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Substrate specificity of lipase B from *Candida antarctica* in the synthesis of arylaliphatic glycolipids

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Abstract

Arylaliphatic glycolipids are known for their pharmaceutical and medicinal properties. We found that a great variety of arylaliphatic esters can be synthesized from non-activated substrates like glucose or the natural occurring drug salicin using lipase B from *Candida antarctica* (CAL-B). However, esters based on aromatic carboxylic acids or unsaturated arylaliphatic acids, like cinnamic acid and its derivatives, which are known to display anticancer activity, could not be obtained. In this work, we performed computer-aided molecular modeling based on data of our work published recently and syntheses of new glycolipids to understand why some substances are accepted by CAL-B while some are not. For this purpose, we investigated the accessibility of the lipase binding site for the arylaliphatic acyl donors as well as the steric interactions between the aglycons of glucosides and the residues of the alcohol binding pocket in order to elucidate potentials and limitations of CAL-B for the synthesis of aromatic glycolipids.

Keywords: *Candida antarctica* lipase B; Aromatic glycolipids; Salicin; Computer-aided molecular modeling

1. Introduction

Aromatic glycolipids are of both medical as well as pharmaceutical interest. The use of glucose esters of unsaturated arylaliphatic acids, like cinnamic acid and its hydroxylated derivatives (e.g., caffeic acid), or sugar esters of aromatic carboxylic acids (e.g., gallic acid) in tumor treatment were described [1–4]. Furthermore, alkyl and phenolic glucosides (e.g., salicin) are known for their antimicrobial, antiviral, and antiinflammatory activities [5–7]. Combining both aromatic glucose esters and phenolic glucosides led to the formation of novel aromatic arylglucoside esters exhibiting putative pharmaceutical effects [6].

These kinds of glycolipids were traditionally isolated from plants like certain *Prunus* sp., *Rheum* sp. and from the family Salicaceae. Be-

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sides extraction, some of these compounds have been obtained by biotransformations using plant cell suspension cultures of *Glycyrrhyza echinata* and *Aconitum japonicum* [8,9] as well as by cofactor-dependent enzymatic synthesis [10] encountered with low yields, undesired side-reactions, and difficult product isolation.

Recently, we exhibited that aromatic glycolipids can be obtained in moderate to good yields in a lipase-catalyzed esterification, which has been limited so far to the synthesis of simple esters derived from glucose and aliphatic long-chain fatty acids [11,12]. With our method, starting from non-activated substrates, both the acyl donor and the sugar component could be varied to a wide extent. Thus, arylaliphatic esters derived from glucose, the natural occurring drug salicin, or ascorbic acid could be synthesized. In all our studies, lipase B from *Candida antarctica* (CAL-B) was the best enzyme in terms of activity and regioselectivity and highest yields were obtained when the reaction was performed in a semi-solid system with low water content using *t*-butanol as an adjuvant [13–15].

CAL-B is a 33 kDa protein. Like most carboxylic esterases of known structure, it consists of the common structural framework such as the α/β-hydrolase fold and the catalytic triad in the active site [16]. A short helix (α5-loop) was identified as the potential lid [17]. However, in contrast to other lipases, no interfacial activation is observed for CAL-B [18]. The catalytical mechanism, thus, is similar to serine proteases forming tetrahedral intermediates [19,20]. The α/β-hydrolase fold comprises a central structural element of seven mostly parallel β-sheets which are surrounded on both sides by amphiphilic helices. The narrow and deep substrate binding pocket (10×4 Å wide and 12 Å deep, Ref. [17]) has, compared to other lipases, very limited available space in the active site pocket and can accordingly be expected to exhibit a high degree of selectivity. X-ray crystallographic studies indicate the active site pocket to be composed of two channels, one hosting the acyl-, the other hosting the alcohol moiety of the substrate. Of these channels, the acyl channel is more spacious than the alcohol channel [17]. The structure of the acyl binding site can be subdivided in a hydrophillic ''bottom'' region, including the catalytic triad, and a hydrophobic ''top'' region, the so-called hydrophobic crevice. The hydrophobic crevice consists of the residues A141, L144, V149 and I285 which are located at the top of the binding site. The bottom of the binding site is mostly hydrophilic and consists of D134, T138, Q157, and the residues of the catalytic triad (S105, D187 and H224). Residues A281, A282 and I285 point toward the alcohol moiety of the substrate and limit the size of the channel. The alcohol binding pocket is defined by the mainly hydrophobic residues W104, L278, A281, A282 and I285 [21].

CAL-B is a very versatile lipase which is catalytically active, even in polar solvents such as *t*-butanol or acetone. When immobilized, CAL-B is highly thermostable and can be used in continuous operation at 60–80°C without significant loss in activity, even after several thousand hours of use [22]. This lipase has been shown in numerous publications to be particularly efficient in both regio- and enantioselective synthesis [23–25].

Although our recently described method for CAL-B catalyzed esterifications allowed access to a wide range of aromatic glycolipids with widespread properties [1,3,7,14,26], the formation of important sugar esters based on aromatic carboxylic acids or unsaturated arylaliphatic acids, which are known to display anticancer activity, could not been achieved. It remained unknown whether this non-reactivity was due to unsuitable reaction parameters or limitations in the substrate specificity of the biocatalyst.

In the present work, we focus on molecular reasons to qualitatively understand the great substrate flexibility of CAL-B, but also the limitations observed toward certain substrates. To interpret both our recent and new experimental results, we applied computer-aided molecular

modeling methods for docking substrate structures into the active site of CAL-B.

2. Methods

2.1. Enzymes and chemicals

Immobilized CAL-B (SP435) was donated by Boehringer Mannheim (Penzberg, Germany). All chemicals were purchased from Fluka (Buchs, Switzerland), except for β-D(+)-glucose (Sigma, St. Louis, MO).

2.2. CAL-B-catalyzed synthesis of glycolipids

The lipase-catalyzed synthesis of glycolipids was performed as described earlier [13–15]. Esterification was performed in a semi-solid system with low water content using *t*-butanol (two weight equivalents of substrates) as an adjuvant, equimolar concentrations of sugar and acyl donor (for details, see below) in the presence of activated molecular sieve (3 Å, 10 mesh, half the weight of substrates). The reaction mixture was incubated in a round bottom flask, placed in an oil bath thermostated to 60°C, and stirred by a magnetic bar (250 rpm) until solidification took place. Reactions were initiated by addition of immobilized CAL-B (1.25–1.5 weight equivalents of substrates) and terminated after 48 h (with glucose) or 32 h (with other sugars). Products were isolated by extraction with 20 ml ethyl acetate:isopropanol (4:1) in case of glucose esters or with 20 ml dichloromethane in case of other sugar esters. After removal of organic solvent in vacuum, the crude products were purified by silica gel chromatography (ethyl acetate:methanol, 10:1). Identity of products was confirmed by NMR-spectroscopy. For the line of arguments, additional aromatic glycolipids had to be synthesized according to the procedure described earlier [14].

NMR shifts: non-marked (acidic part), marked with * (aglycone) or with ′ (sugar moiety).

2.2.1. 6-O-Phenylpropionyl-β-D-glucopyranose (3)

Starting from 5 mmol of glucose and 5 mmol of phenylpropionic acid: 23% yield (351 mg), white solid, R_f: 0.28 (chloroform:methanol:water, 65:15:2); ^{13}C-NMR ([D_6]DMSO): δ = 30.56 (C-2), 39.34 (C-3), 64.49 (C-6′), 69.48 (C-4′), 70.92 (C-2′), 72.51 (C-5′), 73.21 (C-3′), 92.64 (C-1), 126.41 (C-7), 128.57 (C-5, C-6, C-8, C-9), 140.87 (C-4), 172.58 (C=O). Anal. Calcd. for $C_{15}H_{20}O_7$ (312.32): C, 57.69; H, 6.45. Found: C, 58.45; H, 6.59.

2.2.2. 6-O-(3-Hydroxyphenylacetyl)-β-D-glucopyranose (7)

Starting from 5 mmol of glucose and 5 mmol of *m*-hydroxyphenylacetic acid: 18% yield (282 mg), slightly yellow solid, R_f: 0.14 (chloroform:methanol:water, 65:15:2); ^{13}C-NMR ([D_6]DMSO): δ = 40.45 (C-2), 64.42 (C-6′), 69.11 (C-4′), 70.49 (C-2′), 72.09 (C-5′), 72.79 (C-3′), 92.24 (C-1′), 113.72 (C-6), 116.15 (C-4), 119.84 (C-8), 129.20 (C-7), 135.48 (C-3), 157.21 (C-5), 171.45 (C=O). Anal. Calcd. for $C_{14}H_{18}O_8$ (314.29): C, 53.50; H, 5.77. Found: C, 53.54; H, 5.68.

2.2.3. 6-O-p-Chlorophenylacetyl-β-D-glucopyranose (9)

Starting from 5 mmol of glucose and 5 mmol of *p*-chlorophenylacetic acid: 27% yield (447 mg), white solid, R_f: 0.30 (chloroform:methanol:water, 65:15:2); ^{13}*C*-NMR ([D_6]DMSO): δ (ppm) = 40.43 (C-2), 64.86 (C-6′), 69.46 (C-4′), 70.85 (C-5′), 72.51 (C-2′), 73.18 (C-3′), 92.65 (C-1′), 128.56 (C-5, C-7), 131.57-131.92 (C-4, C-8, C-3), 133.76 (C-6), 171.29 (C=O). Anal. Calcd. for $C_{14}H_{17}O_7N$ (332.73): C, 50.54; H, 5.15; Cl, 10.65. Found: C, 50.74; H, 5.15; Cl, 10.71.

2.2.4. 6-O-p-Nitrophenylacetyl-β-D-glucopyranose (10)

Starting from 5 mmol of glucose and 5 mmol of *p*-nitrophenylacetic acid: 12% yield (212 mg), white solid, R_f: 0.26 (chloroform:me-

thanol:water, 65:15:2); ^{13}C-NMR (CD_3OD): δ (ppm) = 41.36 (C-2), 65.52 (C-6′), 70.65 (C-4′), 71.89 (C-5′), 73.82 (C-2′), 74.75 (C-3′), 94.05 (C-1′), 124.45 (C-5, C-7), 131.75 (C-4, C-8), 143.38, 144.09 (C-3, C-6), 172.15 (C=O). Anal. Calcd. for $C_{14}H_{17}O_9N$ (343.29): C, 48.99; H, 4.99; N, 4.08. Found: C, 49.22; H, 4.86; N, 4.48.

*2.2.5. 6-O-Phenylbutyryl-1-octyl-β-D-glucopyranose (**17**)*

Starting from 3.4 mmol of *n*-octylglycoside and 5.9 mmol of 4-phenyl butyric acid: 53% yield (770 mg), amorphous white solid, R_f: 0.59 (chloroform:methanol:water, 65:15:2). ^{13}C-NMR (CD_3OD): δ (ppm) = 14.07 (C-8*), 23.71 (C-7*), 27.89 (C-3*), 28.01 (C-3), 30.51–30.78 (C-2*, 4*, 5*), 33.10 (C-6*), 34.23 (C-2), 36.31 (C-4), 64.56 (C-6′), 71.02 (C-4′), 71.71 (C-1*), 75.00 (C-2′), 75.32 (C-5′), 77.98 (C-3′), 104.41 (C-1′), 126.88 (C-8), 129.53 (C-6, C-7, C-9, C-10), 142.92 (C-5), 175.01 (C=O). Anal. Calcd. for $C_{24}H_{38}O_7$ (438.59): C, 65.73; H, 8.73. Found C, 66.24; H, 8.51.

*2.2.6. 6-O-Phenylbutyryl-(1-phenyl)-β-D-glucopyranoside (**18**)*

Starting from 3.7 mmol of 1-phenyl-β-D-glucopyranoside and 6.2 mmol of 4-phenylbutyric acid: 67% yield (990 mg), amorphous white solid, R_f: 0.52 (chloroform:methanol:water, 65:15:2); ^{13}C-NMR (CD_3OD): δ (ppm) = 27.89 (C-3), 34.33 (C-2), 36.17 (C-4), 64.67 (C-6′), 71.67 (C-4′), 74.78 (C-2′), 75.34 (C-5′), 77.83 (C-3′), 102.11 (C-1′), 117.74 (C-2*, C-6*), 123.47 (C-4*), 126.89 (C-8), 129.44 (C-6, C-7, C-9, C-10), 130.81 (C-3*, C-5*), 142.87 (C-5), 158.93 (C-1*), 174.94 (C=O). Anal. Calcd. for $C_{22}H_{26}O_7$ (402.44): C, 65.66; H, 6.51. Found: C, 65.87; H, 6.65.

*2.2.7. 6-O-Octanoyl-1-[2-(hydroxymethyl) phenyl]-β-D-glucopyranoside (**20**)*

Starting from 5 mmol of 2-(hydroxymethyl)phenyl-β-D-glucopyranoside (D(−)-salicin) and 10 mmol of octanoic acid: 14% yield (297 mg), white solid, R_f: 0.47 (chloroform:methanol:water, 65:15:2); ^{13}C-NMR (CD_3OD): δ (ppm) = 14.44 (C-8), 23.67 (C-7), 26.03 (C-3), 30.12 (C-4, C-5), 32.85 (C-6), 35.04 (C-2), 60.97 (C-7*), 64.57 (C-6′), 71.65 (C-4′), 74.98 (C-2′), 75.52 (C-5′), 77.85 (C-3′), 103.25 (C-1′), 117.08 (C-6*), 123.87 (C-4*), 129.80–132.37 (C-2*, C-3*, C-5*), 156.96 (C-1*), 175.29 (C=O). Anal. Calcd. for $C_{21}H_{32}O_8$ (412.48): C, 61.15; H, 7.82. Found: C, 61.18; H, 7.79.

*2.2.8. 6-O-Lauroyl-1-[2-(hydroxymethyl) phenyl]-β-D-glucopyranoside (**21**)*

Starting from 5 mmol of D(−)-salicin and 10 mmol of lauric acid: 23% yield (532 mg), white solid, R_f: 0.48 (chloroform:methanol:water, 65:15:2); ^{13}C-NMR (CD_3OD): δ (ppm) = 14.47 (C-12), 23.74 (C-11), 26.02 (C-3), 30.22–30.74 (C-4–C-9), 33.08 (C-10), 35.05 (C-2), 60.98 (C-7*), 64.60 (C-6′), 71.66 (C-4′), 74.98 (C-2′), 75.51 (C-5′), 77.84 (C-3′), 103.23 (C-1′), 117.09 (C-6*), 123.85 (C-4*), 129.80–132.36 (C-2*, C-3*, C-5*), 156.98 (C-1*), 175.28 (C=O). Anal. Calcd. for $C_{25}H_{40}O_8$ (468.59): C, 64.08; H, 8.60. Found: C, 64.88; H, 9.02.

*2.2.9. 6-O-Palmitoyl-1-[2-(hydroxymethyl) phenyl]-β-D-glucopyranoside (**22**)*

Starting from 5 mmol of D(−)-salicin and 10 mmol of palmitic acid: 43% yield (1190 mg), white solid, R_f: 0.57 (chloroform:methanol:water, 65:15:2); ^{13}C-NMR (CD_3OD): δ (ppm) = 14.47 (C-16), 23.74 (C-15), 26.00 (C-3), 30.22-30.80 (C-4–C-13), 33.08 (C-14), 35.03 (C-2), 60.98 (C-7*), 64.59 (C-6′), 71.64 (C-4′), 74.96 (C-2′), 75.49 (C-5′), 77.82 (C-3′), 103.22 (C-1′), 117.07 (C-6*), 123.82 (C-4*), 129.78–132.34 (C-2*, C-3*, C-5*), 156.98 (C-1*), 175.23 (C=O). Anal. Calcd. for $C_{29}H_{48}O_8$ (524.69): C, 66.39; H, 9.22. Found: C, 67.88; H, 9.41.

*2.2.10. 6-O-Phenylacetyl-1-[2-(hydroxymethyl)phenyl]-β-D-glucopyranoside (**24**)*

Starting from 5 mmol of D(−)-salicin and 10 mmol of phenylacetic acid: 23% yield (507

mg), white solid, R_f: 0.43 (chloroform: methanol:water, 65:15:2); ^{13}C-NMR (CD_3OD): δ (ppm) = 41.82 (C-2), 60.99 (C-7*), 65.03 (C-6′), 71.52 (C-4′), 74.94 (C-2′), 75.49 (C-5′), 77.77 (C-3′), 103.21 (C-1′), 117.11 (C-6*), 123.91 (C-4*), 127.89 (C-6), 129.46–132.37 (C-2*, C-3*, C-5*; C-4, C-5, C-7, C-8), 136.12 (C-3), 156.95 (C-1*), 173.31 (C=O). Anal. Calcd. for $C_{21}H_{24}O_8$ (404.41): C, 62.38; H, 5.98. Found: C, 63.96; H, 5.90.

*2.2.11. 6-O-Phenylvaleryl-1-[2-(hydroxymethyl)phenyl]-β-D-glucopyranoside (**25**)*

Starting from 5 mmol of D(−)-salicin and 10 mmol of phenylvaleric acid: 28% yield (624 mg), white solid, R_f: 0.51 (chloroform: methanol:water, 65:15:2); ^{13}C-NMR (CD_3OD): δ (ppm) = 25.54, 32.03 (C-3, C-4), 34.81 (C-2), 36.47 (C-5), 60.97 (C-7*), 64.57 (C-6′), 71.62 (C-4′), 74.96 (C-2′), 75.48 (C-5′), 77.81 (C-3′), 103.17 (C-1′), 117.06 (C-6*), 123.88 (C-4*), 126.76 (C-9), 129.32–132.33 (C-2*, C-3*, C-5*; C-7, C-8, C-10, C-11), 143.41 (C-6), 156.92 (C-1*), 175.13 (C=O). Anal. Calcd. for $C_{24}H_{30}O_8$ (446.49): C, 64.56; H, 6.77. Found: C, 66.13; H, 6.99.

2.3. Computer-aided molecular modeling

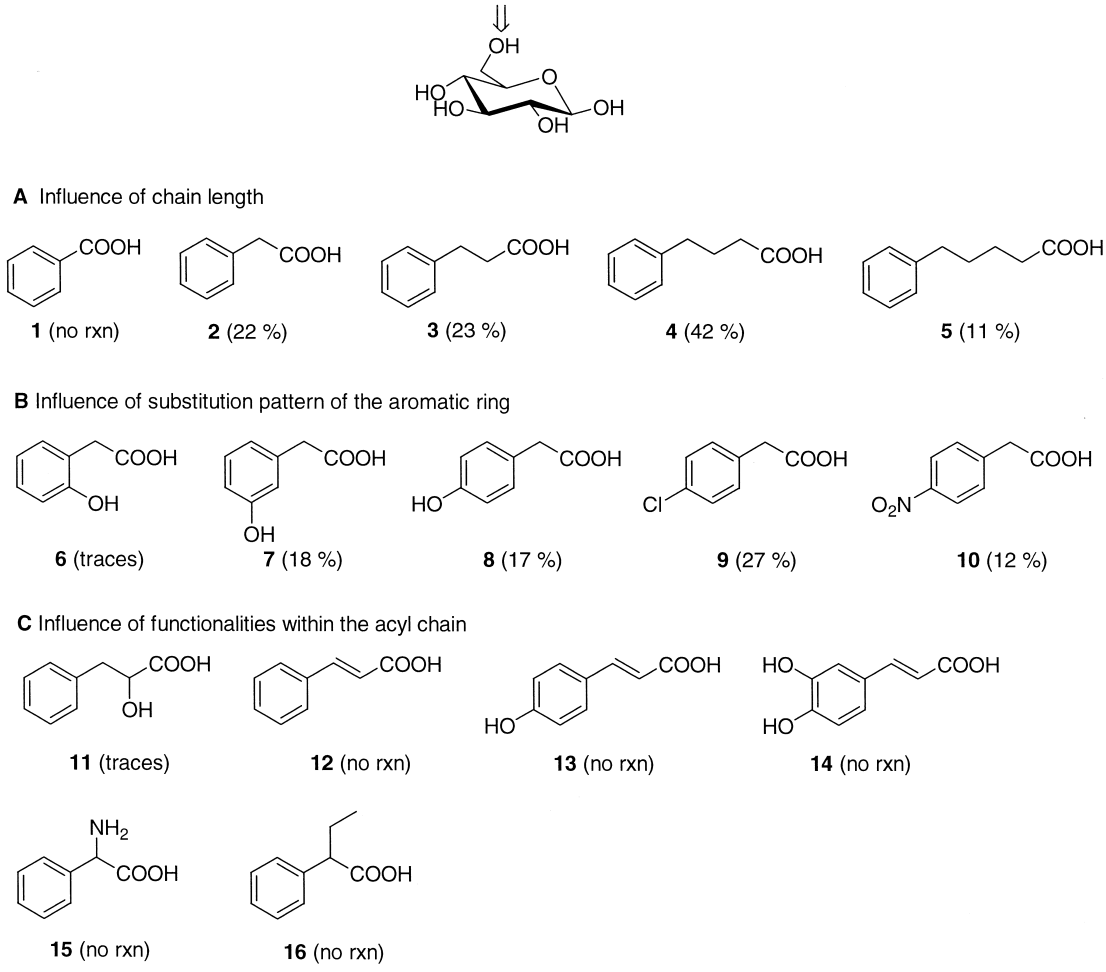
The crystal structure of the open form of CAL-B (PDB-entry 1lbs, Ref. [27]) was obtained from the Protein Data Bank (PDB, Ref. [28]). The binding site was investigated by GRID software [29]. Using the dry-, methyl- and water-probe, hydrophobic, small and non-polar as well as hydrophilic binding regions were identified, respectively. The substrates were created using the SYBYL 6.4 software package (Tripos, St. Louis, MO). The substrate was covalently bound to Oγ of the catalytically active serine S105 mimicking the first tetrahedral intermediate which is supposed to be the rate-limiting step in hydrolysis reactions of organic esters or the esterification of the respective alcohols [30]. The catalytic triad motif is wide spread in nature and was subject to various divergent evolutionary processes [31]. The catalytic mechanism of serine proteases and, therefore, lipases was intensely studied [19,20] and was found to be independent from the reaction medium [32–35]. However, one drawback of the semi-solid phase system is that initial rates cannot be determined accurately. As a consequence, the modeling results can only be qualitatively compared with experimental data based on isolated yields, since effects like mass transfer limitations and substrate solubility could not be taken into account.

Substrate docking was guided by results from GRID calculations and the positioning of the hydrophobic phosphonate inhibitor of 1lbs and related longer chain compounds [21]. Also, results of intense studies of interactions between triacylglycerols and residues in the binding site of CAL-B were taken into account (H. Scheib, unpublished data). To relax the lipase–substrate complexes, the energies were minimized performing 100 Simplexing steps, followed by 100 steps of Powell minimization. The formal charges in the imidazol ring of the catalytic histidine H224 were modified according to Holzwarth et al. [36]. During the course of an energy minimization, only bond lengths, angles and dihedrals are adjusted in order to reach a local energy minimum, while molecular dynamics simulations, ideally, lead to a global energy minimum. However, applying molecular dynamics simulations is a very CPU-time consuming procedure which was considered to be the major drawback in our studies. Therefore, energy minimizations rather than molecular dynamics studies [37,38] were performed. Moreover, since in terms of molecular modeling, a thorough knowledge of the binding geometry and possible interactions between CAL-B and the substrate was available [21,37,38], we preselected the complexes with the most likely substrate configurations in the protein binding site for energy minimization. Our modeling results gave evidence for interactions between the substrate and the protein, but are limited by the number of investigated configurations in the

substrate. Nevertheless, for the benzoic acid acyl component, molecular dynamics simulations were performed as described by Scheib et al. [37] in order to study the complex interactions between the short, aromatic and, therefore, rigid benzoic acid acyl moiety with the residues of the scissile acid binding pocket.

3. Results and discussion

We recently reported that a wide spectrum of aromatic glycolipids could be obtained using CAL-B. Glucose was esterified with several arylaliphatic acids, but also other glucosides like phenylglucoside and salicin were used as alcohol donors [14]. However, we observed that a few arylaliphatic and aromatic carboxylic acids did not react at all or only traces of product were found. In order to elucidate molecular reasons for the latter observation, we performed computer-aided molecular modeling based on recent data and syntheses of new glycolipids to qualitatively judge which substances are accepted by CAL-B in the formation of aromatic glycolipids and which are not.



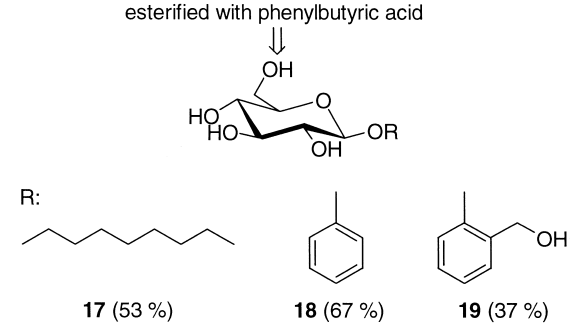
Scheme 1. Acyl donors used in the CAL-B catalyzed synthesis of glucose esters with equimolar reaction mixtures. Yields are given in brackets; traces, product detectable by thin layer chromatography; no rxn., no product detectable. Numbering of compounds refers to produced sugar esters.

The compounds could be divided into two main categories: (i) esters of glucose (**1**–**16**, Scheme 1) and (ii) esters of glycosides (**17**–**19**, Scheme 2, and **20**–**27**, Scheme 3). For computer-aided molecular modeling, substrates of group (i) were used when focused on interactions with the acyl binding pocket, substrates of group (ii) were investigated in order to evaluate the properties of the alcohol binding site.

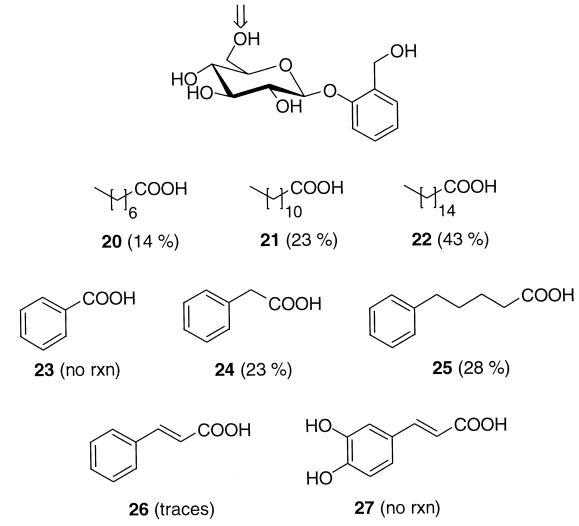
3.1. Acyl binding site / variation of the acyl moiety

Although a broad range of arylaliphatic carboxylic acids could be esterified with glucose, the isolated yields differed considerably. Moreover, some substrates could not be converted at all (Scheme 1). Since we cannot exclude the reactivity to be affected by the more hydrophilic nature of these acyl donors compared to fatty acids, we rather believe that the bulky aromatic substituent, the chain length (**1**–**5**), the substitution pattern at the aromatic ring (**6**–**10**, **13**, **14**), as well as the functionalities (**11**–**16**) between the carboxylic and the aromatic group contributed much more significantly to the reactivity.

To underline this assumption, computer-aided molecular modeling was performed with the substrates binding covalently to Oγ of the catalytically active serine S105 and, thus, mimicking the first tetrahedral intermediate. This was supposed to be the rate-limiting step in hydrolysis reactions of organic esters or the esterification of the respective alcohols [30].



Scheme 2. Glucosides **17**–**19** were acylated with phenylbutyric acid using CAL-B as catalyst. Yields are given in brackets and numbering of compounds refers to produced sugar esters.



Scheme 3. D-(−)-Salicin was esterified with both aliphatic and arylaliphatic acids using CAL-B as catalyst. Yields are given in brackets and numbering of compounds refers to produced sugar esters.

3.1.1. Influence of chain length between carboxylic group and aromatic ring

Hydrophobic van-der-Waals contacts between the substrate and the hydrophobic crevice seemed to be essential for conversion. The phenylacetic and phenylpropionic acid ester substrates (**2**) and (**3**), respectively, were found to be too short to contact the hydrophobic crevice, an aspect contributing to the observed yields of the respective phenylbutyric acid ester (**4**). The latter acyl residue reached the hydrophobic top region of the binding site, although, it has to slightly kink at C4 avoiding contact with L144 in the hydrophobic crevice (Figs. 1 and 2). A correlation between further increase of the chain length and a significantly reduced yield of the sugar monoester of phenylvaleric acid (**5**) (11% vs. 42%) was observed.

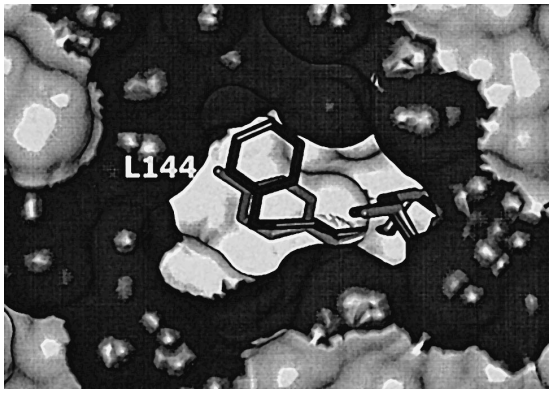


Fig. 1. Acyl binding site of CAL-B with hydrophillic bottom (white) and hydrophobic crevice (dark). The long 6-*O*-phenylvaleryl-β-D-glucopyranoside (black) is forced to kink at L144 while the smaller 6-*O*-*p*-hydroxyphenylacetyl-β-D-glucopyranoside (gray) properly fits into the binding site.

Moreover, fast formation of di-phenylvaleryl-glucose occurred. However, computer-aided molecular modeling underlined this observation such that the voluminous acyl residue of 6-*O*-phenylvaleryl-β-D-glucopyranoside properly fitted into the wide and hydrophobic alcohol binding site. In this orientation, the 2- and 3-hydroxy groups of glucose directed to the acyl binding site and can, therefore, be further esterified. Thus, both phenylbutyric and phenylvaleric acid were converted with high reaction rates coinciding with the fact that most lipases prefer lipophilic substrates [10,39].

Benzoic acid ester (**1**) and its derivative, gallic acid ester (not shown), were not converted at all. For the benzoic acid compound, molecular dynamics simulations were performed in order to study the complex interactions between the short aromatic acyl moiety with the residues of the scissile acid binding pocket. Steric interactions between the bulky phenyl ring of benzoic acid and the methyl group in the side chain of T138 vigorously affected substrate binding. Furthermore, the benzoic acid phenyl ring could not rearrange in the binding site due to its rigid ring geometry and the surrounding residues T40, Q157 and I189. Additionally, even more unfavorable interactions occurred between the non-polar aromatic ring and the polar carboxylate group in D134. We assume a spacer of at least one methylene bridge between the carboxylate group and the aromatic ring is necessary in order to induce a lipase-catalyzed conversion.

3.1.2. Influence of the substitution pattern of the aromatic ring

Derivatives of phenylacetic acid were investigated to study the influence of the substitution pattern at the aromatic ring. It turned out that hydroxy groups in *m*- and *p*-position, as well as a *p*-chloro substitution (**7**–**9**) only have a minor effect on the conversion, since the hydrophobic crevice is wide enough to exclude undesired steric interactions. The polar *p*-nitro group was found to be an unfavorable substituent for the non-polar environment of the acyl binding pocket. In contrast, only traces of the respective sugar ester were formed with *o*-hydroxyphenylacetic acid (**6**). Our modeling studies suggest that the *o*-hydroxy group may serve as a hydro-

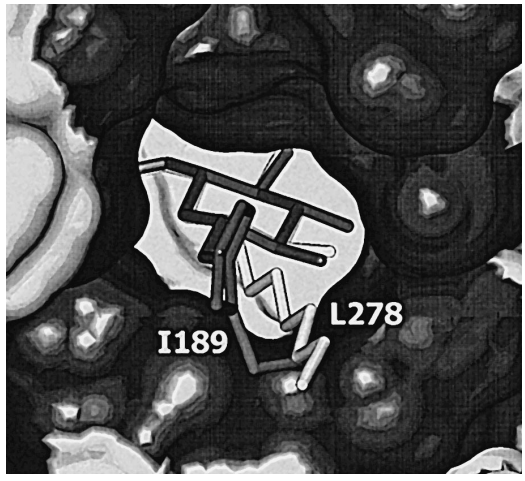


Fig. 2. Alcohol binding pocket of CAL-B with hydrophilic bottom (white) and hydrophobic residues (dark). The hydrophobic aglycons of the glucosides, which were substituted at the β-configurated C-atom of the glucose, were placed in a hydrophobic gap between L278 and I189. The alcohol binding pocket is limited by a rim consisting of hydrophobic amino acid residues which can sterically interact with the *ortho* hydroxymethyl group of the salicin ester (dark).

gen donor to the oxyanion and as a consequence may disturb the charge distribution in this area which is essential for catalysis to occur [32–35].

3.1.3. Influence of functionalities within the acyl chain

As stated above, phenylacetic or phenylpropionic acid could be esterified by CAL-B with glucose leading to compounds (**2**) and (**3**) in acceptable yields (22–23%). In sharp contrast, a simple introduction of a double bond in α-position (**12**–**14**) or an α-substituent (**11**, **15**, **16**) tips the balance from reactivity to non-reactivity.

For α,β-unsaturated compounds (**12**–**14**), computer-aided molecular modeling results suggest that substrate access to the active site is hindered due to reduced flexibility of the acyl residue. The latter does not properly fit into the hydrophobic crevice as compared to saturated analogs. Furthermore, the double bond is in the same position as the aromatic ring system in benzoic acid. As described above, similar effects can be taken into account to describe these observations. Besides steric effects, also electronic influences, especially for both the esters of *p*-coumaric (**13**) and caffeic acid (**14**), were found to be reasonable. The ring hydroxy groups (especially in *para*-position) caused inductive and, above all, mesomeric effects along the delocalized electron system. Therefore, the reactivity of the carboxylic acid group in the cinnamic acid derivatives (**13**) and (**14**) was presumably negatively influenced by the formation of Michael-addition by-products [40]. Our results were underlined by data generated by Guyot et al. [41] who reported a very slow conversion in the esterification reaction of cinnamic acid with aliphatic alcohols, but no reaction with caffeic acid.

However, unsaturated arylaliphatic glucose esters are compounds of pharmaceutical interest. One method to circumvent the problem of non-reactivity in the synthesis of these glucose esters is the CAL-B catalyzed esterification of glucuronic acid with arylaliphatic α,β-unsaturated alcohols yielding closely related esters at reasonable yields [15].

In case of α-substituted arylaliphatic acids, like α-amino phenylacetic acid and α-ethyl phenylacetic acid, conversion did not occur at all. The substrate binding site of CAL-B narrows close to the funnel-like hydrophilic bottom. Both the ethyl and amino groups are bulky substituents interacting stericly with the lipase (especially residue D134 and the catalytically active residues S105 and H224). The non-polar ethyl group might undergo similar interactions with the polar side chain of D134 as described above for benzoic acid.

Surprisingly, the formation of 3-phenyllactyl glucose (**11**) was observed. Together with the successful esterification of lactic acid with different aliphatic alcohols [42] CAL-B seems to be suitable for the esterification of α-hydroxy-substituted carboxylic acids. This is most likely due to two effects: First, the hydroxy group is less voluminous as the respective ethyl or amino group. Second, in the orientation of the hydroxy group as compared to the amino group, the hydroxy group may serve as a hydrogen donor to the negatively charged carboxylate group of D134 and could, therefore, energetically stabilize the binding of phenyllactic acid into the acyl binding site.

3.2. Alcohol binding site

Besides the esterification of glucose with a variety of acyl donors, the synthesis of aromatic esters of glucosides — especially from phenolic glucosides like, e.g., the natural occurring drug salicin — could be of pharmaceutical importance.

In order to demonstrate whether the specificity of CAL-B, which was described for the synthesis of arylaliphatic glucose esters, could be transferred to the esterification of other bulky polyols, different aliphatic and arylaliphatic esters of D-(−)-salicin were synthesized (Scheme 3). Trends in productivity and yield were the same as found for β-D-glucose esters. A correla-

tion between an increased acyl chain length of the carboxylic acid and higher yields was found. Again, the esterification of α,β-unsaturated arylaliphatic and benzoic acid was either encountered with low yields or the reaction did not occur at all.

Thus, glucose and glucosides, seemed to be fixed in a similar orientation in the active site of CAL-B. Molecular modeling results indicated that the most favorable position of the sugar was such that the primary alcohol group directed toward the acyl binding site. Other orientations of the alcohol moiety in the binding site of CAL-B were found to be unlikely, since steric repulsion with residues S105 and H224 of the catalytic triad could occur. Indeed, ^{1}H-NMR and ^{13}C-NMR spectra gave evidence that the acylation proceeded in a highly regioselective manner exclusively at the primary hydroxy group of β-D-(+)-glucose and glucosides, respectively (typical shift in ^{13}C spectra from 61 to 65 ppm). Hence, no acylation at the hydroxymethyl group attached to the aromatic ring in D-(−)-salicin could be detected.

We also evaluated the influence of variation in the aglycon. Experimental data indicated that both yield (37%) and productivity (0.35 to 0.19 mmol product g^{-1} immobilized lipase h^{-1}) in the esterification of phenylbutyric acid with salicin yielding compound (**19**) were significantly lower as compared to *n*-octyl- (**17**) and phenyl-glucosides (**18**) which were isolated at 53% and 67% yield, respectively. To give evidence for these observations, computer-aided molecular modeling revealed that the alcohol binding pocket in CAL-B consists of mainly hydrophobic residues, like A281, A282 and I285 and is sterically limited close to the tetrahedral carbon of the hydrolyzed ester. The alcohol binding site is wide enough to host bulky hydrophobic aglycons when the substituents are in moderate distance from the catalytic machinery. However, it is limited by a rim consisting of residues L144, I189, L278, A282 and V286. The hydrophobic aglycons in glucosides (**17**, **18**), which were substituted at the β-configurated C1 of glucose, were placed in a hydrophobic gap between L278 and I189 (Fig. 2). Introducing polar groups into the aromatic ring system (e.g., hydroxymethyl group in salicin ester **19**) repelled both the polar alcohol moiety and the non-polar residues of the alcohol binding pocket due to steric and electrostatic effects.

Our computer-aided molecular modeling studies were useful to identify and understand interactions between both the substrate and the lipase on a molecular level which are essential for the observed reactions to occur. As a result, a correlation between substrate–lipase interactions and the experimentally determined yields was found. From this, we conclude that binding site properties and interactions between the lipase and the substrate essentially contribute to the product yield. However, the yield depends on several reaction parameters (e.g., relative solubilities of starting materials and products, reaction time, etc.), which are much more complex. Our model gives a simple method at hand to qualitatively rate the reactivity of CAL-B toward the formation of glycolipid substrates.

From these results, the modification of a broader range of natural occurring glycosidic drugs seems reasonable, even for substrates containing an aromatic ring. In case of *meta*- or *para*-substitution along the aromatic ring, the earlier described effects should be less prominent.

The following trends can be summarized from computer-aided molecular modeling investigation of CAL-B-catalyzed synthesis of aromatic glycolipids:

1. A broad range of arylaliphatic carboxylic acids can be esterified with glucose if at least one methylene bridge is present between the carboxylic group and the aromatic ring.
2. Results for the esterification of glucose can be transferred to C1-substituted sugars like D-(−)-salicin.
3. CAL-B is not a suitable catalyst in the esterification of sugars with
   - α,β-unsaturated arylaliphatic acids, like cinnamic acid and its derivatives,

- phenolic acids, like benzoic acid and its derivatives, and
- α-substituted carboxylic acids (with the important exception of an α-hydroxy group).

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