Tetrahedron: *Asymmetry* 23 (2012) 775–779

Contents lists available at SciVerse ScienceDirect

Tetrahedron: *Asymmetry*

journal homepage: www.elsevier.com/locate/tetasy

Resolution of racemic 4-hydroxy-2-cyclopentenone with immobilized penicillin G acylase

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ARTICLE INFO

Article history:
Received 19 April 2012
Accepted 18 May 2012
Available online 15 June 2012

ABSTRACT

Enantiomerically pure 4-oxo-2-cyclopentenyl derivatives were prepared by kinetic resolution with penicillin G acylase (EC 3.5.1.11) immobilized on an epoxy activated polymer. The enzyme selectively hydrolyzes the phenylacetyl ester of the (*S*)-enantiomer to give the (*S*)-alcohol. The enantioselectivity *E* increases from 12.4 in 0.05 M phosphate buffer, to *E* = 100–110 in acetonitrile-buffer (20% v/v) and *E* >200 in diisopropyl ether. The immobilized enzyme is stable and retains >90% of its activity after 10 recycles over one week at room temperature.

1. Introduction

Enantiomerically pure (*R*)- and (*S*)-4-hydroxy-2-cyclopentenones **1** possess three different functional groups in a cyclopentyl molecule and hence are sought after as versatile intermediates for the synthesis of a large number of complex natural products such as prostaglandins, prostacyclins, thromboxane, and nucleosides.[1–3] Several approaches have been made to obtain enantiomerically pure **1**, which include classical resolution via the diastereomer of caronaldehyde,[4] kinetic resolution with lipases[5] and chiral catalysts,[6], and desymmetrization of *meso*-cyclopentenediol or its corresponding diacetate catalyzed by lipase.[7–9] The enantioselective synthesis of **1** has also been achieved with D-tartaric acid as a chiral template.[10] Amongst these approaches, the lipozyme catalyzed enantioselective alcoholysis of (±)-4-oxocyclopenten-2-yl acetate appears to be the most effective (ee 85% at 43% conversion, *E* = 24).[11a] Porcine pancreatic lipase has also been reported to exhibit selectivity toward 4-hydroxy-2-alkyl-2-cyclopentenones during transesterification with vinyl acetate with *E* values near 20, but so far there has been no report on the enantioselectivity toward the unsubstituted substrate.[11b]

Herein we report a better methodology for obtaining enantiomerically pure O-protected 4-oxo-2-cyclopentenyl derivatives using the commercially available enzyme penicillin G acylase (EC 3.5.1.11) immobilized on an epoxy activated polymer (Scheme 1). The enzyme selectively hydrolyzes the phenylacetyl ester of (*S*)-4-hydroxycyclopent-2-en-1-one with excellent enantioselectivity (*E* >200).

2. Results and discussion

Penicillin G acylase (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.[12–14] The enzyme specifically recognizes the phenylacetyl group of amides and esters, but not simple aliphatic groups, such as acetyl. It has also found use in peptide synthesis,[15] resolution of amino acids,[16–18] β-hydroxy-α-amino acids,[19] amino alcohols,[20,21] aryloxycarboxylic acids,[22] a large variety of esters containing aromatic acyl moieties,[23,24] and even secondary alcohols.[25] These reports inspired us to attempt the resolution of 4-hydroxy-2-cyclopentenone **1**. It was quite interesting to discover that penicillin G acylase hydrolyzed the *O*-phenylacetyl derivative **2** and selectively recognized the (*S*)-enantiomer.

2.1. Penicillin G acylase catalyzed hydrolysis of (±) 2 in an aqueous medium

Initially, the hydrolysis of **2** was attempted in a phosphate buffer (50 mM) at pH 8. The hydrolytic reaction showed selectivity toward the (*S*)-substrate. Based on the observed conversion monitored by reverse phase HPLC analysis, and the enantiomeric excess of the unreacted (*R*)-ester, as measured by HPLC analysis on a chiral stationary phase, the enantioselectivity *E* was estimated[26] as *E* = 12.4. However, the reaction could only be carried out in very dilute solutions (<0.3% w/v) due to the low substrate solubility. In order to improve substrate solubility, the addition of a water-miscible organic solvent was attempted.

It is well known that organic co-solvents can change an enzyme selectivity.[27] These changes can occur for a wide variety of reasons.

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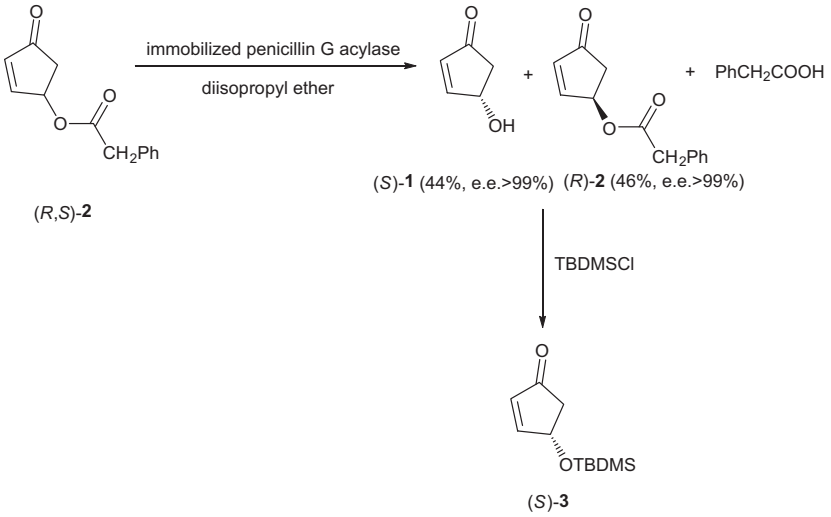
http://dx.doi.org/10.1016/j.tetasy.2012.05.018



Scheme 1. Resolution of (±)-**2** using immobilized penicillin G acylase.

An appropriate solvent can optimize the enzyme conformation and improve its enantioselectivity toward a particular substrate.[28–30] Similarly, different solvents cause different changes in solubility, desolvation of the substrates and the products, and this in turn can cause a change in substrate binding, resulting in a different enantioselectivity.[31,32] It has also been reported that solvent molecules binding at the active site can act as competitive inhibitors to the substrate. This ability of solvent to bind at active sites depends on the nature and structure of the solvent molecules, which varies from solvent to solvent.[33,34] In the case of penicillin G acylase, it has been reported that the effects of co-solvents on the reaction rate are dependent on their polarity and hydrophobicity.[35,36] Hydrophilic polar co-solvents (low log*P* and high ε) accelerate the reaction process while co-solvents with a higher log*P* inhibit the hydrolytic reactions. The effects of such solvent parameters on the enantioselectivity of the enzyme have also been studied for other enzymes,[37] but no general predictable model for the solvent effects is currently available.[38] It appears that each enzymatic reaction with its substrate needs to be studied individually in order to determine the optimum parameters for the best possible enantioselectivity.

In order to select an appropriate co-solvent for our synthesis, hydrolytic reactions were performed in mixtures of water miscible organic solvents, such as acetonitrile, 1,4-dioxane, sulfolane, 1-methyl-2-pyrrolidone, *tert*-butanol, and tetrahydrofuran at a fixed solvent composition of 20% (v/v). The enantiomeric purity of the unreacted ester was determined after 24 h (Table 1). It was observed that amongst all of the solvents tested, acetonitrile was the most appropriate, giving unreacted ester with 91% ee. The determination of the conversion from the peak heights of phenylacetic acid and unreacted phenylacetyl ester in reverse phase HPLC analysis indicated that the enantioselectivity of the enzyme in the solvent mixture had improved greatly (E = 100–110). Our results are in agreement with earlier reports on the improved enantioselectivity of penicillin G acylase in the presence of acetonitrile during the hydrolysis of secondary alcohols[25] and the acylation of amines.[39]

Table 1
Enantioselective hydrolysis of (±)-4-oxocyclopent-2-enyl 2-phenylacetate **2** by immobilized penicillin G acylase in water-miscible organic solvents[a]

S. No	Solvent (20% v/v)	ee (%) of (*R*)-**2**
1	Acetonitrile	91
2	1,4-Dioxane	56
3	Sulfolane	77.4
4	*N*-Methyl-2-pyrrolidinone	45.2
5	*t*-Butanol	No selectivity
6	Tetrahydrofuran	No reaction

[a] Reaction conditions: [substrate] = 10 mg, penicillin G acylase 10 mg, wet (2 units) in a phosphate buffer (50 mM, pH 7.5) containing 20% (v/v) co-solvent. Reaction volume 1 mL. Temperature 30 °C. Reaction period 24 h.

2.2. Hydrolysis of (±)-2 in acetonitrile–water mixtures catalyzed by immobilized penicillin G acylase

In order to determine the optimum solvent composition for the reaction, the effect of acetonitrile concentration on enzyme activity and stability was studied. As observed earlier for co-solvent effects on enzyme activities,[40] the plot of solvent concentration against enzyme activity (after incubation for 24 h) was sigmoidal. The enzyme could tolerate the co-solvent up to 20% (v/v) without losing its activity but lost it completely within a range of 30–40% (Fig. 1).

Figure 2 shows the progress of the reaction at different time intervals in solvent mixtures containing 10–25% acetonitrile. It

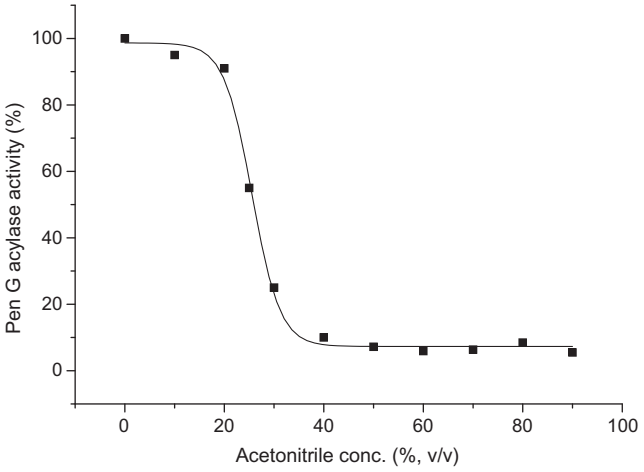


Figure 1. Effect of acetonitrile on the penicillin G acylase activity at 30 °C. Reaction conditions: [substrate] = 10 mg, penicillin G acylase 10 mg (wet, 2 units) in phosphate buffer (50 mM, pH 7.5) containing 0–90% of CH_3CN (v/v). Reaction volume 1 mL. Reaction period 24 h.

was observed that the rate of reaction was slightly faster in solutions containing 10% acetonitrile compared to that in a solution of 20% acetonitrile, while it was very sluggish in 25% acetonitrile, apparently due to denaturation and the loss of enzyme activity. The conversion followed a typical first-order reaction path for an enzymatic reaction occurring under substrate concentration $<K_m$. The apparent first-order rate constants, k_{obsd}, were found to be 0.83 h^{-1} and 0.76 h^{-1}, respectively in 10% and 20% acetonitrile–water mixtures. The enzyme was found to be stable and could be recycled 10 times without a loss of activity in solutions of both 10% and 20% acetonitrile.

Although the results shown in Figure 2 were very encouraging, the solubility of the substrate was still a major concern. Even in solutions containing 20% acetonitrile, only a maximum of 10 mg/mL of the substrate could be dissolved. We attempted to disperse the substrate in the reaction medium by strong stirring, but at higher concentrations the undissolved substrate was adsorbed onto the polymer surface and was not available for enzymatic reaction. A second problem encountered by us was the isolation of hydroxy product (*S*)-**1**, which was highly soluble in water and also quite unstable. Solvent extraction followed by evaporation invariably led to the formation of a dark red polymerized material. Although O-protected derivatives such as terahydropyranyl or TBDMS were stable for long term storage, direct derivatization was not feasible in aqueous solutions. It was thus necessary to devise another strategy that would allow us to use the enzyme in a water-immiscible solvent, where product derivatization and isolation could be easily performed.

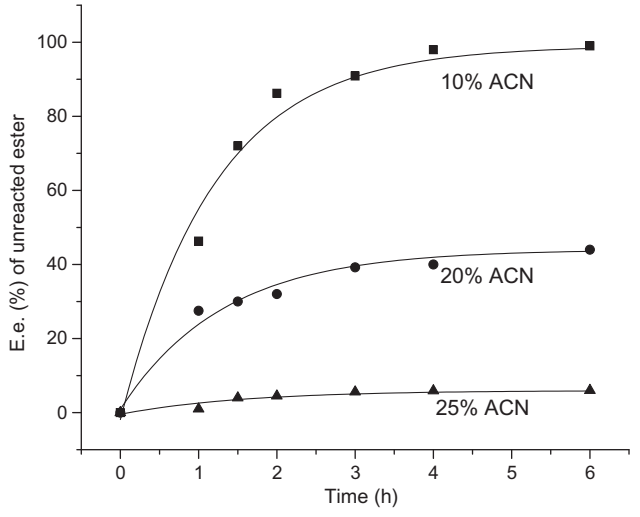


Figure 2. Effect of acetonitrile concentration on the hydrolysis of (±)-4-oxocyclopent-2-enyl 2-phenylacetate **2** by immobilized penicillin G acylase. Reaction conditions: [substrate] = 10 mg, penicillin G acylase 25 mg (wet, 5 units) in phosphate buffer (50 mM, pH 7.5) containing 0–90% of CH_3CN (v/v), reaction volume 1 mL, temperature 30 °C. Reaction period 24 h.

2.3. Reactions in water-immiscible organic solvents

In order to explore the feasibility of using the enzyme in a water-immiscible organic solvent, reactions were performed with suspension of the wet immobilized enzyme in several solvents, such as dichloromethane, chloroform, diisopropyl ether, and toluene. No reaction was observed in dichloromethane, chloroform, and toluene but it did occur in diisopropyl ether, with surprisingly excellent enantioselectivity (*E* >200).

2.4. Resolution of (±) 2 by immobilized penicillin G acylase in diisopropyl ether

In a typical experiment, the wet polymer beads (1 g, 200 units, water content 67% w/w) dispersed with the help of Celite (300 mg) were stirred magnetically with a solution of substrate (500 mg) in diisopropyl ether (10 mL) under a nitrogen atmosphere. The conversion was followed by chiral HPLC analysis. The reaction was found to be inhibited by the product when the ee of the unreacted ester reached 40%. At this stage, the enzyme was replaced, and fresh enzyme and Celite were added and stirring was continued. The reaction was complete in 3 days. The reaction mixture was then separated from the enzyme, after which the enzyme was washed with diisopropyl ether and the combined washings were dried over anhydrous magnesium sulfate and evaporated on a rotavapor. The residue was dissolved in THF and treated with TBDMSCl in order to obtain the corresponding TBDMS-protected alcohol.[41] The (*R*)-phenylacetyl **2** and (*S*)-TBDMS derivative **3** were easily separated by column chromatography and were obtained in 90–92% theoretical yield and ee >99%.

2.5. Stability of immobilized penicillin G acylase

The exposure of an enzyme to an organic solvent is known to cause a loss of enzyme activity due to denaturation.[42] Thus the stability of immobilized penicillin G acylase in suspension of diisopropyl ether is an important factor if it is to be reused. In the resolution process described in Section 2.4, the reaction was found to slow down considerably and needed replacement of the enzyme after 24 h. It was important to know if this was due to enzyme denaturation by solvent molecules. Thus, the enzyme activity of the beads after each cycle of solvent contact for 24 h was studied. Figure 3 shows that penicillin G acylase retains >90% activity even after exposure to the solvent for 10 days.

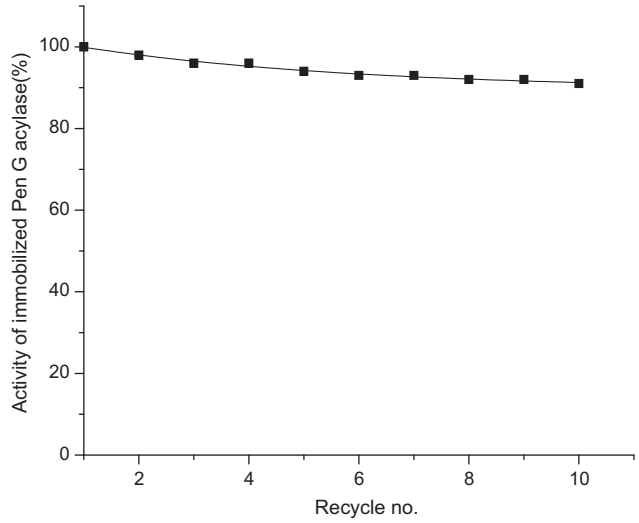


Figure 3. Stability of immobilized penicillin G acylase in diisopropyl ether at 30 °C.

The enzyme activity of the polymer beads recovered after the resolution reaction was also assayed for the sake of comparison. It was observed that neither the reactants nor the products cause any loss of enzyme activity. Also, the presence or absence of Celite has no bearing on the expression of enzyme activity. The most probable reason for reaction slowing down is due to a decrease in substrate concentration caused by hydrolysis. A detailed kinetic analysis is currently in progress.

3. Conclusion

Herein we have reported the efficient resolution of racemic 4-hydroxy-2-cyclopentenone with commercially available immobilized penicillin G acylase. The immobilized enzyme works well in a suspension of water-immiscible organic solvent and can be recycled several times.

4. Experimental

4.1. General

IR spectra were recorded on a Perkin–Elmer RX-1 FT-IR system. ^{1}H NMR (200 MHz) spectra were recorded on a Varian Gemini-200 MHz spectrometer. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on Bruker Avance-300 MHz spectrometer. Optical rotations were measured with a Horiba-SEPA-300 digital polarimeter. Mass measurement was performed on Q STAR mass spectrometer (Applied Biosystems, USA). HPLC analysis was carried out on Varian Pro Star HPLC unit.

The enzyme penicillin G acylase immobilized on polymeric support of average particle size of 150 μm (200 units/g, wet) was a generous gift from M/s KDL Biotech Limited, Savroli, India (e-mail: dra@kdlbiotech.in). (±)-4-Hydroxycyclopent-2-en-1-one **1**, (±)-4-oxocyclopent-2-enyl acetate, and (*R*)-4-oxocyclopent-2-enyl acetate were prepared as described in the literature.[11a] All other reagents and solvents used were of analytical grade obtained from Hi Media and Qualigens, India.

4.2. (±)-4-Oxocyclopent-2-enyl 2-phenylacetate 2

A solution of (±)-4-hydroxycyclopent-2-en-1-one **1** (2 g, 20 mmol), phenylacetic acid 3 g (22 mmol), and 4-dimethylaminopyridine (0.24 g, 2 mmol) in dry DCM was stirred and cooled in an ice bath. A solution of dicyclohexylcarbodiimide (4.6 g, 0.22 mol) in DCM (10 mL) was added to reaction mixture and after stirring for 10 min, the ice bath was removed. The dark-brown reaction mixture was stirred for 3 h at room temperature and filtered. The filtrate was washed with 0.5 M hydrochloric acid (2 × 5 mL) and then with 10% sodium carbonate solution (2 × 5 mL). The organic layer was then dried over anhydrous magnesium sulfate and evaporated on a rotavapor. The dark brown residue was extracted with diisopropyl ether. Removal of the solvent gave 4-oxocyclopent-2-enyl 2-phenylacetate as a pale yellow low melting powder (3.5 g, 81%). Mp 55–56.2 °C. ^{1}H NMR ($CDCl_3$) 7.51 (dd, 1H, J = 5.6, 4.8 Hz), 7.29–7.22 (m, 5H), 6.3(d, 1H, J = 6), 5.8(m, 1H), 3.6 (s, 2H). 2.8 (dd, 1H, J = 6.0, 18.1 Hz), 2.28 (dd, 1H, J = 2.3, 18.1 Hz); ^{13}C NMR ($CDCl_3$) 204.5, 170.7, 158.6, 136.8, 133.1, 128.9, 128.4, 127.0, 72.1, 40.8, 40.6; IR (neat) V_{max}, 2937, 1724, 1660 cm^{-1}; MS (ESI) *m/z* 217 (M+1). HRMS [ESI, (M+H)$^+$]: *m/z* calcd for $C_{13}H_{13}O_3$: 217.0864; found: 217.0859.

4.3. Enzymatic reaction and preparation of (*R*)-2 and (*S*)-3

Polymer beads bearing penicillin G acylase were washed with phosphate buffer (0.05 M, pH 7.5) several times to remove the preservatives and dried by tapping with filter paper. The moist polymer beads (water content 67% w/w determined by Karl-Fisher titration, 1 g, 200 units) were stirred magnetically with a solution of substrate (500 mg, 2.3 mmol) in diisopropyl ether (10 mL). In the presence of an organic solvent, the polymer beads tended to clump and adhere to the walls of container. To overcome this problem, Celite (300 mg) was added which helped in dispersing the polymer beads evenly in the reaction mixture. The reaction was found to be inhibited by the product when the ee of the unreacted ester reached 40% (24 h). At this stage, the enzyme was removed, washed with diisopropyl ether (2 × 5 mL) and the washings were combined with the reaction mixture. The reaction mixture was then concentrated on a rotavapor to 10 mL, after which freshly prewashed and filter paper dried enzyme (1 g) and Celite (300 mg) were added and stirring was continued. After one more repetition with fresh enzyme, the reaction was complete (the ee of the unreacted ester reached >99%) in 3 days. The reaction mixture was separated from the enzyme and the enzyme beads were washed with solvent. The combined enzyme beads along with Celite were suspended in water (25 mL) and filtered through a wire mesh of 105 microns (ASTM Mesh No. 140). The polymer beads with an average diameter of 150 microns stayed on the mesh while the Celite particles passed through. The washed enzyme beads were stored for reuse.

The combined organic extracts were dried over anhydrous magnesium sulfate and the solvent was removed on a rotavapor. The residue consisting of an equimolar mixture of (*S*)-**1** and the unhydrolyzed (*R*)-**2** was dissolved in THF (10 mL). Triethylamine (250 μL, 1.8 mmol), and DMAP (3 mg, 0.24 mmol) were added and the solution was cooled in ice. *t*-Butyldimethylsilyl chloride (170 mg, 1.13 mmol) dissolved in cold THF (2 mL) was added slowly with a syringe. The reaction mixture was stirred overnight at room temperature. Next, THF was removed on a rotavapor, after which HCl (0.5 M, 10 mL) was added and TBDMS derivative (*S*)-**3** was extracted with *n*-heptane (3 × 10 mL). After removal of the heptane on a rotavapor, the residue was chromatographed over silicagel using 20% (v/v) ethylacetate in *n*-hexane. (*S*)-**3** (R_f 0.73), (*R*)-**2** (R_f 0.41). The products (*R*)-**2** and (*S*)-**3** were obtained in 90–92% theoretical yield.

(*R*)-4-Oxocyclopent-2-enyl 2-phenylacetate 2 (230 mg, 46%) $[\alpha]_D^{25} = -40.5$ (*c* 0.5, MeOH); ee >99%, chiral HPLC.

(*S*)-4-((*tert*-Butyldimethylsilyl)oxy)clopent-2-enone 3 (220 mg, 44%)

$[\alpha]_D^{25} = -65.2$ (*c* 1.3, MeOH); lit[43] $[\alpha]_D^{25} = -64.7$ (*c* 1, MeOH); ee >99%, chiral HPLC of corresponding acetate.

4.4. 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% MeOH–water containing 0.1% perchloric acid. Flow rate, 0.7 mL/min. Detection wavelength, 230 nm. Retention times: 4-hydroxy-2-cyclopentenone: 4.99 min; 4-oxocyclopent-2-enyl 2-phenylacetate: 16.3 min; phenylacetic acid: 10.65 min.

4.5. Determination of the enantiomeric purity

Enantiomeric purity of unreacted **2** was determined directly by HPLC analysis on a chiral stationary phase, although that of product alcohol **1** could not be determined directly due to its instability and propensity to undergo polymerization. Similarly, we could not obtain a satisfactory resolution of the TBDMS derivative even when several different chiral HPLC columns and analytical conditions were tried. We were, however, able to obtain excellent separation of both phenylacetyl as well as acetyl derivatives on the same column and under the same analytical conditions. All four peaks were well resolved and it was easy to determine the ee of the reactants and the products accurately. Thus for the determination of enantiomeric purity, a small aliquot (100 μL) of the reaction mixture was collected in an Eppendorf tube, cooled in ice and acetic anhydride (10 μL) was added. The contents were shaken vigorously for 30 min and then sodium carbonate solution (100 μL, 5% in water) was added. The contents were centrifuged for 5 min at 5000 rpm after which the upper organic layer was separated and used directly for chiral HPLC analysis.

4.6. HPLC analysis on chiral stationary phase

The enantiomeric purity was determined by HPLC analysis on Chiralcel AD-H column (250 × 5 mm), Daicel Chemical Industries, Japan. Mobile phase, 3% 2-propanol in hexane. Flow rate, 0.5 mL/min. Detection wavelength, 220 nm. Retention times (*S*)-**2**: 18.7; (*R*)-**2**: 23.2 min. (*R*)-4-Oxocyclopent-2-enyl acetate: 14.8; (*S*)-4-oxocyclopent-2-enyl acetate: 16.6 min.

4.7. Assignment of product configuration

The configuration of *tert*-butyldimethylsilyl derivative **3** was assigned as (*S*)- from the measurement of its specific rotation and comparison with the reported value.[43] By inference, the configuration of the unreacted phenylacetyl derivative was assigned as (*R*).

4.8. Determination of the enzyme activity

Penicillin G acylase activity was assayed from the hydrolysis of benzyl penicillin sodium salt in an aqueous buffer. A solution of benzyl penicillin sodium salt (0.01 M, 10 mL) in 5 mM sodium phosphate buffer (pH 8.0) was placed in a temperature controlled double walled glass vessel maintained at 37 °C. The enzyme (50 mg, wet) was added and the contents were mechanically stirred at 100 rpm. The pH of the reaction medium was maintained at 8.0 by continuous titration with 0.1 M NaOH for 10 min. The enzyme activity is expressed in units where one unit of enzyme is the amount of enzyme which releases 1 μmol of titratable phenylacetic acid per minute under the described conditions. The enzyme activity was found to be 200 units/g.

4.9. Stability of immobilized penicillin G acylase

The enzyme stability in a suspension of the organic solvent was studied by assaying the enzyme activity of the beads after exposure for varying time intervals. In these experiments, a set of 10 vials, each with 100 mg enzyme was prepared. The enzyme beads were stirred with diisopropyl ether (5 mL) for 24 h. After removing the solvent, enzyme activity in the first tube was assayed by a titrimetric method. The other 9 vials were again stirred with a fresh batch of solvent, and after another 24 h, the enzyme in the second vial was assayed for its activity. In this fashion, enzyme stability in diisopropyl ether was determined over 10 days.

Acknowledgements

We thank the Department of Science and Technology and CSIR New Delhi for financial support.

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