that the signals of H-7′ and methoxy group are split into two sets of peaks with equal intensity ($\Delta\nu$ 1−2 Hz) (100 MHz) when the spectrum is measured in benzene-d_6 containing a minimum amount of C_5D_5N to ensure solubility. To avoid the formation of regioisomers, Tanaka et al.[7] synthesized a key intermediate (**2**), which then through a coupling reaction with 2,4,6-trimethoxymethoxyacetophenone formed a chalcone intermediate. Subsequent epoxidation and deprotection, followed by acidic cyclization, provided a regioselective synthesis of diastereoisomeric silybin in 63% overall yield.

Isosilybin (**3**) was isolated by preparative TLC (silica gel, $CHCl_3$−EtOAc−Me_2CO−HCOOH, 8:1:1:0.1), and its structure was first reported by Arnone et al. in 1979.[6] The same split of the ^{1}H NMR signal of H-7′ into a doublet in the solvent toluene-d_8−pyridine-d_5 and the zero optical rotation of 2,3-dehydroisosilybin clearly revealed that isosilybin was a diastereoisomeric mixture.[6] All of ^{1}H NMR data showed that both silybin and isosilybin have the same *trans* conformation of C-2, C-3 and C-7′, C-8′. Tittle et al.[8] first reported the HPLC analysis of silymarin, and the two peaks around t_R 20−25 min were assigned as silybin, but another two peaks between t_R 30−35 min were poorly resolved and reported as dehydrosilybin and marked as x/y,[9] which has now been proved to be isosilybin. A micellar electrokinetic capillary chromatographic (MECC) method recently developed by Ding et al.[10] confirmed the diastereoisomeric nature of silybin and isosilybin. Synthetic silybin[7] and isosilybin[5] were also diastereoisomers. All of these reports confirmed that both silybin and isosilybin are mixtures of diastereoisomers and are difficult to separate. Lotter and Wagner[11] reported the X-ray crystallographic study of silybin and attempted to solve the problems of stereochemistry. Unfortunately, the X-ray study was performed on crystals that were mixture of diastereoisomeric silybin. Because there was no single diastereoisomer obtained previously, all of the spectral data published were derived from diastereoisomeric mixtures. Delfini et al.[12]



Silybin (**1**)



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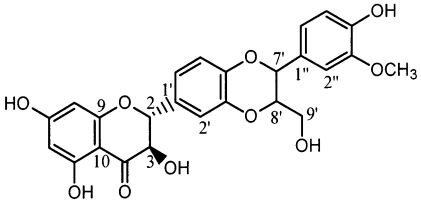

Isosilybin (**3**)

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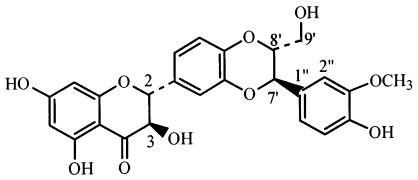
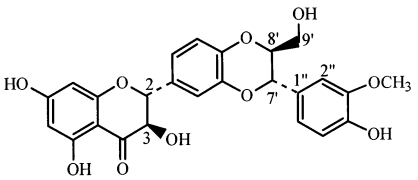
investigated the ^{1}H NMR characteristics of diastereoisomeric silybin, but their assignments of H-2 and H-3 appear to be opposite according to our ^{1}H−^{1}H COSY experiment. Křen et al.[13] synthesized the peracylated monoglycosides of silybin A and silybin B and then through hydrolysis and chromatographic separation obtained small amounts of two diastereoisomers of silybin. However, the ^{1}H and ^{13}C NMR spectral and $[\alpha]_D$ data failed to provide unambiguous information on the stereochemistry. Therefore, the separation and identification of each isomer with correct stereochemistry have been a challenge, particularly in regard to their individual biological activities.

The absolute stereochemistry of 2*R*, 3*R* of silybin was established[2,6] previously by comparison of the CD spectrum of silybin with those of natural 2*R*,3*R*-flavanonols. Therefore, the main focus of the present investigation was to clarify the absolute stereochemistry at C-7′, C-8′ for each diastereoisomer of silybin and isosilybin.

It is well established that isomeric compounds with opposite stereochemistry often produce different biological activities. During our systematic study of the structure−activity relationship of silybin, isosilybin, and other analogues in milk thistle preparations, we have now successfully isolated four diastereoisomers and assigned them as silybin A (**4**), silybin B (**5**), isosilybin A (**6**), and isosilybin B (**7**). Complete assignments of the ^{1}H and ^{13}C NMR signals were obtained from analysis of their ^{1}H−^{1}H COSY, HMQC, and HMBC spectra. The stereochemistry of each isomer was established by an X-ray crystallographic study of isosilybin A in combination with optical rotation measurements of each isomer.



Silybin A (**4**)



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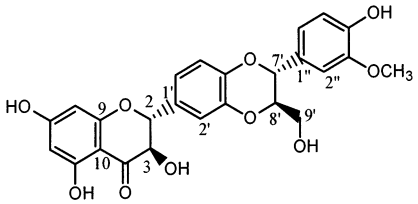
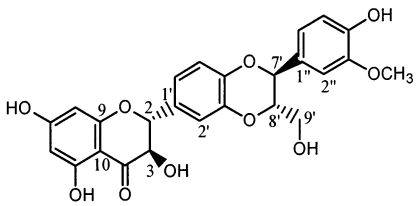


Isosilybin A (**6**)



Isosilybin B (**7**)

Table 1. ^{1}H NMR Data of Silybin A (**4**) and Silybin B (**5**) (300 MHz, Me_2CO-d_6, δ)

position	**4**	**5**
2	5.081 (d, 11.4 Hz)	5.075 (d, 11.4 Hz)
3	4.647 (d, 11.4 Hz)	4.637 (d, 11.7 Hz)
6	5.947 (d, 1.8 Hz)	5.955 (d, 1.8 Hz)
8	5.980 (d, 1.8 Hz)	5.982 (d, 2.4 Hz)
2′	7.150 (d, 2.1 Hz)	7.143 (d, 2.1 Hz)
5′	6.951 (d, 8.1 Hz)	6.956 (d, 8.1 Hz)
6′	7.078 (dd, 8.4, 2.1 Hz)	7.087 (dd, 8.1, 2.1 Hz)
7′	4.993 (d, 8.1 Hz)	4.998 (d, 8.7 Hz)
8′	4.153 (ddd, 6.9, 3.9, 2.7 Hz)	4.148 (ddd, 6.9, 4.5, 2.7 Hz)
9′	3.747 (dd, 12.3, 2.4 Hz)	3.751 (dd, 12.3, 2.7 Hz)
9′	3.515 (dd, 12.3, 3.9 Hz)	3.519 (dd, 12.3, 4.2 Hz)
2″	7.135 (d, 1.5 Hz)	7.143 (d, 2.1 Hz)
5″	6.874 (d, 8.1 Hz)	6.878 (d, 8.1 Hz)
6″	6.976 (dd, 8.4, 1.8 Hz)	6.980 (dd, 8.4, 2.1 Hz)
OMe	3.865 (s)	3.871 (s)

Results and Discussion

The mixture of silybin, isosilybin, silydianin, and several other analogues, which showed almost one single spot on TLC, was successfully separated by silica gel chromatography. The diastereoisomeric silybin can be readily separated from the mixture by recrystallization from CH_2Cl_2−MeOH. A reversed-phase HPLC chromatogram showed a pattern similar to that reported by Tittle et al.[8] The peak at t_R 3.78 min was separated and characterized as silychristin. The peaks corresponding to the diastereoisomeric silybin A (**4**) (t_R 8.03), silybin B (**5**) (t_R 9.01), isosilybin A (**6**) (t_R 11.98), and isosilybin B (**7**) (t_R 13.12) were successfully separated by reversed-phase preparative HPLC.

Silybin A (**4**) was collected by preparative HPLC and obtained as an off-white amorphous powder after evaporating the solvent. Yellowish flat crystals were obtained after recrystallization from aqueous methanol. The ^{1}H NMR spectrum (Table 1) of **4** displayed the typical characteristics of a 5,7-dihydroxy-substituted flavanonol by the signals at δ 5.947 (1H, d, J = 1.8 Hz, H-6), 5.980 (1H, d, J = 1.8 Hz, H-8), 5.081 (1H, d, J = 11.4 Hz, H-2), and 4.647 (1H, d, J = 11.4 Hz, H-3). Six protons in the aromatic region could be attributed to two 1,3,4-trisubstituted aromatic rings, one belonging to the B-ring of a flavanonol unit and another belonging to a cinnamic alcohol group. Four protons at δ 4.993 (1H, d, J = 8.1 Hz, H-7′), 4.153 (1H, ddd, J = 6.9, 3.9, 2.7 Hz, H-8′), 3.747 (1H, dd, J = 12.3, 2.4 Hz, H-9′), and 3.515 (1H, dd, J = 12.3, 3.9 Hz, H-9′) could be assigned to a propanol moiety connected to a dioxane ring.

The ^{1}H−^{1}H COSY spectrum of **4** fully supported the correlations of H-2, H-3, H-7′, H-8′, and H-9′. In turn, *trans* conformations of H-2, H-3 and H-7′, H-8′ were determined by the observed coupling constants (11.4 and 8.1 Hz). The ^{13}C NMR (Table 3) and DEPT spectra showed 25 carbon signals, characterized as one CH_3, one CH_2, 12 CH, and 11 C (Table 1). The stereochemistry at 2*R*, 3*R* was previously reported by Arnone et al.[6] by dehydrogenation of silybin. The conformations of C-7′ and C-8′ were deduced as 7′*R* and 8′*R* by direct comparison of the optical rotation data with that of isosilybin A (**6**), which was determined herein by X-ray crystallography (vide infra).

Silybin B (**5**) was isolated from the isomeric mixture (A/B: 47/53) as an off-white amorphous powder following preparative HPLC separation. Yellow crystals were obtained after recrystallization in MeOH−H_2O. The ^{1}H NMR spectrum (Table 1) of **5** was very similar to that of silybin A (**4**), as evidenced by less than 0.01 ppm differences of the ^{1}H NMR chemical shifts between these two isomers. This result explains why the ^{1}H NMR spectrum of a mixture of silybins A and B looks like that of a single

Table 2. ^{1}H NMR Data of Isosilybin A (**6**) and Isosilybin B (**7**) (300 MHz, Me_2CO-d_6, δ)

position	**6**	**7**
2	5.075 (d, 11.4 Hz)	5.120 (d, 11.4 Hz)
3	4.604 (d, 11.4 Hz)	4.662 (d, 11.4 Hz)
6	5.941 (d, 2.1 Hz)	5.978 (d, 2.1 Hz)
8	5.971 (d, 2.1 Hz)	6.008 (d, 2.1 Hz)
2′	7.145 (d, 1.8 Hz)	7.158 (d, 1.8 Hz)
5′	6.936 (d, 8.7 Hz)	6.949 (d, 8.4 Hz)
6′	7.066 (dd, 8.7, 1.8 Hz)	7.076 (dd, 8.1, 2.1 Hz)
7′	5.007 (d, 7.8 Hz)	5.017 (d, 8.4 Hz)
8′	4.101 (ddd, 6.0, 4.2, 2.4 Hz)	4.148 (ddd, 6.6, 3.9, 2.4 Hz)
9′	3.740 (dd, 12.3, 2.4 Hz)	3.757 (dd, 12.3, 2.4 Hz)
9′	3.500 (dd, 12.3, 4.2 Hz)	3.512 (dd, 12.6, 3.9 Hz)
2″	7.116 (d, 1.8 Hz)	7.135 (d, 1.8 Hz)
5″	6.868 (d, 8.1 Hz)	6.890 (d, 8.4 Hz)
6″	6.969 (dd, 8.4, 1.8 Hz)	6.985 (dd, 8.1, 1.8 Hz)
OMe	3.879 (s)	3.878 (s)

Table 3. ^{13}C NMR Data of Compounds **4**−**7** (75 MHz, Me_2CO-d_6, δ)

position	4	**5**	**6**	**7**
2	83.372	83.311	83.448	83.402
3	72.321	72.366	72.504	72.504
4	197.120	196.977	197.410	195.094
5	164.058	164.012	164.332	164.363
6	95.537	95.644	95.446	95.400
7	168.026	168.393	167.187	167.217
8	96.514	96.621	96.499	96.484
9	163.294	163.218	163.401	163.401
10	100.498	100.391	100.880	100.864
1′	130.507	130.553	130.645	130.690
2′	116.739	116.846	116.846	116.831
3′	144.001	143.955	144.764	144.733
4′	144.398	144.321	143.680	143.726
5′	116.709	116.739	116.678	116.602
6′	121.379	121.272	121.089	121.089
7′	76.518	76.534	76.595	76.564
8′	78.884	78.854	78.854	78.899
9′	61.010	60.949	61.208	61.193
1″	128.447	128.386	128.508	128.523
2″	111.412	111.412	111.290	111.290
3″	147.939	148.000	147.847	147.847
4″	147.373	147.374	147.374	147.374
5″	115.167	115.228	115.136	115.091
6″	120.891	120.860	120.921	120.906
OMe	55.713	55.729	55.698	55.698

compound. The ^{13}C NMR spectra (Table 3) of **4** and **5** were also very similar. We could not find any differences in the chemical shifts greater than 0.06 ppm for C-7′, C-8′, and C-9′. The signals of C-7′, C-8′, and C-9′ were easily assigned from the ^{1}H−^{1}H COSY and HMQC spectra. The optical rotation of **5** ($[\alpha]_D$ −1.07°) showed a significant difference from that of **4** ($[\alpha]_D$ + 20°), indicating that the configurations at C-7′ and C-8′ are opposite. Therefore, we assigned 7′*S*, 8′*S* configurations to silybin B (**5**). It is interesting to note that silybin A and silybin B have almost identical ^{1}H NMR and ^{13}C NMR spectra, but reserved-phase HPLC showed a significant difference from each other in terms of retention times (see Experimental Section).

Isosilybin A (**6**), which occurred in a ratio of about 77:23 to isosilybin B (**7**), was obtained as colorless needle crystals by the same preparative HPLC procedure used above. The HRESIMS established the identity of the molecular formula of **6** as $C_{25}H_{22}O_{10}$. The ^{1}H NMR (Table 2) and ^{13}C NMR (Table 3) spectra of **6** were also almost identical to those of silybins A (**4**) and B (**5**), especially the *trans* conformation of C-2, C-3 and C-7′, C-8′. The assignments of all the protons and carbons were based on the analysis of the ^{1}H−^{1}H COSY and HMQC spectra. The HMBC spectrum of **6** gave clear connectivities between H-2′, H-6′, and C-4′ and between C-4′, C-2″, C-6″, and H-7′. Correlations were also observed between H-5′, H-2′, and C-3′ and between C-3′, C-1″, and H-8′. Apparently, C-7′ of the cinnamic alcohol was connected with C-4′ of the flavanonol unit through oxygen, whereas silybin A and silybin B were connected as C-7′−O−C-3′. The key HMBC correlations of isosilybin A (**4**) are shown in Figure 1. It was reported that silybin (**1**) and isosilybin (**3**) both have the same 2*R*, 3*R* stereochemistry.[4,5,7] Therefore, the stereochemistry of **6** with the configurations of 2*R*, 3*R*, 7′*R*, 8′*R* was successfully assigned by a single-crystal X-ray crystallographic study (Figure 2) in combination with optical rotation data.

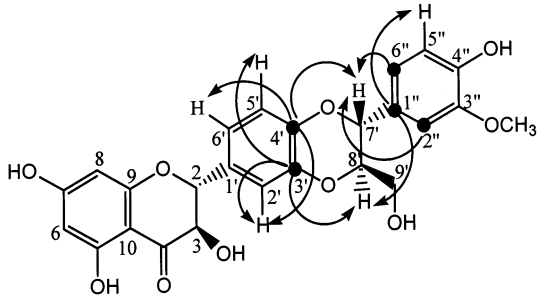


Figure 1. Key HMBC correlations of isosilybin A (**6**).

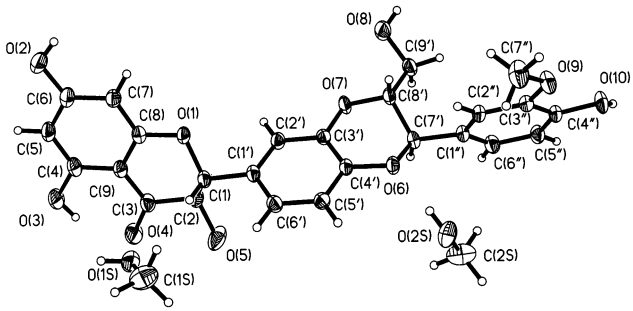


Figure 2. X-ray structure of isosilybin A (**6**).

Isosilybin B (**7**), which had 1.8% of the detectable peaks under the current HPLC conditions, was obtained as colorless hairy crystals by preparative HPLC. The ^{1}H NMR, ^{13}C NMR, and HRESIMS data were almost the same as those of isosilybin A (**6**). A key difference between **7** and **6** was also observed from the optical rotation measurements: for compound **7** the $[\alpha]_D$ was −23.55°, whereas for compound **6** the $[\alpha]_D$ was +48.15°. This significant difference resulted from the different stereochemistry at C-7′ and C-8′ and was identical in terms of the stereochemistry of silybins A and B. Thus, isosilybin B (**7**) was established as 2*R*, 3*R*, 7′*S*, 8′*S*, and it has the same stereochemistry as silybin B (**5**).

In conclusion, silybin A (**4**), silybin B (**5**), isosilybin A (**6**), and isosilybin B (**7**) are two pairs of diastereoisomers with different connectivities at C-7′−O−C-3′ and C-7′−O−C-4′, respectively. These diastereoisomers have very similar ^{1}H and ^{13}C NMR spectra and have no characteristic signals for facile identification of individual isomers. Reversed-phase HPLC can be applied effectively to identify and purify each of these diastereoisomers.

Experimental Section

General Experimental Procedures. Melting points were measured on a Fisher Scientific melting point apparatus and are uncorrected. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. NMR spectra were recorded on a Varian VXR 300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts (δ, ppm) are relative to internal TMS. COSY, HMQC, and HMBC (J = 10, 6.25,

1.25 Hz) were measured with standard pulse programs. ESITOFMSs were measured on a Micromass Platform II mass spectrometer. Analytical HPLC was carried out with a Waters 1525 binary HPLC pump and Waters 2487 dual wavelength detector using an ODS-A column (4.6 mm i.d. × 150 mm) (Waters) with a solvent system consisting of methanol−water (1:1) and a flow rate of 1.0 mL/min at room temperature. The detection was carried out at 254 nm. Preparative HPLC was conducted with a Varian ProStar instrument using an ODS-A column (20 mm i.d. × 400 mm) (Waters), with the same solvent system and detection wavelength as for analytical HPLC and a flow rate of 5.0 mL/min. Silica gel 60 (EM Science) was used for column chromatography eluted by increasing amounts of methanol in dichloromethane. TLC was performed on precoated silica gel 60 F_{254} plates (EM Science).

Plant Material. The seeds of *Silybum marianum* were collected in Panjin, Liaoning Province, People's Republic of China, in September 2000, and identified by one of the authors (D.Y.L.). A voucher of the seeds (NPI-2000-18) is on file and kept in our laboratory. Silymarin, the extract of the seeds of *S. marianum* containing 80% of total flavonolignans, was provided by Natural Pharmacia, International, Inc., Belmont, MA.

Extraction and Isolation. The dried yellow powder of silymarin (80 g) was dissolved in 200 mL of methanol and mixed with silica gel. After removing the solvent under a vacuum, the silymarin absorbed on silica gel was subjected to a silica gel column chromatography using CH_2Cl_2−MeOH as the gradient eluting solvent system (5% → 100% MeOH in CH_2Cl_2). Fractions were collected by the volume (20 mL per fraction) and detected by TLC. Fractions 59−84 showed one spot by TLC, but contained all four diastereoisomers of silybin and isosilybin. Each isomer of silybin and isosilybin was purified from fractions 59−84 by preparative HPLC, using the conditions described above. Silybin A (**4**), silybin B (**5**), isosilybin A (**6**), and isosilybin B (**7**) were separated in the order of t_R value, 8.03, 9.01, 11.98, and 13.12 min, respectively.

Silybin A (4): yellowish flat crystals (MeOH−H_2O); mp 162−163 °C; $[\alpha]_D$ +20° (*c* 0.21, acetone); ^1H and ^{13}C NMR (DMSO-d_6, 300 and 75 MHz) data, see Tables 1 and 3; ESITOFMS *m*/*z* 483 $[M + H]^+$; HRESITOFMS *m*/*z* 483.1298 {calcd for $[M (C_{25}H_{22}O_{10}) + H]^+$, 483.1291}.

Silybin B (5): yellow grain crystals (MeOH−H_2O); mp 158−160 °C; $[\alpha]_D$ −1.07° (*c* 0.28, acetone); ^1H and ^{13}C NMR (DMSO-d_6, 300 and 75 MHz) data, see Tables 1 and 3; ESITOFMS *m*/*z* 483 $[M + H]^+$; HRESITOFMS *m*/*z* 483.1301 {calcd for $[M (C_{25}H_{22}O_{10}) + H]^+$, 483.1291}.

Isosilybin A (6): colorless needle crystals (MeOH−H_2O); mp 201−203 °C; $[\alpha]_D$ +48.15° (*c* 0.27, acetone); ^1H and ^{13}C NMR (DMSO-d_6, 300 and 75 MHz) data, see Tables 2 and 3; ESITOFMS *m*/*z* 483 $[M + H]^+$; HRESITOFMS *m*/*z* 483.1288 {calcd for $[M (C_{25}H_{22}O_{10}) + H]^+$, 483.1291}.

Isosilybin B (7): colorless needle crystals (MeOH−H_2O); mp 236−238 °C; $[\alpha]_D$ −23.55° (*c* 0.31, acetone); ^1H and ^{13}C NMR (DMSO-d_6, 300 and 75 MHz) data, see Tables 2 and 3; ESITOFMS *m*/*z* 483 $[M+H]^+$; HRESITOFMS *m*/*z* 483.1303 {calcd for $[M (C_{25}H_{22}O_{10}) + H]^+$, 483.1291}.

X-ray Diffraction Structure Determination for Isosilybin A (6).[14] Crystal data: $C_{25}H_{22}O_{10}$ $(CH_3OH)_2$; crystal size (mm) 0.12 × 0.08 × 0.06, colorless needle; crystal system monoclinic; space group $P2_1$; unit cell dimensions a = 8.0652(12) Å, b = 8.2841(11) Å, c = 18.893(3) Å; volume 1252.6(3) Å^3; Z = 2; fw 546.49; density (calcd) 1.444 mg/m^3; absorption coefficient 0.115 mm^{-1}; F(000) 572; θ range for data collection 2.54−28.30°; reflections collected 8293; independent reflections 5255 (R_{int} = 0.0369); completeness to θ = 28.30°; absorption correction, none; refinement method full-matrix least-squares on F^2 1.022; final R indices [$I > 2\sigma(I)$] R1 = 0.0439, wR2 = 0.1167; largest difference between peak and hole 0.443 and −0.238 e Å^{-3}.

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Supporting Information Available: HPLC separation of the seed extract of *S. marianum*, photo of a single-crystal of isosilybin A (**6**) under microscope, and X-ray crystallographic characteristics of isosilybin A (**6**). These materials are available free of charge via the Internet at http://pubs.acs.org.

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(14) X-ray data of isosilybin A (**6**) have been deposited at the Cambridge Crystallographic Data Center. Copies of the data can be obtained free of charge on application to the Director, CCDC, 12 Union Rd., Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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