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Highly efficient and enantioselective enzymatic acylation of amines in aqueous medium

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Abstract—A new strategy based on the unique catalytic properties, stability and enantioselectivity of the relatively unknown penicillin acylase from *Alcaligenes faecalis* has been developed for the effective and enantioselective acylation of amines in aqueous medium. In contrast to lipase-catalyzed acylations in organic solvents, the penicillin acylase-catalyzed acylation of amines in aqueous solution is a rapid and chemoselective process leading to a product which can subsequently be deacylated by the same enzyme, imposing secondary enantiocontrol and leading to effective resolution.

1. Introduction

The use of enzymes for the preparation of chiral synthons is one of the most impressive concepts in the cooperation of organic chemistry and enzymology. Serious development in this field was stimulated by employing enzymes in organic media. This approach has significantly broadened the scope of biocatalytic reactions used for chiral discrimination by adding, inter alia esterification and transesterification reactions catalyzed by lipases and esterases to the pool of the synthetic chemoenzymatic methods.[1–7] As a result, the stereoselective acylation of alcohols by lipases in organic solvents has become a useful strategy in the development of chirotechnology.[8]

Effective enzymatic resolution of the more reactive amines in organic solvents is hampered by several problems: (1) their spontaneous acylation with activated acyl donors commonly used in lipase-catalyzed acylations; (2) the need for of stringently anhydrous reaction conditions, as in the presence of even small amounts of water hydrolysis of the acyl donor would prevail; (3) the low catalytic activity of most enzymes in anhydrous organic media; (4) the stability of the product (amide) formed, which, in contrast to esters, cannot be easily hydrolyzed under basic conditions and requires harsh treatment to liberate the free amine.[9] Although lipases have been developed that tolerate anhydrous reaction conditions surprisingly well,[10] spontaneous acylation can be suppressed under special conditions,[9] and acyl donors (such as esters of methoxyacetic or acetic acid), that do not react spontaneously with amines, can be used.[11–13] However, massive amounts of enzyme are required to perform the acylation and the reaction times tend to be long. Moreover, deprotection of the acylated amines remains a serious problem as enzymatic hydrolysis is ineffective.[14]

It would seem that the productivity could be seriously increased by performing the acylation reaction in aqueous solution. To be feasible, such an approach would require an enzyme that is stable and active in alkaline media as well as capable of transferring an acyl group from the acyl-enzyme intermediate to the amine rather than to a water molecule as a competing nucleophile (Scheme 1). In this respect our attention was drawn towards penicillin acylases (PA), which are widely used as industrial catalysts in the biocatalytic production of 6-aminopenicillanic acid.[15] Due to its wide substrate specificity, PA from *E. coli* (PA-*E. coli*) was used also for enantioselective enzymatic hydrolysis of *N*-phenylacetylated α-, β- and γ-amino acids,[16–18] aminophosphonic acids,[19] and effective enzymatic deprotection techniques.[20] Attempts to use PA-*E. coli* for the hydrolytic resolution of several amines have

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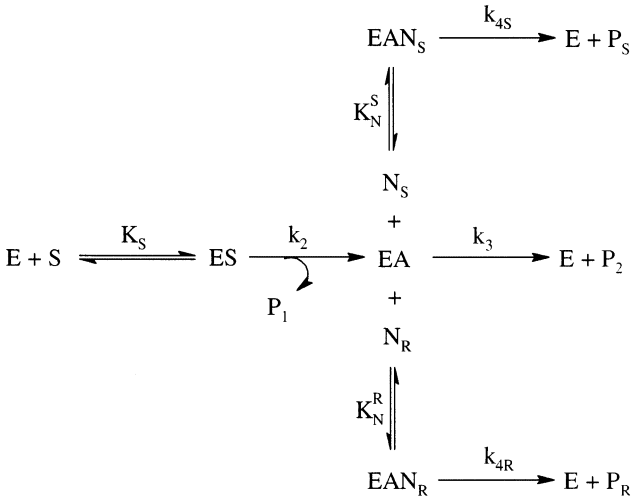


Scheme 1. 'Minimal' kinetic scheme for enzymatic acyl transfer reaction from acyl donor S to the enantiomers of nucleophile (amine) N_S and N_R. E – enzyme, ES – enzyme–substrate complex, EA – acylenzyme intermediate, EAN – acylenzyme–nucleophile(amine) complex, P_1 – first reaction product released at formation of acylenzyme, P_2 – product of acyl donor hydrolysis, P_S and P_R – acyl transfer products to (*S*)- and (*R*)-enantiomers of nucleophile.

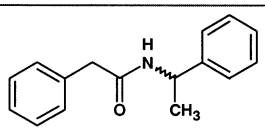
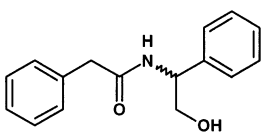
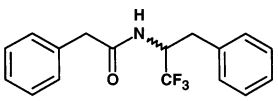
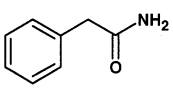
demonstrated surprisingly low stereospecificity[21] (see also Table 1), in contrast to the extremely high stereospecificity observed in the hydrolysis of derivatives of amino acids and their organoelement analogues.[22]

Herein, we report results of our studies on highly efficient and enantioselective acylation of amines catalyzed by relatively unknown penicillin acylase from *Alcaligenes faecalis* (PA-*A. faecalis*) in aqueous medium.†

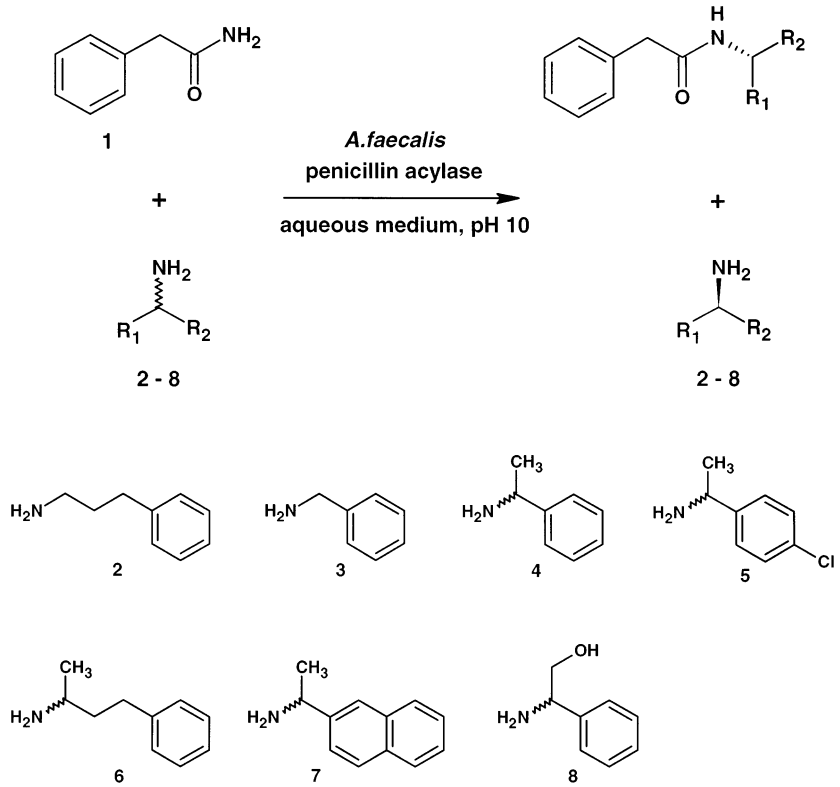
2. Results and discussion

Studying the substrate and stereospecificity of different PAs we have observed that the relatively unknown PA-*A. faecalis* has a much higher enantioselectivity in the hydrolysis of *N*-phenylacetylated amines compared with the well known PA-*E. coli* (Table 1). This enzyme was also much more active towards most substrates, including phenylacetamide **1**, which could serve as a potential acyl donor for the enzymatic acylation of amines. As PA-*A. faecalis* had a broad pH optimum of 8–10 and maintained nearly 80% of its maximum catalytic activity at pH 11,[24] we examined the ability of this enzyme to acylate different amines (Scheme 2). Surprisingly, we found that PA-*A. faecalis*, in contrast to PA-*E. coli*, is able to catalyze effective and enantioselective acylation of amines in aqueous solution (Figs. 1 and 2). Acylation of 3-phenylpropylamine **2** was nearly quantitative (Fig. 1) with 97.5% conversion of amine and 96.5% conversion of **1** to the precipitated product, and the acylation rate (Table 2) was more than an order of magnitude higher compared to the highest reaction rates observed earlier in PA-*A. faecalis*-catalyzed hydrolysis of the most specific substrates.[24] Acylation of benzylamine **3**, as well as other amines (Table 2), was effective but slower compared to the extremely fast acylation of **2**, and had a lower synthesis/hydrolysis ratio (S/H). In the acylation of (*RS*)-1-phenylethylamine **4** the reaction rapidly came to a standstill at 50% conversion when all (*R*)-**4** had been

Table 1. Kinetic parameters and stereospecificity of PA-catalyzed hydrolysis of *N*-phenylacetylated amines. Reaction conditions: pH 7.5, 25°C

Substrate	Enzyme	$k_{cat}/K_M \cdot 10^{-3}$ $M^{-1} \cdot s^{-1}$	E
	PA-*A.faecalis*	1100	310
	PA-*E.coli*	12	10
	PA-*A.faecalis*	1900	670
	PA-*E.coli*	50	95
	PA-*A.faecalis*	100	100
	PA-*E.coli*	2	1.3
	PA-*A.faecalis*	1500	
	PA-*E.coli*	310	

† The development of this work and possibility to regulate enantioselectivity of PA-*A. faecalis* by adding organic solvents was reported earlier.[23]



Scheme 2.

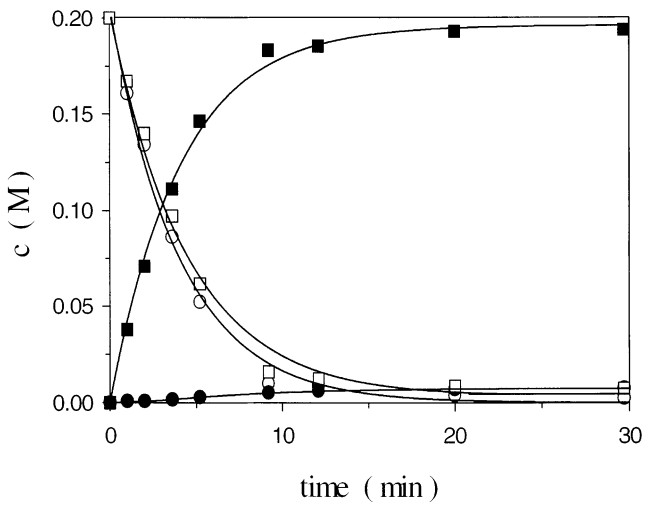


Figure 1. PA-*A. faecalis*-catalyzed acylation of **2** (□) with **1** (○) as an acyl donor; *N*-phenylacetyl-**2** (■), phenylacetic acid (●).

consumed (Fig. 2). Acylation of 1-(4-chlorophenyl)-ethylamine **5** and 2-amino-4-phenylbutane **6** was even more effective taking into account the high S/H ratio. Neither spontaneous hydrolysis of **1**, nor spontaneous acylation of amines at the conditions of enzymatic reaction in the absence of PA-*A. faecalis* was observed, demonstrating obvious advantage of this acyl donor. The products of enzymatic amine acylation could easily be isolated by filtration due to their low solubility. It should be noted, that PA-*A. faecalis* could also be used for subsequent effective deacylation of the obtained amide in contrast to harsh chemical hydrolysis needed at lipase-catalyzed amine resolution. Moreover, a mild enzymatic method to liberate a free amine imposes secondary chiral control for effective resolution (see Table 1).

A kinetic study of the PA-*A. faecalis*-catalyzed acylation of the individual enantiomers of **4**, 1-(2-naphthyl)ethylamine **7**, and phenylglycinol **8** has demonstrated that high enantioselectivity of enzymatic acylation is in good agreement with $e.e._p$ values observed in the acylation of racemic amines (Table 2). It is worth noting that PA-*A. faecalis*-catalyzed acylation of the latter compound was chemoselective and ester formation was not observed with **8**, nor with (*RS*)-1-phenylethan-1-ol.

3. Conclusion

PA-*A. faecalis*-catalyzed acylation of amines is surprisingly efficient and shows a high enantioselectivity for the chiral amines tested. PA-*A. faecalis*-catalyzed resolution of chiral amines could become an alternative to the existing methods due to the very high reaction rates, simplicity of the product isolation and the possibility to hydrolyze the product formed by the same enzyme with high enantioselectivity. The further scope and limitations of this approach are under investigation.

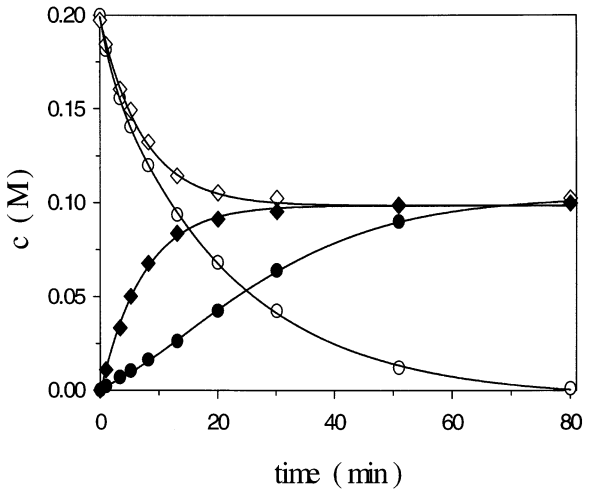


Figure 2. PA-*A. faecalis*-catalyzed acylation of (*RS*)-**4** (◇) with **1** (○) as an acyl donor; *N*-phenylacetyl-**4** (◆), phenylacetic acid (●).

4. Experimental

Solutions of penicillin acylase from *A. faecalis* and from *E. coli* were obtained from DSM Anti-Infectives, Delft, The Netherlands. Concentration of penicillin acylase active sites for both enzyme preparations was determined as described earlier.[24] 2-Amino-4-phenylbutane, 1-(4-chlorophenyl)ethylamine and (*R*)-phenylglycinol were obtained from Acros; (*S*)-phenylglycinol, (*R*)- and (*S*)-1-(2-naphthyl)ethylamine, benzylamine was obtained from Fluka; (±)-1-phenylethylamine and its individual enantiomers were obtained from Sigma; 3-phenylpropylamine was obtained from Aldrich; phenylacetic acid, phenylacetamide and phenylacetylchloride were obtained from Reakhim.

4.1. HPLC analysis

Concentrations of the reactants were determined by HPLC using a Waters M6000 pump, a Chrompack Nucleosil 100 C-18 column (150×4.6 mm, 5 μm) and a Waters M481 LC detector at 210 nm with 7 mM phosphate pH 3.0, containing acetonitrile (26 or 40%, v/v) and a 0.7 g/L of sodium dodecylsulphate, as the eluent. The flow rate was 1.0 mL/min. Retention times (in min) for the eluent with 26% CH_3CN: phenylacetamide (4.3), phenylacetic acid (7.8), *N*-phenylacetyl-phenylglycinol (13.8), phenylglycinol (18); retention times (in min) for the eluent with 40% CH_3CN: phenylacetamide (2.9), phenylacetic acid (4.4), benzylamine (6.1), *N*-phenylacetyl-benzylamine (10.8), 3-phenylpropylamine (10), *N*-phenylacetyl-3-phenylpropylamine (20), 1-phenylethylamine (10.5), *N*-phenylacetyl-1-phenylethylamine (19), 1-(4-chlorophenyl)ethylamine (11.7), *N*-phenylacetyl-1-(4-chlorophenyl)ethylamine (26), 2-amino-4-phenylbutane (12.1), *N*-phenylacetyl-2-amino-4-phenylbutane (31), 1-benzyl-2,2,2-trifluoroethylamine (15), *N*-phenylacetyl-1-benzyl-2,2,2-trifluoroethylamine (38), 1-(2-naphthyl)ethylamine (16.6), *N*-phenylacetyl-1-(2-naphthyl)ethylamine (33.4).

The enantiomeric excess of the products, $e.e._p$, 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-*iso*-propanol (95:5, v/v) as the eluent; retention times, in min: *N*-phenylacetyl-1-phenylethylamine: (*R*)- 42, (*S*)- 55; *N*-phenylacetyl-1-(4-chlorophenyl)ethylamine: (*R*)- 44, (*S*)- 58; *N*-phenylacetyl-phenylglycinol: (*R*)- 58, (*S*)- 49.5; *N*-phenylacetyl-2-amino-4-phenylbutane: (*R*)- 64, (*S*)- 90; or a reversed phase Chiralcel OD-RH column (Daicel Chemical Industries) with CH_3CN–water (6:4, v/v) as the eluent; retention times, in min: *N*-phenylacetyl-2-amino-4-phenylbutane: (*R*)- 8, (*S*)- 12; *N*-phenylacetyl-1-(2-naphthyl)ethylamine: (*R*)- 8.5, (*S*)- 11.5.

4.2. Synthesis of *N*-phenylacetylated amines

Acylation of amines was carried out by phenylacetylchloride in the following way: phenylacetylchloride (2.75 mmol) in acetone (2.75 mL) was added dropwise to the solution of enantiopure amine (2.5 mmol) and potassium bicarbonate (3 mmol) in aqueous acetone (1/1 v/v, 10 mL) with stirring at 0–5°C in an ice bath; the pH value was kept close to 10 by adding 2N aqueous NaOH. Finally the resulting solution was stirred for two more hours at room temperature, pH 10, acetone was evaporated under vacuum and the

Table 2. PA-*A. faecalis*-catalyzed acylation[a] of amines by **1** as an acyl donor: reaction rates, synthesis/hydrolysis ratios and enantioselectivities

Amine	Enantiomer	v_s/E_0[a] (s^{-1})	$(S/H)_0$[b]	E[c]	$e.e._p$[d] (%)
2	–	730	90	–	–
3	–	140	12	–	–
4	*R*	210	7.2	350	98.5 (47.3)
5	*R*	160	11	~1000	99.3 (49)
6	*R*	350	13	120	96.0 (45.1)
7	*R*	240	6.5	250	98.1 (46.8)
8	*S*	130	3.6	>100	98.0 (43)

[a] E_0 – PA-*A. faecalis* concentration in the reaction mixture determined after titration of the active sites;[24] 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 – estimated from the dependence $e.e._p$ versus degree of racemic amine acylation.

[d] $e.e._p$ – product e.e. at indicated (in brackets) degree of racemic amine acylation.

reaction mixture was acidified to pH 7 with 2N aqueous HCl and washed with ethyl acetate (3×10 mL). The organic layer was dried with Na_2SO_4 over 24 h, ethyl acetate after filtration was evaporated under vacuum and *N*-acylated amine was recrystallized from aqueous ethanol.

4.2.1. *N*-Phenylacetyl-(*S*)-1-(2-naphthyl)ethylamine. Yield: 0.520 g, (72%); $[\alpha]_D^{20}=-245$ (*c* 1 MeOH); mp 129–130°C; ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.38 (d, 3H, CH_3), 3.51 (s, 2H, CH_2), 5.18 (m, 1H, CH), 5.58 (d, 1H, NH), 7.13–7.71 (m, 12H, Ar). MS *m*/*z*: 289 (71, M), 274 (20, M–CH_3), 170 (57, M–$PhCH_2C(O)$), 155 (100, $C_{10}H_7CHCH_3$), 127 (55, $C_{10}H_7$), 91 (75, $PhCH_2$), 65 (18).

4.2.2. *N*-Phenylacetyl-(*R*)-phenylglycinol. Yield: 0.446 g, (70%); $[\alpha]_D^{20}=-177$ (*c* 1 MeOH); mp 114–115°C; ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.33 (s, 1H, OH), 3.51 (s, 2H, CH_2Ph), 3.70 (d, 2H, CH_2OH), 4.94 (q, 1H, CH), 6.00 (d, 1H, NH), 7.00–7.30 (m, 10H, Ph). MS *m*/*z*: 237 (23, M–H_2O), 224 (70, M–CH_2OH), 206 (56, M–CH_2OH–H_2O), 136 (24, M–$PhCH_2CO$), 132 (58), 120 (66), 119 (50, $PhCH(NH)CH_2$, $PhCH_2CO$), 106 (99, PhCHNH+H), 91 (100, $PhCH_2$), 77 (73, Ph), 65 (73), 51 (65), 39 (53).

4.3. General procedure for enzymatic acylation

A typical acylation was carried out in a thermostatted cell of a pH-stat (Radiometer RTS-622, Copenhagen, Denmark) at pH 10, 25°C in an aqueous medium (total volume 8 mL) with equimolar amounts of amine and acyl donor **1** in the presence of 0.9–2 μM PA-*A. faecalis* under permanent stirring and pH control by adding 2 M aqueous KOH solution. Reaction products were precipitating in the course of acylation and the maximum yield of amide was achieved in 10–30 min depending on the amine reactivity. Samples of the heterogeneous reaction mixture were prepared by adding an aliquot (50 μL) of the heterogeneous reaction mixture to a portion of the eluent (1.95 mL) in order to dissolve all reactants and to stop the enzymatic reaction, then the resulting solution was subjected to HPLC analysis. Progress curves of the enzymatic acylation were followed up to high degrees of conversion (from 50 to 100% of amine acylation depending on the enantioselectivity of penicillin acylase to racemic amine used). As a rule progress curves for all reaction components (acyl donor, amine, *N*-acylated amine and product of acyl donor hydrolysis) were documented what provided additional control due to the balance of the enzymatic reaction and made possible to follow synthesis/hydrolysis (S/H) ratio in the course of enzymatic acylation of amines; $(S/H)_0$ ratio was determined as a ratio of initial rates for accumulation of both reaction products.

Another aliquot of the heterogeneous reaction mixture was taken in the course of enzymatic acylation and filtrated in order to separate precipitating reaction product from the solution; isolated *N*-acylated amine was dissolved in the eluent and subjected to the chiral analysis. Finally, the reaction product (*N*-acylated amine) was filtrated, suspended in aqueous phosphate buffer (0.01 M pH 7.5, 10 mL) and then extracted by ethyl acetate (3×10 mL). The organic layer was dried with Na_2SO_4 overnight, ethyl acetate was evaporated under vacuum and *N*-acylated amine was recrystallized from aqueous ethanol.

4.3.1. *N*-Phenylacetyl-3-phenylpropylamine. *N*-Phenylacetyl-3-phenylpropylamine was synthesised using the general method with a reaction time of 40 min from equimolar (1.6 mmol) amounts of 3-phenylpropylamine and phenylacetamide by using 7.2 nmol PA-*A. faecalis*. Yield 0.372 g (92%); mp 73–75°C; ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.65 (m, 2H, CH_2), 2.45 (t, 2H, CH_2Ph), 3.12 (q, 2H, CH_2NH), 3.45 (s, 2H, $PhCH_2CO$), 5.22 (br. s, 1H, NH), 6.96–7.30 (m, 10H, Ph); MS *m*/*z*: 253 (42, M), 162 (45, M–$PhCH_2$), 149 (83, $PhCH_2CONHCH_2$+H), 118 (83, $PhCH_2CO$–H), 105 (48, $PhCH_2CH_2$), 91 (100, $PhCH_2$), 77 (42, Ph), 65 (33).

4.3.2. *N*-Phenylacetylbenzylamine. *N*-Phenylacetylbenzylamine was synthesised using the general method with a reaction time of 60 min from equimolar (1.6 mmol) amounts of benzylamine and phenylacetamide by using 8.6 nmol PA-*A. faecalis*. Yield 0.263 g (73%); mp 121–122°C; ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.52 (s, 2H, CH_2Ph), 4.31 (d, 2H, $NHCH_2Ph$), 5.57 (br. s, 1H, NH), 7.03–7.28 (m, 10H, Ph). MS *m*/*z*: 225 (74, M), 132 (37, $PhCH_2CONH$–2H, M–$PhCH_2$–2H), 106 (23, $PhCH_2NH$), 91 (100, $PhCH_2$), 77 (23, Ph), 65 (39).

4.3.3. *N*-Phenylacetyl-(*R*)-1-phenylethylamine. *N*-Phenylacetyl-(*R*)-1-phenylethylamine was synthesised using the general method with a reaction time of 25 min from equimolar (1.6 mmol) amounts of racemic (*RS*)-1-phenylethylamine and phenylacetamide by using 9.6 nmol PA-*A. faecalis*. Yield 0.172 g, (45%); e.e. 98.5%; 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).

4.3.4. *N*-Phenylacetyl-(*R*)-1-(4-chlorophenyl)ethylamine. *N*-Phenylacetyl-(*R*)-1-(4-chlorophenyl)ethylamine was synthesised using the general method with a reaction time of 37 min from equimolar (0.64 mmol) amounts of (*RS*)-1-(4-chlorophenyl)ethylamine and phenylacetamide by using 13.6 nmol PA-*A. faecalis*. Yield 0.079 g (45%); e.e. 99.3%; $[\alpha]_D^{20}=+110$ (*c* 1 MeOH); mp 177–178°C; ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.25 (d, 3H, CH_3), 3.46 (s, 2H, CH_2), 4.96 (m, 1H, CH), 5.45 (d, 1H, NH), 6.97–7.29 (m, 9H, Ar). MS *m*/*z*: 273 (67, M), 154 (25, 4-Cl-$PhCH_2CH(NH)CH_3$), 139 (100, 4-Cl-$PhCH_2CH(NH)$), 103 (80, $PhCH_2C$), 91 (83, $PhCH_2$), 77 (55, Ph), 65 (39).

4.3.5. *N*-Phenylacetyl-(*R*)-1-(2-naphthyl)ethylamine. *N*-Phenylacetyl-(*R*)-1-(2-naphthyl)ethylamine was synthesised using the general method with a reaction time of 20 min from equimolar (1.6 mmol) amounts of (*RS*)-1-(2-naphthyl)ethylamine and phenylacetamide by using 8.8 nmol PA-*A. faecalis*. Yield: 0.218 g (47%); e.e.

98.1%; mp 127–128°C; ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.38 (d, 3H, CH_3), 3.50 (s, 2H, CH_2), 5.19 (m, 1H, CH), 5.58 (d, 1H, NH), 7.13–7.71 (m, 12H, Ar). MS m/z: 289 (24, M), 170 (15, M–$PhCH_2C(O)$), 155 (100, $C_{10}H_7CHCH_3$), 127 (13, $C_{10}H_7$), 91 (26, $PhCH_2$).

4.3.6. *N*-Phenylacetyl-(*R*)-2-amino-4-phenylbutane. *N*-Phenylacetyl-(*R*)-2-amino-4-phenylbutane was synthesised using the general method with a reaction time of 5 min from equimolar (1.6 mmol) amounts of racemic (*RS*)-2-amino-4-phenylbutane and phenylacetamide by using 16 nmol PA-*A. faecalis*. Yield 0.17 g (40%); e.e. 96%; mp 109–112°C; ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.981 (d, 3H, CH_3), 1.54 (m, 2H, CH_2Bz), 2.43 (t, 2H, CH_2Ph), 3.43 (s, 2H, CH_2Ph), 3.92 (m, 1H, CH), 5.00 (d, 1H, NH), 6.97–7.30 (m, 10H, Ph). MS m/z: 267 (5, M), 176 (5, M–$PhCH_2$), 163 (26, M–$PhCH_2CH_2$+H), 117 (13, $PhCH_2CH_2CH$-H), 91 (100, $PhCH_2$), 77 (5, Ph), 65 (7), 44 (22).

4.3.7. *N*-Phenylacetyl-(*R*)-phenylglycinol. *N*-Phenylacetyl-(*R*)-phenylglycinol was synthesised using the general method with a reaction time of 0.5 h from equimolar (1.6 mmol) amounts of racemic (*RS*)-phenylglycinol and phenylacetamide by using 11.5 nmol PA-*A. faecalis*. Yield 26% 0.106 g (26%); e.e. 98%; mp 116–117°C; ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.35 (br. s, 1H, OH), 3.52 (s, 2H, CH_2Ph), 3.69 (br. s, 2H, CH_2OH), 4.94 (q, 1H, CH), 6.01 (d, 1H, NH), 6.99–7.30 (m, 10H, Ph). MS m/z: 237 (29, M–H_2O), 224 (70, M–CH_2OH), 206 (64, M–CH_2OH–H_2O), 132 (64), 120 (66), 119 (51, $PhCH(NH)CH_2$, $PhCH_2CO$), 106 (92, PhCHNH+H), 91 (100, $PhCH_2$), 77 (72, Ph), 65 (72), 51 (64), 39 (47).

Acknowledgements

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