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Asymmetric synthesis of *cis*-3,5-diacetoxycyclopent-1-ene using metagenome-derived hydrolases

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Abstract—Esterases and lipases originating from the metagenome (environmental DNA) were studied for the asymmetric hydrolysis of *cis*-3,5-diacetoxycyclopent-1-ene **1**. Out of 83 enzymes, 35 were found to be active. Subsequent analytical and laboratory scale reactions identified three enzymes showing excellent (−)-preference and one enzyme with excellent (+)-selectivity exceeding even the performance of recombinant or commercial pig liver esterase, which is known as the best biocatalyst for this compound.

1. Introduction

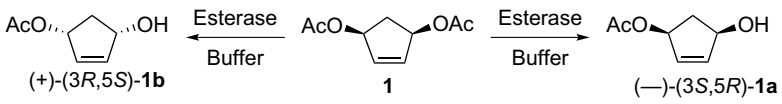
The asymmetric hydrolysis of *cis*-3,5-diacetoxycyclopent-1-ene **1** to the enantiomerically pure monoacetates **1a** or **1b** is an important step in the synthesis of optically active cyclopentanoids (e.g., prostaglandins, prostacyclins, thromboxanes), and enzymatic reactions have been shown to allow access to both enantiomers.[1–4] However, there is a constant need to identify new biocatalysts offering superior properties with respect to enantiopreference, stereoselectivity, activity, and stability.

A very rich source of novel enzymes is the so-called 'metagenome', that is, the collective genomes of ideally all microorganisms in a given habitat.[5] Modern molecular biology methods allow the isolation and cloning of this environmental DNA without the need of cultivation of the corresponding organisms.[6–11] Sophisticated screening approaches have been established to allow access to the enormous resource of microbial biocatalysts.[12] Many new and diverse enzymes have already been isolated from metagenomic libraries[13] to evaluate their potential as industrial biocatalysts, as they needed to be characterized with respect to process relevant parameters, such as activity, stability, and enantioselectivity.

Herein, we report the screening and characterization of novel metagenome-derived hydrolases for their activity and selectivity in the asymmetric hydrolysis of *cis*-3,5-diacetoxycyclopent-1-ene **1** (Scheme 1).

2. Results and discussion

For the identification of new and selective enzymes, 83 esterases from the metagenome were investigated. In addition, a commercial preparation of pig liver esterase



Scheme 1. Asymmetric enzymatic hydrolysis of **1** to yield enantiomerically pure monoacetate **1a** or **1b**.

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(Fluka-PLE) and the recombinant γ-rPLE[14] served as benchmark enzymes.

First, active enzymes were identified by a microtiterplate-based screening using a pH-indicator assay,[15,16] with bromothymol blue. The 35 metagenome-derived enzymes and the PLEs showed activity and were then subjected to analytical scale reactions (10 mM substrate). Conversion and enantiomeric excess of the product were determined using gas chromatography. The results are summarized in Figure 1 and demonstrate that indeed the biocatalysts were active. Moreover, biocatalysts with complementary enantiopreference and very high enantioselectivity even exceeding the performance of PLE could be identified.

Thus, enzymes 7, 10, CE, and CL1 showed the highest enantiomeric excess and were subjected to laboratory scale reactions (Table 1). These experiments confirmed the excellent enantioselectivity and complete conversion of the diacetate into the corresponding monoacetate, which could be isolated either as (−)-**1a** or (+)-**1b** in acceptable yields.

Enzymes 7, 10, and CE, which showed a preference for the (−)-**1a** enantiomer, all belong to family VIII esterases, which are more related to class C β-lactamases than to esterases with the typical α/β-hydrolase fold.[17] The enzyme CL1, similar to the pig liver esterase, is a member of the so-called 'GGGX'-type family of esterases,[18] which were described as being capable of enantioselective conversion of esters of tertiary alcohols.[19,20] However, these two enzymes displayed opposite enantiopreferences.

Thus, since none of the 'GX'-type esterases were found to convert **1** with high enantioselectivity, it seems that 'GGGX-type' esterases and family VIII esterases are especially suited for the enantioselective conversion of this compound. Whether this conclusion can be extended to other cyclopentanoids demands further investigations using different substrates from this group.

Table 1. Results of laboratory scale reactions

Esterase	Purity[a] (% ee_P)	Conversion[a] (%)	Yield (%)
7	95 (−)	99	57
10	97 (−)	100	66
CE	80 (−)	100	52
CL1	97 (+)	100	56

[a] As determined by GC analysis.

3. Conclusions

Herein, we have shown that enzymes derived from environmental DNA exhibit excellent and complementary enantiopreference in the asymmetric hydrolysis of *cis*-3,5-diacetoxycyclopent-1-ene and even surpass the performance of pig liver esterase, which was reported[3] to give only 80.3% ee.

4. Experimental

4.1. General

All chemicals were purchased from Fluka (Buchs, Switzerland) at the highest purity available. All metagenomic esterases were produced by B.R.A.I.N. AG and used as glycerol-stabilized crude cell extracts. Plasmids containing the genes for the esterases A3,[11] CE[11] CL1, and CL2 were obtained from K.-E. Jaeger (Juelich, Germany) and Professor W. Streit (Hamburg, Germany). The commercial PLE preparation was purchased from Fluka (Buchs, Switzerland) and the recombinant γ-PLE was produced as described.[14]

4.2. Analytic methods

Analysis of conversion and enantiomeric purity was performed by gas chromatography on a GC–MS QP2010 (Shimadzu, Japan) using the chiral column

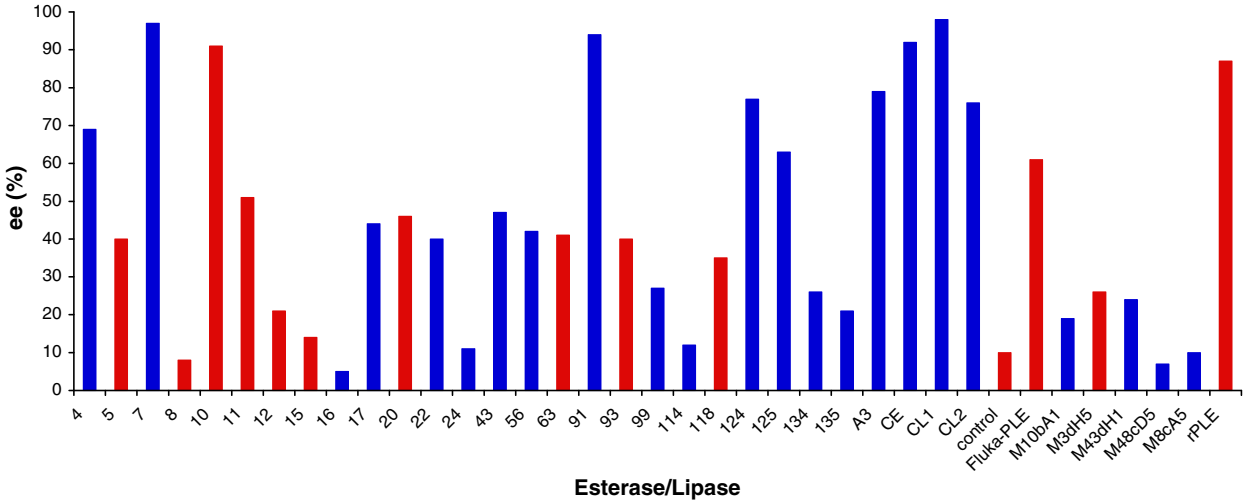

Figure 1. Results of analytical scale reactions using the metagenome-derived enzymes and two PLEs; blue columns: preferred formation of **1b**; red columns: preferred formation of **1a**. Control: sample from an *E. coli* strain carrying the expression plasmid without inset.

Hydrodex®-β-3B (Heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl-β-cyclodextrin)). Separation of **1**, **1a**, **1b** and diol **1c** was carried out at 120 °C (retention times: **1**, 11.2 min; **1a**, 9.7 min; **1b**, 8.8 min; **1c**, 8.5 min). Assignment of absolute configuration was based on polarimetric measurement (POLAMAT A, Carl Zeiss, Germany) and by comparison with literature data.[3]

4.3. pH-Indicator prescreening

This was performed in 96-well microtiter plates (MTP). The assay solutions were prepared by mixing 50 µl of diacetate **1** (0.1 mg/µl in DMSO) with 50 µl of the pH indicator bromothymolblue (1:10 diluted in DMSO) and 120 µl sodium phosphate buffer (5 mM, pH 7.3) per well. Reactions were started by the addition of 30 µl enzyme solutions. Next, the MTPs were covered and incubated at room temperature until a visually monitored color change (blue to yellow) indicated active enzymes. Mixtures without enzyme or substrate served as controls.

4.4. Enzymatic reactions

4.4.1. Analytical scale resolutions. Diacetate **1** was dissolved in a sodium phosphate buffer (50 mM, pH 7.5) to a final concentration of 10 mM. Desymmetrization of the prochiral substrate was started by the addition of an appropriate amount of enzyme in a final volume of 1 ml. The hydrolysis reactions were carried out in a thermomixer (Eppendorf, Germany) at 37 °C. To terminate the reactions, the mixtures were extracted with methylene chloride and the organic phases were dried over anhydrous sodium sulfate. The determination of enantiomeric purity and conversion was performed by gas chromatography. The enantiomeric excess was calculated according to Chen et al.[21]

4.4.2. Laboratory scale resolutions with esterases 7, 10, CE, and CL1. Substrate **1** (100 mg) was dissolved in 9.5 ml of sodium phosphate buffer (50 mM, pH 7.5) and 0.5 ml of ethanol as cosolvent to increase solubility. The reactions were started by adding 250 µl enzyme (for 7, CE or CL1) or 2.5 ml of esterase 10 and mixing at 37 °C. After complete conversion was determined by GC analysis, the reaction mixtures were extracted five times with 10 ml of methylene chloride, the organic layers were combined, dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure affording crystalline product (**1a** or **1b**). Determination of enantiomeric purity and conversion was performed as described above.

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