Enzymatic Hydrolysis of a Prochiral 3-Substituted Glutarate Ester, an Intermediate in the Synthesis of an NK_1/NK_2 Dual Antagonist

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Abstract: An enzymatic process for desymmetrization of the prochiral diethyl 3-[3',4'-dichlorophenyl]-glutarate, **1**, an intermediate in the synthesis of a series of neurokinin (NK) receptor antagonists, has been developed and scaled up. The transformation catalyzed by *Candida antarctica* lipase B in either the free or the immobilized form was carried out at 100 g/L of substrate and proceeded with an average conversion of 97%. In the pilot plant, the process produced 200 kg of **2** in 3 batches with ee >99% and an average isolated yield of 80%. The immobilized enzyme preparation was particularly effective, achieving over 70,000 enzyme turnovers per batch.

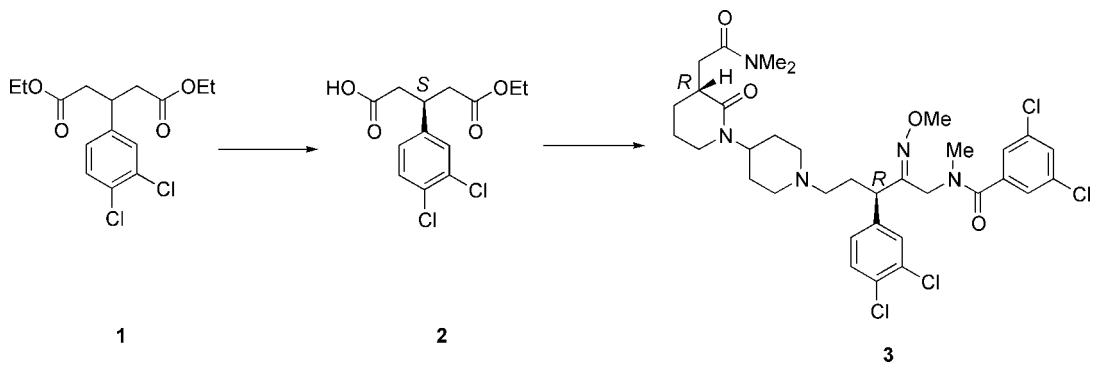
Keywords: *Candida antarctica* lipase; enzymatic desymmetrization; enzymatic hydrolysis; prochiral selectivity

Introduction

Tachykinins are a group of biologically active neuropeptide hormones that are widely distributed throughout the nervous system. They are implicated in a variety of biological processes such as pain transmission, neurogenic inflammation, vasodilation, and secretion.[1] The effect of tachykinins is modulated via the specific G-protein-coupled receptors, NK_1, NK_2, and NK_3 and therefore, non-peptide NK-receptor antagonists are believed to be efficacious in the treatment of a variety of chronic diseases including asthma, bronchospasm, arthritis, and migraine.[2,3,4,5,6]

The detailed investigation of the structure-activity relationship of several non-peptide NK_1/NK_2 antagonists has led to the discovery of a new class of oxime-based NK_1/NK_2 dual antagonists (Scheme 1).[7,8,9] The biological evaluation of one such antagonist, **3**, has revealed that the affinity for the NK_2 receptor resides predominately in the *R,R*-diastereomer.

Here we report a novel approach for establishing the desired stereochemistry of one chiral center based on an enzymatic hydrolysis of the prochiral diethyl 3-[3',4'-dichlorophenyl]glutarate (**1**). It has been shown that the resulting monoglutarate **2** can be then elaborated into **3** by conventional chemical means without racemization.



1 2 3

Scheme 1.

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Results and Discussion

Although the enzymatic desymmetrization of **1** has not been described in the literature, the hydrolysis of similar prochiral glutarate diesters has been studied extensively resulting in the product with the *R*-configuration.[10–16] For example, enantioselective hydrolysis of diethyl 4-chlorophenylglutarate catalyzed by chymotrypsin was exploited in the synthesis of a cholesterol absorption inhibitor, (+)-Sch 54016.[12] The hydrolysis of the corresponding dimethyl ester was employed in the synthesis of the enantiopure (*R*)-baclofen.[15] The enzymatic desymmetrization of yet another derivative, 3-cinnamylglutarate, was instrumental in the synthesis of the macrolide antibiotic rhizoxin.[11]

In an attempt to find a catalyst with *S*-selectivity, the screening of about 200 commercial hydrolases was undertaken revealing several enzymes capable of hydrolyzing **1** to both (*S*)-**2** and (*R*)-**2** with good to excellent enantioselectivity (Table 1). Out of 11 candidates with *pro-S* selectivity, lipase B from *Candida antarctica*, Chirazyme L-2, was selected for further development due to the enzyme's high thermostability, excellent selectivity, and moderate cost.

Reaction Optimization

Even though *Candida antarctica* lipase B appeared to be superior to other catalysts, the degree of conversion of **1** by the immobilized enzyme preparation was relatively poor (Table 1). This low conversion was at least partly attributed to diffusional limitations between the solid immobilized enzyme preparation and the highly insoluble substrate (the solubility of **1** in water is in the micromolar range). In an attempt to improve the reaction rate, the effect of temperature on the initial rate of hydrolysis was examined. It was found that around the melting point of the substrate (36 °C) the initial rate of hydrolysis catalyzed by the immobilized lipase preparation increased by a factor of 7 within a range of only 2 °C (Figure 1, A).

In contrast, the reaction catalyzed by the soluble enzyme exhibited typical Arrhenius behavior characterized by an activation energy of about 91.5 kJ/mole (Figure 1, B). This difference in behavior between the immobilized and the soluble enzyme preparations supports the notion that the rate of hydrolysis catalyzed by the immobilized enzyme is controlled by diffusion at temperatures below the melting point of the substrate (T_m). However, above the T_m, where the

Table 1. Enzymes that hydrolyze diethyl 3-[3',4'-dichlorophenyl]glutarate with high to excellent selectively.

Vendor	Enzyme (mg used)	ee (%)	Product configuration	% conversion
Genzyme	Lipase *Candida cylindracea*	>99	*S*	32
Meito Sangyo	Lipase OF *Candida cylindracea*	89	*S*	13
Novo Nordisk	Novozyme 435: *Candida antarctica*, type B	>99	*S*	59
Novo Nordisk	SP 525 Lipase: *Candida antarctica*, type B	>99	*S*	31
Seikagaki	Lipase: *Rhizopus delemar*	>99	*S*	40
Boehringer	Cholesterol esterase: *Candida cylindracea*	>99	*S*	65
Boehringer	chirazyme L2	>99	*S*	31
Altus	ChiroCLEC CRO	>99	*S*	10
Altus	ChiroCLEC-CR	>99	*S*	19
Altus	Lipase CR: A. Grade 001	>99	*S*	30
Diversa	ESL-001–01	>99	*S*	25
Biocatalyst	Lipase porcine pancreatic	>99	*R*	12
Sigma	Lipase type II porcine pancreas	>99	*R*	5
Solvay	Lipase pancreatic	>99	*R*	7
Sci. Prot. Lab	PEC High Lipase	>99	*R*	17
Sigma	Chymotrypsin type II bovine pancreas	>99	*R*	26
Sigma	Trypsin: porcine pancreas	97	*R*	16
Rohm	Corolase PP: porcine pancreas	>99	*R*	5
Boehringer	chirazyme L7	>99	*R*	20
ThermoGen	ThermoCat E001	95	*R*	27
ThermoGen	ThermoCat E002	90	*R*	10
ThermoGen	ThermoCat E003	97	*R*	39
ThermoGen	ThermoCat E004	95	*R*	22
ThermoGen	ThermoCat E005	96	*R*	30
ThermoGen	ThermoCat E006	96	*R*	37
ThermoGen	ThermoCat E008	95	*R*	38
ThermoGen	ThermoCat E010	95	*R*	30
ThermoGen	ThermoCat E011	95	*R*	31
ThermoGen	ThermoCat E012	96	*R*	32
ThermoGen	ThermoCat E013	93	*R*	22
ThermoGen	ThermoCat E014	94	*R*	35
ThermoGen	ThermoCat E015	94	*R*	26
ThermoGen	ThermoCat E0017B	94	*R*	35
ThermoGen	ThermoCat E019	96	*R*	27
ThermoGen	ThermoCat E020	96	*R*	40
ThermoGen	ThermoCat E027	97	*R*	66

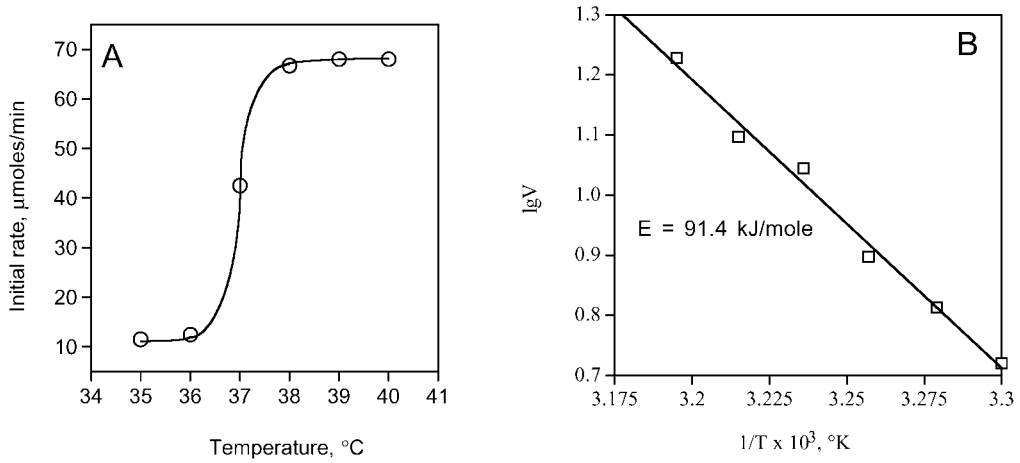

Figure 1. A: The temperature dependence of the initial reaction rate of the hydrolysis of **1** by immobilized *Candida antarctica* lipase B. **B:** The same for the soluble enzyme in the Arrhenius format.

substrate was emulsified, the reaction proceeded efficiently with little or no diffusional limitations.

The low activity exhibited by many enzymes in reactions with xenobiotic substrates and the commonly encountered phenomenon of substrate/product inhibition are considered to be major obstacles to the successful commercialization of biocatalysis. To determine if the hydrolysis of **1** suffered from substrate inhibition, the initial rate of the reaction catalyzed by the soluble enzyme preparation was studied as a function of the substrate concentration in a range between 12 and 300 mM. The results, presented in Eadie-Hofstee format (Figure 2), clearly indicate that within a wide range of substrate concentrations the reaction followed Michaelis-Menten kinetics with no substrate inhibition and an apparent K_M of 88 mM.

Moreover, product inhibition, though not studied specifically, is not likely to be a significant factor as indicated by the greater than 95% conversion of 100 g/L of the substrate (Figure 3, curves a, b).

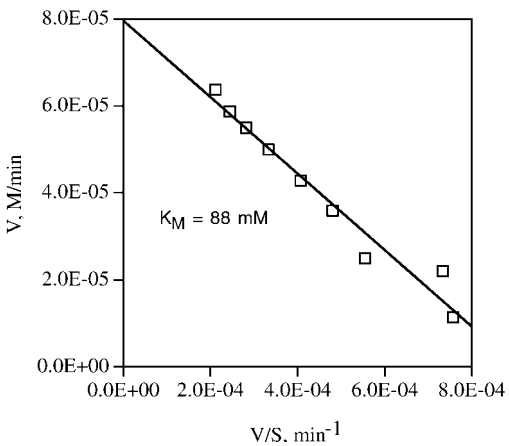

Figure 2. Eadie-Hofstee plot for the hydrolysis of **1** by the soluble *Candida antarctica* lipase B.

While the rate, as expected, depended on the concentration of the catalyst, >95% conversion was achieved within 24 h in the presence of >20 g/L of the immobilized form of the enzyme. However, lowering the enzyme loading to 10 g/L resulted in a significant slow down of the reaction after about 30 h due to inactivation of the catalyst (curve c). This finding was somewhat unexpected in light of the commonly accepted view that *Candida antarctica* lipase B is a highly thermostable enzyme capable of tolerating temperatures up to 80 °C.[10,17] Under our operating conditions, the immobilized lipase preparation lost ~30% of its original activity within the first 18 h. The inactivation of the soluble enzyme was even more pronounced with a half-life of only 15 h at 32 °C, pH 8.0.

The enzyme inactivation profile presented in Figure 4 clearly reveals that at least 2 successive first order processes are responsible for enzyme inactivation. A relatively slow process with a k_{inact} of 0.007 h^{-1} was followed by a much faster process (k_{inact} = 0.028 h^{-1}). It was hypothesized that the oxidation of the Met 72 residue situated near the enzyme's

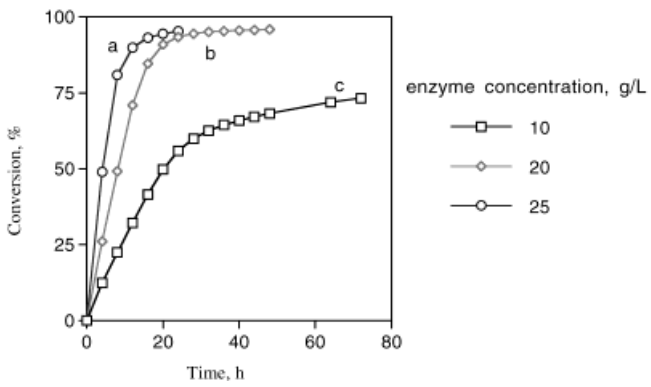

Figure 3. Hydrolysis of **1** by immobilized *Candida antarctica* lipase B at varying enzyme loadings.

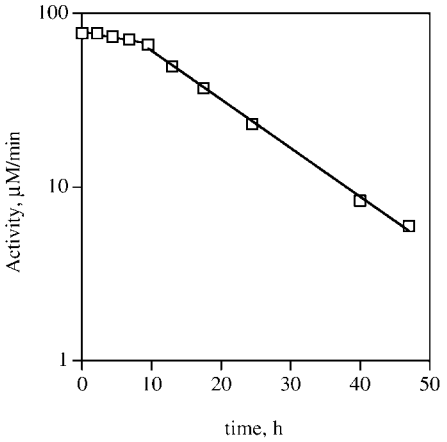

Figure 4. Inactivation of soluble *Candida antarctica* lipase B under the reaction conditions at 32 °C, pH 7.5 (see experimental section for details).

active site might be at least partly responsible for the inactivation.[18] However, exclusion of air did not increase the stability of the lipase to any appreciable extent, removing support for oxygen being involved in this deactivation. Although the exact mechanism of lipase inactivation has not been investigated, one can hypothesize that the relatively low stability of this thermostable enzyme under the process conditions is caused by the unfavorable non-specific interactions between the enzyme and the hydrophobic surface of the highly insoluble substrate. Since the rate of inactivation was found to decrease at more neutral pH, all preparative transformations were carried out at pH 7.5 and 8.0 for soluble and immobilized preparations, respectively.

Product Isolation

Due to the low solubility of the substrate, the high degree of conversion, and the excellent solubility of the product in a basic aqueous medium, product isolation was straightforward and easily amenable to scale-up. Following the removal of the enzyme beads and unreacted diester by filtration, the aqueous reaction mixture was acidified causing the desired mono-ester to precipitate. The precipitate was then recovered by filtration, washed, dried and purified by recrystallization from a TBME/heptane mixture. The enzyme preparation was not recycled due to partial inactivation of the catalyst.

The use of soluble enzyme as catalyst eliminated the need for the enzyme filtration step thereby simplifying the purification procedure even further. Another benefit of using soluble enzyme was lower susceptibility of the process to temperature variation and the slightly higher yield of the isolated product (Table 2). Nevertheless, because of the lower stability of the free enzyme, the overall cost contribution of the soluble catalyst was about twice that of the immobilized enzyme preparation.

Table 2. Comparison of processes catalyzed by immobilized and soluble enzymes on a pilot scale.

	Immobilized enzyme	Soluble enzyme
Substrate loading, (g/L)	100	100
Catalyst/substrate ratio (w/w)	0.125[a]	0.0065[b]
pH	8.0	7.5
Temperature (°C)	38–40	28–32
Reaction time (h)	40–45	40–45
Conversion (%)	92–96	94–98
ee (%)	>99	>99
Enzyme turnovers/batch	~70,000[c]	~15,000[b]
Enzyme cost, $/kg of **2** (no recycling)	200–210	350–360

[a] Based on total weight of the immobilized enzyme.
[b] Based on protein content of the soluble enzyme (~13 mg/mL).
[c] Based on protein content of immobilized enzyme (~1% w/w).

Conclusion

In conclusion, an enzymatic process for the enantioselective hydrolysis of **1** utilizing either soluble or immobilized preparations of *Candida antarctica* lipase B has been developed. About 200 kg of **2** with >99% ee have been produced in 3 batches with ~80% average molar yield.

Experimental Section

Materials and General Methods

Commercially available solvents supplied in bulk quantities and reagents were used without further purification. The course of hydrolysis was followed by reversed-phase HPLC on a Waters Symmetry C_{18} (4.6 × 250 mm) column equilibrated with acetonitrile/water/trifluoroacetic acid mixture (57/43/0.025). The chiral analysis was performed on a Phenomenex Chirex 3005 (50 × 4.6 mm) column equilibrated with hexane/isopropyl alcohol/trifluoroacetic acid mixture (98/2/0.025). ^{13}C and ^{1}H NMR spectra were determined using a Varian XL-300. MS data were obtained on a Perkin Elmer SCIEX API-150 MS. Optical rotation was measured on a JASCO DIP-1000.

Chirazyme L-2 was purchased from Boehringer Mannheim (Penzberg, Germany). It was supplied as a solution (~13 mg enzyme/mL) with an activity of 12,000 U/mL (920 U/mg enzyme) in the tributyrin assay at 25 °C, and as a solid (immobilized preparation) with activity of ~8,000 U/g (~1% w/w enzyme loading).

The conversion and enantioselectivity of the enzymatic hydrolysis of **1** were determined as follows: the enzyme preparations (1 – 30 mg) were placed in vials containing 50 mM sodium phosphate buffer pH 7.0 (0.9 mL) and the reactions initiated by the addition of solution of **1** (0.25 g/mL) in ace-

tone (0.1 mL). Following 48 hours of incubation at 30 °C with agitation at 225 rpm, the reactions were stopped by adjusting the pH to ~2.0 with 6 N HCl. The samples were extracted with ethyl acetate (2 mL) and analyzed for conversion and selectivity by the reverse-phase and chiral HPLC, respectively.

The lipolytic activity was determined by measuring the initial rate of hydrolysis of tributyrin at 25 °C as follows: tributyrin (3 mL) was added to a 100-mL thermostated cuvette containing 0.1 M KCl (50 mL) and the mixture emulsified by high speed stirring with a stirrer bar. The reaction was initiated by the addition of the lipase (15 U). The rate was monitored on a pH-stat by maintaining pH 7.5 by automatic addition of 0.1 M NaOH.

The Michaelis constant was determined by measuring the initial rate of hydrolysis of **1** in a range of concentrations between 12 and 300 mM at 32 °C in 25 mM potassium phosphate buffer. The pH was maintained at 7.5 by the automatic addition of 0.5 M NaOH.

The lipase inactivation was determined as follows: **1** (40 g) was placed into a 500-mL flask containing 25 mM potassium phosphate buffer (180 mL) and the free lipase (10 mL, 1.4×10^5 units of activity). The reaction mixture was agitated at 32 °C at pH 7.5. Samples were withdrawn periodically, and the remaining lipolytic activity was determined in a standard tributyrin assay as described above.

Pilot-Scale Preparation of (*S*)-Ethyl 3-[3',4'-Dichlorophenyl]glutarate (2) using Chirazyme L-2 Adsorbed on Beads

Diethyl 3-[3',4'-dichlorophenyl]glutarate (**1**; 50 kg) was dispersed in 10 mM sodium phosphate buffer pH ~8.0 (450 L) with agitation at 40 °C in a 1200-L glass-lined reactor equipped with an in-vessel pH probe. The reaction was initiated by the addition of 6.25 kg of Chirazyme L-2 (adsorbed on beads). The pH was maintained between 7.9 and 8.1 by the addition of 0.5 M NaOH. A 93% conversion yield was achieved at 38 – 40 °C following 43 hours of incubation. The hydrolysis was stopped by removing the enzyme by filtration. The enzyme filter cake was washed with 100 mM phosphate buffer pH ~8.0 (~ 125 L). The filter cake wash and filtrate were combined in an 800-L reactor and the pH was adjusted to ~4.2 using 10% H_2SO_4 followed by isolation of the precipitated product by filtration. The acid precipitate was rinsed *in situ* with water (~100 L) and dried on trays in an air dryer, yielding 40.5 kg product in >99% enantiomeric excess. The dried cakes from two batch reactions (~79 kg), both made with the fresh supply of the enzyme, were mixed with 6 kg of Supercel and TBME (320 L). Insoluble material and Supercel were removed by filtration in a sparkler and rinsed with TBME (110 L). The rinse was combined with the filtrate and concentrated 2 – 3-fold by vacuum evaporation. The TBME concentrate was mixed with *n*-heptane (640 L) and vacuum evaporated to remove the TBME. Removal of TBME resulted in precipitation of the desired *S*-monoester. The precipitated slurry was filtered and dried; yield: 75.2 kg (82.1%; >99% ee). $[\alpha]_D^{20}$: + 6.9 (*c* 1.0075, EtOH). ^{13}C NMR (DMSO-d_6): δ = 172.52, 171.00, 144.38, 130.72, 130.28, 129.71, 129.06, 128.18, 59.84, 40.32, 38.65, 37.39, 13.96; ^{1}H NMR (DMSO-d_6): δ = 7.55 (d, *J* = 2.0 Hz, 1H), 7.50 (d, *J* = 8.0 Hz, 1H), 7.26 (dd, *J* = 2.0 Hz, 1H), 3.92 (dd, *J* = 2.0 Hz, 2H), 3.37 – 3.96 (m, 1H), 2.48 – 2.76 (m, 4H), 1.05 (t, *J* = 6.8 Hz, 3H). Anal. calcd. for $C_{15}H_{14}Cl_2O_4$: C 51.17, H 4.62; found: C 51.33, H 4.67.

Pilot-Scale (kg) Preparation of (S)-Ethyl 3-[3',4'-Dichlorophenyl]glutarate (2) using Soluble Chirazyme L-2

Diethyl 3-[3',4'-dichlorophenyl]glutarate (**1**; 6.5 kg) was dispersed in 25 mM sodium phosphate buffer pH ~8.0 (~60 L) with agitation at 30 °C in a 200-L glass-lined reactor equipped with an in-vessel pH probe. The reaction was initiated by the addition of 3.25 L of Chirazyme L-2 (liquid). The reaction pH was maintained between 7.4 and 7.6 was by the addition of 0.5 M NaOH. The reaction was terminated after 45 h (97% molar yield) by reducing the pH to ~4.2 using 10% H_2SO_4, followed by isolation of the precipitated product by filtration. The acid precipitate was rinsed *in situ* with water (~13 L) and dried on trays in an air dryer, yielding 5.8 kg product (>99% ee). Dried cakes from two batch reactions (~11.4 kg) were suspended in TBME (50 L) and Supercel (2 kg) was added. Insoluble material and Supercel were removed by filtration and rinsed with TBME (11 L). The rinse was combined with the filtrate and concentrated 2 – 3-fold by vacuum evaporation. The TBME concentrate was mixed with ~110 L of n-heptane and vacuum evaporated to remove the TBME. Removal of TBME resulted in precipitation of the desired *S*-monoester **2**. The precipitated slurry was filtered and dried; yield: 9.7 kg (81.4%; >99% ee).

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