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Kinetic Resolution of β-Amino Esters by Acylation Using Immobilized Penicillin Amidohydrolase

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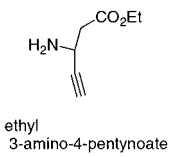
Abstract:

Penicillin amidohydrolase [EC 3.5.1.11] was used to resolve stereoisomers of a β-amino acid ester (ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate) by phenylacetylation. After screening commercially available sources of the immobilized enzyme, one was found to be significantly more efficient, and this was developed at 1-L scale reaction. The effects of phenylacetic acid concentration, β-amino acid ester concentration, and pH on bioconversion rates and side reactions were examined. The enzymatic reaction was monitored off-line by naphthoylation of samples and chiral analytical chromatography. The best conditions for the bioconversion were pH 5.7, 28 °C, and 14 000 units of enzyme activity per liter. The phenylacetic acid concentration was set at 50 g/L (0.37 M), and the amine at 100 g/L (0.47 M). Under these conditions, yields of the desired (*S*)-amino acid ester were on the order of 90% with ee's of 95% or greater in less than 12 h. This process, along with a slight modification, was tested through 15 cycles at 0.4-L scale, and was scaled to 70 L. Recycle results extrapolated to approximately 25 reaction cycles before the enzyme lost 50% of its initial activity. Through three runs at 70 L, overall yield of (*S*)-amine was 42.7 ± 0.6%, overall yield of (*R*)-phenylacetyl amide was 47.2 ± 1.8%. The average ee of the amine (two runs) was 98.1 ± 0.4%, for the amide the ee was 99.5 ± 0.2%.

Introduction

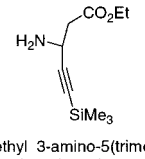
Pharmacia developed a pseudopeptide designed to inhibit platelet adhesion. In the synthesis of this pseudopeptide a chiral synthon, ethyl 3(*S*)-amino-4-pentynoate, was used. To prepare the enantiomerically pure synthon, ethyl 3(*R*,*S*)-amino-5-(trimethylsilyl)-4-pentynoate was resolved as the mandelate salt. Other options, including asymmetric synthesis were considered. While the crystallization as the mandelate salt produced satisfactory enantiomeric purity, the yields were low, less than 50% of the desired isomer was recovered. Asymmetric synthesis utilized expensive reagents but was under development. As an option to these procedures, enzymatic resolution using penicillin acylase was proposed.

Penicillin G acylase (PGA, EC 3.5.1.11, penicillin amidase or penicillin amidohydrolase) is a serine hydrolase with a high specificity (low substrate tolerance) for the acyl side chain (phenylacetyl) but a low selectivity (high substrate tolerance) for the amino side chain.[1] The enzyme comprises two subunits, α and β, each of which may have activity.[2] Mechanistically, the enzyme is believed to produce an acyl-enzyme intermediate like the serine proteases.[3] Although penicillin G acylase has traditionally been used to hydrolyze penicillin G,[4] it has also been used to resolve other amino compounds.[5] Screening for penicillin acylases and selection of the substrate amino ester (nucleophile) for the biocatalytic resolution are reported. The kinetic study of the enzymatic resolution of ethyl 3-amino-4-pentynoate (amine **1**) and ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate (amine **2**) is reported. The effects of pH, amine and phenylacetic acid concentrations, and agitation are described. One-liter scale bioconversions for two different amines (*tert*-butyl 3-amino-5-(trimethylsilyl)-4-pentynoate or amine **3** and amine **2**) are also presented. Additional details may be found elsewhere.[6]



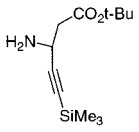
ethyl
3-amino-4-pentynoate

1



ethyl 3-amino-5(trimethylsilyl)
4-pentynoate

2



t-butyl 3-amino-5(trimethylsilyl)
4-pentynoate

3

Experimental Section

I. Analytical. *Reversed Phase (RP-HPLC) Chromatography.* Aqueous samples for RP-chromatography were diluted into an equal volume of acetonitrile, except for amine

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3, for which samples were diluted with two to four volumes of acetonitrile.

Chiral Chromatography. The chiral purity of both phenylacetyl amide and unacylated amines were determined using a 25 cm × 4.6 mm Pirkle covalent column ((*S*,*S*) Whelk-O 1 from Regis Technologies, Inc., Morton Grove, IL 60053). Mobile phase was 20−30% 2-propanol in *n*-hexane, flow was 1.25 mL/min, with detection at 255 nm. Standards were run to confirm retention times. For chiral analysis of phenylacetyl derivatives, 2 mL of the reaction suspension were extracted with 10 mL of ethyl acetate, the ethyl acetate was extracted with 2 mL of 5% $NaHCO_3$, dried, and evaporated in vacuo. The residue was dissolved in 1 mL of 2-propanol and injected onto the chiral column. To analyze the unacylated amines, 2 mL of the bioreaction were extracted with 2 mL of methylene chloride. The methylene chloride layer was extracted three times with 100 μL of 2.5 N NaOH and dried with sodium sulfate. Two drops of 1-naphthoyl chloride were added, and the solution was mixed. After 5 min samples were diluted 10-fold with 2-propanol and analyzed. The reaction was assayed after a few hours and then every hour as the bioconversion neared completion.

Determination of Bioactivity. Enzyme activity was assessed according to a procedure supplied by Roche Diagnostics (Roche Test Method 5407, edition 9). The reaction was run in 1 mM potassium phosphate, pH 8.0, at 28 °C. Penicillin G (potassium salt) was dissolved in water (all water used in all studies was either HPLC grade or Milli Q water) to 100 mg/mL (solutions had to be prepared daily). After equilibration of both solutions at 28 °C, 0.4 mL of 50 mM potassium phosphate, pH 8.0 was added to 20 mL of the penicillin G solution, and then immobilized penicillin G acylase (approximately 20 units) was added to initiate the reaction. The reaction was stirred magnetically and maintained at 28 °C in a jacketed 50-mL beaker. The reaction pH was controlled at 8.0 by the addition of 0.10 M NaOH, using a Radiometer PHM82 and ABU80 autoburet. One unit was defined as that activity which consumed 1 μmol of base per min at 28 °C.

Titration of Amines. Amines **1, 2**, and **3** were titrated using a Radiometer PHM82 and ABU80 autoburet. Standardized sodium hydroxide solutions were used to back-titrate from the hydrochloric acid solutions of the amines.

II. Selection of Substrate, Nucleophile, and Enzyme. *Selection of Acyl Donor and Nucleophile.* The selection of acylating agent was made for the resolution of a specific amine by the three different enzymes, penicillin-G-amidase-450 (Roche Diagnostics, formerly Boehringer Mannheim, immobilized enzyme 129 units/g, lot 13811543-07), Eupergit penicillin acylase-600 (Rohm Pharma immobilized enzyme 83 units/g, lot 0440339025), and Pen-G acylase SpA (Recordati immobilized enzyme, lot 950-1613). In these experiments, immobilized enzyme that had been washed with water was added to the enzyme reactor. To 25 or 30 mL of Milli Q water or 0.05 M phosphate buffer was added 1.2−1.5 mmol of amine **1** with magnetic stirring to dissolve the amine. Washed enzyme was then added to a final concentration of 0.13 or 0.26 units/mL. After the addition of enzyme the pH was adjusted to 6.0 with 0.5 N NaOH and maintained at that pH with 2.5 N NaOH during the reaction. Between 1.8 and 4.5 mmol of ethyl phenylacetate was added periodically throughout the first 5 h incubation. Samples were analyzed by RP-HPLC.

tert-Butyl 3-amino-5-(trimethylsilyl)-4-pentynoate (Amine 3). Phenylacetic acid, 20.36 g (150 mmol), was suspended in 900 mL of water and then titrated to pH 6.44 with 2.5 N NaOH. To this clear solution was added 16.0 g (39 mmol) of amine **3** (tosylate salt). Roche PGA-450 (5147 units) was washed with water and added to the stirred suspension at room temperature. Samples were assayed by RP-HPLC. After 92 h the bioconversion was harvested.

Enzyme Screening. Phenylacetic acid was used as the donor, and amine **1** or **2** was used as the acceptor. Enzymes used were PGA-450 (Roche Diagnostics GMBH, Mannheim, Germany, lots 13811543-07 and 13811557-07); Eupergit PcA-600 (Rohm Pharma immobilized enzyme, lot 044033-9025, Rohm GMBH, Chemische Fabrik, D 64275 Darmstadt); Pen-G acylase (Recordati immobilized enzyme, lot 950-1613, Unita Biochimici De. Bi., 20060 Cassina De Pecchi, Milano).

Amine **1** or **2** (1.5 or 5 mmol) or the respective hydrochloride salt was added to a bioreactor in a 25 °C water bath. Milli Q water (15 or 50 mL) was added, and the mixture was stirred to dissolve the amine or salt. For reactions in which amine **2** was used, the pH was adjusted to 2.0 to dissolve the amine. Phenylacetic acid was added to a final concentration of 147 mM, and the pH was adjusted to either 6.0, 6.5, 7.0, or 7.5 with 2.5 N NaOH and 1 N HCl, and no further pH adjustments were made throughout the reaction. Washed, immobilized enzyme (0.5 or 5 units/mL) was then added to the reactor. The final volume was diluted to 30 or 100 mL with Milli Q water. The 30-mL reactions were stirred magnetically, and the 100-mL reactions were stirred mechanically.

III. Parameter Optimization. *Thirty-Milliliter Screening Experiments.* Approximately 600 mg of phenylacetic acid (4.4 mmol) were dissolved in 20 mL of water by adding 2.5 N NaOH or NH_4OH with magnetic stirring at room temperature until dissolved. To this solution was added about 1.5 mmol of amine **2** salt (hydrochloride) in 10 mL and the pH adjusted to 6.0 or the target pH. Immobilized enzymes were washed in water immediately prior to adding to initiate bioconversion. Only the pH-rate profile, substrate screens, and enzyme screens were determined at this scale, using 0.5 unit/mL immobilized enzyme for the pH-rate profile and 0.13 to 5.2 units/mL for the substrate and enzyme screens.

Effect of Agitation. One-liter bioconversions were performed with amine **2** (47 mM) and phenylacetic acid (147 mM), pH 5.3−5.5. Using a jacketed, unbaffled glass round-bottom reactor (10 cm diameter × 15 cm height) equipped with an overhead stirrer and 2.5-cm marine prop, agitation was set at low, medium, and high settings. These corresponded to approximately 200, 300, and 400 rpm, respectively. At the low setting, no vortexing was apparent, although the enzyme was evenly suspended initially. At the medium setting there was a vortex (about 2 cm deep), and

at the high setting the vortex reached the impeller. The bioconversion was initiated by adding 500 units PGA-450 to one liter of the phenylacetic acid-amine **2** solution.

IV. Scale-Up. *One Hundred and Fifty-Milliliter Bioconversions.* Amine **2** (15 g, 70 mmol) was dissolved in 1 N hydrochloric acid, and the pH was adjusted to 2.0 or less. This solution was diluted to 97.5 mL with water and extracted twice with MTBE. Residual MTBE was removed in vacuo, and then the pH was readjusted to 5.4 with 2.5 N NaOH. Phenylacetic acid was dissolved in 5 N NaOH or 5 M NH_4OH, and the pH adjusted to approximately 6.0 with 2.5 N NaOH or 1 N HCl. Both solutions were equilibrated at 28 °C and then mixed with PGA-450 to initiate bioconversion. With these preset conditions, the final pH was close to 5.7. For 100-mL bioconversions, proportional amounts of reagents were used. Bioconversions were carried out in 250-mL unbaffled round-bottom flasks. Reactions were agitated by magnetic stirring sufficient to evenly suspend the immobilized enzyme throughout the course of the reaction. This scale was used for determining the effect on rates of phenylacetic acid concentration and recycle stability. For the phenylacetic acid studies, 0.5 units/mL enzyme was used. For the recycle stability, 14 units/mL were used.

One-Liter Bioconversions. Amine **2** was dissolved in 1 N HCl and extracted with MTBE. Residual MTBE in the aqueous layer was removed under vacuum. This solution was titrated with 2.5 M NH_4OH or 2.5 N NaOH to pH 4.5−5.5 and thermally equilibrated. Phenylacetic acid, neutralized with NH_4OH or NaOH (generally 5 N), was thermally equilibrated with the enzyme, and then the extracted amine **2** solution was added and the pH adjusted to the target pH. Samples were taken for reversed phase (RP-HPLC) and chiral HPLC analysis, and the pH was maintained at the target pH with 1 N HCl, 2.5 M NH_4OH, or 2.5 N NaOH. This scale was used to study the effect of amine concentration on rates, using PGA-450, 500 units/L..

Workup. The completed bioreactions were chilled and adjusted in two steps to pH 8.0 with 2.5 M $KHCO_3/K_2CO_3$ (1.25 M each) or 2.5 M NH_4OH, and extracted twice with one-half volume of chilled MTBE. The MTBE was removed by vacuum filtration from the top of the bioconversion mixture. The organic layer was extracted with 0.5 N HCl, a little more than a 10% excess of acid being used. The pH was checked, and if necessary, additional acid was added to lower the pH to 2.0 or less. The combined aqueous extracts were extracted once with 400 mL of MTBE which was added back to the initial MTBE layer. The combined MTBE layers were extracted once with 100 mL of water, which was discarded. The MTBE layer was dried with sodium sulfate and evaporated, or evaporated without drying. Evaporation was at approximately 50 Torr using a 40 °C water bath. The acidic aqueous layer was stirred with 500 mL of MTBE, and the pH was adjusted to between 7.8 and 8.2 with 2.5 N NaOH. After separation of the phases, the aqueous was extracted a second time with 500 mL of MTBE. The combined MTBE layers were dried with sodium sulfate (50 g) and evaporated at approximately 50 Torr using a 40 °C water bath. This process flow is diagrammed in Figure 1.

Seventy-Liter Bioconversions. Bioconversions were performed on 70-L scale in a baffled, open, stainless steel tank (jacketed; 105 L working volume), equipped with a bottom filter screen. Agitation was at 300 rpm (marine prop). The phenylacetate salt of amine **2** was prepared by adding a 0.43 M solution of phenylacetic acid in heptane to 0.43 M amine **2** in heptane at 50 °C. The suspension was cooled to 4 °C and the salt allowed to crystallize for 1 h. The salt was collected by centrifugation and washed with heptane. Recovery of salt was 98%. For bioconversion, 6.1 kg of this amine **2** salt was dissolved in 70 L of water at 25 °C. The pH was adjusted (as needed) to 5.4−5.6 and 525 000 units of PGA-450 was added to initiate reaction. After 20 to 22 h, 35 L of chilled (refrigerated, 4 °C) MTBE was added followed by 7.9 L of 2.5 M $KHCO_3/K_2CO_3$ (1.25 M each). The suspension was pumped out of the tank through the filter screen, leaving the wet enzyme in the tank. The layers were separated in a 120-L glass separatory funnel, and the aqueous suspension was re-extracted with 21 L of chilled MTBE and the aqueous layer discarded. The combined organic extracts were extracted with 19.8 L of 0.5 N HCl. After separation of the layers, the organic layer was re-extracted with 14 L of 10 mM HCl. The combined aqueous layers were extracted with 17.5 L of chilled MTBE. After separation, the MTBE extracts were combined, dried over sodium sulfate, and evaporated using a rotary evaporator. The temperature of the aqueous layer was lowered to 10 °C, 35 L of chilled MTBE were added, and the pH was adjusted to 7.9 with 2.5 M $KHCO_3/K_2CO_3$. After separation of the layers the aqueous layer was re-extracted with 17.5 L of MTBE. The combined MTBE layers were dried with sodium sulfate and evaporated. The recovered enzyme was used for three cycles.

Results and Discussion

I. Analytical. *Endpoint calculation*:

A_S = area of the naphthoylated (*S*)-amine peak

A_R = area of the naphthoylated (*R*)-amine peak

$$\%\ (R)\ \text{converted} = \left(1 - \left[\frac{A_R}{A_S}\right]\right) \times 100$$

Since it was known that the (*S*)-amine was acylated in the bioreaction much more slowly than the (*R*)-amine, the (*S*)-amine acted as an internal standard and was used to estimate the "zero time" concentration of the (*R*)-amine in each analytical sample. This assumption lead to an inherent uncertainty of less than 1% for individual assays. Individual rate determinations typically had standard errors of 2−6%, although between-run variation was higher, especially with amine **1**. For activity measurements against penicillin G, the standard errors were 1−2%.

Titration of Amines. In Figure 2 are shown titration curves for amines **1**, **2**, and **3**. From these curves, approximate pK_a of 6.8−6.9 for amine **2**, 6.9 for amine **1**, and 6.4−6.5 for amine **3** were determined. Under the mildly acidic conditions of the chiral resolution, the higher the pK_a the lower the concentration of nucleophile (unprotonated amine). It was, therefore, the low pK_a of these amines which allowed chiral resolution at pH 6 or less.

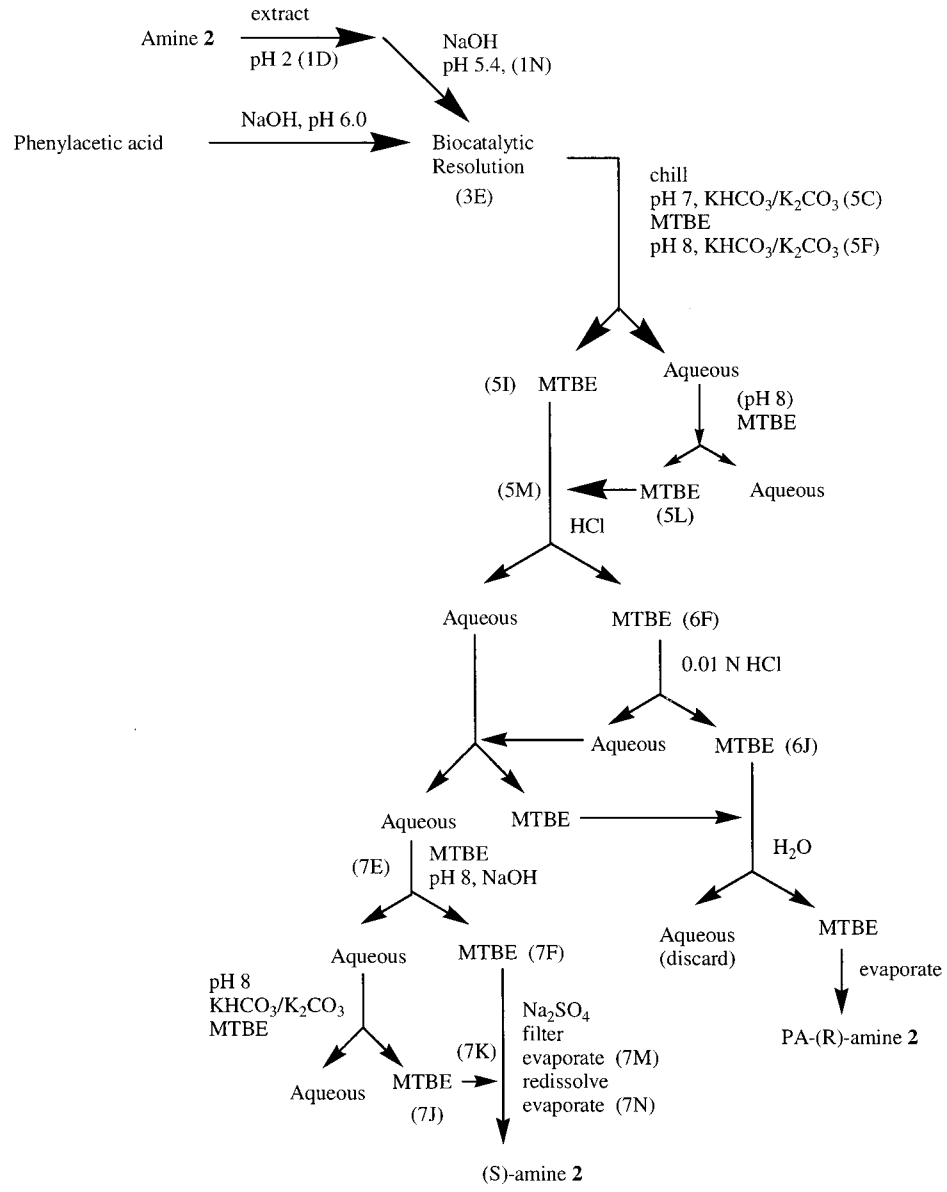

Figure 1. **Flow diagram for workup of bioconversions.**

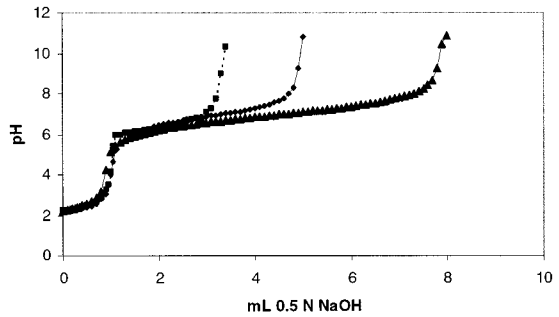

Figure 2. **Titration of amines with 0.5 N NaOH; (▲) amine 1; (◆) amine 2; (■) amine 3.**

II. Selection of Substrate, Nucleophile, and Enzyme. *Selection of Substrate (Acyl Donor) and Nucleophile.* Two substrates (acyl donors) and three nucleophiles were tested. The substrates were ethyl phenylacetate and phenylacetic acid, and the nucleophiles were ethyl 3-amino-4-pentynoate or its hydrochloride (amine **1**), ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate or its hydrochloride (amine **2**), and *tert*-butyl 3-amino-5-(trimethylsilyl)-4-pentynoate or its hydrochloride (amine **3**). Since unreliable initial rates were obtained when ethyl phenylacetate was used, phenylacetic acid was the preferred substrate (acyl donor).

Table 1. **Rate of reaction for different nucleophiles at room temperature with PGA-450 (average of three trials**

nucleophile	reactions	rate, μmoles/unit/h
amine **1**	pH = 6.0	2.7 ± 1.4
amine **2**	pH = 6.0	0.73 ± 0.10
amine **3**	pH = 6.0	0.54 ± 0.24

Using phenylacetic acid as the acyl donor, experiments were run varying only the amine (nucleophiles). Table 1 presents the specific reaction rates of the different nucleophiles. The initial specific rate for the experiment using amine **1** ranged from 1.8 to 4.3 μmol/unit/h compared to initial rates of 0.27−0.70 μmol/unit/h for the experiments using amine **3**. For amine **2**, the initial rates ranged from 0.67 to 0.81 μmol/unit/h.

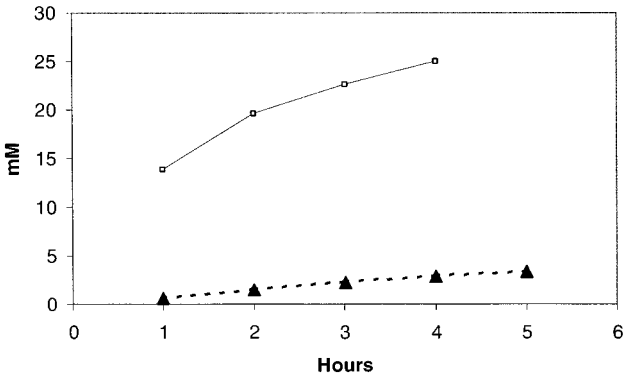

Figure 3. **Relative rates of acylation of (*S*)-amine 1 (▲) and (*R*)-amine 1 (□).**

The slower rates of acylation of amines **2** and **3** were due to their solubility in water which is lower than that of amine **1**. Acylation of amine **1** was studied at pH 6.0 at low enzyme concentration (0.13 units/mL) and pH 5.7 at high enzyme concentration (1.3 units/mL). Figure 3 records the time-dependent formation of (*R*)- and (*S*)-phenylacetyl amides of amine **1**. The rate of acylation for (*R*)-isomer was only 5-fold faster than that of (*S*)-isomer under these conditions (3.65 mM/h vs 0.7 mM/h). For amine **2**, the ratio of relative acylation rates for the stereoisomers was over 100; therefore, little acylation of the (*S*)-stereoisomer occurred. On the basis of the much greater selectivity for this nucleophile, amine **2** was chosen for development.

Enzyme Screening. Enzymes from eight sources were screened. The best three were examined in more detail. A comparison of the pH effect on the initial rates of reactions for three enzymes is shown in Table 2. PGA-450 (Roche Diagnostics) had the fastest initial rate at a pH of 6.0, and its activity decreased with either decreasing or increasing pH. The same was true for Pen-G acylase (Recordati SpA); however, the rate for Eupergit PcA 600 (Rohm Pharma) increased with increasing pH with a maximum at pH 7.0.

The PGA-450 and Pen-G acylase were retested. Initial rates were very comparable (Table 3). However, with the Recordati enzyme, to achieve 98% ee, it was necessary to allow the reaction to proceed for 30 to 40 h. Furthermore, while assay of the PGA-450 indicated 97% retention of activity, the Recordati enzyme activity was found to retain only 66% of the original activity. On this basis, PGA-450 was the enzyme chosen for the study, and Pen-G acylase (Recordati) selected as a backup enzyme.

III. Parameter Optimization. *pH-Rate Profile.* The pH dependence of the rate of acylation of amine **2** is shown in Figure 4. Although data from two different amine **2** concentrations are included, it is clear that the rate was maximal near pH 6.0. The decrease in rate with decreasing pH below 6 was probably due in part to increasing amounts of protonated amine and to the pH dependence of the enzymatic kinetic mechanism (optimum pH 7−9 for hydrolysis).[7] The decrease in rate above pH 6 was probably due in part to decreasing proportions of protonated phenylacetic acid and to competition from the reverse reaction, deacylation of (*R*)-phenylacetylamine **2**.

Agitation. There were three concerns involving agitation. The first entailed diffusion of the substrates to the immobilized enzyme. If the kinetics were diffusion-limited at low agitation, then increasing the agitation should increase the rate of acylation of amine **2**. The second centered on the ability to sample homogeneously, and the third consisted of possible degradation of the enzyme support, resulting in loss of enzymatic activity as well as increased difficulty in workup. When bioconversions were subjected to differing levels of agitation (Figure 5), it was apparent that once a certain agitation was achieved, the bioconversion rate was not especially sensitive to agitation. At very low agitation the apparent dependence on agitation was found to be due to sampling inhomogeneity since increasing the agitation immediately increased the apparent concentration of phenylacetylamine **2** to a concentration similar to that observed for the other agitation trials at the same reaction time. Small-scale (150 mL and smaller) reactions were performed using magnetic stirring. This ground the enzyme support to fine particles, dramatically decreasing its reusability. At no time with any of the reactions agitated by overhead stirring was degradation of the particles observed. However, since the benefits of increasing the agitation were small, high agitation rates were avoided.

Phenylacetic Acid Concentration. Experiments in which phenylacetic acid concentration was varied suggested that PGA-450 did not exhibit Michaelis−Menten kinetics toward phenylacetic acid. Since minimizing the phenylacetic acid concentration would affect cost, the dependence of the rate of bioconversion on phenylacetic acid concentration was determined (Figure 6). At low amine **2** concentration, it appeared that increasing concentrations of phenylacetic acid inhibited the bioconversion, by as much as 80%. When higher concentrations of amine **2**, the inhibition was much less. The rates observed at 100 mg/mL amine **2** were consistent with 98% conversion of the (*R*)-amine **2** in 11 h, using 14 units/mL enzyme.

Effect of Amine 2 Concentration. The kinetic behavior of the rate data for the (*R*)-amine **2** concentration could be either first-order (nucleophilic attack of the acyl-enzyme intermediate) or obey Michaelis−Menten kinetics. In Figure 7 are shown data for four concentrations of amine **2**, expressed as the (*R*)-stereoisomer. The data did not support first-order, but neither was it clear that the rate data followed Michaelis−Menten kinetics. Regardless, evaluation of these data by double reciprocal plot (Figure 8) suggested that V_{max} for 0.52 units/mL PGA-450 was 2.0 mM/h (8% cv) and the $K_{m(app)}$ was 20 mM (31% cv).

Endpoint Decision. One of the goals was to obtain (*S*)-amine **2** with an ee >99%. However, minimizing the losses of the (*S*)-amine **2** due to overacylation counterbalanced this. In Figure 9 are shown two bioconversions which were followed closely as the endpoint neared. In these instances, the % (*S*)-amine **2** reached a maximum and then decreased slowly. This may be explained by either slow acylation of

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Table 2. **Comparison of Penicillin Amidases (Acylases), pH effect[a]**

supplier	enzyme name	rate μmoles/unit/h pH 5.4	pH 5.6	pH 6.0	pH 6.5	pH 7.0	pH 7.5
Roche Diagnostics	PGA-450 (129 units/g)	0.80	1.65	2.97	2.61	2.54	–
Rohm Pharma	Eupergit PcA 600 (83.3 units/g)	–	–	0.61	1.45	2.63	0.98
Recordati SpA	Pen-G Acylase (121.5 units/g)	–	2.07	3.31	3.49	1.93	–

[a] Reaction was conducted in a 30-mL incubation mixture containing 1.5 mmol of amine **2**, 4.4 mmol of phenylacetic acid, and 15 units of enzyme. The formation of *N*-phenylacetyl amide at pH 6.5 and 7.0, and 25 °C was measured by reversed phase HPLC.

Table 3. **Comparison of PGA-450 and Pen-G Acylase in a 100-mL bioreactor containing 32 mmol amine 2, 36 mmol phenylacetic acid, and 750 units of enzyme, pH 5.6, 33 °C[a]**

supplier	enzyme name	units/g	reaction time, h	rate, μmol/unit/h	ee %
Roche Diagnostics	PGA-450	129	17.5	2.57	97.9
Recordati SpA	Pen-G Acylase	121.5	42.7	1.83	98.1
			30.7	1.98	98.4

[a] The formation of *N*-phenylacetyl amide was measured by reversed phase HPLC.

the (*S*)-amine **2** or by deacylation of (*R*)-phenylacetylamine **2** or both. Since the net acylation of (*R*)-amine **2** was rapidly diminishing once the percent conversion reached 98% (ee = 96%), it was decided to set the endpoint at 98% consumption of the (*R*)-amine **2** to maximize yield of (*S*)-amine **2**. The targeted endpoint was achieved in 9−11 h.

IV. Scale-Up. *One-Liter Bioconversions.* On the basis of the previous experience, the bioconversion was demonstrated at 1-L scale under the conditions 28 °C, 100 g/L amine **2**, 40 g/L phenylacetic acid, and pH 5.7. In the first trial it was difficult to achieve the targeted 98% conversion of the (*R*)-amine **2**. Since the phenylacetic acid was not in a large excess, the starting concentration of phenylacetic acid was increased to 50 g/L for the second and third trials. As part of these demonstrations, the workup was also modified to utilize potassium bicarbonate, and a mixture of potassium bicarbonate/potassium carbonate to change the pH of the completed bioconversion, along with sodium hydroxide to neutralize both the phenylacetic acid and the amine **2** hydrochloride. The flow diagram for this process is shown in Figure 1. Yield data, percent desilylation, and ee are shown in Table 4. The ee of the isolated amine and amide agreed with high stereoselectivity of the acylation for the (*R*)-amine **2**. The yields were 85−90% of the expected (*S*)-amine, and the yields of the (*R*)-amide combined with that of the amine corresponded to 94% mass balance.

Seventy-Liter Pilot Runs. Three cycles using recovered enzyme were performed at the Roche (formerly Boehringer Mannheim) facility in Penzberg, Germany. Data are summarized in Table 5. Since the pH was set midway between the pK_a of amine **2** and phenylacetic acid, and they were present in equimolar concentrations, the pH did not change during the reaction. In the first run temperature control was lost, and the temperature decreased during the run from 25 to 19 °C. This caused the chiral purity of the unacylated (*S*)-amine to be lower than expected. In the remaining runs the temperature control was maintained, and the expected chiral purity was achieved. Overall yields of the desired (*S*)-amine averaged 85% (43% of starting racemate), with a chiral purity of 99% (excluding first run).

The use of the phenylacetic acid salt of amine **2** had three effects. First, preparing and isolating the salt from heptane removed most of the impurities generated in synthesizing amine **2**. Second, it simplified the process since otherwise the additional steps of extracting the amine in strongly acidic solution and evaporation of the residual solvent were required. And third, due to its low packed density, use of the phenylacetate salt lowered the throughput from 0.47 to 0.25 M. This last was offset by the process simplification, however.

The workup was designed to take advantage of the differing solubility in water of amine **2** in basic or acidic media. Since the amide product was insoluble in water (∼0.01 g/L), it was necessary to solubilize it to recover the enzyme, which was accomplished with MTBE in the reactor. At 70-L scale decantation of the organic layer was impractical. However, the bottom screen permitted facile solid/liquid separation. Separation of the immiscible liquids and back-extraction of both phases gave good recovery of both amide and amine. Since the solubility of water in MTBE is high,[8] residual water was a concern. This was dealt with by drying with sodium sulfate prior to evaporation. At larger scale alternative means may have to be devised. In several early experiments ammonium hydroxide was used in place of sodium hydroxide or potassium carbonate/bicarbonate. This resulted in partial desilylation of amine **2**. Although the extent was small, never more than 5%, changing to less nucleophilic bases gave better control of the desilylation. The main concern was the stability of the enzyme in the presence of MTBE. However, the enzyme was determined to be stable enough for cost-effective use.

Amine 3. One trial with amine **3** (tosylate salt) was performed to help decide the choice of nucleophile to be resolved. Although this was performed at lower concentration than the 1-L bioconversions for amine **2**, some comparisons can be made. On the basis of the first few hours of reaction, it was expected that this reaction would have been completed in 16−21 h, approximately double that required for amine **2**. This probably reflected the lower solubility of the *tert*-

(8) Riccick, J. A.; Bunger, W. B.; Sakano, T. K. *Organic Solvents: Physical Properties and Methods of Purification*, 4th ed.; Techniques of Chemistry, Vol. 2; Wiley: New York, 1986.

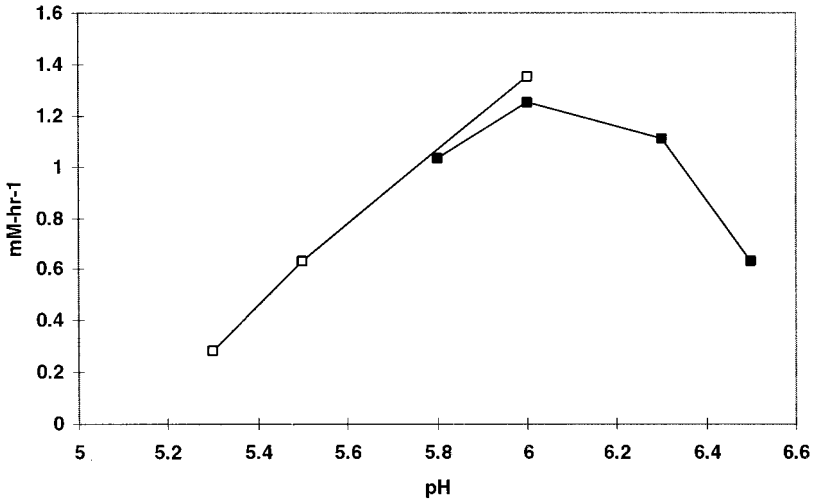

Figure 4. **pH dependence of the rate of bioconversion: (■) 10 g/L amine 2; (□) 20 g/L amine 2.**

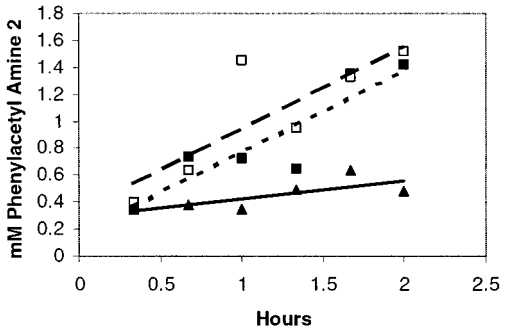

Figure 5. **Effect of agitation on acylation of amine 2; (▲) low agitation (200 rpm); (■) medium agitation (300 rpm); (□) high agitation (400 rpm).**

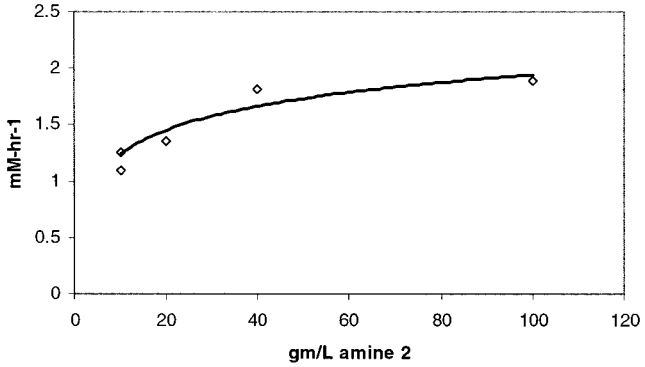

Figure 7. **Dependence of rate of bioconversion on amine 2 concentration.**

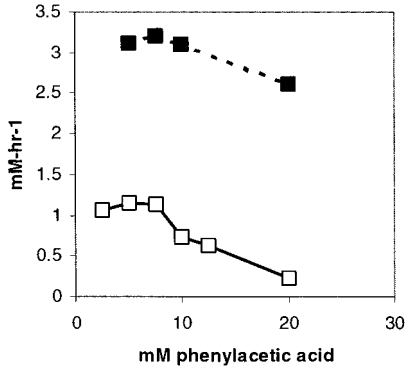

Figure 6. **Dependence of bioconversion rate on phenylacetic acid concentration; (□)10 g/L amine 2; (■) 100 g/L amine 2.**

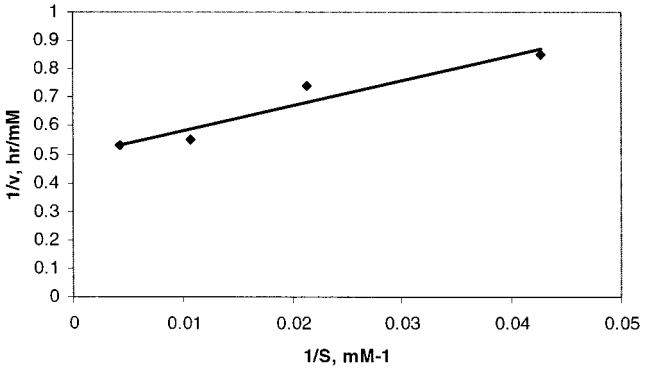

Figure 8. **Double reciprocal plot, 1/rate vs 1/[(*R*)-amine 2].**

butyl ester (free base) in water, and this projected longer reaction time was a justification for selecting amine **2** over amine **3** for resolution. Overall, this reaction gave about 12 mmol (53% yield) of (*S*)-amine, with an optical purity of 98.6%.

Recycle Stability. Cost analysis indicated the need for recycle of the enzyme. Bioactivity of the PGA-450 was monitored before and after bioconversions (Table 6). After one cycle, there was only a small apparent loss of bioactivity. When enzyme from one cycle was recovered and used in a second (and third) bioreaction, the bioactivity after three cycles was significantly diminished relative to the initial bioactivity. Whether this was due to the handling in the bioconversion or some physicochemical aspect of the bioconversion itself was not determined.

In a separate experiment, the stability of PGA-450 was tested over a total of 185 reaction hours in five consecutive cycles. These reactions were terminated on the basis of reversed phase HPLC analyses of samples taken throughout the reaction. Endpoint times varied greatly, between 23 and 48.5 h. Initial rates were determined from the first 2 h of the reaction when amide concentrations were low. Extrapolation of the specific activity data (Figure 10) suggested loss of 50% of the activity in 10 or 11 cycles. In another experiment the half-life was extrapolated to 18−20

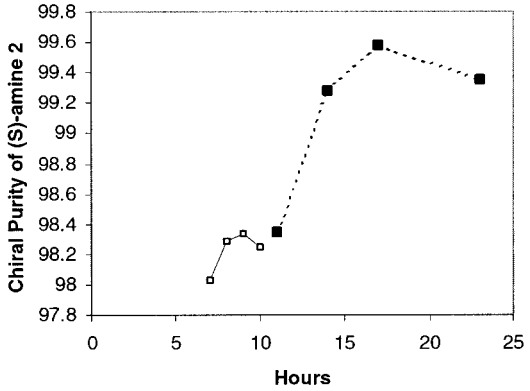

Figure 9. **Chiral purity of (*S*)-amine 2 near the endpoint of the bioconversion; trial A (□); trial B (■).**

Table 4. **Yield and purity data for 1-L bioconversions (h = time at which 98% of the (*R*)-amine had been acylated, % desiliylated = % of areas from an HPLC assay)**

	h	yield, g	% ee	% desilylated
		(*S*)-Amine **2**		
first 1 L	10	43.6	96.61	1.17
second 1 liter	8.5	46.1	97.85	0.47
third 1 L	11	40.6	97.70	2.34
		(*R*)-Phenylacetyl Amide **2**		
first 1 L		74.8	99.37	0.75
second 1 liter		79.1	99.37	1.51
third 1 L		65.2	99.50	2.48

Table 5. **Yield, chiral purity, and mass balance for 70-L pilot runs[a]**

	(*S*)-amine		(*R*)-amide		
trial	yield, %	% ee	yield, %	% ee	mass balance, %
3B-1	85	91.1	90	99.6	87.5
3B-2	84	98.4	97	99.3	90.6
3B-3	87	97.7	96	99.6	91.4

[a] Conditions were 0.25 M amine **2**, 0.25 M phenylacetic acid, 7500 units/L PGA-450, 25 °C.

Table 6. **Bioactivity of immobilized penicillin amidohydrolase after biocatalytic resolution of amine 2; average of three trials for the bioactivity**

	initial bioactivity	final bioactivity
one cycle, trial 1	148.85 ± 1.55 units/g	145.47 ± 1.44 units/g
one cycle, trial 2	148.06 ± 0.65 units/g	145.16 ± 0.67 units/g
three cycles	147.15 ± 0.68 units/g	102.18 ± 1.31 units/g

cycles (data not shown), and when the process was scaled up to 70 L at Roche Diagnostics in Penzberg, 25 cycles were shown to be plausible. As part of the work performed at Roche, 15 consecutive cycles were performed at 400-mL scale using the same conditions as used in the pilot runs. Extrapolation of these data indicated 20−25 runs could be performed before 50% activity loss was incurred. The incremental cost contribution of the enzyme at the scale chosen for the cost analysis indicated that after 15 cycles

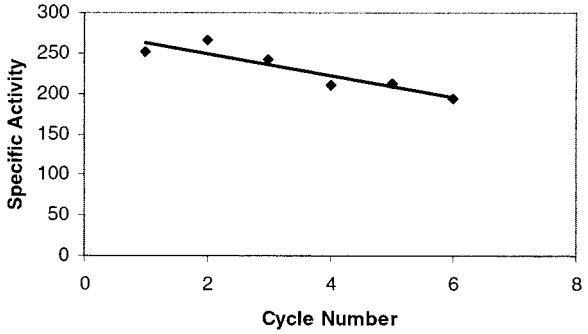

Figure 10. **Activity as a function of cycle number.**

the enzyme contributed less than 5% to the cost of the resolution. So at 20 or more cycles, the enzyme cost contribution was acceptable.

Conclusions

Since it was the (*S*)-stereoisomer of amine **2** which was needed, resolution by acylation permitted a simpler process than resolution by deacylation. The (*R*,*S*)-β-amino ester, 3(*R*,*S*)-amino-5-(trimethylsilyl)-4-pentynoate (amine **2**), was resolved by stereoselective, enzymatic acylation with phenylacetic acid at pH 5.7, 28 °C. The enzyme used was Roche Diagnostics immobilized penicillin amidohydrolase (PGA-450). The amine loading was 100 g/L using 50 g/L phenylacetic acid. At this pH, because the amine **2** had a pK_a of less than 7, substantial free base was available to participate as a nucleophile in deacylating the acyl enzyme intermediate of penicillin amidohydrolase. The pH-rate profile of the enzyme exhibited a maximum at or a little above pH 6, and the effect of agitation was shown to be minimal. The effect of phenylacetic acid concentration on the rate of acylation of amine **2** appeared to be inhibitory at low concentrations of amine **2** (10 g/L) with little or no effect at 100 g/L amine **2**. Since the net rate of acylation diminished dramatically near the endpoint, and >99% acylation of the (*R*)-amine **2** was extremely difficult to achieve, it was decided to terminate the reaction before completion, at approximately 98% acylation of the (*R*)-amine **2**. The bioconversion was scaled to 70 L for three runs, from which was isolated 43−46% molar yield as (*S*)-amine **2**, with 96−98% enantiomeric excess.

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