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# Resolution of racemic 2-chlorophenyl glycine with immobilized penicillin G acylase

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ABSTRACT

Racemic 2-chlorophenyl glycine has been resolved to obtain (*S*)-α-amino-(2-chlorophenyl)acetic acid with >99% enantiomeric purity via enantioselective hydrolysis of its *N*-phenylacetyl derivative with penicillin G acylase immobilized on Eupergit C. The resolution can be conveniently performed in water at substrate concentration of 10% in 5 h with an enzyme:substrate ratio of 1:5 (w/w), and the enzyme can be recycled >25 times without any loss of activity. The unwanted (*R*)-enantiomer can be easily racemized for recycling.



## 1. Introduction

(*S*)-2-Amino-(2-chlorophenyl)ethanoic acid [(*S*)-2-chlorophenyl glycine] **1** is an important intermediate in the synthesis of Clopidogrel, a potent oral antiplatelet agent often used in the treatment of coronary artery disease, peripheral vascular disease, and cerebrovascular disease.[1] The drug is marketed by Bristol-Myers Squibb and Sanofi-Aventis under the trade names Plavix and Iscover, and by Sun Pharmaceuticals under the trade name Clopilet. It is also used, along with aspirin, for the prevention of thromboembolism after placement of intracoronary stent.

Although the synthesis of clopidogrel can be carried out via several routes, most routes utilize either the 2-chloromandalate or 2-chlorophenyl glycine derivatives as starting materials. The final product is usually made in racemic form and resolved via fractional crystallization with a resolving agent such as camphor sulfonic acid.[1–7] Economically, the use of an enantiomerically pure reagent at the start of a synthetic sequence is more cost effective and less polluting. Therefore recently, strategies for the preparation of enantiomerically pure 2-chloromandalate using enzymatic routes, such as kinetic resolution using hydrolases[8,9] or enantioselective reduction of a prochiral ketoacid,[10] have been successfully demonstrated. The resolution of racemic 2-chlorophenyl glycine has been carried out by a classical method using D-camphor sulfonic acid in rather low yield (42% of theoretical value).[11] Alternatively, the methyl ester of 2-chlorophenyl glycine can be resolved with (+)-tartaric acid.[12] Although enantioselective hydrolysis of 2-chlorophenyl glycine amide by microbes possessing amidase activity has been reported,[13,14] the low solubility of the amide in water and necessity of fermenting a specific bacterial strain limit the application of this process on a large scale.

Herein, we report a simple methodology for obtaining enantiomerically pure (*S*)-α-amino-(2-chlorophenyl)acetic acid on a multigram scale, which is based on the principle of enantioselective hydrolysis of an *N*-phenylacetyl derivative of a racemic amino acid with the commercially available enzyme penicillin G acylase (E.C.3.5.1.11) immobilized on Eupergit C.[15] The enzyme selectively hydrolyzes only the (*S*)-enantiomer to give the (*S*)-amino acid with ee >99%. It is possible to carry out the reaction at a substrate concentration as high as 20% and recycle the enzyme >25 times without loss of activity (Scheme 1).

## 2. Results and discussion

Penicillin G acylase (penicillin amidohydrolase, EC 3.5.1.11) cleaves the acyl chain of penicillins to yield 6-amino penicillanic acid (6-APA) and the corresponding organic acid, and is used industrially, mainly for the production of 6-APA and semi-synthetic antibiotics.[16–18] It is also useful in peptide synthesis[19] and in resolution of secondary alcohols,[20] amino alcohols,[21,22] amino acids,[23–25] β-hydroxy-α-amino acids,[26] aryloxycarboxylic acids,[27] etc. The stereochemical preference of penicillin G acylase toward the L-enantiomer of amino acids such as 2-phenyl glycine and 4-hydroxyphenyl glycine is well known.[28] However, hydrolases generally do not show high selectivities towards *ortho*-substituted substrates due to steric hindrance,[9,29–31] and it is necessary to screen several enzymes and manipulate the reaction conditions and substrate structure in order to achieve the required enantioselectivity. It was gratifying to discover that penicillin G acylase recognized only the (*S*)-enantiomer of *N*-phenylacetyl derivative of 2-chlorophenyl glycine for hydrolysis.

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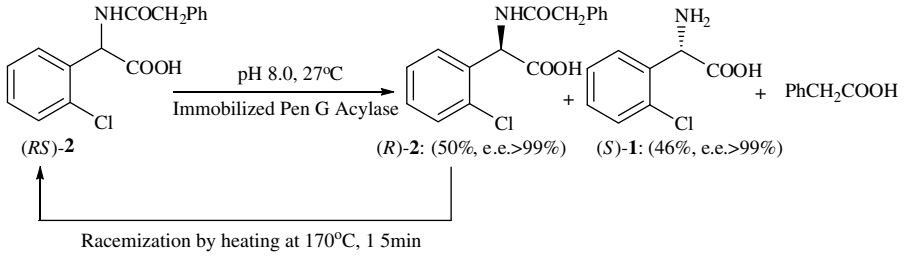


**Scheme 1.**

### 2.1. Effect of substrate concentration on reaction rate and enantioselectivity

In our earlier studies on the resolution of 2-aminobutanol,[21] we observed that the enantiomeric purity of the product was dependent on the substrate concentration and conversion due to high apparent *Km* value ($Km_{app}$) for the substrate and moderate *E* value of 44. Thus, the effect of substrate concentration on the rate and enantioselectivity of the reaction was studied. To determine the *E*-value, experiments were conducted with enantiomerically pure (*R*)- and (*S*)-amides, separately, and it was confirmed that the enzyme did not accept the (*R*)-enantiomer ($E \gg 200$).

In the second experiment, the enzyme concentration was held constant at 50 mg in a reaction volume of 5 mL. Substrate concentrations were varied from 0.5% (w/v) to 20% (w/v). Reactions were conducted in glass vials with magnetic stirring. It was observed that the enantioselectivity of the reaction did not change with substrate concentration and the hydrolytic reaction stopped at 48–50% conversion, even after prolonged contact (24 h) with the enzyme. A typical reaction curve of conversion versus time is shown in Figure 1.

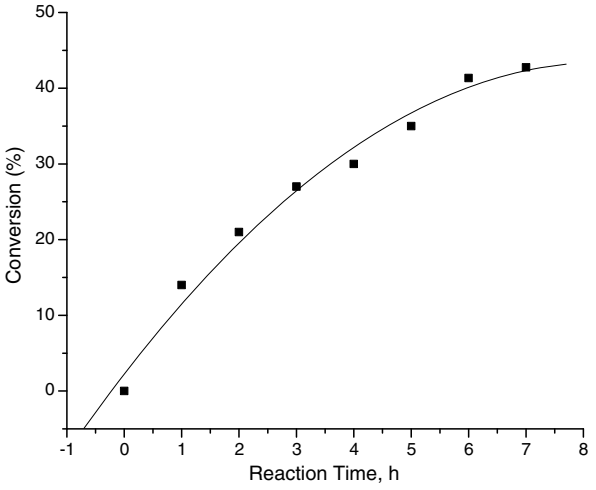


**Figure 1.** Hydrolysis of 2-(2-chlorophenyl)-2-[(2-phenylacetyl)amino]acetic acid **2** by penicillin G acylase immobilized on Eupergit C in aqueous solution at pH 8.0, 27 °C. [(*R,S*)-**2**] = 0.33 M, 10% (w/v). Immobilized enzyme, 50 mg, in 5 mL reaction volume.

The reaction followed the usual Michaelis–Menten kinetics (Fig. 2). Using nonlinear regression analysis of the initial rates, the apparent $V_{max}$ ($V_{max,app}$) and apparent *Km* ($Km_{,app}$) were found to be 19 mM/min/g and 74 mM, respectively (correlation coefficient $r = 0.988$).

### 2.2. Preparative scale enzymatic resolution

From the kinetic data, it was observed that the reaction velocity reached a plateau of 0.8 mM/min at a substrate concentration of about 0.3 M (9% w/v) (Fig. 2). Thus, the preparative resolution was carried out at a substrate concentration of 10%. With a substrate to enzyme ratio of 5:1 (w/w), the reaction typically reached about 48% conversion in 5 h. The enzyme was filtered, and the aqueous solution was cooled, acidified to pH 2, and extracted with dichloromethane. The resolved (*S*)-amino acid was recovered by evaporating the aqueous layer to dryness and purified (yield 46%, 92% of theoretical value; ee >99%). The dichloromethane layer was evaporated to obtain unreacted (*R*)-**2** along with phenylacetic acid. This was extracted several times with boiling cyclohexane to remove the phenylacetic acid. The residual amide was finally recrystallized to obtain pure (*R*)-**2**. For a sample of the amide with ee >99%, the enzymatic reaction was allowed to proceed overnight.

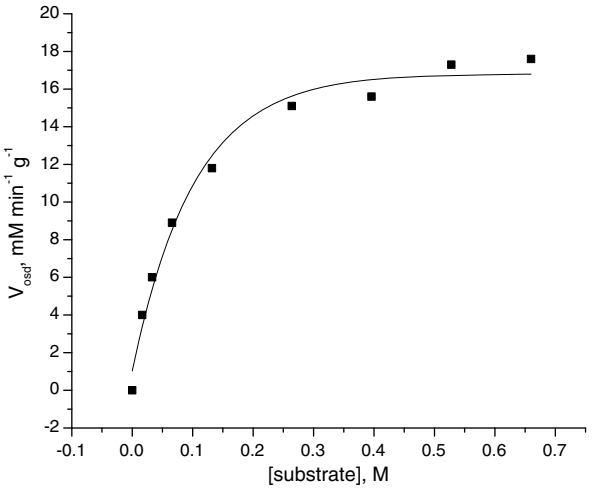


**Figure 2.** Effect of substrate concentration on the initial rate of hydrolysis of **2** by penicillin G acylase immobilized on Eupergit C in aqueous solution at pH 8.0, 27 °C.

### 2.3. Enzyme recycle

Enzyme recycle studies were carried out in an Amicon stirred cell as a representative filter reactor equipped with a mechanical stirrer. The total reaction volume was 50 mL, and reactions were carried out using a 10% substrate solution and 1 g of immobilized enzyme. The reactants were stirred at 80–100 rpm. The reaction was followed by HPLC and stopped after 5 h when the rate of reaction became considerably slow. The reactants were then filtered out, and next batch was carried out with a fresh substrate solution using the same enzyme. Studies with 25 recycles showed no loss of enzyme activity or enantioselectivity.

### 2.4. Racemization of (*R*)-2

Racemization of the unwanted (*R*)-enantiomer was carried out by the method reported by Grabley.[32] A mixture of phenylacetic

acid and (*R*)-**2** recovered after enzymatic reaction was heated in an oil bath at 170 °C. The melt was held at this temperature for 15 min, and then cooled. Measurement of the specific rotation indicated complete racemization. No decomposition products were found in HPLC analysis. The racemization was confirmed by HPLC analysis on a chiral stationary column.

## 3. Conclusion

The present methodology provides an excellent alternative to the existing route for the resolution of 2-chlorophenyl glycine with almost quantitative yield and consistent enantiomeric purity of >99%. The enzyme penicillin G acylase is commercially available in large quantities at a reasonable price and can be recycled several times. Although we have carried out reactions at 10% substrate concentration, it is possible to use a substrate concentration as high as 20%. Considering the observed $V_{max}$ of 19 mM/min/g, a judicious design of a bioreactor can provide an excellent process on an industrial scale.

## 4. Experimental

2-Chlorophenyl glycine was purchased from Aldrich. Immobilized Penicillin G acylase was a gift from M/s KDL Biotech Ltd, Savroli village, India. HPLC analyses were carried out on Hewlett Packard HP1090 unit with diode array detector and HP CHEM STATION software. Curve fittings were performed with Graph Pad Prism version 5, GRAPH PAD Software, San Diego, California, USA.

### 4.1. 2-(2-Chlorophenyl)-2-[(2-phenylacetyl)amino]ethanoic acid 2

The racemic amino acid **1** (18.56 g, 0.1 mol) dissolved in 4 M NaOH (50 mL) was stirred in an ice-bath. Phenylacetyl chloride (17 g, 0.11 mol) was added dropwise with 4 M NaOH (50 mL) with vigorous stirring. After complete addition, the reactants were stirred overnight and extracted with dichloromethane. The aqueous layer was then cooled in ice and acidified with 6 M HCl. The precipitated phenylacetyl derivative was filtered, washed with cold water, dried, and finally recrystallized from ethylacetate–hexane to obtain **2** (28 g, 92%). Mp 208–209 °C. $^1$H NMR ($CDCl_3$ + DMSO-$d_6$, 200 MHz): $\delta$ ppm 3.50 (s, 2H, CO**CH$_2$**Ph), 5.71 (d, 1H, $J$ = 7.3 Hz **CH**COOH), 7.14–7.42 (m, 9H, aromatic), 8.00 (d, 1H, $J$ = 7.3 Hz, –**NH**). $^{13}$C NMR ($CDCl_3$ + DMSO-$d_6$, 200 MHz): $\delta$ ppm 176.1 (Ph-**CO**, 175.2 (**CO**OH), 140.3, 138.3, 134.3, 134.0, 133.0, 131.8, 131.2 aromatic), 58.7 (**CH**–NH), 44.0 (Ph**CH$_2$**).

### 4.2. Enzymatic reaction

Substrate **2** (5 g, 16.5 mmoles) was suspended in water (20 mL) and dissolved with the addition of 5% ammonia solution. The pH was adjusted to 8.0 with 2 M HCl and the volume was finally made up to 50 mL with water. The enzyme (1 g wet) was added and the reaction mixture was stirred at room temperature (27 °C) in an Amicon stirred ultrafiltration cell, model 8200 at 80–100 rpm. The reaction was followed by reverse phase HPLC. Typically, the reaction stopped at approximately 48% conversion in 5 h. The reactants were filtered out, and the enzyme was washed with water (3 × 5 mL) and the combined aqueous solution was cooled in ice and acidified with 6 M HCl to pH 2. The precipitated mixture of (*R*)-phenylacetylamino acid **2** and phenylacetic acid **3** was extracted with dichloromethane (3 × 10 mL) and the solvent was evaporated. Phenylacetic acid was recovered from the mixture by extracting repeatedly with boiling cyclohexane (1.0 g, 47.5%). The residue gave (*R*)-phenylacetylamino acid **2** (2.5 g, 50%; mp 210 °C. Ee >99%; $[\alpha]_D^{25} = -96$ (*c* 1, chloroform).

The amino acid (*S*)-**1** was recovered by evaporating the aqueous solution on a rotavaporator after adjusting the pH to 7.0, redissolving in hot isopropanol to remove salts, and evaporating the isopropanol layer (1.4 g, 92% of theoretical value). The enantiomeric purity of the product was determined by chiral HPLC analysis after converting it to its phenylacetyl derivative. $[\alpha]_D^{25} = +89$ (*c* 1, 1 M HCl),[33] (lit.[11] +115.6 (*c* 1, 1 M HCl). Ee >99%. $^1$H NMR ($D_2O$, 200 MHz : $\delta$ ppm 4.68 (br s, 2H, **NH$_2$**), 4.94 (br s, 1H, **COOH**), 5.20 (s, 1H, **CH**COOH), 7.40–7.64 (m, 4H, aromatic).

### 4.3. Reverse phase HPLC analysis

The hydrolysis of phenylacetyl derivative **2** was followed by reverse phase HPLC. Column C-8 (250 × 5 mm), Chrompack, The Netherlands. Mobile phase, 50% acetonitrile–water containing 0.1% perchloric acid. Flow rate, 0.7 ml/min. Detection wavelength, 220 nm. Retention times: **1**: 4.14; phenylacetic acid 7.44; **2**: 9.55 min.

#### 4.3.1. HPLC analysis with chiral stationary phase

Enantiomeric purity was determined by HPLC analysis on Chiralcel AD-H column (250 × 5 mm), Daicel Chemical Industries, Japan. Mobile phase, 6% 2-propanol in hexane containing 0.1% trifluoroacetic acid. Flow rate, 1 ml/min. Detection wavelength, 220 nm. Retention times (*S*)-**2**: 44. 38; (*R*)-2: 48.06 min.

## Acknowledgment

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