Exploration of Thermophilic Esterases/Lipases for Asymmetric Desymmetrization of Norbornane Derivatives

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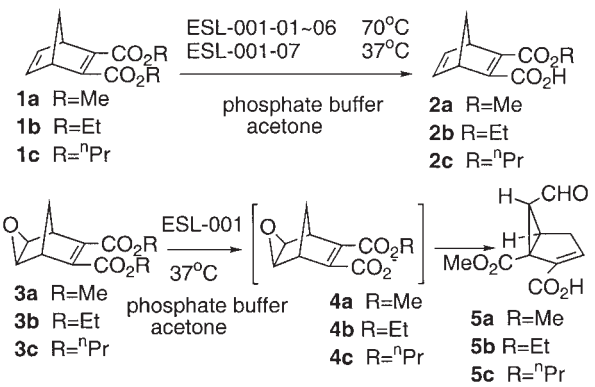
Asymmetric monohydrolysis of a series of norbornane derivatives by a thermophilic esterase/lipase library was examined. Three esterases/lipases showed high enantioselectivities toward dialkyl bicyclo[2.2.1]hepta-2,5-diene-2,3-dicarboxylates with high chemical yields.

Enzymatic asymmetric hydrolysis has been a versatile tool in organic synthesis. Recently thermophilic enzymes, cloned from a diverse range of microorganisms in extreme environments of temperature, pH, osmotic and hydrostatic pressure, and salt concentrations have been showing new types of reactivities.[1] Such enzymes, referred to as "thermophiles" or "extremozymes," are expected to be more thermally robust and more stable in organic solvents, and therefore their synthetic utility is expected, especially in industrial processes. However, despite their expected unique utility, the application of these extremozymes to organic synthesis is still limited to only a few examples.[2] As one instance, we recently reported the first example of desymmetrization by asymmetric monohydrolysis of symmetric diesters by a thermophilic esterase/lipase, ESPESL 1864.[3] This enzyme successfully monohydrolyzed a series of dialkyl bicyclo[2.2.1]hepta-2,5-diene-2,3-dicarboxylates with high chemical and optical purities.

Here we have extended the possibilities to other thermophilic lipases/esterases as well as substrate diesters. We investigated the thermophilic esterase/lipase library CloneZyme,™ ESL-001, which consists of seven enzymes, most of whose optimal temperatures are around 70 °C.[4] One of the enzymes in this library, ESL-001-02, is identical to ESPESL 1864,[5] for which we had found remarkable utility earlier.



Scheme 1.

Therefore, applying this library, we investigated enzymatic desymmetrization of norbornane derivatives, dialkyl bicyclo[2.2.1]hepta-2,5-diene-2,3-dicarboxylates, **1a–c**, and dialkyl 5,6-epoxybicyclo[2.2.1]hept-2-ene-2,3-dicarboxylates, **3a–c** (Scheme 1). Norbornane derivatives have been known to be versatile for synthesis of a variety of natural products owing to their uniquely strained skeletons, and several derivatives have been obtained enzymatically.[6] These diesters are easily and inexpensively prepared by a single-step Diels–Alder reaction, and therefore the corresponding half-esters would also be versatile chiral building blocks. The reactivities of these enzymes were also compared with more common enzymes such as pig liver esterase and lipases from *Candida rugosa* (Scheme 1).

Using the CloneZyme™ esterase/lipase library, the monohydrolyses of these substrates were carried out at the temperatures indicated in Scheme 1, which showed optimum reactivities. Table 1 summarizes the results of the yields and optical purities. The absolute configuration of the predominant enantiomer was determined as depicted in the scheme for all the cases tried here, based on the sign of the specific rotation values.[7, 8a]

Among the lipases/esterases in this library, three enzymes (ESL-001-01, 02, and 07) exhibited quite high reactivity toward dialkyl bicyclo[2.2.1]hepta-2,5-diene-2,3-dicarboxylates, **1a–c**, affording high chemical yields and optical purities, which were $> 99\%$ ee in all the diesters. The high reactivity of ESL-001-02 is as expected from our recent experiments.[3] The reaction times required for monohydrolysis of **1a–c** using ESL-001-02 were only 1.5 hours to 2 hours. Enzyme ESL-001-01 required a longer time (one day) than did ESL-001-02, although the chemical yields and optical purities were still excellent. However, when the reactions were conducted at 37 °C with these enzymes, with all other conditions remaining the same, the reactions required a far longer time (2–3 days). This enhancement of reaction time is beneficial for this class of thermophilic esterase/lipases. Enzymes ESL-001-01 and 07 exhibited slightly higher reactivities toward **1c** than did ESL-001-02. The remaining CloneZyme™ enzymes (ESL-001-03, 04, 05, and 06) showed no reactivity to these diesters. All these enzymes produced half-esters **2a–c** that possess the same absolute configurations as depicted in Scheme 1. We have reported that more common esterases, pig liver esterases and lipases AY from *Candida rugosa*,[9] showed only modest reactivity toward these diesters and that the optical purities were also low; lipases A, AK, D, F-AP15, G, M, PS, or S[9] induced no reactions after incubation for 48 hours,[3] indicating the expected versatility in these enzymes described here.

Dialkyl 5,6-epoxybicyclo[2.2.1]hept-2-ene-2,3-dicarboxylates, **3a–c**, is known to undergo a characteristic rearrangement to afford **5a–c** in the buffer medium.[8] As these compounds appeared to be prone to decomposition at 70 °C, the optimum reactivities were observed at 37 °C for monohydrolyses of these diesters. Among the enzymes in the library, only ESL-001-02 showed reactivity, giving **5a** and **5c** in significantly higher optical purity than did pig liver esterase, although the chemical yields and the optical purities were lower than those of **2a–c**. It appears that the epoxy group hinders the activity of these enzymes.

Table 1. Yields and Optical Purities after Asymmetric Monohydrolysis of **1a–c** and **3a–c** by the Thermophilic Enzyme Library

	1a	**1b**	**1c**	**3a**	**3b**	**3c**
Enzyme	%yield (%ee)	%yield (%ee)	%yield (%ee)	%yield (%ee)	%yield (%ee)	%yield (%ee)
ESL001-01	92 (> 99)	83 (> 99)	84 (> 99)	N.R.	N.R.	N.R.
ESL001-02	98 (> 99)	93 (> 99)	72 (> 99)	66 (79)	60 (55)	61 (55)
ESL001-03	N.R.[a)]	N.R.	N.R.	N.R.	N.R.	N.R.
ESL001-04	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.
ESL001-05	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.
ESL001-06	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.
ESL001-07	89 (> 99)	80 (> 99)	79 (> 99)	N.R.	N.R.	N.R.
Pig liver esterase[b)]	> 99 (32)	89 (38)	> 99 (71)	> 99 (47)	> 99 (65)	75 (28)
Lipase AY from *Candida rugosa*[b),c)]	61 (78)	44 (41)	48 (14)	35 (7)	N.R.	N.R.

a) N.R. = no reaction. b) Reactions with these enzymes were all performed at 37 °C. Part of these data have been published in Refs. 3 and 8a. c) Lipases A, AK, D, F-AP15, G, M, PS, or S[9] showed no reactivities for any of the diesters under the same conditions.

In summary, we found that several enzymes in the thermophilic esterase/lipase library possess remarkable enantioselectivity toward dialkyl bicyclo[2.2.1]hepta-2,5-diene-2,3-dicarboxylates, **1a–c**. These enzymes may constitute unique biocatalysts with unique specificities and reactivities; therefore, synthetic utility is expected.

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Experimental

The typical procedure for monohydrolysis of diesters **1a–c** and **3a–c**, was as follows: The diester (0.1 mmol) was dissolved in 0.1 mL of acetone and 10 mL of sodium phosphate buffer solution (0.1 M, pH7.5), and 20 μL of the enzyme solution (1 mg/50 μL) was added to the reaction mixture. This reaction mixture was incubated at the indicated temperature until the consumption of the starting material was observed by TLC, cooled in a water-bath, adjusted to pH 10 with 1 M NaOH, and immediately washed with ethyl acetate (× 3); this extract was discarded. The reaction mixture was immediately acidified with 1 M HCl solution (pH = 3), extracted with ethyl acetate (× 3), washed with brine, dried over Na_2SO_4, concentrated in vacuo, and purified by silica-gel column chromatography.

The monohydrolyzed products obtained were identified by ^{1}H and ^{13}C NMR as well as by IR spectra, referring to these data we reported earlier.[3,8] ^{1}H NMR at 300 MHz, and ^{13}C NMR at 75 MHz spectra were measured as solutions in $CDCl_3$ using TMS as an internal standard. The IR spectra were recorded on an FTIR instrument.

Enantiomeric excesses of the products were determined by gas chromatography with chiral stationary phase, CycloSil B, at 160–170 °C, under isothermal conditions using calibration curves.[10] The enantiomeric excesses for **5a–c** were determined by referring to the specific rotation values for optically pure **5a–c**, which we reported earlier.[7]

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4 CloneZyme,™ ESL-001 was purchased from Sigma. The optimal temperature of ESL-001-07 appears to be in the range of 37–50 °C, while there is a report that this enzyme reacts at 75 °C as well.[2d]

5 ESPESL1864 is commercially available from Diversa Corp. Both ESPESL 1864 and ESL-001-02 are assigned BD 1864. The BD number identifies all the proprietary enzymes made available outside Diversa Corporation.

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9 The origins of these lipases are as follows: A-*Aspergillus niger*, AK-*Pseudomonas fluorescens*, D-*Rhizopus oryzae*, F-AP15-*Rhizopus oryzae*, G-*Penicillium camembertii*, M-*Mucor javanicus*, PS and S-*Burkholderia cepacia*. All these lipases were supplied by Amano Enzyme U.S.A. Co. Ltd.

10 The retention times for both enantiomers for **2a–c** are as follows. In all the compounds, **2a–c**, the enantiomers produced in these enzymatic monohydrolyses have the longer retention times. **2a**: 24.69–24.70 minutes and 25.70–25.71 minutes. **2b**: 30.70–30.80 minutes and 31.68–31.69 minutes. **2c**: 29.29–29.30 minutes and 30.40–30.41 minutes.