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An 'easy-on, easy-off' protecting group for the enzymatic resolution of (±)-1-phenylethylamine in an aqueous medium

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Abstract—A new approach has been developed for the biocatalytic resolution of (±)-1-phenylethylamine in 100% aqueous medium based on two integrated enzymatic steps: protection and deprotection of the reactive amine enantiomer catalyzed by the same enzyme—penicillin acylase from *Alcaligenes faecalis*. An 'easy-on, easy-off' protecting group has been introduced using (*R*)-phenylglycine amide as the acyl donor. (*R*)-Phenylglycyl-substituted (*R*)-1-phenylethylamine was poorly soluble and precipitated at enzymatic acylation in an alkaline medium (pH 10–11), driving the synthesis towards high yields. Conversely at pH <7.5, its solubility was continuously increasing, which rendered the subsequent deacylation by the same enzyme highly efficient. In contrast to the resolutions, which employ one biocatalytic step, the new approach made it possible to exploit two sequential enantioselective enzymatic reactions implementing a double enantioselectivity control. Effective enzymatic resolution of (±)-1-phenylethylamine in an aqueous medium was performed with (*R*)-phenylglycine amide as an acyl donor using the suggested approach.

1. Introduction

Recently a new strategy based on the unique catalytic properties, stability and enantioselectivity of the relatively unknown penicillin acylase from *Alcaligenes faecalis* (*Alcaligenes*-PA) has been suggested for the effective enantioselective acylation of amines in aqueous media.[1] Further development has shown the possibility to regulate the enantioselectivity of the enzymatic amine acylation by adding organic cosolvents.[2] Very high reaction rates, chemoselectivity and simple product isolation advantageously distinguish *Alcaligenes*-PA-catalyzed amine acylation in aqueous medium from the lipase or subtilisin-catalyzed amine acylations in organic solvents.[3–8] When comparing applications of different enzymes in the resolution of amines, it should be noted that biocatalytic amine acylation in organic solvents proceeds with a relatively low reaction rate,[4] requires strictly anhydrous reaction conditions and is complicated by nonenzymatic acylation. Moreover, the hydrolysis of the acylated ('reactive') amine enantiomer requires harsh reaction conditions to liberate the free amine,[6] because enzymatic splitting using lipase or subtilisin is ineffective.[9,10]

In this respect, the development of the amine resolution using enzyme(s) for both protection and deprotection steps could have serious advantages. Such a strategy of preparing individual amine enantiomers would encompass three steps: (1) stereoselective enzymatic acylation of the more reactive enantiomer (e.g., the (*R*)-enantiomer as in *Alcaligenes*-PA-catalyzed acylations) in the reaction mixture containing racemic amine; (2) separation of nonacylated (*S*)-amine from *N*-acylated (*R*)-enantiomer followed by (3) the enantioselective enzymatic hydrolysis of the latter in order to liberate the enantiomerically pure (*R*)-amine.

Herein we present a new biocatalytic approach for enzymatic resolution of (±)-1-phenylethylamine in an aqueous medium, which integrates two sequential enzymatic steps: protection and deprotection catalyzed by the same enzyme—*Alcaligenes*-PA using (*R*)-phenylglycine amide as acyl donor.

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2. Results and discussion

Recently we have shown the possibility of highly efficient *Alcaligenes*-PA-catalyzed stereoselective acylation of amines in aqueous medium using phenylacetamide as the acyl donor.[1] As a result, the 'reactive' amine enantiomer is acylated, whereas the other one remains intact. It should be noted that phenylacetyl derivatives of hydrophobic amines are poorly soluble in water and due to this circumstance amine acylation in aqueous medium can proceed with a high yield leading also to a simple and effective isolation of the product by filtration. At the same time, successive hydrolysis of this poorly soluble phenylacetyl derivative of amines represents a quite difficult problem. In addition to the low substrate solubility in water, such a hydrolysis is complicated due to the effective inhibition by the hydrolysis product—phenylacetic acid,[11] which is a well soluble compound and, therefore, its accumulation slows down the process sharply [see, e.g., hydrolysis of *N*-phenylacetyl-(*R*)-1-phenylethylamine in Fig. 1, dashed line].

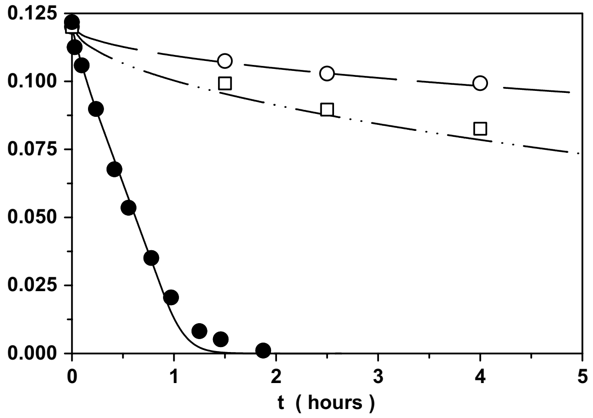


Figure 1. *Alcaligenes*-PA-catalyzed deprotection of the 'reactive' amine enantiomer; hydrolysis of: *N*-(*R*)-phenylglycyl-(*R*)-1-phenylethylamine in 100% aqueous medium (●, solid line), *N*-phenylacetyl-(*R*)-1-phenylethylamine in 100% aqueous medium (○, dashed line) and in 20% (v/v) methanol (□, dash-dotted line). Reaction conditions: 1.5 μM *Alcaligenes*-PA, pH 7.5, 25 °C.

Slow rates of *Escherichia*-PA-catalyzed hydrolysis of phenylacetyl derivatives of amines and alcohols because of their low solubility were noted in some earlier publications.[12–14] In order to improve the solubility of acyl derivatives of amines, alchohols and carbinols the hydrolysis was carried out in water-miscible organic solvents.[15–17] The solubility of *N*-phenylacetyl-1-phenylethylamine, for example, can be increased in such a system, but the productivity of hydrolysis cannot be seriously improved (Fig. 1, dash-dotted line). As another option, racemic substrates were used, which contain hydrophilic group in the acyl moiety, such as pyridylacetyl-,[18] *p*-amino-[19] and *p*-hydroxy-phenylacetic acid derivatives.[20] However, in all these cases the hydrolysis product (pyridylacetic acid, *p*-amino- or *p*-hydroxyphenylacetic acid) is far more soluble than a corresponding initial substrate and binds in the enzyme's active site nearly as effectively as phenylacetic acid; hence, hydrolysis of the partially dissolved substrate is very strongly suppressed by the accumulating product.[21]

Keeping in mind the overall two-step biocatalytic procedure (enzymatic acylation and subsequent enzymatic deacylation), it would be optimal to have such a protecting group that the protected amine would be poorly soluble at the acylation conditions (making acylation effective and product separation easy), but would be well soluble at the hydrolysis conditions, and, as an ideal property, the hydrolysis product would be a weak, poorly soluble inhibitor which does not complicate a deprotection of an 'active' amine enantiomer. The (*R*)-phenylglycyl group, with a p*K* value close to 7, turned out an excellent candidate as (*R*)-phenylglycyl-1-phenylethylamine appeared to be poorly soluble at *Alcaligenes*-PA-catalyzed acylation conditions (pH 10) and precipitated in the course of the enzymatic reaction, driving synthesis towards high yields in aqueous medium, whereas at pH below 7.5 (*R*)-phenylglycyl-1-phenylethylamine appeared to be well soluble substrate, rendering *Alcaligenes*-PA-catalyzed hydrolysis effective at these conditions. The solubility of (*R*)-phenylglycyl-1-phenylethylamine at pH 7.5 surpasses that of *N*-phenylacetyl-1-phenylethylamine by approximately 2 orders of magnitude. Moreover, (*R*)-phenylglycine [the product of (*R*)-phenylglycyl-1-phenylethylamine hydrolysis] is a weak inhibitor of penicillin acylase (Table 1) with a limited solubility (approximately 10 mM at these conditions, Fig. 2).

Table 1. *Alcaligenes*-PA-catalyzed hydrolysis of *N*-(*R*)-phenylglycyl-1-phenylethylamine [*N*-(*R*)-PG-PEA] and *N*-phenylacetyl-1-phenylethylamine [*N*-Phac-PEA]: kinetic parameters, enantioselectivity and substrate solubility (pH 7.5, 25 °C)

Substrate	$k_{cat}/K_M \times 10^3$ $(M\,s)^{-1}$	*E*	Solubility (mM)
N-(*R*)-PG-(*R*)-PEA	5.1	1700	22
N-Phac-(*R*)-PEA	1100	310	0.65

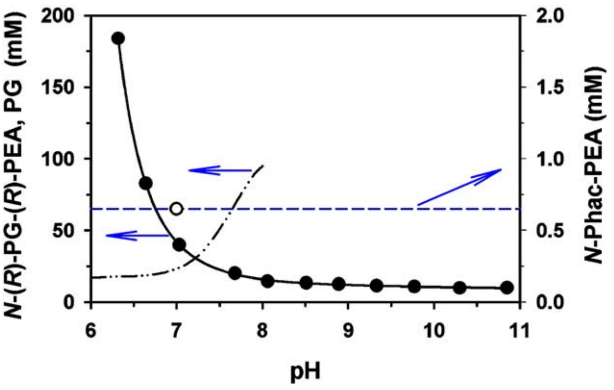


Figure 2. pH-dependencies for solubility of (*R*)-phenylglycyl-1-phenylethylamine [(*R*)-PG-PEA, solid line], (*R*)-phenylglycine[22] (PG, dash-dotted line) and *N*-phenylacetyl-1-phenylethylamine (*N*-Phac-PEA, dashed line) at 25 °C.

In addition to the inhibition/solubility benefits, the (*R*)-phenylglycyl-protecting group brings some more advan-

Table 2. Comparison of *Alcaligenes*-PA-catalyzed acylation of (±)-1-phenylethylamine by (*R*)-phenylglycine amide [(*R*)-PGA] and phenylacetamide (PAA) as acyl donors: reaction rates, synthesis/hydrolysis ratios and enantioselectivities (pH 10, 25 °C)

Acyl donor	Active amine enantiomer	v_S/E_0[a] (s^{-1})	$(S/H)_0$[b]	E[c]
(*R*)-PGA	*R*	100	11.4	>1000
PAA	*R*	210	7.2	350

[a] E_0—*Alcaligenes*-PA concentration in the reaction mixture determined after titration of the active sites;[11] v_s—initial rate of enzymatic amine acylation.

[b] $(S/H)_0$—synthesis/hydrolysis ratio determined as a ratio of initial rates for accumulation of both reaction products.

[c] E—enantioselectivity of an enzymatic acylation calculated from the dependence ee_p versus degree of racemic amine acylation.

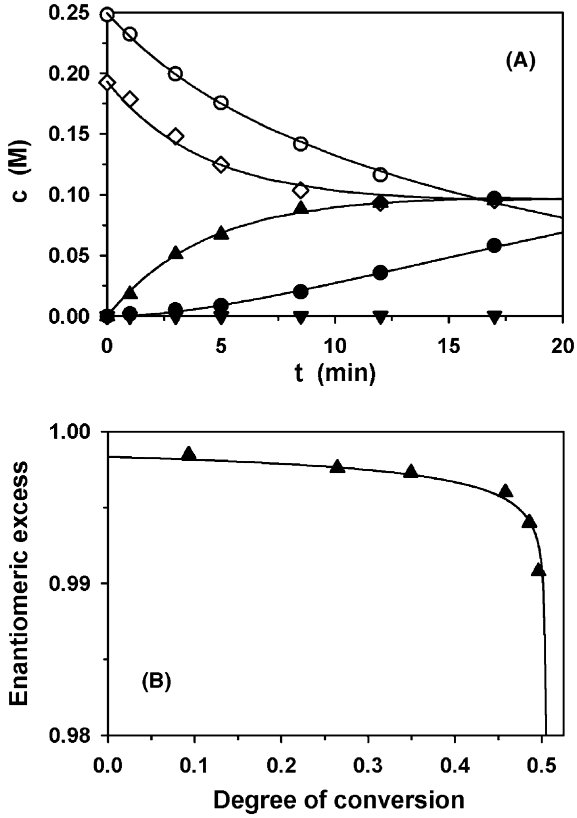
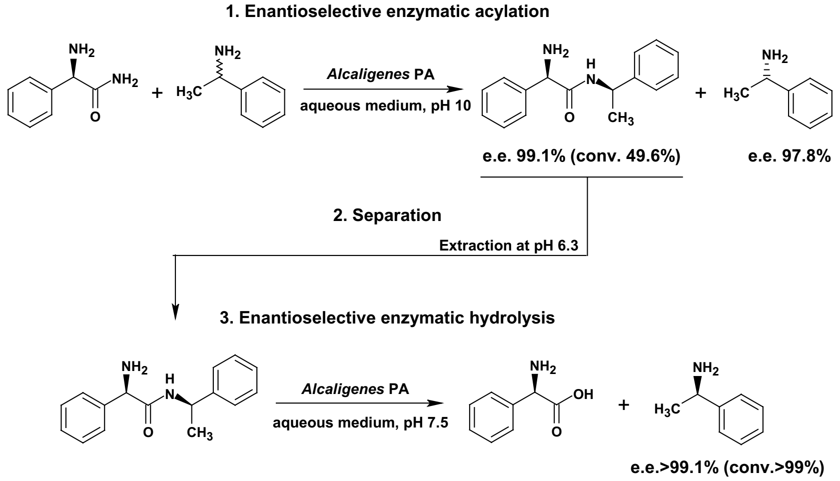


Figure 3. *Alcaligenes*-PA-catalyzed acylation of (±)-1-phenylethylamine (◇) with (*R*)-phenylglycine amide (○) as the acyl donor; *N*-(*R*)-phenylglycyl-(*R*)-1-phenylethylamine (▲), *N*-(*R*)-phenylglycyl-(*S*)-1-phenylethylamine (▼), (*R*)-phenylglycine (●). Reaction conditions: 3.0 μM *Alcaligenes*-PA, pH 10, 25 °C. (A)—Time course of the reaction; (B)—enantiomeric excess of the precipitated *N*-(*R*)-phenylglycyl-(*R*)-1-phenylethylamine (▲) versus degree of a racemic amine acylation; the solid line correspond to the enantiomeric excess of a product calculated for a biocatalytic acylation with enantioselectivity equal to 1200.

tages: surprisingly, the use of (*R*)-phenylglycine amide as the acyl donor instead of phenylacetamide leads to a remarkably improved enantioselectivity (E >1000) and a higher synthesis/hydrolysis ratio (S/H >11) of the *Alcaligenes*-PA-catalyzed amine acylation (Table 2). In much the same manner the structure of the protecting group influences the kinetics and enantioselectivity of the *Alcaligenes*-PA-catalyzed hydrolysis of acylated amines (Table 1). These results undoubtedly demonstrate the tight relationship between the *Alcaligenes*-PA acyl group binding pocket and the nucleophile binding subsite. A further investigation is needed to study a mechanism and a scale of available effects. Our results show that (*R*)-phenylglycine amide is an excellent acyl donor for resolution of (±)-1-phenylethylamine and other hydrophobic amines. A preparative resolution has been performed according to Scheme 1; the kinetics of the acylation is presented in Figure 3A. Synthesized *N*-(*R*)-phenylglycyl-(*R*)-1-phenylethylamine (Fig. 3B) was quantitatively separated from the intact (*S*)-1-phenylethylamine by extraction and subjected to the *Alcaligenes*-PA-catalyzed hydrolysis leading to a free (*R*)-1-phenylethylamine. Both enzymatic steps were fast and highly enantioselective. Although *N*-(*R*)-phenylglycyl-(*R*)-1-phenylethylamine seems to be a less reactive substrate than *N*-phenylacetyl-(*R*)-1-phenylethylamine if one compares k_{cat}/K_M values of their hydrolysis (Table 1),



Scheme 1. *Alcaligenes*-PA-catalyzed resolution of (±)-1-phenylethylamine by an 'easy-on, easy-off' approach, which integrates an enantioselective enzymatic acylation of a racemic amine using (*R*)-phenylglycine amide as the acyl donor and a successive deprotection of the acylated amine enantiomer.

its preparative conversion (Fig. 1, solid line), however, proceeds more effectively due to a much higher substrate solubility and a lower sensitivity to the inhibition by the product (Table 2). As a result, the resolution is very effective and both enantiomers of 1-phenylethylamine are produced with a high yield and enantiomeric excess (see Scheme 1).

3. Conclusion

Introduction of the 'easy-on, easy-off' (*R*)-phenylglycyl-protecting group allowed us to integrate the enantioselective *Alcaligenes*-PA-catalyzed acylation of (±)-1-phenylethylamine and the enantioselective *Alcaligenes*-PA-catalyzed hydrolysis of its acylated (*R*)-enantiomer into a two-step aqueous biocatalytic amine resolution process. The suggested approach provided a key to improve enantioselectivity and effectivity of biocatalytic stages and solve a problem, which had arisen using phenylacetamide as an acyl donor for a resolution of hydrophobic amines.

A new strategy has been developed for the biocatalytic resolution of (±)-1-phenylethylamine in 100% aqueous medium based on two sequential enzymatic steps: protection and deprotection of the reactive amine enantiomer catalyzed by the same enzyme—*Alcaligenes*-PA. The biocatalytic resolution utilizing the 'easy-on, easy-off' (*R*)-phenylglycyl-protecting group is highly productive, ecologically friendly and involves quite simple technological solutions. In fact it has a triple chiral control (two enantioselective biocatalytic steps and a diastereomeric crystallization) resulting in high yields and enantiomeric purity of the products. In contrast to the relatively slow biocatalytic processes in an organic medium, *Alcaligenes*-PA-catalyzed (±)-1-phenylethylamine acylation and deacylation in an aqueous medium is fast and efficient, so that answers the principal requirements for a large-scale resolution of enantiomers. The scope of this strategy is under investigation.

4. Experimental

Solution of penicillin acylase from *A. faecalis* was obtained from DSM Anti-Infectives, Delft, The Netherlands. Concentration of *Alcaligenes*-PA active sites was determined as described earlier.[11] (*R*)-Phenylglycine, racemic (±)-1-phenylethylamine and its individual enantiomers were obtained from Sigma; (*R*)-phenylglycine amide from DSM, phenylacetic acid from Aldrich; phenylacetylchloride from Fluka, phenylacetamide from Reakhim.

4.1. HPLC analysis

Concentrations of the reactants and enantiomeric excess of *N*-(*R*)-phenylglycyl-1-phenylethylamine were determined by HPLC using a Waters M6000 pump, a reversed phase Phenomenex Luna 5u C18(2) column (250 × 4 mm, 5 μm) and a Waters M481 LC detector at 210 nm with 7 mM phosphate pH 3.0, containing acetonitrile (60%, v/v) and 0.68 g/L of sodium dodecylsulfate, as an eluent. The flow rate was 0.5 mL/min. Retention times (in min): phenylacetamide (5.5), phenylacetic acid (7.8), *N*-phenylacetyl-1-phenylethylamine (13.6), (*R*)-phenylglycine (5.0), (*R*)-phenylglycine amide (7.4), 1-phenylethylamine (10.4), *N*-(*R*)-phenylglycyl-1-(*R*)-phenylethylamine (22.4), *N*-(*R*)-phenylglycyl-(*S*)-1-phenylethylamine (25.0).

The enantiomeric excess of the *N*-phenylacetyl-1-phenylethylamine was determined by HPLC using a Waters 590 pump and a Waters 486 UV detector at 215 nm and the flow rate 0.6 mL/min on a Chiralcel OD column (Daicel Chemical Industries) with hexane–isopropanol (95:5, v/v) as an eluent. Retention times (in min): *N*-phenylacetyl-(*R*)-1-phenylethylamine (42), *N*-phenylacetyl-(*S*)-1-phenylethylamine (55).

The enantiomeric excess of 1-phenylethylamine was determined by HPLC using a Waters M6000 pump and a Waters M481 LC detector at 210 nm and the flow rate 0.5 mL/min on a Crownpak CR(−) column (Daicel Chemical Industries) with H_2O–$HClO_4$ (pH 2.0) as an eluent. Retention times (in min): (*R*)-1-phenylethylamine (23.0), (*S*)-1-phenylethylamine (29.1).

4.1.1. Preparation of samples for HPLC analysis. Samples of the heterogeneous reaction mixture were prepared diluting an aliquot (50 μL) of the heterogeneous reaction mixture in 1.95 mL of an eluent in order to dissolve all reactants and to stop the enzymatic reaction. An aliquot of the resulting solution was further diluted by an eluent and subjected to HPLC analysis; when we needed to perform chiral analysis of 1-phenylethylamine 2 M KOH was added to the remaining solution to adjust the pH to 12 and amine was extracted by dichloromethane. The organic layer was washed by a solution of $HClO_4$ (pH 2.0), dichloromethane was evaporated under vacuum and an aqueous solution of an amine was subjected to a chiral analysis. The progress curves for all reaction components (acyl donor, 1-phenylethylamine, *N*-acylated amine and product of acyl donor hydrolysis) were documented what provided additional control due to the balance of the enzymatic reactions.

In order to determine enantiomeric excess of a formed *N*-phenylacetyl-(*R*)-1-phenylethylamine another aliquot of the heterogeneous reaction mixture was taken in the course of enzymatic acylation, filtrated and washed to separate the precipitating reaction product from the solution. After drying the isolated *N*-phenylacetyl-(*R*)-1-phenylethylamine was dissolved in the eluent and subjected to the chiral analysis.

4.2. Synthesis of *N*-(*R*)-phenylglycyl-(*S*)-1-phenylethylamine

N-(*R*)-Phenylglycyl-(*S*)-1-phenylethylamine was synthesized using *t*-butyl oxycarbonyl protection in the following way: to an aqueous solution (150 mL) of (*R*)-PG (25 mmol) and triethylamine (35 mL, 250 mmol) di-*t*-butyl dicarbonate (6.55 g, 30 mmol) in 1,4-dioxane (50 mL) was added and the mixture was stirred for 2h at 60 °C. The mixture was concentrated under vacuum, cooled with an ice-salt bath, and the pH was adjusted to 7.0 with 40% HCl. The precipitated salt *N*-Boc-(*R*)-PG·Et_3N was filtrated, washed with a minimum volume of water and dried under P_2O_5. The prepared triethylamine salt of *t*-butylcarbamate (20 mmol) was dissolved in a dry chloroform (100 mL), and ethyl chloroformate (2.4 mL, 25 mmol) was slowly added to the mixture cooled with an ice-salt bath. Then a solution of (*S*)-PEA (20 mmol) in chloroform (30 mL) containing triethylamine (3 mL, 21 mmol) was added and the mixture was stirred at room temperature overnight. The solution was washed consecutively with diluted HCl, sodium bicarbonate solution and water. Evaporation under vacuum yielded a yellowish oil. Diethyl ether saturated with a hydrochloric gas was added to the oil under stirring and the hydrochloric salt of *N*-(*R*)-PG-(*S*)-PEA was precipitated.

4.2.1. *N*-(*R*)-Phenylglycyl-(*S*)-1-phenylethylamine hydrochloride. Yield 50% (2.9 g); mp 123–125 °C; ^{1}H NMR (300 MHz, DMSO): δ 1.25 (d, 3H, CH_3), 4.89 (m, 1H, CH), 5.09 (br s, $C_\alpha H$), 7.20–7.66 (m, 10H, Ph), 8.82 (br s, 3H, NH_3Cl), 9.37 (d, 1H, NH).

4.3. Enzymatic acylation of a racemic (±)-1-phenylethylamine by (*R*)-phenylglycine amide

The acylation of (±)-1-phenylethylamine was carried out in a thermostated cell of a pH-stat (Titrino 719, Metrohm, Switzerland) at pH 10, 25 °C in an aqueous medium (total volume 10 mL) with 2.0 mmol racemic amine and 2.5 mmol (*R*)-phenylglycine amide as an acyl donor in a presence of 30 nmol *Alcaligenes*-PA under permanent stirring and pH control by adding 2 M KOH solution. Reaction product was precipitating in a course of acylation and its maximum yield was achieved in 17 min; then 2 M KOH was added to the reaction mixture to adjust the pH to 12 in order to inactivate enzyme. Then the reaction mixture was acidified by H_3PO_4 and (*R*)-1-phenylglycyl-1-phenylethylamine was extracted by dichloromethane (4 × 3 mL) at pH 6.3. The organic layer was evaporated under vacuum and *N*-acylated amine was recrystallized from water.

4.3.1. *N*-(*R*)-Phenylglycyl-(*R*)-1-phenylethylamine. Yield 42% (0.214 g); ee 0.991; mp 112–113 °C; ^{1}H NMR (300 MHz, DMSO): δ 1.37 (d, 3H, CH_3), 2.22 (br s, 2H, NH_2), 4.40 (s, 1H, $C_\alpha H$), 4.92 (m, 1H, CH), 7.12–7.43 (m, 10H, Ph), 8.43 (d, 1H, NH). MS *m*/*z*: 120 (7.1, $PhCH_2CH(NH)CH_3$), 106 (100, $PhCHNH_2$, $PhCH_2CH_3$), 79 (50), 77 (39, Ph), 51 (18).

4.3.2. *N*-Phenylacetyl-(*R*)-1-phenylethylamine. *N*-phenylacetyl-(*R*)-1-phenylethylamine was synthesized in 25 min from equimolar (1.6 mmol) amounts of racemic (±)-1-phenylethylamine and phenylacetamide with 9.6 nmol *Alcaligenes*-PA. Yield 45% (0.172 g); ee 0.985; mp 117–118 °C; ^{1} H NMR (250 MHz, $CDCl_3$): δ 1.29 (d, 3H, CH_3), 3.47 (s, 2H, CH_2), 5.01 (m, 1H, CH), 5.49 (d, 1H, NH), 7.04–7.29 (m, 10H, Ph). MS *m*/*z*: 239 (62, M), 120 (49, $PhCH_2CH(NH)CH_3$), 105 (100, $PhCCH_3$), 91 (75, $PhCH_2$), 77 (68, Ph), 65 (61).

4.4. Enzymatic hydrolysis of *N*-(*R*)-phenylglycyl-(*R*)-1-phenylethylamine

The hydrolysis of obtained *N*-(*R*)-phenylglycyl-(*R*)-1-phenylethylamine (0.84 mmol) was carried out in a closed thermostated cell of a pH-stat (Titrino 719, Metrohm, Switzerland) at pH 7.5, 25 °C in 0.02 M KH_2PO_4 aqueous solution (total volume 7 mL) in the presence of 10.5 nmol *Alcaligenes*-PA under permanent stirring and pH control by adding 2 M KOH solution for 2 h.

4.5. Enzymatic hydrolysis of *N*-phenylacetyl-(*R*)-1-phenylethylamine

The hydrolysis of *N*-phenylacetyl-(*R*)-1-phenylethylamine (0.60 mmol) was carried out in a closed thermostated cell of a pH-stat (Titrino 719, Metrohm, Switzerland) at pH 7.5, 25 °C in 0.02 M KH_2PO_4 (aqueous or containing 20% (v/v) methanol, total volume 5 mL) in the presence of 7.5 nmol *Alcaligenes*-PA under permanent stirring and pH control by adding 2 M KOH solution for 5 h.

4.6. Solubility measurements

The saturated aqueous solutions of *N*-(*R*)-phenylglycyl-(*R*)-1-phenylethylamine were prepared by stirring a corresponding suspension during 40–60 min in a thermostated cell of a pH-stat (Titrino 719, Metrohm, Switzerland) at 25 °C and an appropriate pH kept constant by an automated titration with HCl. Then the suspensions were centrifuged and the isolated supernatants were subjected to HPLC analysis after dilution with an eluent to determine solubility value at each particular pH.

The solubility of *N*-phenylacetyl-1-phenylethylamine in water and in 20% (v/v) methanol was determined in the same mode by stirring a corresponding suspension for 1 h in a closed thermostated cell of a pH-stat at pH 7.0, 25 °C.

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