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Penicillin acylase-catalyzed resolution of amines in aqueous organic solvents

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Abstract

Penicillin acylase from *Alcaligenes faecalis* catalyzes the enantioselective acylation of amines with phenylacetamide in a kinetically controlled reaction in water at pH 11. Addition of cosolvent to the reaction mixture significantly improved the enantioselectivity in most cases. Penicillin acylase from *E. coli* also catalyzed the phenylacetylation of amines, but an order of magnitude less efficiently than with the enzyme of *A. faecalis*. Amine resolution via kinetically controlled acylation competes effectively with hydrolysis of *N*-acylated compounds and constitutes a synthetically useful alternative to existing lipase-based methods.

1. Introduction

Following the first reports of the resolution of chiral amines via protease-[1] or lipase-[2]catalyzed acylation in organic media these reactions have been extensively studied, especially with lipases.[3] A serious shortcoming of these reactions is, however, the low turnover rate and, hence, catalyst productivity observed in organic media. More recently Bayer[4] and BASF[5] scientists achieved dramatic improvements in rates by employing an ester of methoxyacetic acid as the acyl donor, thus paving the way to commercialization of lipase-catalyzed resolution of amines.

Penicillin acylase (EC 3.5.1.11) is a readily available enzyme with potentially interesting resolving capability. It is selective for phenylacetic acid derivatives in its acyl donor binding site and is used industrially in the hydrolytic cleavage of penicillin G into 6-aminopenicillanic acid (6-APA) and phenylacetic acid.[6] Characterization of penicillin acylase from *E. coli* established that its nucleophile subsite is not particularly selective for β-lactam nuclei, but that a very wide range of nucleophiles is accepted.[7–10] It became clear that the nucleophile binding subsite is

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enantioselective for α-amino acids and their derivatives,[9,11–13] α-amino alcohols,[10] α-amino nitriles,[10] β- and γ-amino acids,[14–17] 1-aminoethylphosphonic and 1-aminoethylphosphonous acids[18] and several simple amines.[19]

Penicillin acylase is quite sensitive to organic solvents and is inactive in anhydrous media, in contrast with lipases and proteases. Therefore, it is preferably used in aqueous medium although stabilized preparations, such as cross-linked aggregates[20] and cross-linked crystals[21] of *E. coli* penicillin acylase, have occasionally been used in organic media. Hence, the use of penicillin acylase in amine resolution has largely been restricted to the hydrolysis of *N*-phenylacetyl-amines.[11,14,16,17,19,22,23] The alternative approach, a kinetically controlled acylation in water, has not received much attention. 1-Carbacephem compounds have been resolved using penicillin acylases from a variety of sources[24] and phenylglycine has been resolved via acylation with methyl 4-hydroxyphenylacetate.[25]

Recently we reported a study of the characterization of the relatively unknown penicillin acylase from *A. faecalis*.[26] This enzyme was discovered in the late 1960s[27] and its gene was later cloned and sequenced.[28,29] It was found to be much more thermostable than the acylase from *E. coli*, which was attributed to the presence of a disulfide bridge.[28] Penicillin acylase from *A. faecalis* has a broad pH optimum, showing at least 80% of the maximum activity in the pH range 7–11, whereas the *E. coli* enzyme has its optimum between pH 6 and 9.[30] Subsequently, we showed that the *A. faecalis* enzyme efficiently acylates amines at high pH in aqueous medium using phenylacetamide as the donor. Surprisingly the acylation of 1-phenylethylamine was highly enantioselective,[31] whereas this amine is poorly resolved by the penicillin acylase from *E. coli*.[19] Herein we report an investigation of the resolving capacity of *A. faecalis* penicillin acylase for several amines in a kinetically controlled acylation reaction in water/cosolvent mixtures and compare its activity with the corresponding enzyme from *E. coli* (Fig. 1).

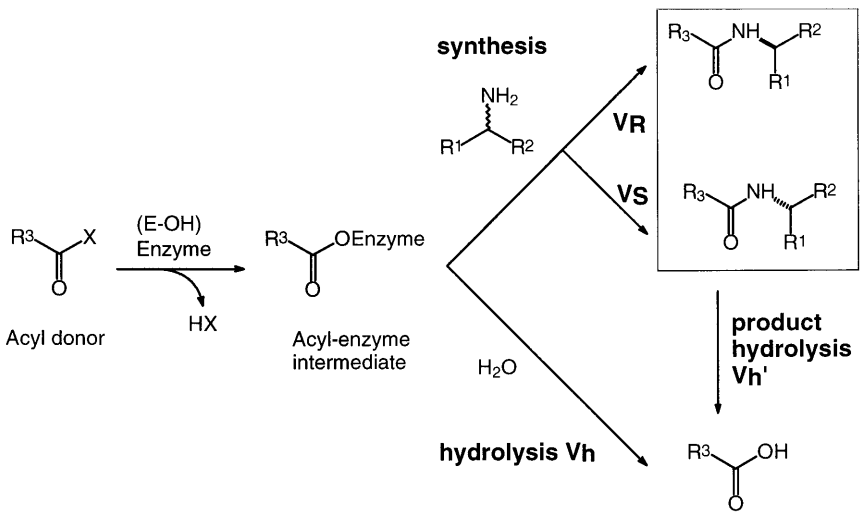


Figure 1. Enzymatic resolution of amines by kinetically controlled acylation. The ratio between V_R and V_S determines the enantioselectivity of the process; the ratio between $V_h+V'_h$ and V_R+V_S determines the required excess of acyl donor

2. Results and discussion

A range of amines were acylated by phenylacetamide in the presence of the penicillin acylase from *A. faecalis* or *E. coli* under kinetic control (Table 1). The reactions with the *A. faecalis* enzyme were performed at pH 11 to fully deprotonate the amine. The stability of the *E. coli* penicillin acylase, however, was low at this pH (data not shown), hence the reactions with this enzyme were performed at pH 10. The performance of the penicillin acylases from *A. faecalis* and *E. coli* were compared with regard to the enantioselectivity, rate and the ratio of synthesis to hydrolysis (S/H). *E. coli* penicillin acylase displayed low enantioselectivities with all the amines tested, with an enantiomeric ratio (E) ranging from 2 to 12. In contrast, *A. faecalis* penicillin acylase was highly enantioselective (E>100) with amines **1** and **4**, which both contained a phenyl substituent. Both enzymes exhibited poor enantioselectivity with the aliphatic amines **2** and **3** and with aminoindane **5**. We conclude that aromatic substituents are generally, but not always, favorable for enantioselectivity. Another important difference between the two enzymes was observed with regard to competing hydrolysis: the synthesis/hydrolysis (S/H) ratios were much higher with the *A. faecalis* penicillin acylase. Similarly, the rates observed with the *A. faecalis* enzyme were an order of magnitude higher than those with *E. coli* penicillin acylase (see Table 1). It is clear from these results that with regard to rate, enantioselectivity and S/H ratio the *A. faecalis* enzyme is superior.

Small amounts of acetonitrile already affected the enantioselectivity of the *A. faecalis* penicillin acylase mediated acylation as shown in Table 1; other solvents, such as *tert*-butyl alcohol and ethanol, were found to have a similar effect (data not shown). 2-Amino-4-phenylbutane could be resolved on a preparative scale in acetonitrile–water (1:3, v/v) yielding the *N*-phenylacetylated amine with 98% ee. In general, the presence of acetonitrile increased the preference for the (*R*)-enantiomer of the amines, which resulted in the case of 2-aminoheptane in an inversion of the chiral preference upon adding cosolvent. It is a frequently observed phenomenon in stereoselective reactions that the nature of the solvent changes the enantioselectivity of the enzyme,[32] which was explained by a different solvent effect for both enantiomers on the energy of binding in the active site.[33]

A model has been constructed, based on hydrolytic studies, for the binding of chiral nucleophiles in the acyl acceptor binding subsite of penicillin acylase from *E. coli*, as shown in Fig. 2.[19,22,34] Negatively charged or hydrophilic groups close to the stereogenic center were found to bind in subsite ρ3, causing the high enantioselectivity for amino acids and α-amino alcohols.[10,22] Furthermore, a slight preference of subsite ρ3 was observed for binding aromatic structures. Clearly, the preference of the ρ3 binding site of *A. faecalis* penicillin acylase for aromatic structures is much higher than in the case of *E. coli*.

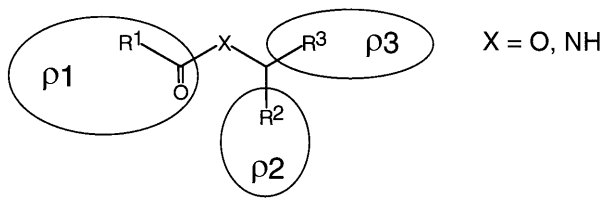
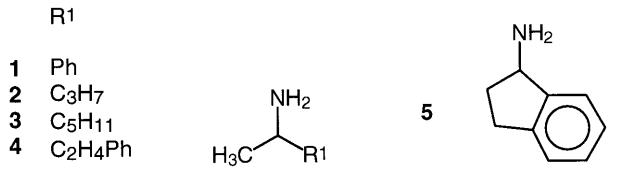


Figure 2. Model of the configuration of the favored substrate for *E. coli* penicillin acylase

Table 1
Resolution of amines by kinetically controlled acylation using penicillin acylase



	E. coli (pH 10)			*A. faecalis* (pH 11)								
	Water			Water			10% ACN			25% ACN		
Amine	E^a	S/H[b]	V_i^c	E^a	S/H[b]	V_i^c	E^a	S/H[b]	V_i^c	E^a	S/H[b]	V_i^c
1	4 (*R*)	0.3	0.3	>100 (*R*)	2	23		N.d.			N.d.	
2	3 (*S*)	0.2	0.8	9 (*R*)	2.4	12	14 (*R*)	3	38	10 (*R*)	3.5	17
3	7 (*S*)	0.6	1.2	1.7 (*S*)	2	24	1.6 (*R*)	2	23	2.6 (*R*)	2	25
4	2 (*R*)	0.5	0.14	110 (*R*)	4	19	220 (*R*)	4	21	400 (*R*)	4	13
5	12 (*R*)	0.06	0.03	2.8 (*R*)	0.3	1.8	3.5 (*R*)	0.2	1.3	4.8 (*R*)	0.1	0.6

[a] The *E* value has been calculated based on the enantiomeric purity of the product at 10–30% conversion; in case of compound **5**, which was slowly converted, the *E* value was calculated at 2–6% conversion. The absolute configuration of the product was determined by HPLC; standards of pure *R*-phenylacetylated amines were prepared by lipase-catalyzed aminolysis of methyl phenylacetate.

[b] S/H is defined as the initial ratio between amide formation and formation of phenylacetic acid.

[c] The initial rate of amide formation in μmol per unit per hour.

The reaction rates achieved with *A. faecalis* penicillin acylase in the resolution of amines by kinetically controlled acylation are high compared with resolution by amide hydrolysis reported for *E. coli* penicillin acylase.[19] The low hydrolysis rates are partially due to the poor solubility of *N*-phenylacetylated amines. The low solubility, however, is advantageous in a synthetic process as the reaction product can easily be obtained from the reaction mixture by filtration.

3. Conclusions

The *A. faecalis* penicillin acylase mediated acylation of amines is surprisingly efficient and shows a high enantioselectivity for α- and β-aryl amines. The enzyme tolerates the presence of cosolvents, which in most cases improves the enantioselectivity of the acylation process. On the basis of these results, we conclude that our penicillin acylase-catalyzed resolution of chiral amines has commercial potential as an alternative to analogous lipase-mediated resolutions.

4. Experimental

Penicillin acylase solution from *A. faecalis* and from *E. coli* were obtained from DSM Anti-Infectives, Delft, The Netherlands. One unit (U) of enzyme is defined as the amount of enzyme that hydrolyzes 1 μmol of penicillin G per min at pH 8.0 and 34°C. Immobilized *C. antarctica* lipase-B (Novozym 435) was a gift from NOVO Nordisk, Bagsvård, Denmark. 2-Heptylamine, 2-amino-4-phenylbutane and phenylacetylchloride were obtained from Acros, 2-pentylamine and 1-aminoindane from Fluka and 1-phenylethylamine from Sigma.

4.1. HPLC analysis

Concentrations of reactants were analyzed by HPLC using a Waters M6000 pump, a Nucleosil C-18 column and a Shimadzu SPD-6A UV detector at 215 nm. The eluent was prepared by adjusting the pH of a 1 g/l solution of sodium dodecylsulfate in methanol–water (60:40, v/v) to 3.5 with phosphoric acid. The flow rate was 1.0 ml/min. Retention times (in min) were as follows: phenylacetamide (3.3), phenylacetic acid (3.9), *N*-phenylacetyl-α-phenylethylamine (6.1), *N*-phenylacetyl-2-pentylamine (6.1), *N*-phenylacetyl-2-amino-4-phenylbutane (10.5), *N*-phenylacetyl-2-heptylamine (13.3), α-phenylethylamine (14.0) and 2-amino-4-phenylbutane (25.1).

The enantiomeric excess of the products was determined by HPLC using a Waters 590 pump, a Chiralcel OD column (Daicel Chemical Industries) and a Waters 486 UV detector at 215 nm. The eluent consisted of hexane–isopropanol (95:5, v/v). The flow rate was 0.6 ml/min. Retention times (in min) were as follows: *N*-phenylacetyl-α-phenylethylamine (42 *R*, 55 *S*), *N*-phenylacetyl-2-aminoheptane (20 *R*, 23 *S*), *N*-phenylacetyl-2-aminopentane (23 *R*, 25 *S*), *N*-phenylacetyl-1-aminoindane (48 *R*, 78 *S*) and *N*-phenylacetyl-2-amino-4-phenylbutane (64 *R*, 90 *S*).

The enantiomeric excess of *N*-phenylacetyl-2-amino-4-phenylbutane was also determined by HPLC using a Waters 590 pump, a reversed phase Chiralcel OD-RH column (Daicel Chemical Industries) and a Waters 486 UV detector at 215 nm. The eluent consisted of acetonitrile–water (6:4, v/v). The flow rate was 0.6 ml/min and retention times were 8 (*R*) and 12 (*S*) min.

4.2. Enzymatic acylation

Phenylacetamide (2 mmol) and amine (0.8 mmol) were suspended in 4 ml water or water/organic solvent mixture at 0°C and the pH was lowered to 11 using 3 M sulfuric acid. Penicillin acylase solution from *A. faecalis* (83 U) was added and under continuous stirring the pH was kept constant at 11 by automatic titration using 2 M NaOH. Resolutions using *E. coli* penicillin acylase were performed at pH 10 and with 166 U of enzyme.

Samples for reversed-phase HPLC analysis were prepared by dissolving an aliquot of the reaction mixture in water–methanol (3:2, v/v), acidified with phosphoric acid. Samples for chiral normal phase HPLC analysis were prepared by diluting the reaction mixture with 50 ml water and extraction with 50 ml hexane; the hexane layer was washed with 50 ml water and twice with 50 ml 1 M HCl and dried over Na_2SO_4.

4.3. Preparative resolution of 2-amino-4-phenylbutane

To 40 ml water/acetonitrile (3:1) at 0°C phenylacetamide (0.81 g, 6 mmol) and 2-amino-4-phenylbutane (1.2 g, 8 mmol) were added and the pH was brought to 11 with 3 M sulfuric acid. *A. faecalis* penicillin acylase solution (166 U) was added and the mixture was stirred for 2.5 hours, while the pH was kept constant with 2 M NaOH. The acetonitrile was evaporated under reduced pressure and the precipitate was filtered off and consisted of 0.93 g (*R*)-*N*-phenylacetyl-2-amino-4-phenylbutane (3.5 mmol, 43.5%) with an ee of 97%. $[\alpha]_D$ +18.7 (*c* 1, ethanol).

4.4. Synthesis of N-phenylacetylated amines

The amine was dissolved in water–1,2-dimethoxyethane (1:1, v/v) and the solution was cooled in an ice–salt bath. An 1.1-fold excess of phenylacetylchloride, dissolved in diethyl ether, was added to the solution, while the pH was kept at 11 using 5 M NaOH. The organic solvent was removed by evaporation under reduced pressure. A crude product was obtained by filtration and was recrystallized from water/ethanol.

Phenylacetamide was prepared by dropping phenylacetylchloride (Acros) in 25% ammonia at 0°C and recrystallizing the precipitated product in water.

N-Phenylacetyl-1-phenylethylamine. Yield 61%. Mp 111°C. ^{1}H NMR (300 MHz): δ 1.35 (d, 3H, CH_3), 3.46 (s, 2H, CH_2), 5.90 (q, 1H, CH), 7.17–7.33 (m, 10H, aromatic protons), 8.38 (d, 1H, NH). ^{13}C NMR (300 MHz): δ 169.04, 144.58, 136.41, 128.85, 128.10, 128.04, 126.47, 126.16, 125.82, 47.79, 42.20, 22.45. MS: 239 (M, 55), 105 (100), 91 (35), 77 (20), 65 (15).

N-Phenylacetyl-2-heptylamine. Yield 45%. Mp 68°C. ^{1}H NMR (300 MHz): δ 0.83 (t, 3H, CH_2CH_3), 1.01 (d, 3H, $CHCH_3$), 1.1–1.4 (m, 8H, $(CH_2)_4$), 3.36 (s, 2H, CH_2CO), 7.18–7.32 (m, 5H, aromatic protons). ^{13}C NMR (300 MHz): δ 169.10, 136.71, 128.75, 128.03, 126.11, 44.05, 42.49, 40.29, 35.97, 31.06, 25.16, 21.98, 20.74, 13.79. MS: 233 (M, 55), 162 (40), 142 (35), 136 (20), 100 (20), 91 (95), 65 (25), 57 (100).

N-Phenylacetyl-2-pentylamine. Yield 49%. Mp 62°C. ^{1}H NMR (300 MHz): δ 0.83 (t, 3H, CH_2CH_3), 1.01 (d, 3H, $CHCH_3$), 1.16–1.42 (m, 4H, $(CH_2)_2$), 3.37 (3, 2H, CH_2CO), 7.18–7.34 (m, 5H, aromatic protons). ^{13}C NMR (300 MHz): δ 169.09, 136.68, 128.75, 128.04, 126.11, 43.81, 42.44, 40.28, 38.21, 20.72, 18.74, 13.73. MS: 205 (M, 20), 162 (20), 92 (100), 71 (70), 65 (35), 44 (95).

N-Phenylacetyl-2-amino-4-phenylbutane. Yield 89%. Mp 109°C. ^{1}H NMR (300 MHz): δ 1.06 (d, 3H, C*H*$_3$), 1.58–1.72 (m, 2H, CHC*H*$_2$), 2.42–2.60 (m, 2H, CH$_2$C*H*$_2$), 3.40 (d, 2H, C*H*$_2$CO), 3.66–3.80 (m, 1H, C*H*), 7.10–7.34 (aromatic protons), 7.96 (d, 1H, N*H*). ^{13}C NMR (300 MHz): δ 169.23, 141.79, 136.67, 128.76, 128.11, 128.07, 126.15, 125.54, 43.83, 42.52, 37.87, 31.68, 20.65. MS: 267 (M, 30), 163 (40), 132 (35), 117 (20), 105 (20), 91 (100), 44 (40).

N-Phenylacetyl-1-aminoindane. Yield 80%. Mp 159°C. ^{1}H NMR (300 MHz): δ 1.72–1.82, 2.33–2.42, 2.74–2.84, 2.88–2.98 (m, 4H, C*H*$_2$C*H*$_2$), 3.46 (d, 2H, C*H*$_2$CO), 5.25 (q, 1H, C*H*), 7.10–7.40 (aromatic protons), 8.47 (d, 1H, N*H*). ^{13}C NMR (300 MHz): δ 169.85, 143.90, 142.81, 136.50, 128.84, 128.10, 127.30, 126.24, 126.21, 124.40, 123.72, 53.53, 42.26, 32.94, 29.62. MS: 251 (M, 30), 136 (60), 117 (100), 91 (50), 65 (15).

*4.5. Synthesis of (*R*)-*N*-phenylacetylated amines*

Amines **2**, **3**, **4** and **5** (1 g) and a half equivalent methyl phenylacetate were dissolved in 25 ml diisopropylether containing 1 g 4 Å molecular sieves and 0.5 g immobilized *C. antarctica* lipase B (Novozym 435). The reaction mixture was shaken at 40°C for 1 day in a closed reaction vessel. The enzyme and molecular sieves were removed from the reaction mixture by filtration and were washed with ethyl acetate. The organic fractions were washed with aqueous HCl and dried over Na_2SO_4. The solvent was evaporated and the crude products were recrystallized from hexane. In all cases enantiopure products were obtained.

Acknowledgements

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