Identification of a metagenome-derived esterase with high enantioselectivity in the kinetic resolution of arylaliphatic tertiary alcohols

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35 metagenome-derived esterases bearing a GGG(A)X motif were screened for activity and enantioselectivity in the hydrolysis of a range of tertiary alcohol acetates. Most of the active esterases showed little or no enantioselectivity in the hydrolysis of the terpinyl acetate, linalyl acetate and 3-methylpent-1-yn-3-yl acetate. However, one esterase showed excellent enantioselectivity ($E > 100$) in the kinetic resolution of 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate as confirmed by a preparative scale reaction.

Introduction

Optically active tertiary alcohols are an important class of organic compounds with valuable applications in organic synthesis.[1] For instance, the tertiary α-acetylenic alcohols 2-phenylbut-3-yn-2-ol **4a** and 1,1,1-trifluoro-2-phenylbut-3-yn-2-ol **5a** were recently used for the synthesis of A_{2A} receptor antagonists that were shown to be orally active in a mouse catalepsy model.[2]

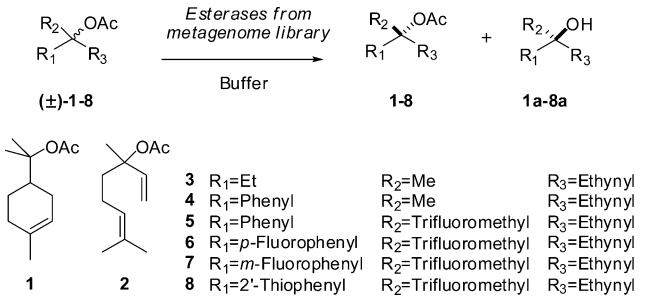
Carboxylester hydrolases (lipases, EC 3.1.1.3; esterases, EC 3.1.1.1) represent a class of versatile biocatalysts for the preparation of enantiopure compounds, especially optically pure secondary alcohols and also, to a smaller extent, for the resolution of primary alcohols and carboxylic acids.[3] In contrast, tertiary alcohols (TAs) are not accepted as substrates by almost all carboxylester hydrolases due to their sterically demanding structure. We recently identified that a GGG(A)X-motif located in the oxyanion hole near the active site region determines activity towards tertiary alcohols and that lipases or esterases with the much more common GX-motif are inactive.[4] We also reported that the enantioselectivity of the active enzymes is rather low, but could be substantially improved by rational protein design or addition of DMSO as cosolvent.[5]

In commercial hydrolases, the GGG(A)X-motif is rather rare and therefore we could only study a handful of enzymes (*i.e.* lipase from *Candida rugosa*, pig liver esterase, acetylcholine esterases) and a recombinant esterase from *Bacillus subtilis*[5] for their activity and enantioselectivity towards tertiary alcohols. The recently developed methods to access the 'non-culturable' biodiversity by the metagenome approach substantially extend the number of available biocatalysts. Most importantly, it has already been shown that new subclasses of enzymes can be identified which show a very broad sequence diversity and have unique properties. For instance, more than 130 novel nitrilases were discovered from environmental DNA libraries, compared to less than 20 nitrilases identified previously by classical cultivation methods,[6] and the metagenome-derived nitrilases showed unique and useful activities. Similarly, we have previously described two studies of the activities of metagenomic lipases and esterases, demonstrating novel properties of these enzymes.[7]

Here we extend our survey of esterases and lipases from environmental DNA libraries to investigate the activity and enantioselectivity on tertiary alcohol acetates of a number of novel enzymes bearing the GGG(A)X-motif.

Results

35 esterases from environmental DNA libraries and identified as containing the GGG(A)X-motif were studied for activity in the conversion of the tertiary alcohol acetates terpinyl acetate **1**, linalyl acetate **2**, 3-methylpent-1-yn-3-yl acetate **3** and 2-phenylbut-3-yn-2-yl acetate **4** (Scheme 1) using a high-throughput screening method based on the quantification of acetic acid released upon enzymatic activity[8] (Fig. 1). This revealed that many of the esterases were active and that the activity was dependent on the substrate used. The lack of activity of some enzymes was related to the fact that not all metagenome-derived enzymes expressed well in *E. coli*, as these enzymes also exhibited very low activity in a standard activity test using umbelliferyl butyrate as substrate (data not shown).



Scheme 1 Acetate esters **1–8** and the corresponding tertiary alcohols **1a–8a** used in this study.

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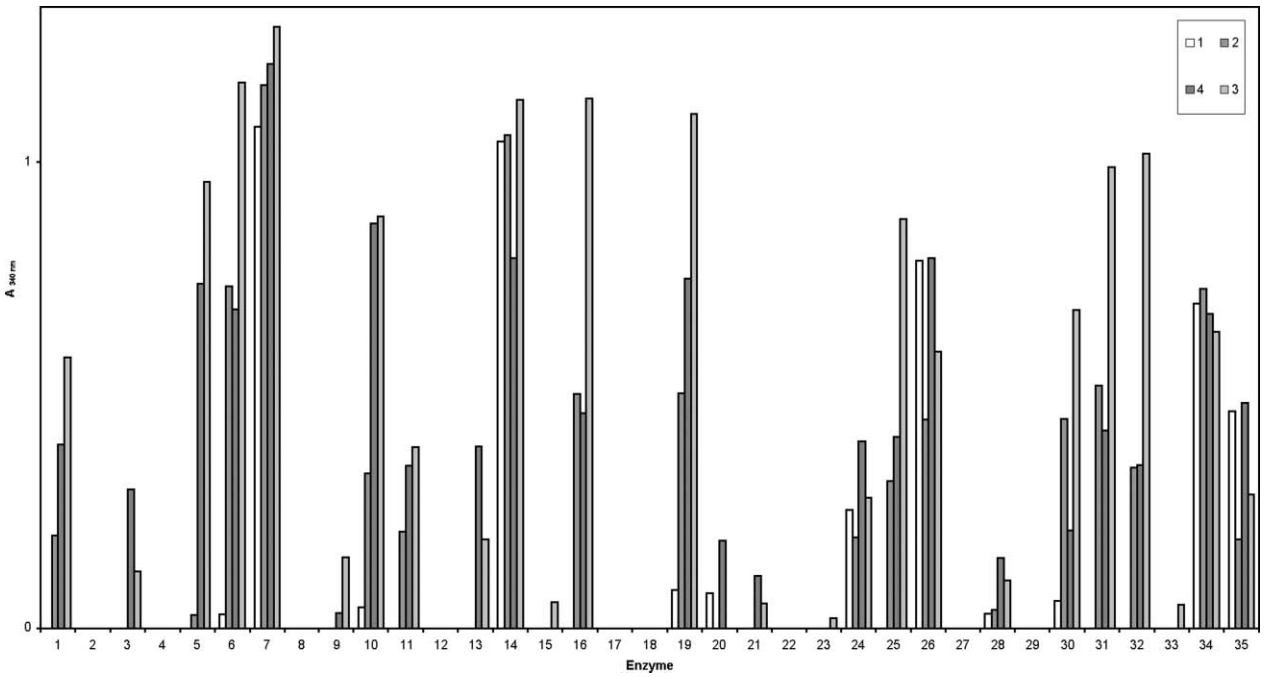

Fig. 1 Preliminary activity screening of the GGG(A)X-hydrolases in the hydrolysis of **1–4**.

Enzymes identified to be active were further studied in the kinetic resolution of the acetates **1–5**, with special focus on the enantioselectivities of the biocatalysts (Table 1). Using acetate **1** as substrate, only two enzymes showed any measurable enantioselectivity, and $E = 2$ is far too low to be of synthetic utility. Compound **1** is a challenging substrate for use in kinetic resolution studies due to the distance of the stereocenter of **1** from the carboxylic carbon atom and the cyclic structure of the terpinyl group (Scheme 1), and so low E values are not unexpected. Compound **2** was hydrolyzed by six esterases, but again enantioselectivities were low and never exceeded $E = 5$ (Table 1). It is noteworthy, however, that one esterase (19) showed opposite enantiopreference to the other

Table 1 Enantioselectivity of GGG(A)X esterases in the kinetic resolution of tertiary alcohols **1–8**

				Enantiomeric excess		
Enzyme	Time/h	Substrate	Conversion (%)[a]	(% ee_S)[a]	(% ee_P)[a]	E[b]
9	24	**1**	64	33	18	2 (−)
14	24	**1**	77	33	7	2 (−)
1	24	**2**	32	17	35	3 (+)
11	24	**2**	58	62	43	5 (+)
6	24	**2**	10	6	54	4 (+)
16	24	**2**	14	8	49	3 (+)
19	24	**2**	25	16	47	3 (−)
34	24	**2**	62	69	41	5 (+)
3	0.5	**4**	26	22	61	5 (*R*)
6	0.5	**4**	67	65	31	4 (*R*)
26	0.5	**4**	27	20	49	4 (*R*)
34	0.5	**4**	40	19	28	2 (*S*)
1	1	**5**	75	99	32	2 (−)
3	1	**5**	81	31	7	1.5 (−)
6	1	**5**	21	21	78	10 (−)
11	1	**5**	33	25	50	4 (−)
14	1	**5**	80	43	43	2 (−)
19	0.25	**5**	22	13	45	3 (−)
26	24	**5**	10	2	20	1.5 (−)
31	1	**5**	21	7	27	2 (−)
34	24	**5**	43	65	88	31 (+)
34	24	**6**	61	86	54	9
34	24	**7**	10	7	67	6
34	24	**8**	81	99	23	6

[a] Determined by chiral GC analyses. [b] In the case of **1** and **2**, calculated from conversion and ee_P. In the case of **3–8**, calculated from ee_S and ee_P.

enzymes. None of the enzymes tested exhibited enantioselectivity towards **3** (data not shown).

Acetate **4** undergoes rather rapid non-enzymatic, S_N1-mediated hydrolysis[1,5] in aqueous media, which can substantially lower the optical purity of substrate and product, and so it was of no surprise that none of the esterases tested showed high enantioselectivities for this substrate. In contrast, the trifluorinated analog **5** is stable in buffer[5] and indeed we were pleased to find that two esterases, 34 ($E = 31$) and 6 ($E = 10$), exhibited moderate enantioselectivities for this substrate. Interestingly, esterase 34 preferentially converted (*S*)-**4** and (+)-**5**, while the other metagenome-derived enzymes investigated had opposite enantiopreferences for both substrates, in common with the highly selective BS2 esterase mutant G105A described previously.[5]

The gene encoding esterase 34 stems from an environmental sample isolated at 106 °C and a pH of 6. However, kinetic resolution studies with 34 and using **5** as substrate over a range of temperatures revealed that above 50 °C the activity of the enzyme decreased markedly (Fig. 2). The enantioselectivity of 34 on **5** was also affected by temperature and decreased above 30 °C. Variation of the pH also had a substantial effect on enantioselectivity (Fig. 2), which was found to be maximal at pH 6 ($E = 89$ at 50% conversion), although this was not the optimal pH for activity. Combining the optimal temperature (30 °C) and pH (6) for esterase 34 at a reaction scale of 150 mg resulted in excellent enantioselectivity ($E > 100$ at 35% conversion). Addition of cosolvents like dimethyl sulfoxide, dimethyl formamide or *t*-butanol did not further increase the enantioselectivity of the esterase (data not shown).

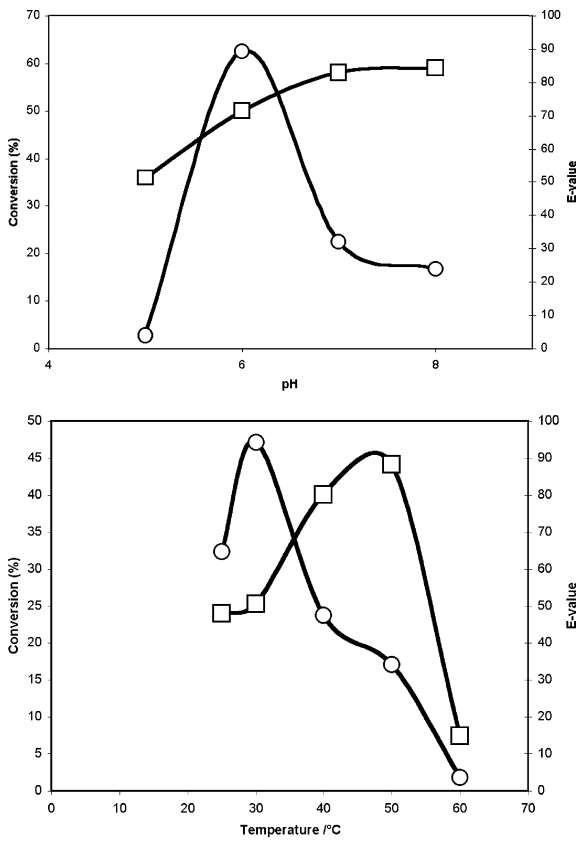

Fig. 2 Influence of pH (measured at 40 °C) and temperature (measured at pH 7.5) on enantioselectivity (circles) and conversion (squares) using esterase 34 in the kinetic resolution of **5**. Data were determined after 24 h.

The enantioselectivity of esterase 34 in the kinetic resolutions was further studied using substrates **6**, **7** and **8** (Table 1). The nature and position of substituents on the aromatic ring were observed to have a strong influence on the activity of the enzyme. Compounds **6** and **8** were converted with higher activity than **5**, whereas acetate **7** was only converted at a low level. The enantioselectivities of the enzyme on these substrates were low.

Discussion

Several new metagenome-derived esterases with activity towards the hydrolysis of tertiary alcohol acetates were identified in an activity-based screening approach. All of the enzymes tested share a GGG(A)X motif, which is believed to confer activity on these sterically hindered substrates by expanding the active site around the oxyanion hole. Most of the active esterases showed no significant enantioselectivity in the hydrolysis of the acetates of the tertiary alcohols terpinyl acetate **1**, linalyl acetate **2** and 3-methylpent-1-yn-3-yl acetate **3**. However, esterase 34 demonstrated moderate preference for hydrolysis of the (+)-enantiomer of 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate **5**. Optimization of the reaction conditions resulted in excellent enantioselectivity of esterase 34 ($E > 100$) on this substrate. This enzyme did not show good enantioselectivity in the hydrolysis of several closely related substrates.

The variation of enantioselectivity of an enzyme across a range of substrates observed here indicates that successful identification of a useful biocatalyst for a given synthetic problem can be enhanced by having a large initial pool of enzymes to screen. The metagenomic era is greatly expanding the pool of available biocatalysts and can be expected to also expand the scope of enzyme applications.

Experimental

All chemicals were purchased from Sigma (Steinheim, Germany) unless stated otherwise. Compounds **1–8** were prepared as described previously.[4,5] Separation protocols, retention times and the column types used for the chiral gas chromatographic analysis of acetates **1–8** and their corresponding alcohols **1a–8a** were performed as described.[4,5] All esterases were discovered and produced by Verenium Corporation (San Diego, USA) and used as lyophilized crude cell lysates.[9]

Preliminary screening

Screening with the acetic acid assay was performed in microtiter-plate format as described previously.[8] The test kit for the determination of released acetic acid was from R-Biopharm GmbH (Darmstadt, Germany) and used according to the manufacturer's protocol. Enzyme solution (20 μL, 2–5 mg lyophilisate per mL) from the production plate and substrate solution (20 μL, 50 mg mL^{-1}) were added to a mixture (150 μL) of the test kit components. The increase in NADH after 10 min was monitored at 340 nm by using Fluostar Galaxy or Fluostar Optima spectrophotometers (BMG, Offenburg, Germany).

General procedure for esterase-catalyzed small-scale resolutions

To a stirred solution of substrate (15 mM) in phosphate buffer (100 mM, pH 7.5), the esterase solution (2–10 mg mL^{-1}) was added to a final volume of 1 mL. The reaction mixture was stirred in a thermoshaker (Eppendorf, Hamburg, Germany) at 37 °C and 1400 rpm. Samples were taken after 1, 5 and 24 h. The reaction samples were extracted twice with 400 μL dichloromethane. The combined organic layers were dried over anhydrous sodium sulfate and the organic solvent was removed under nitrogen. Enantioselectivity and conversion were calculated according to Chen *et al.*[10]

Kinetic resolution of 5 in preparative scale using esterase 34

3.2 mg lyophilisate of esterase 34 with an activity of 12.6 U mg^{-1} in the hydrolysis of *p*-nitrophenyl acetate was added to a stirred solution of **5** (0.62 mmol) in phosphate buffer (100 mM, pH 6.0) to a total volume of 50 mL. The reaction mixture was stirred for 15 min at 30 °C and then extracted three times with 20 mL dichloromethane. The organic layers were combined and washed with brine (2 × 20 mL), water (2 × 20 mL) and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. Purification by column chromatography (hexane–ethylacetate) yielded acetate **5** and alcohol **5a** with a conversion of 35% and an *E*-value of $E > 100$.

(+)-1,1,1-Trifluoro-2-phenylbut-3-yn-2-ol (**5a**): yield: 30% (38 mg); 97% ee.

(−)-1,1,1-Trifluoro-2-phenylbut-3-yn-2-yl acetate (**5**): yield: 51% (76 mg); 53% ee.

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