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Regioselective alcoholysis of silybin A and B acetates with lipases

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ABSTRACT

Large screening identified lipase AK to be capable of the selective deacetylation of pentaacetyl silybins A and B to yield 3,5,20,23-tetra-*O*-acetyl-silybins A and B, and 3,20,23-tri-*O*-acetyl-silybins A and B, respectively. Deacetylation occurred at phenolic OH groups, only. These new compounds prepared from the optically pure silybins can serve as new stereochemically pure synthons for selective silybin modifications.

1. Introduction

The chemistry of flavonoids, analogously to that of other polyols like carbohydrates, is complicated by the need for complex protection/deprotection strategies. Further problems stem from the high sensitivity of flavonoids towards oxidative agents and alkaline conditions, and their tendency to form complexes with some metal cations. This strongly limits the use of certain protection/deprotection strategies. The use of enzymes can circumvent these limitations, both in terms of selectivity and mild conditions; and is also compatible with food and drug applications.

Lipases, which are obvious candidates for the selective derivatization of flavonoids, have been often used for selective modifications of various natural compounds [1,2].

Most of the so far described lipase-catalyzed acylations were focused on flavonoid glycoside acylations where the acyl preferentially substitutes the primary OH group of the glycone (typically glucose) [3–6]; other positions at the flavonoid moiety are seldom hit [5,7]. This was also demonstrated by molecular modeling using the CalB (*Candida antarctica* lipase B) and series of quercetin glycosides (e.g. rutin, isoquercitrin) [8] showing that the only position susceptible to enzymatic acylation on the aglycone part (quercetin) is its 3′-OH. A comprehensive review of enzymatic acylations of flavonoids has been recently published by Chebil et al. [9].

Studies dealing with selective lipase-catalyzed deacylations are scarce and are mostly limited to quercetin, catechin and morin. Lambusta et al. [7] has demonstrated partial deacetylation of quercetin and catechin pentaacetates with three lipases (from *Pseudomonas cepacia*, *Mucor miehei* and *Candida rugosa*). Another study dealing with lipase-catalyzed (*P. cepacia* lipase) deacetylation of luteolin, kaempferol, kaempferide, and quercetin peracetates, found 7- and 4′-acetates to be preferentially removed in these types of compounds [10].

However, the use of lipases for the modification of flavonoids is complicated by technical limitations, e.g. low solubility in water (but also in highly nonpolar solvents) that is circumvented by the use of suitable organic solvents and by another, often neglected fact, that many flavonoids are potent inhibitors of lipases. This effect has been demonstrated e.g. with 3-hydroxyflavone, 5-hydroxyflavone, catechin or kaempferol that even display their potential as inhibitors of lipases [11], or some flavonoids contained in citruses [12] and in medicinal plants [13].

The flavonolignan silybin (**1**) (CAS No. 22888-70-6) is the major component of the silymarin complex extracted from the seeds of *Silybum marianum* (L.) Gaertn. (*Carduus marianus* L., Asteraceae) (milk thistle) [14] occurring as an equimolar mixture of two diastereomers, silybin A (**1a**), and silybin B (**1b**) (Fig. 1). We have recently developed a preparatory-scale chemoenzymatic separation method [15–17], which made it possible to study biological activities with optically pure diastereomers of silybin and also to perform chemical transformations with optically pure and well defined compounds.

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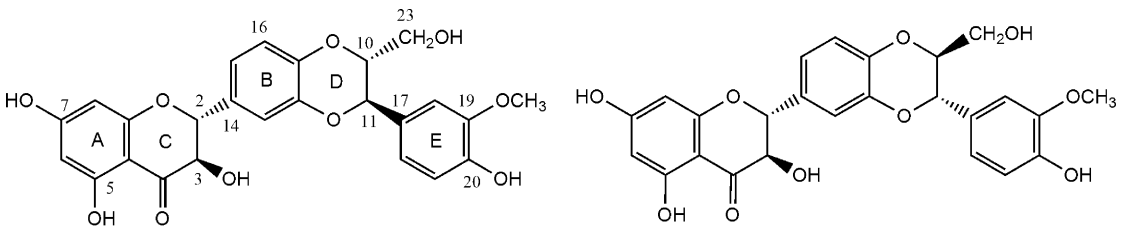


Fig. 1. Diastereomeric silybins (**1a** and **1b**).

Silybin is currently advocated for the treatment of cirrhosis, chronic hepatitis, and liver diseases associated with alcohol consumption and environmental toxin exposure [18], as it is an efficient antioxidant and chemoprotectant. Silybin is considered to be very safe and no serious adverse effects have been reported [19]. Other beneficial effects of silybin include chemopreventive as well as hypocholesterolemic, cardioprotective, and neuroprotective effects [20,21]. For example, silybin is in phase-II clinical trials in the US for the treatment of prostate adenocarcinoma [22]. Silybin activities are linked to a large number of effects at the cellular and molecular level, such as estrogenic activity, the modulation of drug transporters (P-glycoprotein) [23] and a specific action on DNA expression *via* the suppression of nuclear factor-κB [20,24]. The importance of silybin is demonstrated, for example, by the high frequency of relevant papers (ca 200 per year).

Silybin has a unique flavonoid structure, differing from most known flavonoids so far functionalized with lipases. It does not have a double bond at position 2,3 (like quercetin or rutin), which makes it very sensitive towards oxidation even under weak alkaline conditions [25]. Being a flavonolignan, it possesses a lignan moiety with a primary alcoholic 23-OH group. These structural features intrinsically create four stereocenters and this makes silybin chemistry much more complicated than in other more simple flavonoids.

The importance of regioselectively acylated derivatives of silybin (prepared mostly by chemical synthesis) has been well established. Several acyl derivatives of silybin have significantly better biological activity than silybin [26–28], but the synthesis of derivatives other than the 23-*O*-acyl is quite problematic due to poor regioselectivity and low yields [26].

In this work, we report the screening of a library of commercially available hydrolases (lipases, proteases, and acylases) for the regioselective deacylation of peracetylated silybins. An optimized protocol for obtaining optically pure 3,5,20,23-tetra-*O*-acetyl-silybins and 3,20,23-tri-*O*-acetyl-silybins using lipase AK from *Pseudomonas* sp. is described.

2. Experimental

2.1. General methods

NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (600.23 MHz for ^{1}H, 150.93 MHz for ^{13}C at 30 °C) in DMSO-d_6 (99.9 at.% D, Sigma–Aldrich, Steinheim, DE). The residual signal of the solvent was used as an internal standard (δ_H 2.500 ppm, δ_C 39.60 ppm). NMR experiments: COSY, HSQC, and HMBC were performed using the manufacturer's software. ^{1}H NMR and ^{13}C NMR spectra were zero-filled to fourfold data points and multiplied by a window function prior to Fourier transformation. A two-parameter double-exponential Lorentz–Gauss function was applied for ^{1}H to improve resolution and line broadening (1 Hz) was applied to obtain a better ^{13}C signal-to-noise ratio. Chemical shifts are given on a δ-scale with digital resolution justifying the reported values to three (δ_H) or two (δ_C) decimal places.

2.2. HPLC

Chromatographic analyses were performed on a Shimadzu Prominence system (Kyoto, JP) consisting of a DGU-20A mobile phase degasser, two LC-20AD solvent delivery units, a SIL-20AC cooling autosampler, CTO-10AS column oven and SPD-M20A diode array detector with semi-micro cell volume (2.5 μl). Shimadzu LC solution software (Kyoto, JP) was used to collect and process chromatographic data.

A Chromolith Performance RP-18e monolithic column (100 mm × 3 mm i.d., Merck, DE) was used with solvent system A: MeCN/MeOH/H_2O/HCO_2H (2/37/61/0.1, v/v/v/v) and B: 100% MeCN. The optimal gradient was 0–4 min 0% B, 4–25 min 0–40% B, 25–26 min 40% B, 26–28 min 40–0% B; flow rate 1.2 ml/min. The column oven temperature was set to 25 °C and the samples were kept at 20 °C in the autosampler. The PDA data was acquired in the 200–350 nm range and the 285 nm signal was extracted.

2.3. Chemistry

2.3.1. 3,5,7,20,23-Penta-O-acetyl-silybin (2)

Natural silybin (mixture **1a** and **1b**, 1:1) (**1**, 500 mg, 1.04 mmol) was acetylated using the standard acetylation procedure (Ac_2O/pyridine 4 ml, 1:1, v/v, overnight) [29], which yielded 705 mg (98.2%) of the title compound **2** as a yellow amorphous solid. Acetylations of pure silybins A (**1a**) and B (**1b**), prepared as described in [17], were performed analogously yielding peracetates **2a** and **2b**, respectively. The ^{1}H and ^{13}C NMR data for **2a** and **2b**, which was compatible with their structures, is given in supplementary material.

2.3.2. Screening of enzymatic alcoholysis of 3,5,7,20,23-penta-O-acetyl-silybin (2)

The screening of lipases for the alcoholysis of silybin peracetate **2** was accomplished by dissolving **2** (5 mg, 7.2 μmol) in a mixture of *tert*-butyl methyl ether (MTBE) or toluene/*n*-butanol (1.1 ml, 10:1, v/v) containing the respective lipase (5 mg; see Table 1). The reaction mixture was incubated at 45 °C and 450 rpm in a Thermomixer (Eppendorf, DE), reaction progress was monitored by TLC (mobile phase: $CHCl_3$/toluene/acetone/HCO_2H—80:10:5:1) and HPLC. No diastereomeric selectivity, e.g. discrimination between **2a** and **2b**, was observed in any enzymatic deacetylation.

2.3.3. Preparative reactions catalyzed by lipase AK

3,5,7,20,23-Penta-*O*-acetyl-silybin (**2**, 100 mg, 0.144 mmol) was dissolved in a mixture of MTBE/*n*-butanol (22 ml, 10:1, v/v) with the addition of lipase AK from *Pseudomonas* sp. (Amano, JP) (100 mg). The reaction mixture was incubated in a 50 ml Falcon tube at 45 °C under shaking (450 rpm) for 48 h. The residue after filtration and evaporation was purified by column chromatography on silica gel (mobile phase: $CHCl_3$/acetone/toluene/HCO_2H—95:5:5:1) yielding 3,20,23-tri-*O*-acetyl-silybin (**3**, 9 mg, 15.3%) and 3,5,20,23-tetra-*O*-acetyl-silybin (**4**, 42 mg, 77.9%), both as yellowish amorphous

Table 1
Hydrolases tested for silybin peracetate (**2**) alcoholysis.[a]

Enzyme	Source	Producer	MTBE	toluene
Lipase AK	*Pseudomonas* sp.	*Amano*	●	●
Lipase PS	*Pseudomonas cepacia*	*Amano*	●	●
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.*		
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*		●
CAL-B	*Candida antarctica* (Lip. B)	*Novozymes*	●	
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: MTBE (48 h), toluene (150 h); ●—conversion > 2%.

solids; some unreacted **2** was recovered (yields are given in relation to the substrate consumed). **3**: MALDI MS (*m*/*z*) 631 $[M+Na]^+$ (calc. 631.1), **4**: 673 $[M+Na]^+$ (calc. 673.2). Optically pure 3,20,23-tri-*O*-acetyl-silybin A (**3a**, 8 mg, 21.5%) and 3,20,23-tri-*O*-acetyl-silybin B (**3b**, 20 mg, 28.9%), 3,5,20,23-tetra-*O*-acetyl-silybin A (**4a**, 24 mg, 70.5%) and 3,5,20,23-tetra-*O*-acetyl-silybin B (**4b**, 40 mg, 63.1%) were prepared analogously as above. ^{1}H and ^{13}C NMR data for compounds **3**, **3a**, **3b**, and **4**, **4a**, **4b** are given in supplementary material.

3. Results and discussion

The aim of this study was to investigate selective deprotection of silybin peracetate with the possibility of preparing synthons for further silybin derivatization. The field of silybin chemistry is experiencing dynamic progress triggered by recent biological discoveries [21] as well as the availability of optically pure silybins [16,17] and their congeners. For the chemical synthesis of selectively protected derivatives of silybin, at least two types of (orthogonal) protecting groups need to be used.

The acetate group has been chosen for this acylation mainly for its high yields and feasibility—acylation with longer acyls gives considerably lower yields [16,26]. Large screening of lipases and proteases in the presence of peracetylated silybin (**2**) under various reaction conditions showed that only a limited array of enzymatic activities accept this compound as a substrate. From the 24 hydrolases tested, only eleven were active, with very low (ca 2%) conversion in most cases. Better results were achieved with the lipases PS, subtilisin and Acylase Amano 3000, where the conversion ranged from 5% to 10%. Only lipase AK gave good enough conversion rates (40%) to be used in preparatory-scale reactions.

All active lipases yielded the same major products, *i.e.*, 3,20,23-tri-*O*-acetyl-silybin (**3**) and 3,5,20,23-tetra-*O*-acetyl-silybin (**4**) (Scheme 1), whose structures were determined mainly by NMR spectroscopy. As all OH signals are visible in the ^{1}H NMR spectra of acetyl silybins measured in DMSO-d_6 and their experimental assignment by HMBC is available, the absence of some of them (compared to the parent compound) provides the first hint of the acetylation site. The downfield acylation shift is observable with the C-3 or C-23 protons. The heteronuclear coupling between H-3 or H-23 and the acetyl carbonyl is a direct proof of acetylation at these positions. The acetylation of hydroxyls at C-5 and C-7 is manifested by the chemical shift changes of C-5, C-6, C-7, C-8, and C-4a. Acetylation of hydroxyl at C-20 causes substantial changes in the carbon chemical shifts at C-19, C-20, and C-21. Furthermore, it was also possible to detect a four-bond heteronuclear correlation between the methyl protons of the acetyl group and acetylated carbon, which is direct evidence for its substitution.

Kinetic studies (Fig. 2) have proved that the products are formed sequentially, the first product being tetraacetate **4b**. Careful monitoring of the reaction and appropriate termination enables to obtain tetraacetate **4b** in ca 50% purity or triacetate **3b** in ca 90% purity, which facilitates purification. The time course of the reactions with the respective pure diastereomers does not differ substantially. Time profile of the reaction may slightly vary with different batches of Lipase AK.

Phenolic esters are readily cleaved by basic hydrolysis, in general they are more easily hydrolyzed than those of aliphatic alcohols. Basic hydrolysis of peracetylated silybin is, however, non-selective, yielding a complex mixture. Moreover, the presence of a base leads to fast decomposition of the silybin molecule. Therefore, basic hydrolysis is impracticable with silybin. Quantitative hydrolysis of silybin esters is usually achieved by refluxing the corresponding

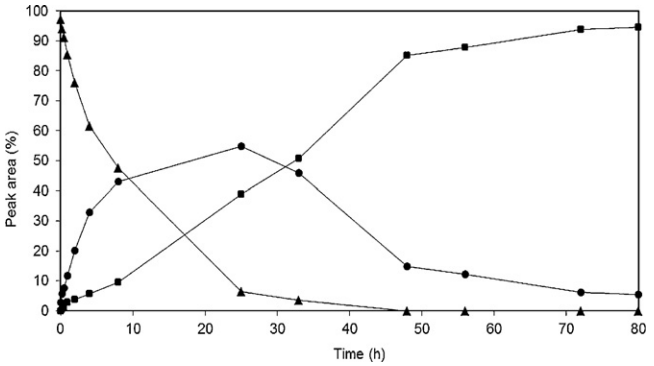
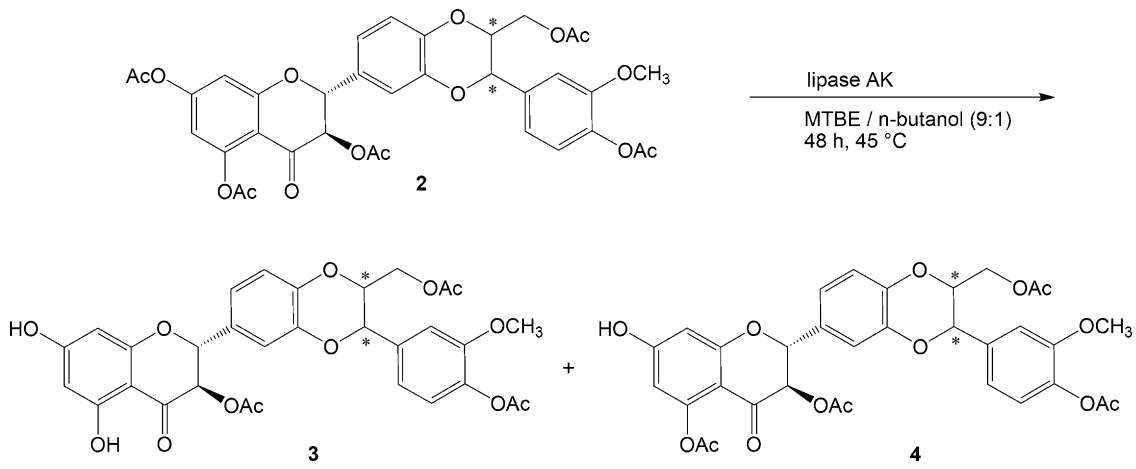


Fig. 2. Time course study of hydrolysis of **2b** (▲) at 45 °C in MTBE/*n*-butanol by lipase AK, products **3b** (■) and **4b** (●) were obtained.



Scheme 1.

ester in an ethanolic solution of conc. HCl. This method is, however, again non-selective and naturally cannot be used for selective hydrolysis. Better selectivity has been observed during attempts to remove the TBDMS group (at 23-OH) in the presence of acetates at the remaining positions of the silybin molecule [25]. The application of either an ion exchanger (Dowex 50W-X8) in MeOH or a Lewis acid ($BF_3.Et_2O$) in $CHCl_3$ led to selective hydrolysis of the TBDMS group together with the acetates at the 5- and 20-OH groups of the silybin molecule. This observation hints that these two positions are the most sensitive to acidic hydrolysis. In contrast to these chemical methods, the biotransformation method we have developed selectively cleaves the acetate at 7-OH (partially also at 5-OH). The difference between the result of the acidic and the enzymatic hydrolysis of silybin acetates is probably caused by a better (sterical) accessibility of the acetate at C-7 for the enzyme compared to other positions. Accordingly, existing studies of regioselective deacylation of simpler flavonoids (quercetin, morin) reported regioselective deacylation of the 7-OH position of the flavonoid skeleton [7,10,30].

As we used natural silybin (being an equimolar mixture of two diastereomers) for the screening we also observed the possibility of diastereomeric discrimination that was demonstrated with Novozym 435 in reactions with silybin-23-acetate [16]. However, no discrimination was observed by HPLC analyses in any positive reaction. This might also be due to the fact that alcoholysis occurred exclusively at positions C-5 and C-7, which are distant from the chiral centers at C-10, C-11. Therefore, we could use the same reaction conditions for the preparation of stereoisomerically pure synthons from silybins A and B and the corresponding tri- and tetraacetates **3a**, **3b**, and **4a**, **4b**, respectively, were isolated for the first time.

4. Conclusions

A high-yielding method for the preparation of silybin tetraacetate **4** was developed. This reaction was also tested on the optically pure silybins, yielding the respective tetraacetates **4a** and **4b** at a reasonable yield of ca 70%, whereas no diastereomeric discrimination was observed. Most of the lipases tested exhibited only limited activity towards peracetylated flavonoid **2**; however all active enzymes gave a similar spectrum of products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.04.007.

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