New Insight into the Biosynthesis of Flavanolignans in the White-Flowered Variant of *Silybum marianum*

Sz. Nyiredy[1,*], Zs. Samu[2], Z. Szücs[1], K. Gulácsi[3], T. Kurtán[3], and **S. Antus[3,4,†]**

[1]Research Institute for Medicinal Plants, P.O. Box 11, H-2011 Budakalász, Hungary; [2]Béres Pharmaceuticals, 2-4 Mikoviny, H-1037 Budapest, Hungary; [3]Department of Organic Chemistry, University of Debrecen, P.O. Box 20, H-4010 Debrecen, Hungary; and [4]Research Group for Carbohydrates of Hungarian Academy of Sciences, University of Debrecen, P.O. Box 94, H-4010 Debrecen, Hungary

Abstract

It has been demonstrated that besides the known flavanolignan constituents of the white-flowered variant of *Silybum marianum*, (–)-silandrin A (3a) **and (–)-isosilandrin A** (4a)**; their trans-benzodioxane diastereomers, (–)-silandrin B** (3b) **and (–)-isosilandrin B** (4b)**, are also produced by the plant. Moreover, the isolation of their cis-benzodioxane diastereomers, (–)-isocisilandrin** (5) **and cisilandrin** (6)**, confirm that the previously proposed biosynthetic pathway involving a nonselective *O*-β coupling is correct.**

Introduction

Mariendistel (*Silybum marianum* L. Gaertn) has been used as a folk medicine, particularly as a drug with a hepatoprotective activity since ancient times (1). The chemistry of its constituents, isolated from the fruits of different *Silybum* species originating from various parts of Europe, has been the subject of numerous research projects since the middle of the past century. Thus, the presence of flavanolignans in the purple-flowered variant of this plant was recognized in the fifties (2) and sixties (3,4), and a systematic study on its flavanolignan constituents led to the isolation of (+)-silybin (**1a**,**b**) (5,6), which is the most active constituent of *Legalon* (Madaus AG, Köln), used in the therapy of liver diseases (7,8). The isolated (+)-silybin was proven to be a 1:1 mixture of diastereomers [(+)-silybin A (**1a**):(–)-silybin B (**1b**) = 1:1] on the basis of 1H and 13C NMR, X-ray crystallography (9,10), and its biomimetic synthesis (11,12). Besides (+)-silybin (**1a**,**b**), its regioisomer, (+)-isosilybin, was also isolated from this source and found also a 1:1 diastereomeric mixture [(+)-isosilybin A (**2a**), (–)-isosilybin B (**2b**)] (11–13).

According to Freudenberg's hypothesis (14), shared later by many other authors (15–18), these compounds may be formed biosynthetically from (+)-taxifolin (**7a**) and coniferyl alcohol (8) in an oxidative process catalyzed by peroxidase enzyme as shown in Figure 1. The corresponding neutral phenoxy (**7a*R***, **7a*R****) and quinone methide (8*R* 8*R**) radicals are formed first, followed by an *O*-β coupling which is neither regio- nor enantioselective. The final step of the biosynthesis is a thermodynamically controlled nucleophilic attack of the OH group on the quinone methide system of intermediate **9** to furnish the 2,3-*trans*-substituted 1,4-benzodioxane skeletons of **1a**,**b** and **2a**,**b**. The *O*-β coupling step is not enantioselective, which was proven by the analytical HPLC separation of the diastereomeric (+)-silybin A

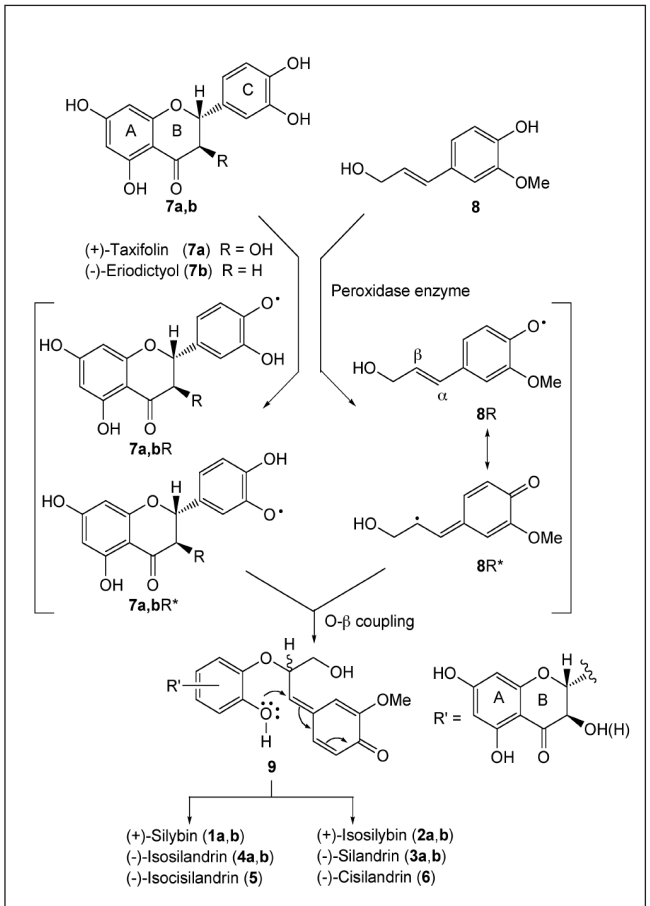


Figure 1. Biosynthetic pathways of 1,4-benzodioxane-type flavanolignans.

* In memory of Prof. Dr. Szabolcs Nyiredy (1949–2006).

† Author to whom correspondence should be addressed.

(**1a**) and (–)-silybin B (**1b**), and (+)-isosilybin (**2a**), and (–)-isosilybin (**2b**) (19–21), as well as by their isolation using a preparative reversed-phase high-performance liquid chromatography (RP-HPLC) method (22,23).

During a systematic study of a population of more than one hundred *Silybum* plants, Stieber et al. (24) recognized that the substitution pattern of the flavanolignan constituents obtained from the seeds of a white-flowered variant of *Silybum marianum* differs markedly from that of the purple-flowered type. This led to the isolation of (–)-silandrin (**3a**) (25) and (–)-isosilandrin (**4a**) (26), whose structures were determined as 3-deoxyisosilybin and 3-deoxysilybin, respectively, and their biosynthesis may involve (–)-eriodictyol (**7b**) instead of (+)-taxifolin (**7a**). We have also reported that in contrast to (+)-silybin (**1a,b**) and (+)-isosilybin (**2a,b**), (–)-isosilandrin (**4a**) and (–)-silandrin (**3a**) are not a mixture of the corresponding diastereomers, but instead are optically pure compounds with (2*S*, 2'*R*, 3'*R*)-configuration (26,27). Moreover, it was also observed that (–)-silandrin (**3a**) possessed a more effective inhibitory activity in carbon tetrachloride-treated cytotoxicity of rat hepatocytes than (+)-silybin (**1a,b**) (28) and, similarly, its regioisomer [(–)-**4a**] showed stronger inhibitory activity on the superoxide anion release by human polymorphonuclear leukocytes (26). Recently, two studies (29,30) using diastereomerically pure isolated (+)-silybin A (**1a**), (–)-silybin B (**1b**), (+)-isosilybin A (**2a**), and (–)-isosilybin B (**2b**) have demonstrated that these flavanolignan stereoisomers showed fundamentally different activity in human prostate carcinoma in vitro and in vivo, and on the prevention of skin cancer. Thus it seemed promising to reexamine the (–)-silandrin (**3a**) and (–)-isosilandrin (**4a**) content of the white-flowered Silybum marianum L. to find out whether the O-β coupling step of their biosynthesis is indeed enantioselective. This implies a search for their other possible diastereomers **3b** and **4b** in the flavanolignan extract.

Experimental

Chemicals and sample treatment

For analytical HPLC, tetrahydrofuran, acetonitrile, methanol (Merck, Darmstadt, Germany), and water (RIMP, Budakalász, Hungary) were of gradient HPLC quality. Light petroleum (b.p. 40–60°C) for degreasing, methanol for extraction, and glacial acetic acid (Reanal, Budapest, Hungary) were of analytical grade. For the normal-phase (NP) purification and fractionation, the solvents (analytical grade) were obtained from Reanal, and the eluents of the RP separations were prepared using LiChrosolv solvents (Merck).

The fruits of white-flowered *Silybum marianum* L. were collected in Budakalász (Hungary), degreased as described in the German Pharmacopoeia (31), and subsequently extracted with MeOH. This extract was concentrated to dryness under reduced pressure.

HPLC operating conditions

Method A

A 250 × 2 mm 5 µm Hypersil ODS analytical column (Knauer, Berlin, Germany) and a mixture of tetrahydrofuran–acetonitrile–methanol–water–glacial acetic acid (14.5:7.0:8.5:70.0:5.0) as eluent were applied. The eluent flow rate was 0.21 mL/min. Three microliters methanolic solution of the dry extract were injected. The column thermostat was set to 40°C and the detection was performed at 288 nm. This separation was optimized by the "PRISMA" model (32,33).

Method B

A 250 × 4 mm 5 µm Prontosil-200 C30 analytical HPLC column (Bischoff, Leonberg, Germany) and a mixture of methanol–water–formic acid (55:45:0.1) as eluent were applied. The eluent flow rate was 1.0 mL/min. Five microliters methanolic solution of the dry extract were injected. The column thermostat was set to 20°C, and the detection was performed at 288 nm.

Isolation of compounds

Purification of the extract was achieved by normal-phase vacuum liquid chromatography. The extract was applied to the top of a layer (2 cm high, 10 cm in diameter) of 15 µm particles TLC-Kieselgel 60 G (Merck, Darmstadt, Germany) and the elution was carried out by a mixture of chloroform–ethyl-acetate–methanol–diethyl-ether (73.9:19.3:2.3:4.5) until colorless eluate. All of the eluate was concentrated to dryness under reduced pressure. This purified extract was fractionated by normal-phase medium pressure liquid chromatography (NP-MPLC). A 920 × 70 mm TLC-Kieselgel 60 G (Merck) glass column was packed with a slurry of 1690 g 15-µm particles made with chloroform. After loading purified extract to the top of the column, the elution was carried out at a flow rate of 1.0 mL/min using the same mixture as eluent as in the case of the vacuum liquid chromatographic purification. The fractions containing the compounds of interest were combined and evaporated to dryness. The flavanolignans were isolated from these fractions by reversed-phase medium pressure liquid chromatography (RP-MPLC). A 460 × 26 mm Kromasil KR 100 C_{18} (Eka Chemicals, Bohus, Sweden) glass column was packed with a slurry of 220 g 16 µm particles made with the eluent. A mixture of tetrahydrofuran–methanol–water = 13.3/34.6/52.1 was used as eluent at a flow rate of 4.0 mL/min. The detection was performed at 288 nm, and the fractions containing flavanolignans were collected, combined, and evaporated to dryness. For the MPLC separations, a model no. 681 pump (Büchi, Flawil, Switzerland), a 2210 dual wavelength detector (HP, Waldbronn, Germany), and 7000 Ultrarac fraction collector (LKB, New Jersey) were used. The final purification of each compound was achieved by semipreparative HPLC on an HP1084 instrument (HP, Waldbronn, Germany) using a 250 × 2 mm 5 µm particle Eurospher-100 C_{18} (Knauer, Berlin, Germany) column and the same eluent as described for RP-MPLC, at a flow rate of 8.0 mL/min. The column thermostat was set to 40°C.

Results and Discussion

The components of the methanolic extract of the white-flowered *Silybum marianum* L. were studied by HPLC using Method

A. The chromatographic profile of the separation is shown in Figure 2. The major components of the crude extract, eluated at a retention times of 5.92, 18.02 and 21.30 min, could be easily identified as (+)-silymonin (25), (not a 1,4-benzodioxane-type flavanolignan constituent of *Silybum marianum*), (–)-isosilandrin (**4a**), and (–)-silandrin (**3a**), respectively.

In order to achieve a better separation of the stereoisomers, Method B was developed and the resulting chromatogram is shown in Figure 3. In this method, the peaks of the *trans*-stereoisomers, silandrin (**3**) and isosilandrin (**4**), could be resolved to 2-2 peaks: (–)-silandrin A (**3a**), (–)-silandrin B (**3b**), and (–)-isosilandrin A (**4a**), (–)-isosilandrin B (**4b**), respectively. Moreover (as it can be seen in both Figures 2 and 3), two other minor components are also separated from the *trans*-stereoisomers. The similarity of the on-line UV spectra of the compounds eluted before (–)-isosilandrin (**4a**) and (–)-silandrin (**3a**) and numbered in Figure 2 as **5** and **6** suggested that these compounds may be their stereoisomers. They could be isolated as crystalline substances with m.p. 172–175 and 198–200°C, respectively, by normal and reversed-phase MPLC and semipreparative HPLC under the conditions given earlier. The structure elucidation of these minor components **5** and **6** has been accomplished by the combination of different spectroscopic methods (nuclear magnetic resonance, infrared, mass spectrometry, circular dichroism), and they were determined as *cis*-stereoisomers of the corresponding regioisomers (–)-**4a** and (–)-**3a**, respectively (34). According to their stereochemical relationship with (–)-isosilandrin (**4a**) and (–)-silandrin (**3a**), (–)-**5**, and (–)-**6** have been named as (–)-isocisilandrin and (–)-cisilandrin. The presence of these *cis*-stereoisomers has clearly indicated that a quinone methide-type intermediate **9** must be formed in the course of the biosynthesis in the fruits of different *Silybum* species. According to the significant difference of the thermodynamic stability of *cis*- and *trans*-stereoisomers (**4a** >> **5**, **3a** >> **6**), the *trans*-substituted 1,4-benzodioxanes (**3a**, **4a**) are formed as major components besides the corresponding *cis* ones (**5**, **6**), which indeed were found to be minor components as shown in Figures 2 and 3.

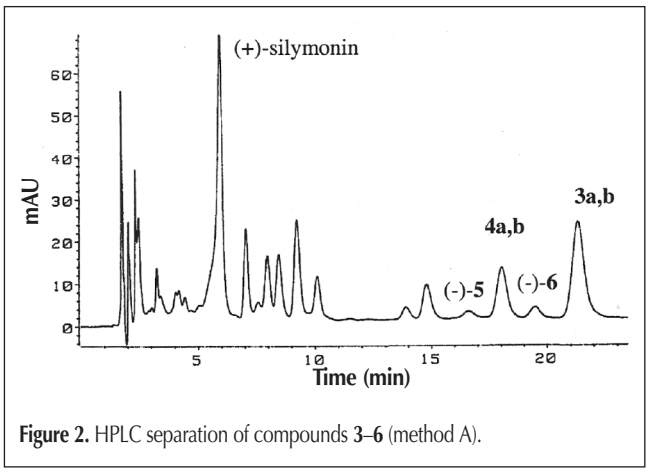


Figure 2. HPLC separation of compounds **3**–**6** (method A).

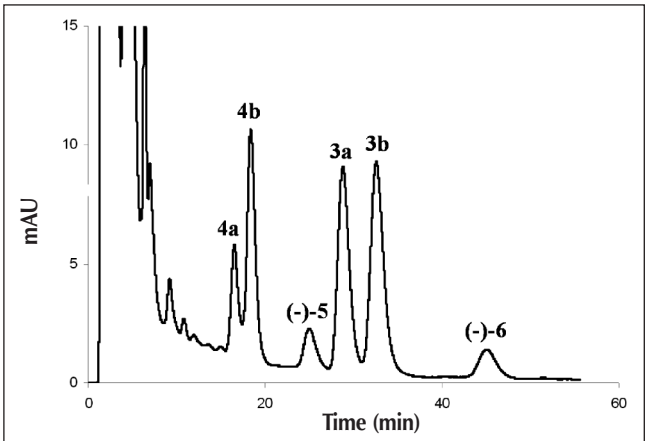
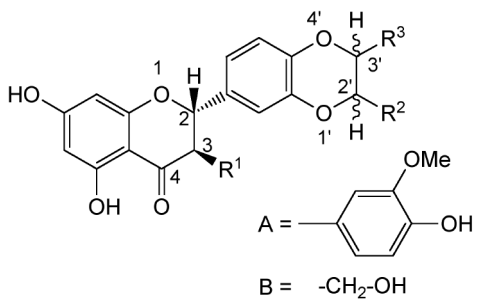


Figure 3. HPLC separation of compounds **3**–**6** (Method B).

Conclusion

As a summary, we have shown that by the proper selection of the stationary phase and eluent, the diastereomers of 1,4-benzodioxane-type flavanolignans [(–)-silandrin A (**3a**), (–)-silandrin B (**3b**); (–)-isosilandrin A (**4a**), (–)-isosilandrin B (**4b**)], formed in the white-flowered variant of *Silybum marianum*, could be separated analytically. Moreover, the *cis* isomers of silandrin A (**3a**) and isosilandrin A (**4a**) were isolated for the first time and named as (–)-isocisilandrin and (–)-cisilandrin. This results have clearly indicated that the biosynthesis of 1,4-benzodioxane constituents take place without regio- and enantioselectivity both in the purple and white-flowered-variant of *Silybum marianum*. The isolation of 1,4-benzodioxane-type constituents of the white-flowered variant of *Silybum marianum* in larger scale and the examination of their biological activities are in progress.



		R^1	R^2	R^3	Configuration
(+)-Silybin A	(**1a**)	OH	A	B	(2*R*,2'*R*,3*R*,3'*R*)
(-)-Silybin B	(**1b**)	OH	A	B	(2*R*,2'*S*,3*R*,3'*S*)
(+)-Isosilybin A	(**2a**)	OH	B	A	(2R,2'R,3R,3'R)
(-)-Isosilybin B	(**2b**)	OH	B	A	(2*R*,2'*S*,3*R*,3'*S*)
(-)-Silandrin A	(**3a**)	H	B	A	(2*S*,2'*R*,3'*R*)
(-)-Silandrin B	(**3b**)	H	B	A	(2*S*,2'*S*,3'*S*)
(-)-Isosilandrin A	(**4a**)	H	A	B	(2*S*,2'*R*,3'*R*)
(-)-Isosilandrin B	(**4b**)	H	A	B	(2*S*,2'*S*,3'*S*)
(-)-Isocissilandrin	(**5**)	H	A	B	(2*S*,2'*R**,3'*S**)
(-)-Cisilandrin	(**6**)	H	B	A	(2*S*,2'*S**,3'*R**)

Figure 4. Structures of flavanolignans (**1**–**6**).

Acknowledgments

The authors thank the Hungarian Scientific Research Fund (OTKA, T-049436, K-68429, NI-61336) for financial support.

References

1. K. Westphal. Gallenfuntionen und Gallenleiden. Springer Verlag, Berlin, 1931, p. 306.
2. H. Schindler. Active substances in pharmaceutical plants; methods to determine plant tinctures; contributions to a supplementary issue of the homoiopathic pharmacopeia. *Arzneim.-Forsch. (Drug Res.)* **2:** 291–296 (1952).
3. B. Janiak and R. Hänsel. Phytochemisch-pharmakognostische Untersuchungen über Fructus cardui Mariae. *Planta Med.* **8:** 71–84 (1960).
4. H. Wagner, L. Hörhammer, and M. Seitz. Chemische Wertbestimmung eines Silymarin-haltigen Flavonoidkonzentrates aus Silybum marianum (L.) Gaertn. *Arzneim.-Forsch. (Drug Res.)* **18:** 696–698 (1968).
5. A. Pelter and R. Hänsel. The structure of silybin (silybum substance E6), the first flavonolignan. *Tetrahedron Lett.* 2911–2916 (1968).
6. H. Wagner, L. Hörhammer, and R. Münster. Zur Chemie des Silymarins (Silybin), des Werkprinzips der Früchte von Silybum marianum (L.) Gaertn. (Carduus marianus L.). *Arzneim-Forsch. (Drug Res.)* **18:** 688–696 (1968).
7. G. Vogel. New Natural Products and Plant Drugs with Pharmacological, Biological and Therapeutical Activity. Springer Verlag, Berlin-Heildelberg-New York, 1977, p. 249.
8. R. Saller, R. Meier, and R. Brignoli. The use of silymarin in the treatment of liver diseases. *Drugs* **61:** 2035–2063 (2001).
9. M. Delfini, M.E. DiCacco, E. Gaggelli, A. Lepri, and G. Valensin. 1H NMR delineation of confromational parameters of constituents of silybum marianum in solution. *Spectrosc. Lett.* **23:** 1007–1025 (1990).
10. H. Lotter and H. Wagner. Zur Stereochemie von Silybin. *Z. Naturforsch.* **38c:** 339–341 (1983).
11. L. Merlini and A. Zanarotti. Biomimetic Synthesis of Natural Silybin. J. Chem. Soc. Chem. Commun. Issue or page numbers required for publication. 695 (1979).
12. L. Merlini, A. Zanarotti, A. Pelter, M.P. Rochefort, and R. Hänsel. Benzodioxans by oxidative phenol coupling. Synthesis of silybin. *J. Chem. Soc. Perkin Trans.* **1:** 775–778 (1980).
13. A. Arnone, L. Merlini, and A. Zanarotti. Constituents of silybum marianum. Structure of isosilybin and stereochemistry of silybin. *J. Chem. Soc. Chem. Commun.* 696–697 (1979).
14. K. Freudenberg and A.C. Neish. Constitution and Biosynthesis of Lignins. Springer Verlag, New York, 1968.
15. H. Erdtman. Dehydrierungen in der Coniferylreihe. II. Dehydrodiisoengenol. *Justus Liebigs Ann. Chem.* **503:** 283–294 (1933).
16. R. Hänsel and H. Rimpler. Structure of silybin: synthetic studies. *Dtsch. Apotk. Ztg.* **108:** 1985 (1968).
17. R. Schrall and H. Becker. Callus-und Suspensionskulturen von Silybum Marianum. *Planta Med.* **32:** 27–32 (1977).
18. L. Merlini and A. Zanarotti. A biogenetically patterned synthesis of (–)-eusiderin. *Tetrahedron Lett.* 3621–3622 (1975).
19. G. Tittel and H. Wagner. Hochleistungsflüssigchromatographie von Silymarin. II: Quantitative Bestimmung von Silymarin aus Silybum marianum durch Hochleistungsflüssigchromatographie. *J. Chromatogr.* **153:** 227–232 (1978).
20. H. Mascher, C. Kikuta, and R. Weyhenmeyer. Diastereomeric separation of free and conjugated silibin in plasma by reversed-phase HPLC after specific extraction. *J. Liquid Chromatogr.* **16:** 2777 (1993).
21. B. Rickling, B. Hans, R. Kramarczyk, G. Krumbiegel, and R. Weyhenmeyer. Two high-performance liquid chromatographic assays for the determination of free and total silibinin diastereomers in plasma using column switching with electrochemical detection and reversed-phase chromatography with ultraviolet detection. *J. Chromatogr. B* **670:** 267 (1995).
22. N.C. Kim, T.N. Graf, Ch.M. Sparacino, M.C. Wani, and M.E. Wall. Complete isolation and characterization of silybins and isosilybins from milk thistle (Silybum marianum). *Org. Biomol. Chem.* **1:** 1684–1689 (2003).
23. D.Y.W. Lee and Y. Lin. Molecular structure and stereochemistry of silybin A, silybin B, isosilybin A, and isosilybin B, isolated from silybum marianum (Milk Thistle). *J. Nat. Prod.* **66:** 1171–1174 (2003).
24. G. Stiber, I. Szilágyi, and P. Tétényi. Hatóanyagtartalmi és összetételbeli különbségek a Silybum genus két fajában (Silybum marianum (L.) Gaertn és Silybum eburneum coss. et durr.). *Herba Hung.* **16:** 55 (1977).
25. I. Szilágyi, P. Tétényi, S. Antus, O. Seligmann, V.M. Chari, M. Seitz, and H. Wagner. Struktur von Silandrin und Silymonin, zwei neuen Flavanolignanen aus einer weissblühenden Silybum marianum Varietät. *Planta Med.* **43:** 121–127 (1981).
26. Zs. Samu, Sz. Nyiredy, E. Baitz-Gács, Zs. Varga, T. Kurtán, Z. Dinya, and S. Antus. Structure elucidation and antioxidant activity of (–)-isosilandrin Isolated from silybum marianum L. Chem. *Biodiversity* **1:** 1668–1677 (2004).
27. S. Antus, E. Baitz-Gács, G. Snatzke, and T.S. Tóth. Synthesis and circular dichroism of steroids with 1,4-benzodioxane chromophore: on the absolute configuration of (–)-silandrin. *Liebigs Ann. Chem.* 633–641 (1991).
28. H. Hikino, Y. Kiso, H. Wagner, and M. Fiebig. Antihepatotoxic actions of flavonolignans from silybum marianum fruits. *Planta Med.* **50:** 248–250 (1984).
29. P.R. David-Searles, Y. Nakanashi, N.C. Kim, T.N. Graf, N.H. Oberlies, M.C. Wami, M.E. Wall, R. Agrawal, and D.J. Kroll. Milk thistle and prostate cancer: differential effects of pure flavonolignans from silybum marianum on antiproliferative end points in human prostate carcinoma cells. *Cancer Res.* **65:** 4448–4456 (2005).
30. M. Pliskova, J. Yondracek, V. Kren, R. Guzak, P. Sedmera, D. Walterova, J. Psotava, V. Simanek, and M. Machala. Effects of silymarin flavonolignans and synthetic silybin derivatives on estrogen and aryl hydrocarbon receptor activation. *Toxicology* **80:** 215–230 (2005).
31. Deutsches Arzneibuch 10. (DAB 10.) 1991 Nachtrag, 1993, Band 3. Monographie über Mariendinstelfrüchte
32. Sz. Nyiredy, K. Dallenbach-Tölke, and O. Sticher. The PRISMA optimization system in planar chromatography. *J. Planar Chromatogr.* **1:** 336– Page numbers required for publication. (1988).
33. Sz. Nyiredy, W. Wosniok, H. Thiele, and O. Sticher. PRISMA model for computer-aided HPLC mobile phase optimization based on an automatic peak identification approach. *J. Liquid Chromatogr.* **14:** 3077 (1991).
34. S. Antus, K. Gulácsi, T. Kurtán, E. Baitz-Gács, Z. Szücs, and Zs. Fatér. Unpublished results.

Manuscript received July 10, 2007;
Revision received August 31, 2007.