Enzymatic Kinetic Resolution of Silybin Diastereoisomers[⊥]

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Received November 20, 2009

In nature, the flavonolignan silybin (**1**) occurs as a mixture of two diastereomers, silybin A and silybin B, which in a number of biological assays exhibit different activities. A library of hydrolases (lipases, esterases, and proteases) was tested for separating the silybin A and B diastereomers by selective transesterification or by stereoselective alcoholysis of 23-*O*-acetylsilybin (**2**). Novozym 435 proved to be the most suitable enzyme for the preparative production of both optically pure silybins A and B by enzymatic discrimination. Gram amounts of the optically pure substances can be produced within one week, and the new method is robust and readily scalable to tens of grams.

The flavonolignan silybin (**1**, Scheme 1) (CAS No. 22888-70-6) is the major component (ca. 30%) of the silymarin complex extracted from the seeds of *Silybum marianum* (L.) Gaertn. (*Carduus marianus* L., Asteraceae; milk thistle). Besides silybin, silymarin contains its congeners, such as isosilybin, silychristin, silydianin, 2,3-dehydrosilybin, and taxifolin,[1,2] and about 20−30% of an undefined polymeric phenolic fraction.[3] Silybin attracts increasing attention, reflected in a high number of papers published recently (over 100 papers per year,[1] e.g., 185 papers in 2008 (ISI−Web of Science)).

Silybin and the other silymarin flavonolignans (isosilybin, silychristin, and silydianin) are biogenetically formed by the oxidative (radical) coupling of coniferyl alcohol to the catechol moiety of taxifolin. This reaction is not stereospecific,[4] and thus all respective flavonolignans occur in the silymarin complex in diastereomeric pairs (always the *trans*-configuration) in proportions of ca. 1:1.

Natural silybin is an approximately equimolar mixture of the two diastereomers silybin A (**1a**, Scheme 1), (2*R*,3*R*)-2-[(2*R*,3*R*)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one, and silybin B (**1b**, Scheme 1), (2*R*,3*R*)-2-[(2*S*,3*S*)-2,3-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2,3-dihydro-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one (this systematic numbering is not congruous with the customary numbering of the silybin skeleton as given in Scheme 1), whose analytical separation is quite feasible,[5] but preparative separation is extremely complicated. The absolute configuration of both silybins A and B was determined recently.[6,7]

Numerous authors denote both silybin diastereomers "A" and "B" without knowing their configuration, usually according to the order of elution observed in HPLC analyses. Optical rotation measurement is the easiest method to assign the absolute configuration of both compounds: natural silybin (a mixture of **1a** and **1b** in a ca. 1:1 ratio; **1b** is usually slightly prevalent) has an $[\alpha]_D^{23}$ +11.4 (*c* 0.29, acetone),[8] silybin A (**1a**) has an $[\alpha]_D^{23}$ +20.0 (*c* 0.21, acetone), and silybin B (**1b**) has an $[\alpha]_D^{23}$ −1.07 (*c* 0.28, acetone).[6]

Typical applications for which milk thistle preparations have been used since ancient times include mostly treatments of liver diseases and gastrointestinal tract problems.[9] Silymarin/silybin and their preparations are currently also advocated for the treatment of cirrhosis, chronic hepatitis, and liver diseases associated with alcohol consumption and environmental toxin exposure.[10]

Pharmacological, medicinal, and phytochemical literature include many conflicting reports on the effects of silymarin and/or silybin.[11,12] The main reason for the controversy and uncertainty of effects is the variable composition of silymarin preparations used in these studies, caused by the use of nonstandard silymarin (complex extract) and silybin (defined compound) preparations and confusing these terms.[1,13]

Recently silybin/silymarin has received attention due to its alternative beneficial activities that are not directly related to its hepatoprotective and/or antioxidant (radical scavenging) effects.[1,14] These include mostly anticancer and chemopreventive actions, as well as hypocholesterolemic, cardioprotective, and neuroprotective activities. Great interest has been focused on the use of silybin for the treatment of various disorders of the prostate including adenocarcinoma that led to the start of clinical tests (phase II) in the U.S. These activities are linked to the discovery of numerous new effects of silybin and its derivatives at the cellular and molecular levels, such as estrogenic activity, modulation of drug transporters (P-glycoprotein), and specific action on DNA expression via the suppression of nuclear factor-κB (NF-κB).[1,15] The molecular mechanism of the antioxidant action of silybin has been described by us recently.[16,17]

Aspects of the optical purity of silybin and other flavonolignans from the silymarin complex have been largely neglected. However, when silybin is used for applications other than as a mere antioxidant in an isotropic milieu, e.g., in solution reacting with nonchiral radicals, its stereochemistry plays an extremely important role, and the respective biological activities also need to be studied with optically pure compounds.

New data on the pharmacological activity of pure silybins A and B appeared recently and clearly demonstrated the different biological activities of the two silybin diastereomers **1a** and **1b**. We have recently presented proof that **1b** interacts with an estrogenic receptor, whereas its diastereomer **1a** is inactive.[18] Later, a paper on the activity of pure silybins A and B, isosilybins A and B, and other silymarin components on human prostate carcinoma demonstrated that isosilybin B is the most effective in suppressing the topoisomerase IIα gene promoter in DU145 cells.[15] Silybin B was the most active in the G1 cell cycle accumulation of DU145 cells.

A major problem hampering studies with optically pure silybin is the extremely complicated separation of its diastereomers. So far, only separations by HPLC or semipreparative HPLC have been used to obtain milligram amounts of these compounds. Some authors even claim to have achieved preparative separation; however they end up with some 10−20 mg of pure silybins.[19] Recently,

[⊥] This paper is dedicated to Prof. Bruno Danieli, Università degli Studi di Milano, on the occasion of his 70th birthday.

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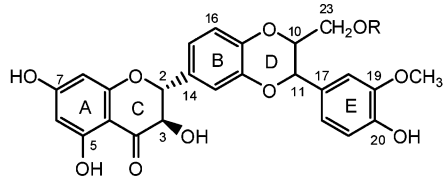
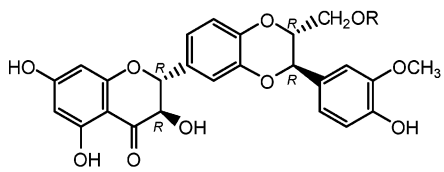
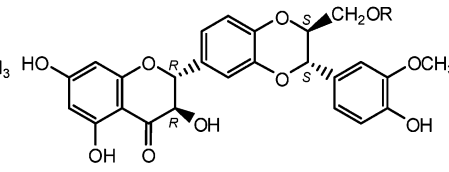
10.1021/np900758d
Published on Web 03/18/2010

Scheme 1. Silybin Diastereomers and Acyl Derivatives



1 R = H, Silybin
2 R = Ac, 23-*O*-Acetylsilybin





1a R = H, Silybin A
2a R = Ac, 23-*O*-Acetylsilybin A
3a R = C_3H_7CO, 23-*O*-Butyrylsilybin A
4a R = $C_7H_{15}CO$, 23-*O*-Octanoylsilybin A

1b R = H, Silybin B
2b R = Ac, 23-*O*-Acetylsilybin B
3b R = C_3H_7CO, 23-*O*-Butyrylsilybin B
4b R = $C_7H_{15}CO$, 23-*O*-Octanoylsilybin B

another study aiming at the preparative separation of silybins A and B and other silymarin congeners by HPLC in larger quantities has been published.[20] However, this is mere repetition and optimization of the previously published HPLC methods on a larger scale (e.g., 154 injections/repetitions), and the authors admit that "... it is possible to generate gram quantities of each diastereoisomer within a few months ...".[20] Moreover, this method uses quite expensive separation materials (e.g., HPLC grade RP-18 silica). This is unacceptable not only for the pharmacological production of these compounds but also for preclinical studies in animals, and emphasizes the need for an efficient and practical method for the preparative-scale separation of the silybins. Recently, we have published an alternative method for separation of silybins A and B via their glycosides.[21] This method provides reasonable yields (60−90%); however, it requires extensive flash chromatography of silybin A and B glycosides, whose separation is critical, and its scalability is limited to ca. 10 g amounts. In addition, the purity of silybins reaches a diastereomeric excess (de) of only 97%, whereas higher optical purity is required for exact measurements. Therefore, a more simple and less labor-demanding method for the silybin separation is still required.

Lipases and proteases proved to be highly efficient in the chiral separation of a plethora of racemates.[22] In addition, enzymatic acylations of flavonoids have been used for various compounds and systems.[23] However, most of the work has been carried out with glycosylated flavonoids, and there are few data available for the enzymatic acylation or deacylation of the respective aglycones. Silybin, being a flavonolignan, has however rather distinctive features distinguishing it from most other flavonoids, e.g., the presence of a primary alcoholic group enabling a variety of derivatizations.[17] Moreover, enzymatic acylation of silybin can be a suitable alternative method to chemical synthesis for the production of acylsilybins with anti-influenza virus activities.[24]

Using our previous expertise both in enzymatic kinetic resolutions[25] and in the biocatalyzed regioselective modification of polyhydroxylated natural compounds,[26] we have opted to employ this methodology toward the preparative separation of silybins A and B in sufficient amounts and with high diastereomeric purity. Our preparative method is robust, feasible, and scalable and should boost further fruitful research into the promising bioactivities of optically pure silybins.

Results and Discussion

Synthesis of 23-*O*-Acylsilybins. Various methods were tested for the synthesis of 23-*O*-acetylsilybin (**2**). Acylation of the 23-OH group of silybin was achieved using the respective acyl chloride or anhydride under $BF_3 \cdot OEt_2$ catalysis. Application of this method for the synthesis of acetate **2** gave ca. 60% yields with excellent regioselectivity at C-23. To study the influence of the size of the acyl chain on enzymatic discrimination, butyryl (**3**) and octanoyl (**4**) derivatives were also synthesized in ca. 30% yields. The yields of longer chain derivatives were considerably lower, and thus they were not employed in the preparative reactions.

Screening of Hydrolases for Silybin Acetylation and 23-*O*-Acetylsilybin Alcoholysis. Twenty-six different commercially available hydrolase preparations, including lipases, proteases, and acylases (Table 1), were tested on an analytical scale (solvent MTBE, 1 mL final volume) for their ability to perform the transesterification reaction of silybin (**1**) to 23-*O*-acetylsilybin (**2**) in the presence of vinyl acetate and also for the alcoholysis reaction of **2** yielding compound **1** using *n*-butanol. This screening was designed to obtain a rapid evaluation of the ability of respective hydrolases to form or to cleave acetate **2** regardless of chiral discrimination. Therefore, previously established reaction conditions suitable for hydrolase activity were used.[27] A set of small-scale reactions was maintained for at least 48 h and monitored in parallel by TLC. Further evaluation of the results was performed by HPLC analyses, which allowed us to identify "positive" reactions, i.e., those giving conversions >2% after 48 h (Table 1).

Enzymes not active under the given conditions, i.e., giving conversion <2% after 48 h, were retested using acetone as co-solvent. No significant change in conversion values was observed; therefore these biocatalysts were excluded from further studies. The inhibition of some esterases by flavonoids is well known,[28] and this might also be the case with silybin. A detailed study of such phenomena would be beyond the scope of this work. Stereoselectivity in the transesterification reactions ("pseudo"-*E* values, Table 2, entries 1−9) of the nine active enzymes was evaluated by HPLC for conversion values < 50%.[29]

The estimated *E* values for the transesterification reaction were generally quite low. The best result was obtained with lipase A; however, the enzyme was poorly active on this substrate with ca. 8% conversion after 48 h. In contrast, both *C. rugosa* lipase and Novozym 435 gave very good conversions, and quite significantly, they showed opposite diastereoselectivity (Figure 1A,B, $E = 4.4$ and 2.6, respectively). Lipase from *C. rugosa* preferentially yielded silybin A acetate **2a** (Figure 1A), whereas Novozym 435 showed a preference for the acetylation of silybin B, thus giving the enriched acetate **2b** (Figure 1B). The identity of the respective silybin diastereomers was determined by cochromatography with authentic samples[8] and confirmed with optical rotation and circular dichroism measurements (Supporting Information, Table S1 and Figure S1).

Table 1. Screening of Hydrolases for Silybin (**1**) Transesterification[a] and 23-*O*-Acetylsilybin (**2**) Alcoholysis[b]

enzyme	source	producer	transesterification	alcoholysis
lipase AK	*Pseudomonas* sp.	Amano		
lipase PS	*Pseudomonas cepacia*	Amano	●[c]	
wheat germ lipase	Wheat germ	Sigma		
lipase D	*Rhizopus delemar*	Amano		
lipase L	*Candida lipolytica*	Amano		
lipase M	*Mucor javanicus*	Amano		
lipase F-AP15	*Rhizopus oryzae*	Amano		
lipase N	*Rhizopus niveus*	Amano		
lipase R	*Penicillium roquefortii*	Amano		
lipase CV	*Chromobacterium viscosum*	Amano	●	
Rhizopus japonicus lipase	*Rhizopus japonicus*	Biocatalysts Ltd.		
Mucor miehei lipase	*Mucor miehei*	Amano		
lipase CE	*Humicola lanuginosa*	Amano		
lipase A	*Aspergillus niger*	Amano	●	●
lipase GC	*Geotrichum candidum*	Amano		
Candida rugosa lipase	*Candida rugosa*	Sigma	●	●
PPL	Porcine pancreas	Sigma		
Novozym 435	*Candida antarctica* (lip. B)	Novozymes	●	●
CAL-A	*Candida antarctica* (lip. A)	Novozymes	●	
Alcaligenes sp. lipase	*Alcaligenes* sp.	Neptune	●	●
subtilisin	*Bacillus subtilis*	Sigma	●	●
protease N	*Bacillus subtilis*	Amano		●
Proleather	*Bacillus subtilis*	Amano		
protease P	*Bacillus subtilis*	Amano		●
acid protease II	*Rhizopus niveus*	Amano		●
acylase Amano 3000	*Aspergillus* sp.	Amano	●	●

[a] Reaction conditions: silybin (**1**, 5 mg, 10.4 μmol) was dissolved in a mixture of MTBE (1 mL) and vinyl acetate (0.1 mL, 1.08 mmol), respective enzyme (50 mg of powder or 5 mg of Novozym 435) was added, and the suspensions were shaken at 45 °C for 48 h and monitored by TLC and HPLC. [b] Reaction conditions: 23-*O*-acetylsilybin (**2**, 5 mg, 9.7 μmol) was dissolved in a mixture of MTBE (1 mL) and *n*-butanol (0.1 mL, 1.09 mmol), respective enzyme (50 mg of powder or 5 mg of Novozym 435) was added, and the suspensions were shaken at 45 °C for 48 h and monitored by TLC and HPLC. [c] (●) Conversion >2% after 48 h.

Table 2. Evaluation of Diastereoselectivity of Selected Hydrolases for Silybin (**1**) Transesterification[a] and 23-*O*-Acetylsilybin (**2**) Alcoholysis[a]

enzyme	reaction type	time [h]	conversion [%]	E^b
lipase PS		48	39.7	1.6 (B)
lipase CV		48	14.4	2.4 (A)
lipase A		48	8.4	**5.5 (B)**
Candida rugosa lipase		3	16.8	**4.4 (A)**
Novozym 435	transesterification	3	32.9	**2.6 (B)**
lipase CAL-A		48	33.0	2.0 (A)
Alcaligenes sp. lipase		18	47.9	1.0 (-)
subtilisin		48	37.2	1.7 (B)
acylase Amano 3000		48	2.9	1.7 (B)
lipase A		48	5.3	1.8 (B)
Candida rugosa lipase		5	29.0	**21.1 (A)**
Novozym 435		3	33.7	**6.5 (B)**
Alcaligenes sp. lipase		48	3.4	1.5 (B)
subtilisin	alcoholysis	48	2.6	1.1 (B)
protease N		48	4.0	1.1 (B)
protease P		48	14.7	1.1 (B)
acid protease II		48	4.3	1.2 (B)
acylase Amano 3000		48	8.3	1.4 (B)

[a] Reaction conditions were the same as in Table 1. Reactions were analyzed by HPLC at conversions values < 50% at a given time. [b] "Pseudo"-*E* values were calculated according to Chen et al.[29] Numbers in bold denote best selectivities, and the preferred diastereomer for each reaction is shown in parentheses.

Alcoholysis reactions gave more interesting results (Table 2, entries 10−18). The best results were observed with *C. rugosa* lipase and Novozym 435; both enzymes exhibited high activity in the alcoholysis of 23-*O*-acetylsilybin. Conversions of ca. 30% were reached in a few hours. These two enzymes exhibited a significantly better diastereoselectivity than in transesterification reactions, showing again opposite diastereospecificity. *C. rugosa* lipase produced enriched silybin A (**1a**) with $E = 21.1$ (Figure 1C), while Novozym 435 preferentially cleaved acetate **2b**, thus producing diastereomerically enriched silybin B (**1b**, Figure 1D) with $E =$ 6.5. Therefore, we focused on the optimization and scale up of alcoholysis reactions catalyzed by *C. rugosa* lipase and Novozym 435.

23-*O*-Acylsilybin Alcoholysis Reactions Catalyzed by *C. rugosa* Lipase and Novozym 435. The influence of the acyl chain length on the conversion and selectivity of alcoholysis reactions was tested. Alcoholysis of 23-*O*-butyrylsilybin (**3**) and 23-*O*-octanoylsilybin (**4**) by *C. rugosa* lipase and Novozym 435 was carried out using the same protocol as for the 23-*O*-acetyl derivative **2** (Table 3). Both enzymes exhibited similar behavior, and the best diastereoselectivity was observed with **2**. With **3**, the reactions were faster, but slightly less selective. Octanoate **4** gave low selectivity and conversion rates. This, together with the higher yields in the preparation of **2**, demonstrated that further work should be done with acetyl derivatives.

Influence of Co-solvents on 23-*O*-Acetylsilybin Alcoholysis Reactions Catalyzed by *C. rugosa* Lipase and Novozym 435. The solvent may significantly influence enzyme selectivity,[30] and accordingly, the alcoholysis reactions were performed in different reaction media. Conversion with Novozym 435 was observed in the presence of all the tested co-solvents with the exception of acetonitrile and acetone (Table 4). The best results, both conversion and *E*, were obtained with toluene, MTBE, and *tert*-amyl alcohol, similar conversions and *E* values being achieved after 3−5 h. No significant improvement in *E* was observed when the temperature was lowered to 25 °C. In the case of *C. rugosa* lipase, only toluene (27% conversion after 4 h, $E = 40$) and MTBE (29% conversion after 5 h, $E = 21$) were suitable solvents.

Scale-up of the Enzymatic Process. For the scale-up, various batches of the two enzymes were tested. However, only the first and rather old batch of *C. rugosa* lipase (Sigma, lot 074K0685) was able to perform the alcoholysis reaction with the appropriate activity and selectivity, whereas two fresher batches (Sigma, lots 1392670 and 1401362) exhibited negligible activity and selectivity.

The presence of various isoenzymes in commercial *C. rugosa* lipase preparations has been often described,[31] and specifically, it was demonstrated that a contaminant esterase activity might be

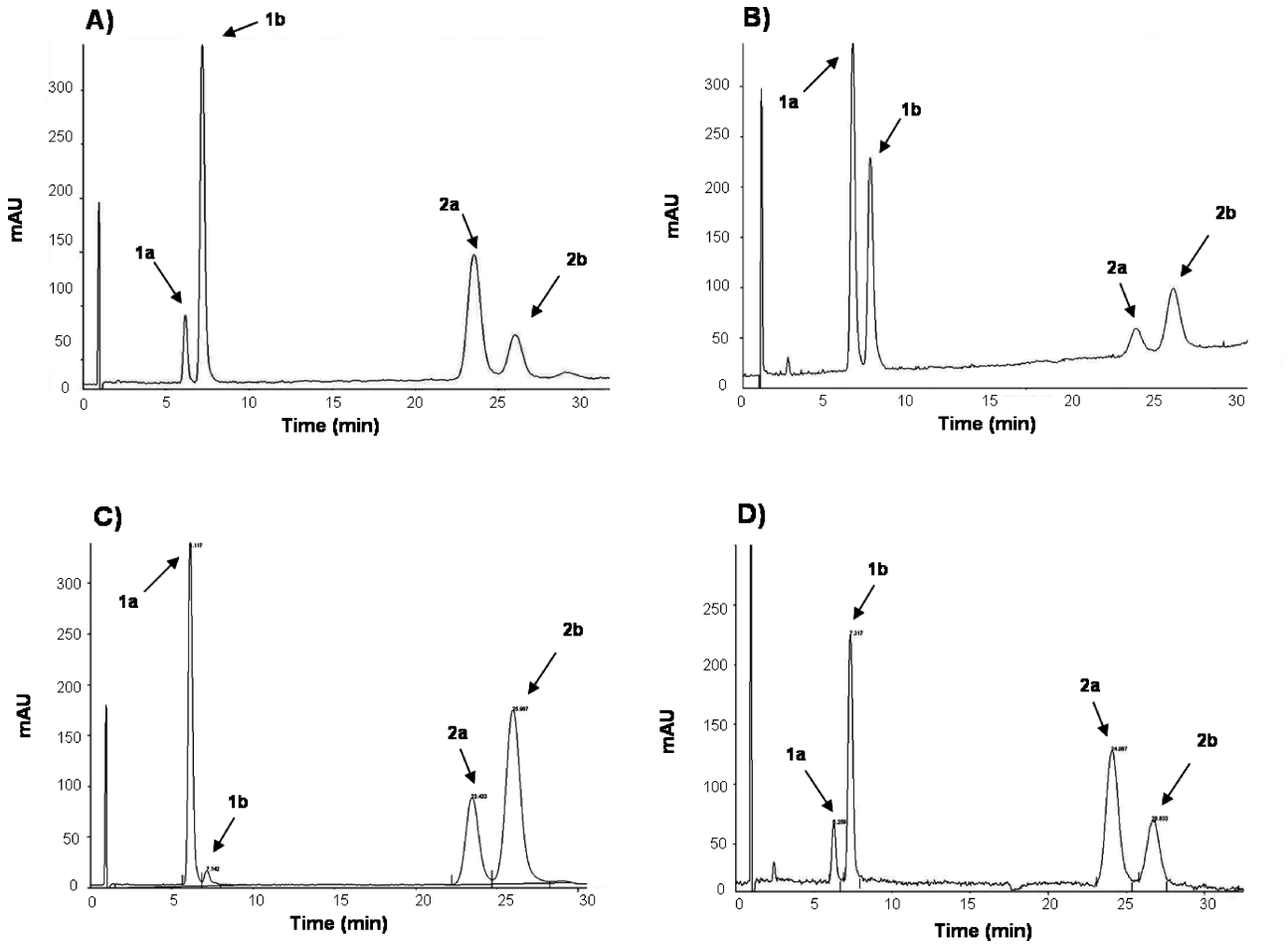


Figure 1. Transacetylation reactions of silybin (**1**) and alcoholysis reactions of 23-*O*-acetylsilybin (**2**) catalyzed by *C. rugosa* lipase (A and C, respectively) and Novozym 435 (B and D, respectively). **1a**, silybin A; **1b**, silybin B; **2a**, 23-*O*-acetylsilybin A; **2b**, 23-*O*-acetylsilybin B. HPLC: Chromolith Speed ROD, RP-18e analytical column (50 × 4.6 mm), mobile phase CH_3CN/MeOH/H_2O/TFA, 2:37:61:0.1, flow rate 0.9 mL/min at 25 °C, detection at 285 nm.

Table 3. Diastereomeric Selectivity of *C. rugosa* Lipase and Novozym 435 toward 23-*O*-Acylsilybins in Alcoholysis Reactions: Influence of Acyl Length[a]

enzyme	substrate	time [h]	conversion [%]	*E*
C. rugosa lipase	23-*O*-acetylsilybin (**2**)	5	29	21.1
	23-*O*-butyrylsilybin (**3**)	1	22	14.7
	23-*O*-octanoylsilybin (**4**)	29	13	3.4
Novozym 435	23-*O*-acetylsilybin (**2**)	3	34	6.5
	23-*O*-butyrylsilybin (**3**)	1	25	5.3
	23-*O*-octanoylsilybin (**4**)	29	7	4.1

[a] Reaction conditions: Respective acylsilybin (9.7 μmol) was dissolved in a mixture of MTBE (1 mL) and *n*-butanol (0.1 mL, 1.09 mmol), *C. rugosa* lipase (50 mg of powder) or Novozym 435 (5 mg) was added, and the suspension was shaken at 45 °C.

Table 4. Screening of Organic Co-solvents in Alcoholysis of **2** Catalyzed by Novozym 435

co-solvent[a]	time (h)	conversion (%)	*E*
toluene	5	40	7.7
MTBE	3	34	6.5
THF	5	13	1.9
dioxane	5	6	3.6
tert-amyl alcohol	4	46	7.0
acetonitrile	24	0	0
acetone	24	0	0

[a] Reaction conditions: 23-*O*-Acetylsilybin (**2**, 5 mg, 9.7 μmol) was dissolved in a mixture of co-solvent (1 mL) and *n*-butanol (0.1 mL, 1.09 mmol), Novozym 435 (5 mg) was added, and the suspensions were shaken at 45 °C and 250 rpm.

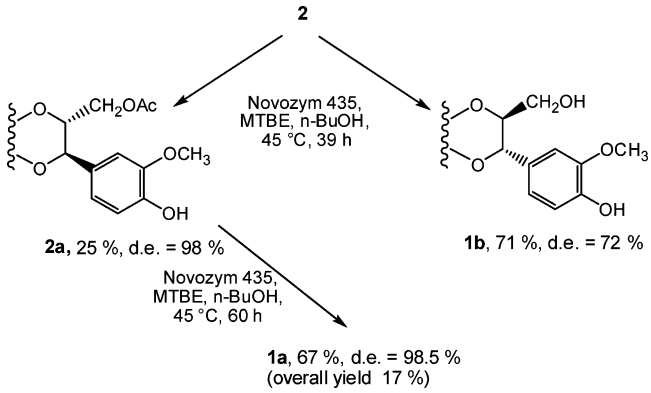
responsible for the observed enantioselectivity.[32] We assumed that the efficient alcoholysis of **2** catalyzed by the "old" *C. rugosa* lipase preparation could have been due to a contaminant hydrolase and not to the major lipase.

On the contrary, various batches of Novozym 435 exhibited reasonable reproducibility. Three distinct batches differed in their activities by approximately 20%, but the selectivity of the alcoholysis was completely retained. Thus, further optimization and scale-up of separation of the silybin stereomers was performed with this biocatalyst and silybin acetate **2**.

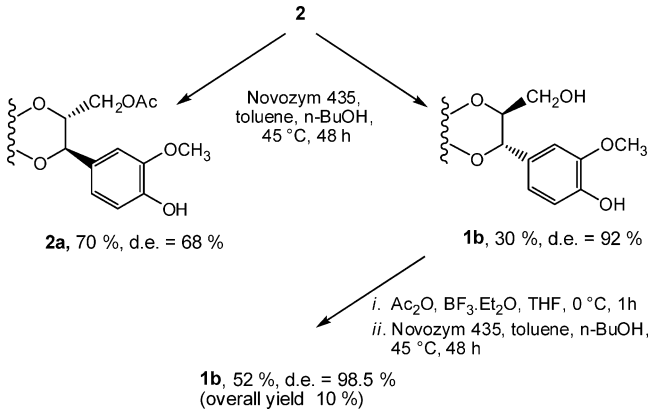
Preparative Separation of Silybin Stereomers with Novozym 435. To develop a suitable method of separation of **1a** and **1b** to a purity of over 95% de, by Novozym 435-catalyzed alcoholysis, a range of conditions and several solvent systems were tested. For verification of the scalability of the results obtained during the previous screening, 300 mg batches of acetate **2** were used during the optimization of reaction conditions.

The composition of the reaction mixtures using MTBE or toluene as co-solvents and the optimal amounts of enzyme at 45 °C were analyzed by HPLC for 72−84 h. The reaction in MTBE/*n*-butanol proceeded with good diastereospecificity toward **2b** during the first 24 h, but later the second diastereomer (**2a**) started to be slowly cleaved. However, we reasoned that the extension of the reaction time for another 15−24 h, depending on the activity of different batches of Novozym 435 under these conditions, could be exploited

Scheme 2. Optimized Preparative Method for Production of Silybin A (**1a**)



Scheme 3. Optimized Preparative Method for Production of Silybin B (**1b**)



to recover the unreacted **2a** with high enough diastereomeric purity to subsequently prepare pure **1a** as the product of a deacetylation reaction.

Alcoholysis of **2b** in the toluene/*n*-butanol mixture was more selective compared to the reaction in MTBE/*n*-butanol, as **2a** was only slightly cleaved over 72 h, independently of the remaining amount of **2b**. However, even after 84 h of reaction, quantitative cleavage of **2b** did not occur. This makes these conditions unsuitable for obtaining pure **2a**, whereas they could be used for recovering optically enriched **1b**.

The methods for the production of **1a** and **1b** with high diastereomeric purity (Schemes 2 and 3, respectively) were then optimized on a preparative scale.

Silybin A (**1a**) was prepared from highly pure **2a** (de = 98%) recovered from the previously described alcoholysis reaction in MTBE/*n*-butanol (Scheme 2). Acid-catalyzed deacetylation should be used to avoid basic conditions (e.g., Zemplén deacetylation), causing decomposition and rapid oxidation of silybin derivatives. However, acidic hydrolysis also leads to a slight decomposition of silybin and the formation of unknown impurities. Therefore, enzymatic alcoholysis of pure **2a** using an excess of Novozym 435 (75% (w/w), catalyst/substrate **2a**) was used to deacetylate the product in a clean way, which further slightly improved optical purity of **1a**. The yield of this method is good, mostly due to the fact that a single chromatographic separation is employed. The overall yield is 17%, which matches 34% of the theoretical yield, as the starting **2** contains ca. 50% of each diastereomer. Additionally, enriched silybin B (**1b**, 71%, de = 72%) can be recovered.

For silybin B an alternative method employing toluene instead of MTBE as a co-solvent (Scheme 3) was used. A single-step separation directly yielded silybin B (**1b**) in 30% yields, but with somehow lower diastereomeric purity (de 92%). The method can be repeated to obtain high-purity silybin B (**1b**), which lowers the overall yield of the reaction sequence including the second acetylation and alcoholysis to ca. 10% but allows the recovery of the target product with a de greater than 98% (Scheme 3). The remaining 23-*O*-acetylsilybin B (**2b**, de 79%) may be subjected to further alcoholysis, affording thus another portion of pure **1b** (de > 95%).

Conclusions

We present an elegant chemoenzymatic method for the separation of silybins A and B using concerted methods of chemical acylation and enzymatic alcoholysis catalyzed by Novozym 435 (lipase B from *C. antarctica*). The method is robust, uses nontoxic and inexpensive chemicals that are all commercially available, and is fully scalable. Such a method enables multigram quantities of silybins A and B to be produced in the laboratory. The procedure greatly increases the availability of optically pure silybins, allowing biological experiments aimed at determining the molecular targets of silybins.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Jasco P-2000 polarimeter (27 °C, c = 0.3, acetone) (Cremella, IT), and CD spectra were recorded on a Jasco J600 spectropolarimeter (27 °C, 1 mg/mL, MeOH) (Cremella, IT) interfaced to a personal computer for data acquisition and processing. NMR 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 30 °C. Chemical shifts were referenced to the residual solvent signal (δ_H 7.265, δ_C 77.00; δ_H 2.50, δ_C 39.60). All 2D NMR experiments (HOM2DJ, gCOSY, TOCSY, HMQC, and HMBC) were performed using the standard manufacturer's software. The sequence for 1D-TOCSY experiments was obtained through the Varian User Library; the gHMQC sequence was obtained from the Varian Application Laboratory in Darmstadt (DE). Positive-ion electrospray ionization (ESI) mass spectra were recorded on a LC^{Q}DECA spectrometer (ThermoQuest, San Jose, CA). HRMS measurements were performed on a commercial APEX-Ultra FTMS instrument equipped with a 9.4 T superconducting magnet and a Dual II ESI/MALDI ion source (Bruker Daltonics, Billerica, MA) using electrospray ionization. The interpretation of mass spectra was done using the software package DataAnalysis version 3.4 (Bruker Daltonics, Billerica, MA). HPLC analyses were carried out on a Shimadzu Prominence LC analytical system consisting of a Shimadzu LC-20AD binary HPLC pump, a Shimadzu SIL-20AC cooling autosampler, a Shimadzu CTO-10AS column oven and a Shimadzu SPD-20MA diode array detector (Shimadzu, Kyoto, Japan). A monolithic column Chromolith SpeedROD, RP-18e, 50 × 4.6 mm equipped with a guard column (5 × 4.6 mm) (Merck, DE) with an isocratic mobile phase of CH_3CN/MeOH/H_2O/TFA, 2:37:61:0.1, was used (flow rate 0.9 mL/min at 25 °C, detection at 285 nm (+ scan 200−365 nm)). t_R (silybin A) = 6.1 min, t_R (silybin B) = 7.1 min, t_R (23-*O*-acetylsilybin A) = 23.5 min, t_R (23-*O*-acetylsilybin B) = 25.9 min.

Materials. Silybin (mixture of both diastereomers A (**1a**) and B (**1b**), ca. 1:1) was kindly provided by Dr. L. Cvak (TEVA Czech Industry, TAPI division, Opava, CZ). Lipases from *Pseudomonas cepacia* (lipase PS), *Pseudomonas* sp. (lipase AK), *Rhizopus delemar* (lipase D), *Candida lipolytica* (lipase L), *Mucor javanicus* (lipase M), *R. oryzae* (lipase F-AP15), *R. niveus* (lipase N), *Penicillium roquefortii* (lipase R), *Chromobacterium viscosum* (lipase CV), *Mucor miehei*, *Humicola lanuginosa* (lipase CE), *Aspergillus niger* (lipase A), and *Geotrichum candidum* (lipase GC) and proteases from *Bacillus subtilis* (protease N, Proleather, and protease P), and *R. niveus* (acid protease II) were from Amano (Amano Enzyme Europe Ltd., Oxfordshire, UK), as well as the *A. niger* acylase Amano 3000. *Candida antarctica* lipase A (CAL-A) and lipase B (Novozym 435) were from Novozymes (Bagsvaerd, DK). Lipases from wheat germ, *Candida rugosa*, and porcine pancreas (PPL) and subtilisin A from *Bacillus* sp. (subtilisin Calsberg) were from Sigma; *R. japonicus* lipase was from Biocatalysts Ltd. (Cardiff, UK). All other reagents were of analytical grade from Sigma-Aldrich.

23-*O*-Acetylsilybin (2). Compound **2** was prepared under optimized conditions according to a reported procedure.[18] Dry silybin (**1**, 1 g, 2.07 mmol) was dissolved in THF (50 mL), and the solution was cooled to 0 °C. Acetic anhydride (1.5 mL, 15.86 mmol) and $BF_3 \cdot Et_2O$ (1.0

mL, 50% solution in Et_2O) were added, and the reaction mixture was stirred for 1 h at 0 °C. The reaction mixture was then diluted with a saturated aqueous solution of $NaHCO_3$ and extracted with EtOAc (2 × 100 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated. Flash chromatography (chloroform/acetone/formic acid, 95:5:1) yielded the title compound **2** (654 mg, 60%) as a white, amorphous solid: $[\alpha]_D^{27}$ and CD spectra, see Supporting Information (Table S1, Figure S1); ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz), see Supporting Information (Tables S2, S3); ESI MS *m*/*z* 525 $[M + H]^+$.

23-*O*-Butyrylsilybin (3). Dry silybin (**1**, 1 g, 2.07 mmol) was dissolved in a mixture of CH_3CN/CH_2Cl_2, 1:1 (100 mL). Butyryl chloride (0.215 mL, 2.07 mmol) and $BF_3 \cdot Et_2O$ (0.62 mL, 2.48 mmol, 50% (v/v) solution in Et_2O) were added, and the mixture was stirred for 2 h at 0 °C. After this period, additional $BF_3 \cdot Et_2O$ (0.62 mL, 2.48 mmol, 50% (v/v) solution in Et_2O) was added and the stirring continued for 1 h at room temperature. The mixture was then diluted with a saturated ice-cold solution of $NaHCO_3$ (150 mL) and briefly stirred (ca. 10 min). The solution was extracted with EtOAc (2 × 150 mL), and the organic phase was washed with brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. Flash chromatography on silica gel ($CHCl_3$/acetone/HCO_2H, 95:5:1) yielded the title compound (**3**, 321 mg, 28%): ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz), see Supporting Information (Tables S2, S3); HRMS (MALDI) *m*/*z* 552.1615 (calcd for $C_{29}H_{28}O_{11}$ (M^+), 552.1632).

23-*O*-Octanoylsilybin (4). Octanoyl chloride was prepared by the addition of oxalyl chloride (10.4 mL, 20.81 mmol, 2 M solution in CH_2Cl_2) to a solution of octanoic acid (1 g, 6.93 mmol) in dry CH_2Cl_2 (20 mL) under Ar. The mixture was stirred for 3 h at room temperature, and the solvent was evaporated, yielding octanoyl chloride. Octanoyl chloride (169 mg, 1.04 mmol) and $BF_3 \cdot Et_2O$ (0.310 mL, 1.14 mmol, 50% (v/v) solution in Et_2O) were added to a solution of dry silybin (**1**, 0.5 g, 1.04 mmol) dissolved in CH_3CN/CH_2Cl_2, 1:1 (50 mL), under Ar, and the mixture was stirred for 2 h at 0 °C. Additional $BF_3 \cdot Et_2O$ (0.310 mL, 1.24 mmol, 50% (v/v) solution in Et_2O) was added, and the reaction mixture was stirred for 1 h at room temperature and monitored by TLC ($CHCl_3$/acetone/HCO_2H/toluene, 12:2:1:1). The reaction was stopped by dilution with a saturated ice-cold solution of $NaHCO_3$ (50 mL) and briefly stirred. The products were extracted with EtOAc (2 × 50 mL), and the organic phase was washed with saturated NaCl solution, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. Flash chromatography ($CHCl_3$/acetone/HCO_2H, 95:5:1) yielded the title compound (**4**, 0.189 g, 30%) as a white, amorphous solid: ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz), see Supporting Information (Tables S2, S3); HRMS (MALDI) *m*/*z* 608.2241 (calcd for $C_{33}H_{36}O_{11}$ (M^+), 608.2258).

Screening of Hydrolases for 23-*O*-Acetylsilybin (2) Alcoholysis. To a solution of **2** (5 mg, 9.7 μmol) in *tert*-butylmethyl ether (MTBE, 1 mL) were added *n*-butanol (0.1 mL, 1.09 mmol) and the respective hydrolase preparation (50 mg of powder, except for Novozym 435, where 5 mg was added), and the suspensions were shaken at 45 °C and 250 rpm for 48 h. Reactions were monitored by TLC ($CHCl_3$/acetone/HCO_2H, 9:2:1) and HPLC. Only reactions with a conversion of over 2% within 48 h were repeated and analyzed by HPLC for conversions < 50% in order to evaluate the "pseudo"-*E* values of the respective reactions from the diastereoisomeric excess of substrates (de_S) and products (de_P).[29]

Screening of Hydrolases for Silybin (1) Transacetylation. Vinyl acetate (0.1 mL, 1.08 mmol) and a suitable amount of a commercially available hydrolase preparation (50 mg of powder, with the exception of Novozym 435, for which 5 mg was used) were added to a solution of **1** (5 mg, 10.4 μmol) in MTBE (1 mL), and the suspensions were shaken at 45 °C and 250 rpm for 48 h. Reactions were monitored by TLC and HPLC as previously described, and those giving conversions < 2% after 48 h were discarded. "Positive" reactions (conversions > 2% in 48 h) were repeated and analyzed by HPLC at conversions < 50% in order to evaluate the "pseudo"-*E* values of the respective reactions.

Examination of the Influence of the Acyl Length in the Alcoholysis Reactions Catalyzed by *C. rugosa* Lipase and Novozym 435. 23-*O*-Butyrylsilybin (**3**) and 23-*O*-octanoylsilybin (**4**) were subjected to alcoholysis reactions catalyzed by *C. rugosa* lipase and Novozym 435 using the same protocol previously described for the 23-*O*-acetyl derivative **2**. Conversions were evaluated by HPLC (Chromolith SpeedROD, RP-18e, 50 × 4.6 mm, (Merck); flow rate 1 mL/min at 25 °C, detection 285 nm) with the following isocratic mobile phases: **3**, CH_3CN/MeOH/H_2O/TFA, 24:30:46:0.1; **4**, CH_3CN/MeOH/H_2O/TFA, 31:27:42:0.1. The diastereoisomeric excess of the respective silybin stereoisomers (de_P) with conversion < 50% was evaluated by HPLC using the same stationary phase (column) and CH_3CN/MeOH/H_2O/TFA, 2:37:61:0.1, as a mobile phase. The "pseudo"-*E* values were evaluated from the conversion and de_P values of the corresponding reactions.[29]

Alcoholysis reactions of **2** catalyzed by *C. rugosa* lipase and Novozym 435 were performed in the presence of various organic solvents (i.e., MTBE, toluene, THF, dioxane, *tert*-amyl alcohol, CH_3CN, acetone) using the same reaction conditions and controlled by HPLC as previously described for the hydrolase screening. The "pseudo"-*E* values were evaluated from the de_S and de_P values of the respective reactions.

Silybin A (1a). Novozym 435 (652 mg, ≥10 000 U/g, 15% w/w, catalyst/substrate **2**) was added to a solution of 23-*O*-acetylsilybin (**2**, 4.34 g, 8.29 mmol) in MTBE/*n*-BuOH, 9:1 (160 mL), and the mixture was shaken at 45 °C and 650 rpm for 48 h. After enzyme removal, the solution was evaporated and the crude mixture purified by column chromatography ($CHCl_3$/acetone/HCO_2H, 90:10:1), yielding silybin B (**1b**, 2.84 g, 71%, de = 72%), which could be recovered, and 23-*O*-acetylsilybin A (**2a**, 1.07 g, 25%, de = 98%). The resulting 23-*O*-acetylsilybin A (**2a**, 1.07 g, 2.04 mmol) was dissolved in MTBE/*n*-BuOH, 9:1 (40 mL), Novozym 435 (803 mg, ≥10 000 U/g, 75% (w/w), catalyst/substrate **2**) was added, and the mixture was shaken at 45 °C and 650 rpm for 60 h. The enzyme was filtered off, solvents were evaporated, and the crude mixture was purified by column chromatography ($CHCl_3$/acetone/HCO_2H, 90:10:1), yielding silybin A (**1a**, 0.66 g, 67%, de = 98.5%), $[\alpha]_D^{27}$ + 16.3 (*c* 0.3, acetone); CD spectra, see Supporting Information (Figure S1).

Silybin B (1b). Novozym 435 (400 mg, ≥10 000 U/g, 30% w/w, catalyst/substrate **2**) was added to a solution of 23-*O*-acetylsilybin (**2**, 1.2 g, 2.49 mmol) in toluene/*n*-BuOH, 10:1 (44 mL), and the resulting mixture was shaken at 45 °C and 650 rpm for 48 h. The enzyme was then removed by filtration, the resulting filtrate was evaporated to dryness, and the solid residue was purified by column chromatography ($CHCl_3$/acetone/HCO_2H, 90:10:1), yielding silybin B (**1b**, 330 mg, 30%, de = 92%) and 23-*O*-acetylsilybin A (**2a**, 840 mg, 70%, de = 68%).

The resulting silybin B (**1b**, 330 mg, de = 92%) was reacetylated as previously described, yielding optically enriched 23-*O*-acetylsilybin B (**2b**, 215 mg, 0.446 mmol, 60%, de = 92%). This silybin acetate was again treated with Novozym 435 (65 mg, 30% w/w, catalyst/substrate) in toluene/*n*-BuOH, 10:1 (15 mL), and the mixture was shaken at 45 °C and 650 rpm for 48 h. The enzyme was removed and the filtrate evaporated and chromatographed ($CHCl_3$/acetone/HCO_2H, 90:10:1) to yield silybin B (**1b**, 112 mg, 52%, de = 98.5%), $[\alpha]_D^{27}$ +4.0 (*c* 0.3, acetone); CD spectra, Supporting Information (Figure S1). 23-*O*-Acetylsilybin B (**2b**, 99 mg, 46%, de = 79%) was also obtained.

Acknowledgment. This work was supported by grant P207/10/0288 from the Czech Science Foundation, ESF COST Chemistry actions CM0701 and CM0602 (grants MSMT OC08049 and LC06010), by institutional research concept AV0Z50200510, and by the bilateral Czech-Italian Inter-Academic Project between CNR and AVČR (D.M. and V.K.).

Supporting Information Available: OR and CD data of **1**, **1a**, **1b**, **2**, **2a**, and **2b** and ^{1}H and ^{13}C NMR data of **2**, **3**, and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP900758D