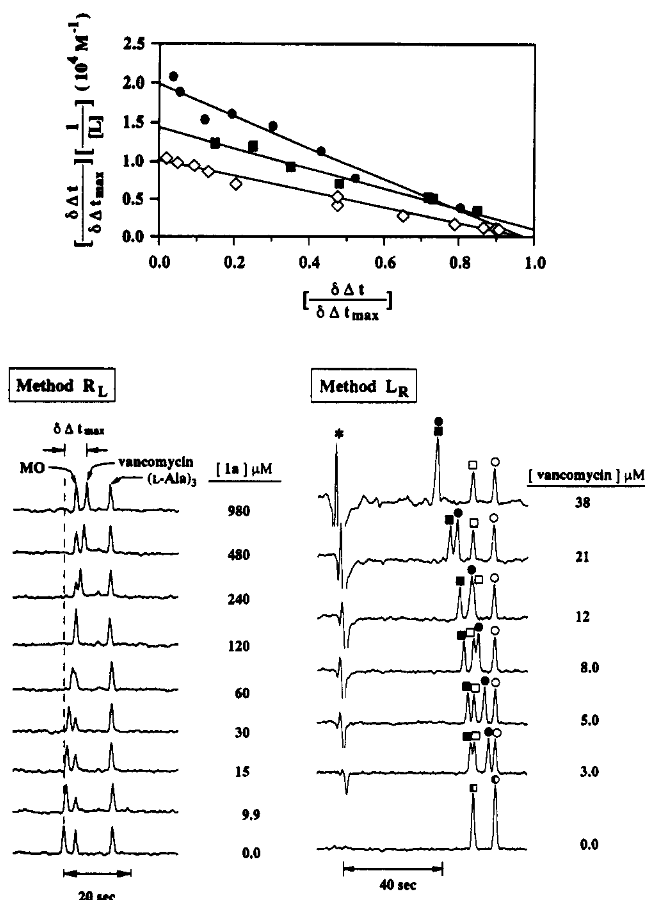

Figure 1. Method R_L: affinity capillary electrophoresis (ACE) of vancomycin in 10 mM sodium phosphate buffer (pH 7.1) containing various concentrations of N-acetyl-D-Ala-D-Ala (**1a**) (◇). The neutral marker mesityl oxide (MO) and the tripeptide L-Ala-L-Ala-L-Ala were used as internal standards. Method L_R: ACE of N-Fmoc-Gly-D-Ala-D-Ala (**4a**) (●), N-Fmoc-Gly-D-Ala-D-Ala-D-Ala (**5a**) (■), N-Fmoc-Gly-L-Ala-L-Ala (**4b**) (○), and N-Fmoc-Gly-L-Ala-L-Ala-L-Ala (**5b**) (□) in 20 mM sodium phosphate buffer (pH 7.5) containing various concentrations of vancomycin. The asterisk (*) indicates the position of the peak for unidentified neutral species carried through the capillary by electroosmotic flow. The total analysis time in each experiment was ~2.5 min (method R_L) and ~4.0 (method L_R) at 30 kV using a 45-cm (inlet to detector), 50-μm uncoated fused silica capillary. The graph is a Scatchard plot of the data according to eq 1.

set of measurements determined values of K_b for **4a**, **4b**, **5a**, and **5b**.[7]

By measuring the appearance time (t) of the peak due to vancomycin (method R_L) or to peptides (method L_R) as a function of the concentration of additive (L or R) present in the CE buffer, it was possible to determine binding constants (K_b). Equation 1 gives a convenient

$$(\delta\Delta t/\delta\Delta t_{max})(1/[B]) = K_b - K_b(\delta\Delta t/\delta\Delta t_{max}) \quad (1)$$

form for Scatchard analysis: here $\Delta t_{[B]}$ is the difference between appearance time of the species of interest and the internal standard at concentration [B] of the additive in buffer, $\delta\Delta t = \Delta t_{[B]} - \Delta t_{[B]=0}$, and $\delta\Delta t_{max}$ is the value of $\delta\Delta t$ at saturating concentrations of B. Table I summarizes results.

Figure 1 shows Scatchard plots (eq 1) derived from both R_L and L_R experiments. The values of K_b for **1a** and **4a** obtained using method R_L compare well with those obtained by method L_R. The values of binding constants measured by ACE also fall in the range of those values obtained from other assays.[8-10]

Affinity capillary electrophoresis has several advantages as a method of measuring binding constants. First, it requires only small quantities of receptor and ligand. Second, when using method R_L, neither high purity for the receptor nor an accurate value of its concentration is required. Third, when using method L_R, it may be possible to measure binding constants of several ligands to a single receptor in the same set of experiments. When used with relatively low molecular weight species, the adsorption of the capillary wall that complicates experiments with proteins is unlikely to occur.[11]

Acknowledgment. Y.-H.C. thanks Hans A. Biebuyck for helpful discussions and comments about the work, Professor Erich C. Blossey, and Luis Z. Avila for helpful suggestions on the manuscript.

Supplementary Material Available: Experimental details for the preparation of 1-5 (7 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(7) Vancomycin absorbs strongly at 200 nm. High concentrations (≥50 μM) decreased the S/N in the L_R method. We used Fmoc-derivatized peptides to increase sensitivity.

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(11) In an R_L experiment, partial adsorption of the receptor on the wall of the capillary has no influence on the measured binding constants, provided that its extent is independent of the concentration of L. The equilibration between solution and capillary wall simply changes the effective mobility, but not the form of the Scatchard analysis. In an L_R experiment, concentration-dependent adsorption may have more potential to cause error. It should, however, be detectable in most cases in nonlinearity in the Scatchard plot.

Biocatalytic Resolution of Tertiary α-Substituted Carboxylic Acid Esters: Efficient Preparation of a Quaternary Asymmetric Carbon Center

Christopher Yee, Todd A. Blythe, Thomas J. McNabb, and Alan E. Walts*
Genzyme Corporation, One Kendall Square, Cambridge, Massachusetts 02139
Received April 28, 1992

Summary: A method for resolving a variety of tertiary α-substituted carboxylic acid esters employing a previously undescribed enzyme is reported.

Scalemic tertiary α-substituted carboxylic acids and their derivatives such as those described here are of considerable scientific and medical significance. Pharmaceuticals comprising optically active α-methyl α-amino and α-methyl α-hydrazino acids function as inhibitors of amino acid decarboxylase enzymes,[1] and other α-alkyl α-hetero-

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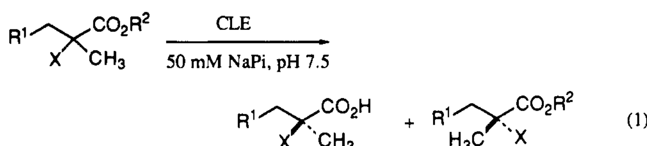
atom substituted acids display desirable biological activity,[2] utility as synthetic intermediates,[3] or utility as research tools.[4] Traditional chemical methods for preparing these compounds include asymmetric synthesis[5] and diastereomeric resolution.[3a,6] The use of biocatalysts to resolve certain compounds in this class has recently met with some success. Specific enzymes to resolve α-methyl α-hydroxy and α-methyl α-(benzyloxy) esters,[7] α-methyl α-amino esters and amides,[8] and α-methyl α-(acylamino) acids[9] have been reported, although enzyme activity, selectivity, or specificity are often limited.

We report in this paper initial studies with a novel enzyme which we term *Candida lipolytica* esterase (CLE). This previously undescribed enzyme is a minor component in commercial preparations of *C. lipolytica* lipase (CLL)[10] and is highly effective in resolving a range of tertiary α-substituted esters (eq 1). CLE accepts substrates con-



taining α-alkyl and α-benzyl moieties and tolerates a variety of heteroatom α-substituents including hydrazino, hydrazido, amino, amido, and hydroxyl groups. In most cases the enantiomeric excess of resolved products exceeds 98–99% at 50% substrate conversion,[18a] rendering CLE a biocatalyst of significant practical utility by providing a general method to resolve a structurally diverse range of tertiary α-substituted esters (eq 1).[11]

Preliminary screening studies[12] directed toward resolution of α-methyl α-hydrazino and α-hydrazido esters suggested a crude preparation of *C. lipolytica* lipase[15] for further investigation. Results for the use of this biocatalyst on the milligram scale[16] are shown in Table I. *C. lipolytica* esterase enzyme tolerates a range of heteroatom α-substituents, including hydrazino, hydrazido, amino, amido and hydroxyl groups, as well as a range of substituents at R^1. The results for compounds **1** and **2** are particularly noteworthy since a method for biocatalytic resolution of hydrazino compounds of this general structure has not been previously available. The enantiomeric excess of the recovered esters generally exceeds 98–99% at 50% conversion,[18a] indicating *E* values[13] in the range of 450–1050. In practice the enzymatic hydrolysis proceeds to 50% conversion and stops,[18] allowing isolation of the remaining ester with excellent enantiomeric excess. In the resolution of substrates **1** and **2** the product carboxylic acid obtained at 50% conversion was also analyzed and found to be of high enantiomeric excess (>99%).

A representative experimental procedure on a multigram scale is presented for the resolution of racemic **4**. Ethyl α-methylphenylalanate (48 g, 0.23 mmol) is suspended in 2.5 L of 50 mM sodium phosphate buffer (pH 7.4). A solution (530 mL) of *C. lipolytica* esterase[10,17] is added to the substrate suspension and stirred at ambient temperature. The reaction progress is monitored by HPLC,[14a] and

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(12) Screening experiments were conducted with commercially available hydrolases including lipases from porcine pancreas, *Pseudomonas fluorescens*, *Candida cylindracea*, *Mucor javanicus*, *Rhizopus arrhizus*, and *Rhizopus delemar*, pig liver and horse liver esterase, α-chymotrypsin, subtilisin Carlsberg, penicillin-G acylase, and hog kidney acylase, using compounds **1** and **2** as substrates. These enzymes were not pursued because of poor hydrolytic activity or enantioselectivity. We note that α-chymotrypsin and subtilisin showed activity toward **1** and **2**. The remaining starting material at >50% conversion was generally of 50–60% ee, with enzyme specificity for hydrolysis of the (*R*)-isomer.

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(14) (a) The course of the enzymatic hydrolyses was followed by periodic withdrawal of aliquots for analysis by HPLC on a C-18 column. Hydrolysis reactions of compounds **1** and **2** were monitored at 280 nm, compounds **3–8** at 254 nm, and compound **9** at 210 nm. (b) The enantiomeric excesses of compounds in Table I were determined by HPLC analysis of camphanic hydrazide derivatives[20] or 2,3,4,6-tetra-*O*-acetylglucopyranosyl-β-D-glucopyranosyl thiourea derivatives[21] of amino esters, or by analysis on a chiral HPLC column (compounds **5** and **7**). Details of these analyses and assignment of absolute configuration are given in the supplementary material. (c) The enantiomeric excesses for the product acids **3–9** were not determined. (d) Satisfactory spectral and analytical data were obtained for all compounds listed in Table I (see supplementary material).

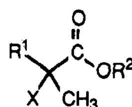
(15) In preliminary experiments we have succeeded in purifying to homogeneity the active enzyme species present in crude *C. lipolytica* lipase. The enzyme is present as a minor component (300–700 μg/g crude lipase) and does not show lipase activity toward triolein or olive oil. We propose that this active species be named *C. lipolytica* esterase (CLE). A detailed account of enzyme purification and characterization studies is in preparation.

(16) In a typical experiment the substrate (50–250 mg) was suspended in 5–25 mL of 50 mM sodium phosphate buffer at room temperature and treated with 0.5–2.5 g of crude *C. lipolytica* lipase. The reaction progress was monitored by HPLC[14a] and typically ceased following ca. 50% substrate conversion. The remaining ester was isolated by extracting the reaction mixture with methylene chloride or chloroform, and the ee analysis by HPLC was carried out.[14b] We have also used purified *C. lipolytica* esterase (CLE)[15,17] to resolve substrates **1** and **4** with ee's exceeding 98–99%.

(17) For large-scale work the crude *C. lipolytica* lipase is dissolved prior to use in 50 mM HEPES buffer (ca. 440 mg of crude powder·mL^{-1}) at pH 7.4 and dialysed (12000–14000 MW cutoff) against 50 mM sodium phosphate containing 1 mM EDTA (pH 7.4). Ultrafiltration (10000 MW cutoff) of the dialysate yields 580 mL of crude enzyme concentrate (protein concentration ca. 8 mg·mL^{-1}). We estimate the concentration of the active enzyme[15] to be 130–300 μg·mL^{-1}.

(18) (a) We have observed that the resolution rate with CLE slows dramatically after ca. 45% substrate conversion is reached. Care must therefore be taken to ensure that the 50% conversion required for maximum ee of recovered ester is reached. In resolutions of compounds **1** and **2** the substrate conversion was difficult to quantify precisely and was 50 ± 1%. The >98% ee reported for recovered esters **1** and **2** is likely less than >99%, as seen for **3–7**, due to difficulty in achieving true 50% substrate conversion. Similar difficulties in achieving 50% conversion have been noted in the use of acylase I to resolve *N*-acetyl amino acids.[9a,19] (b) In control experiments we have observed that α-amino and α-hydrazino esters are susceptible to chemical hydrolysis in aqueous solution at pH 4–8. This chemical hydrolysis is not significant for reaction times of up to 8 h and can be controlled by using sufficient quantities of enzyme to achieve this reaction time. Alternatively, the *N*-acetyl derivatives have been found to be completely stable under the reaction conditions and may be preferred in cases where the reaction rate is unacceptably slow.

Table I. Results for Resolutions with *C. lipolytica* Esterase[14]



compd	R^1	X	R^2	% convn[18]	% ee recovered ester	% ee[14c] recovered acid
1	CH_3O / HO	$-NHNH_2$	n-Bu	50	>98[18a]	>99
2	CH_3O / HO	$-NHNHCOCH_2Ph$	Et	50	>98[18a]	>99
3	CH_3O / CH_3O	$-NH_2$	Et	50	>99	-
4		$-NH_2$	Et	50	>99	-
5		$-NHCOCH_3$	Et	50	>99	-
6		$-NH_2$	n-Bu	50	>99	-
7	CH_3O / HO	-OH	Et	50	>99	-
8	N / H	$-NH_2$	n-Bu	60	95	-
9		$-NHCOCH_3$	Et	50	92	-

when enzymatic hydrolysis stops after 50% substrate conversion (6 h) the reaction mixture is washed twice with 1000 mL of $CHCl_3$ to extract the remaining ester. The combined organic extracts are dried and concentrated under vacuum to yield (*R*)-4 (18 g, 37.4% yield) with >99% ee.[14b]

The stereochemistry of (*R*)-4 was confirmed by acetylation to give the acetamide with $[\alpha]^{20}D = -52.2°$ ($c = 1$, $CHCl_3$) which compares favorably with that reported for >95% ee (*R*)-5 ($[\alpha]D = -47.8°$ ($c = 1$, $CHCl_3$)).[5c] The enantiospecificity of resolutions of compounds 1 and 2 was confirmed by HPLC comparison of carbidopa derived from resolved 1 and 2 with authentic USP samples.[14b] By analogy to the results with compounds 1–2 and 4–5 we suggest that CLE hydrolyzes the (*S*)-isomer of the additional compounds delineated in Table I.

The high level of enantioselectivity observed for hydrolyses by *C. lipolytica* esterase of a range of tertiary α-substituted esters is rare for enzyme reactions of presumably unnatural substrates. The enzyme tolerates a number of heteroatom α-substituents and shows an apparent preference for an α-methyl group as well as an aromatic ring. Efforts to define further the substrate specificity and to characterize this unique enzyme are underway and will be reported in due course.

Supplementary Material Available: Experimental procedures and characterization data for the compounds listed in Table I (13 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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