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Preparation of D-amino acids by enzymatic kinetic resolution using a mutant of penicillin-G acylase from *E. coli*

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Abstract—We have demonstrated for the first time that D-glutamine (D-Gln) and D-glutamic acid (D-Glu) can be efficiently obtained in high ee (97% and 90%, respectively) by enzymatic kinetic resolution of D,L-Gln and D,L-Glu. This was achieved by enantioselective conversion of the L-enantiomers to their *N*-phenylacetyl derivatives in aqueous solution, using a mutant of penicillin-G acylase (PGA) from *E. coli* and phenylacetic acid methylester as the acyl donor. Kinetic modeling studies suggest that the high ee values obtained are both due to a strong enantiopreference for the L-amino acid in the deacylation step of the covalent enzyme intermediate, as well as to completeness of conversion that is transiently obtained as a result of the distinct preference of the mutant PGA for phenylacetic acid methylester over the *N*-phenylacetyl-L-amino acid product. For the other amino acids tested (Asn, Asp, and Ser), the highest ee values that were obtained for the remaining D-enantiomer are moderate (50–80%) because of lower enantioselectivity in the enzyme deacylation step and due to less complete conversion of the L-amino acid caused by competition for the active site between the acyl donor and the *N*-phenylacetyl-L-amino acid that is produced. The results demonstrate that the mutated PGA has great potential for the production of optically active D-amino acids by kinetic resolution.
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1. Introduction

Enantiopure D-amino acids are widely used as intermediates in the production of semi-synthetic antibiotics, agrochemicals, and novel pharmaceuticals,[1–5] but their industrial synthesis is not straightforward.[6] Although methods for the asymmetric synthesis of amino acids have been described, these have never been turned into economically viable processes. On the other hand, there are several cost-efficient synthetic methods for the production of racemic amino acids, which can be readily combined with biocatalytic technologies to give the enantiopure D-enantiomers. For instance, some D-amino acids can be cost-effectively produced from the corresponding D,L-hydantoins with the D-hydantoinase-carbamoylase system[7] or from D,L-amino acid amides using an L-amidase,[8] followed by chemical amide hydrolysis. D-Amino acids are also industrially accessible by

enzymatic hydrolysis of the *N*-acylated racemates with an L-amino acid acylase.[9]

Penicillin-G acylase (PGA) is currently one of the acylases most widely applied industrially, not only for the production of semi-synthetic antibiotics[7] but also for the synthesis of enantiomerically pure L-amino acids through enantioselective enzymatic hydrolysis of *N*-phenylacetyl-D,L-amino acids in aqueous solution. If a D-amino acid is the desired product, an additional chemical hydrolysis step is required. Cole[10,11] showed that PGA can also be applied in the synthetic direction, while Zmijewski et al.[12] demonstrated that PGA is a suitable catalyst for carrying out kinetically controlled acylations in the resolution of racemates. This conversion is based on the differences in efficiencies with which the covalent acyl-enzyme intermediate, in which the nucleophilic serine is acylated, reacts with the enantiomers of an amino acid. When such a kinetic phenylacetylation is performed in aqueous solution with wild-type PGA from *E. coli* (EC 3.5.1.11), the *N*-phenylacetyl-L-amino acids can be obtained in high ee but the ee values obtained for the remaining D-amino acids are low. This is mainly

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due to the fact that the acyl-enzyme intermediate may be subject not only to deacylation by the nucleophilic amino acid but also can be deacylated by water. Furthermore, hydrolysis of the produced *N*-phenylacetyl-L-amino acids can also occur. Both these competing processes can reduce the completeness of conversion of the substrate enantiomer that must be acylated and thereby reduce the ee that can be reached (see Fig. 1).

One solution to the hydrolysis problem is to perform the enzymatic phenylacetylation in organic media, which we demonstrated by the preparation of D-phenylglycine and D-*p*-hydroxyphenylglycine methylesters.[13] The drawbacks of this method for industrial production are the use of environmentally unfriendly organic solvents on a large scale, the fact that the amino acids must first be transformed into esters or amides to render them soluble in organic solvent, and the generally lower enantioselectivity of PGA in organic solvent than in aqueous solution.[14] A second possible solution is to improve the kinetic parameters of the wild-type PGA by site-directed mutagenesis. Recently, we studied a similar PGA-catalyzed acylation reaction, namely the condensation of D-phenylglycine amide with 6-aminopenicillanic acid to give the important semi-synthetic β-lactam antibiotic ampicillin.[15] When comparing the kinetic properties of several mutants with those of wild-type PGA, we found that the F24A mutant (with Phe changed to Ala on position 24 of the β subunit) possesses a threefold higher ratio between the rate of acyl transfer to the nucleophile and the rate of acyl transfer to water, as well as a twofold higher ratio between the maximum concentration of ampicillin and the level of D-phenylglycine at that point of conversion. Furthermore, this mutant still has a sufficiently high activity for preparative conversions.[15] Thus, we reasoned that this F24A mutant might also behave as an improved catalyst in PGA-catalyzed kinetic resolutions of amino acids through enantioselective acylation in aqueous solution using phenylacetic acid methylester as the acyl donor.

2. Results and discussion

The F24A mutant of PGA was applied to the resolution of D,L-Gln, D,L-Glu, D,L-Asn, D,L-Asp, and D,L-Ser. For these five amino acids no direct methods have been published so far to obtain the D-enantiomers in one step via L-selective acylation of the racemate or through D-selective hydrolysis of the D,L-hydantoin. To test these conversions, 1 M aqueous solutions of the racemates were reacted with 1.3–1.8 equiv phenylacetic acid methylester at pH = 9–9.6 in the presence of F24A mutant PGA at 25 °C. At regular time intervals, samples were taken and the ee values of both the *N*-phenylacetyl-L-amino acid products and of the remaining D-amino acids, were determined by chiral HPLC. Conversions and *E* values were subsequently calculated[16] on the basis of these experimentally determined ee values.

The results show that when Gln was the amino acid substrate used, the ee of the remaining D-enantiomer (ee_D) initially showed a rapid increase with time due to preferential enantioselective acylation of L-Gln (Fig. 2). A maximum was reached after 3 h, after which the ee_D decreased slowly. This is due to the hydrolysis of the *N*-phenylacetyl-L-amino acid, as evident from the concomitant reduction of the degree of conversion of total free amino acid. The maximal ee_D that was transiently obtained during the resolution of Gln was surprisingly high, peaking at a value of 97% for ee_D (Table 1). This is a tremendous improvement compared to the maximal ee_D obtained with wild-type PGA (13%). Under similar conditions, a high maximal ee_D (90%) was also obtained for Glu (Table 1). For the other amino acids tested (Asn, Asp, and Ser) the *N*-phenylacetyl-L-amino acids were obtained in high ee (ee_L >93% in all cases, Table 1), but the maximal ee_D values for the remaining free D-amino acids were only moderate (50–80%). In the case of Asn and Asp, the amount of acyl donor was increased from 1.3 to 1.8 equiv, although the maximal ee_D hardly improved. Neither an increase of the initial amino acid
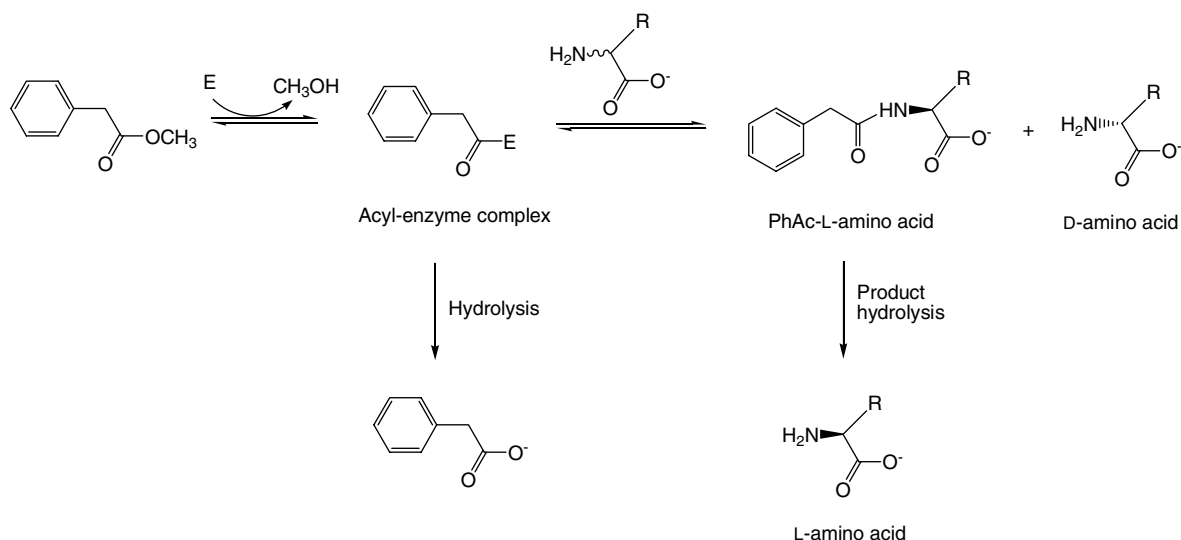

Figure 1. Kinetic resolution of D,L-amino acids by enantioselective acylation of the L-enantiomers using PGA and phenylacetic acid methylester. The vertical arrows indicate (undesirable) hydrolysis reactions. *E* = enzyme.
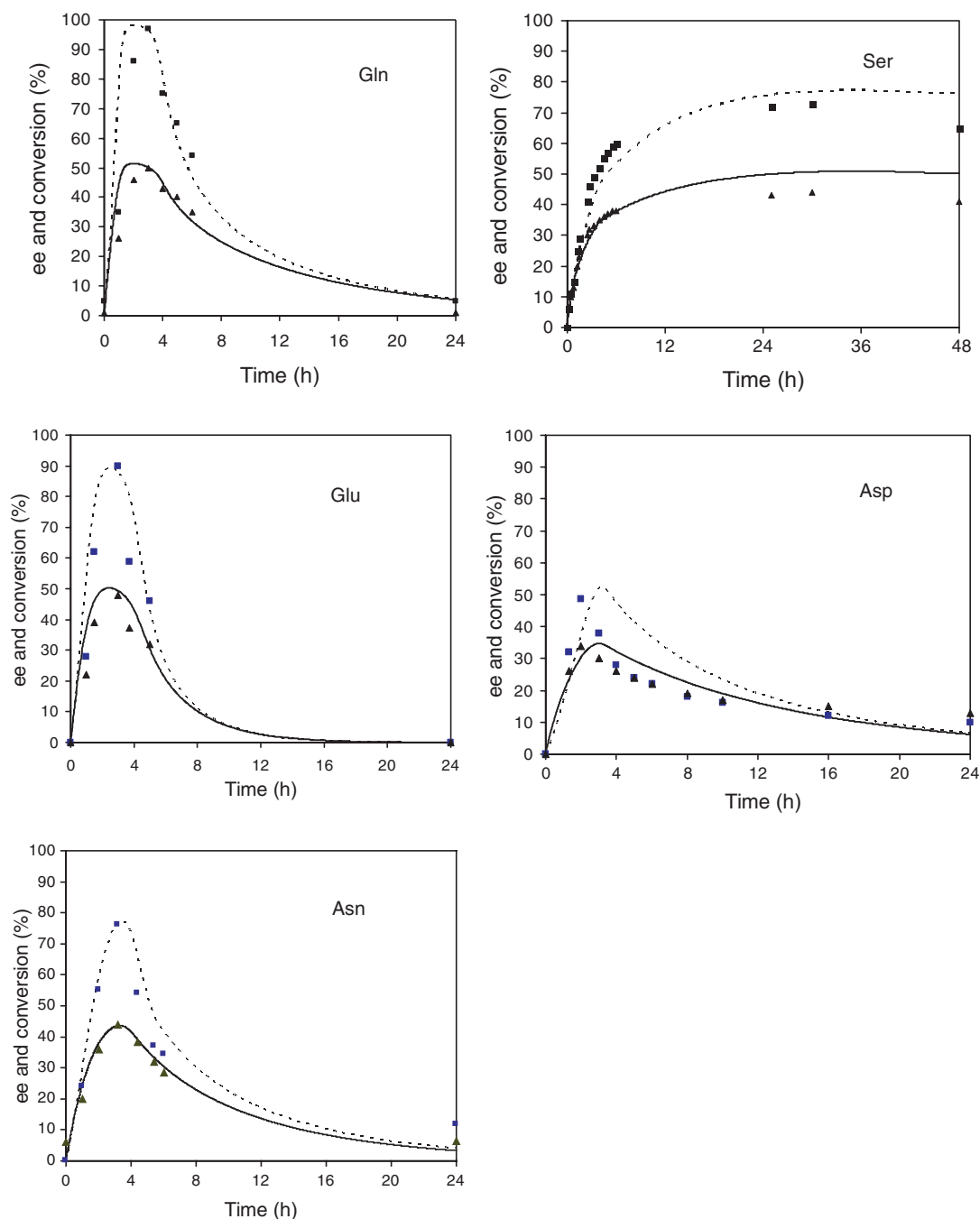
Figure 2. Plots of conversion and ee_D versus time for the phenylacetylation for various amino acids with F24A mutant PGA. Symbols (■, ee_D; ▲, conversion) indicate experimental values, lines represent simulated data, using the lumped parameters given in Table 2.

concentration from 1 to 2 M, nor a variation of the temperature between 5 and 30 °C altered the maximum ee_D value that was reached.

In order to obtain a better understanding of the kinetics of these F24A mutant PGA-catalyzed resolutions, we developed a computer model that describes conversion as a function of steady-state kinetic parameters. The model consists of a set of ordinary differential equations that describe the conversion of the synthetic coupling products P_L and P_D, the hydrolysis product A, and the free enzyme (see Fig. 3). The model contains three

lumped steady-state kinetic parameters, α, β_0 and γ (Table 2), that govern the course of a kinetic resolution in terms of ee and c in time and can be regarded as relative preference for product as compared to acyl donor (α), concentration-dependent nucleophile (N_D, N_L) reactivity (β_0), and maximum reactivity of the nucleophile at saturating concentrations of nucleophile as compared to water (γ). The values that were obtained by fitting (see Experimental) may not be unique solutions, but do show how the lumped kinetic parameters are related to the ee and yield that are obtained in PGA-catalyzed kinetic resolutions.

Table 1. Enzymatic resolution of racemic D,L-amino acids using the F24A mutant of PGA from *E. coli* as well as wild-type PGA as the biocatalyst and phenylacetic acid methylester as acyl donor in aqueous solution at 25 °C (for further details, see Section 4)

PhAc-L-amino acid   D-amino acid
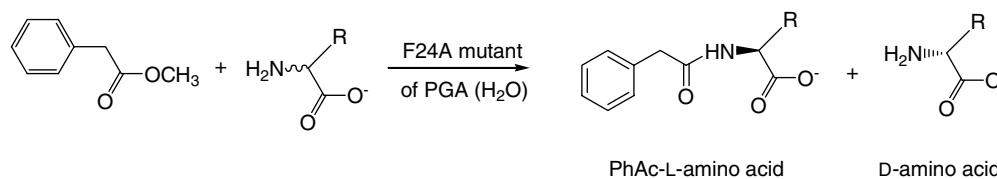
Amino acid	R	Acyl donor (equiv)	Enzyme	pH	ee_D max (%)	ee_L[a] (%)	Conversion[a,b] (%)	E value[c]
Gln	$(CH_2)_2CONH_2$	1.3	F24A	9.0	97	>99	49.5	810
Gln	$(CH_2)_2CONH_2$	1.3	Wild-type	9.4	13	99	12	48
Glu	$(CH_2)_2CO_2H$	1.3	F24A	9.0	90	>99	48	250
Asn	CH_2CONH_2	1.3 (1.8)	F24A	9.4	76 (78)	97 (97)	44 (45)	140
Asp	CH_2CO_2H	1.3 (1.8)	F24A	9.6 (9.4)	48 (56)	95 (91)	34 (38)	46
Ser	CH_2OH	1.8	F24A	9.4	73	93	44	60

[a] Measured at maximal ee_D.
[b] Of the racemic amino acid.
[c] Calculated using the ee_L and ee_D values at maximal ee_D value.
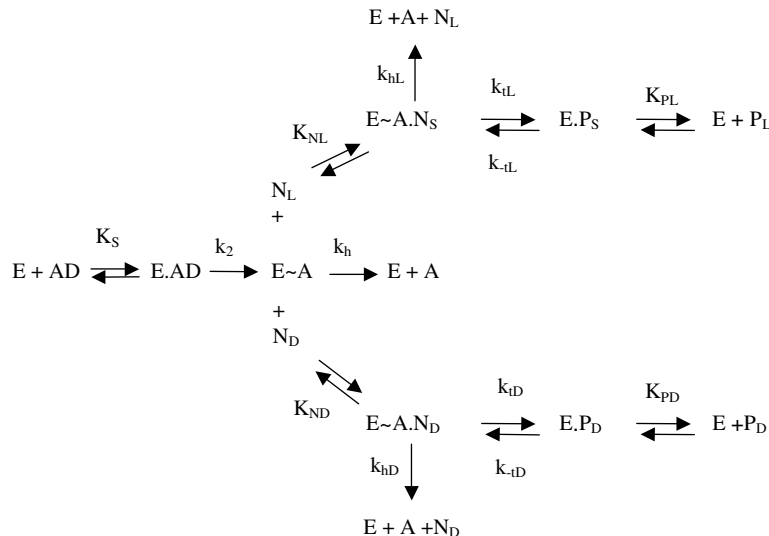
Figure 3. Kinetic scheme for a PGA-mediated resolution of amino acids in water. AD = acyl donor; N = nucleophilic amino acid; P = phenylacetylated product; A = phenylacetic acid; E = enzyme.

Table 2. Steady state parameters obtained by a Monte Carlo type numerical fitting of the experimental data to the model described in the text

	α	β_0 (mM^{-1})	γ
L-Gln	2.5	30	0.05
D-Gln	2.5	1	3
L-Ser	12.5	300	0.05
D-Ser	125	56	0.053
L-Glu	2.5	30	0.05
D-Glu	2.5	2	1.5
L-Asp	0.033	3.75	0.33
D-Asp	5	2	2
L-Asn	0.125	8.75	0.29
D-Asn	10	2	1

The definition of the parameters is given in the experimental.[19,20]

The high enantioselectivity for Gln conversion is in agreement with a high ratio (30) between the β_0 values for L- and D-Gln, which reflects a much higher reactivity (as a nucleophile) of the L-enantiomer as compared to the D-enantiomer. The same L-preference is caused by the low ratio of the γ values (0.017), which indicates that the preference for L-Gln as the deacylating enantiomer prevails at high amino acid concentration. The low value for α_L reflects a low tendency of the F24A mutant PGA to react with the N-phenylacetyl-L-Gln produced, allowing conversion of L-Gln to proceed to completion by accepting phenylacetic acid methylester even when product starts to accumulate, and contributing to the high ee_D. With Gln, after 2.5 h the reverse reaction became important. Hydrolytic degradation of N-phenylacetyl-L-Gln was accompanied by the release of free L-Gln, which caused the degree of conversion c and ee_D to drop drastically.

The kinetic resolution experiment with the F24A mutant PGA and D,L-Ser followed a similar trend, but the ee and c obtained were significantly lower. The time course

of ee and c with D,L-Ser could also be fitted using the model, and when comparing the lumped parameters obtained for the enantiomers of Gln and Ser it appeared that the lower quality of the kinetic resolution of Ser can be explained by a lower L-selectivity of the enzyme in the deacylation reaction, as reflected by β_{0L}/β_{0D} ratios of 30 and 5 for Gln and Ser, respectively. The γ_L/γ_D ratios predicted by the model were 0.017 and 1, respectively, which also indicates a better enantioselectivity in the deacylation of the enzyme coupled to the L-enantiomer in the case of Gln than with Ser, since a low value for γ means a high tendency to react at saturating concentration. Besides the lower ee_D obtained with Ser as the nucleophile, it is also remarkable that there is no drop in the ee during the course of the experiment, unlike with Gln. This can be explained by higher β_0 values and lower γ values for Ser (γ should be low for good conversion), which indicate a low tendency of the acyl-enzyme to undergo hydrolysis, implying that the acyl donor is not rapidly depleted. The incompleteness of conversion as in the case of Ser can be explained by the relatively high value for α ($\alpha_{L,Ser}/\alpha_{L,Gln} = 5$), which reduces c even if the acyl donor is still present. This phenomenon appears as product inhibition, and can possibly be influenced by selecting another acyl donor or by further mutagenesis. The progress of the kinetic resolution with Glu was similar to that obtained with Gln, although the maximal ee_D obtained was significantly lower, which is in agreement with the smaller difference between the β_0 values and the γ values for the enantiomers of this substrate, as compared to Gln. The resolution with Asp was even worse, and the differences between the β_0 and γ values, which mainly determines enantioselectivity, was even smaller in this case.

3. Conclusion

Herein, we have shown that D-Gln and D-Glu can be obtained in one step in high ee (97% and 90%, respectively) in an enzymatic kinetic resolution of their racemates. This was achieved by enantioselective conversion of the L-enantiomers to the N-phenylacetyl derivatives with phenylacetic acid methylester as an acyl donor in aqueous solution by using an F24A mutant of PGA from E. coli. The high ee_D values are mainly due to the significantly suppressed hydrolysis rate of N-phenylacetyl-L-Gln and N-phenylacetyl-L-Glu, respectively, compared to wild-type PGA. Although, for the other amino acids tested (Asn, Asp, and Ser), the intrinsic E values of the F24A mutant are also high and the N-phenylacetylated L-enantiomers can be obtained in high ee (>93%), because the maximal ee_D values are only moderate (50–80%), the hydrolysis rate of the N-phenylacetyl-L-amino acid products is still too high and the conversion of the amino acid nucleophile is complete. A kinetic model describing conversion in time appears suitable for the numerical simulation of the outcome of the kinetic resolutions of amino acids with PGA, and can explain differences in enantioselectivity in terms of variations in nucleophile reactivity and competition between substrate and product at the nucleophile

binding site of the acyl-enzyme intermediate. The model may be used as a tool to direct experiments aimed at improving conversions. For example, in the case of D,L-Gln and D,L-Glu, engineering of PGA should be aimed at extending the period of time at which a high ee and c are maintained, for example, by means of additional mutagenesis that leads to suppression of hydrolysis of the acyl donor. For the current experiments, the kinetic modeling studies confirm the more distinct preference of the F24A mutant PGA for phenylacetic acid methylester as compared to the N-phenylacetyl-L-amino acid in the case of conversion of Gln as compared to the case with Ser as the nucleophile. The present results are of significant industrial importance especially for the cost-efficient production of D-Gln and D-Glu. These D-amino acids can now be obtained in good ee in one single step from the racemate with a simple work-up.

4. Experimental

4.1. Materials

The F24A mutant of PGA was prepared as described earlier.[15] The wild-type PGA was obtained from DSM Anti-infectives (Delft, The Netherlands). D,L-Gln, D,L-Glu, D,L-Asn, D,L-Asp, D,L-Ser, and phenylacetic acid methylester were obtained from Fluka and used without further purification.

4.2. Enzymatic acylation reactions

To a mixture of 25 mL of a 1 M solution of the D,L-amino acid in water, which had been adjusted to pH = approximately 9 with 2 M aqueous NaOH, and 1.3–1.8 equiv of phenylacetic acid methylester, was added 2.2 g of the PGA mutant (or of the wild-type PGA). The mixture was stirred at 25 °C and during the reaction the pH was maintained at approximately 9 by titration with 2 M aqueous NaOH using a pH stat apparatus. To monitor the reaction, a 1 mL aliquot was withdrawn from the reaction mixture with regular time intervals and diluted 10-fold with 50 mM phosphate buffer (pH = 2.7). The resulting sample was used for both HPLC analysis 1 (ee determination of the amino acid substrates) and HPLC analysis 2 (ee determination of the N-phenylacetylated products).

4.3. Synthesis of the N-phenylacetyl-D,L-amino acid reference compounds

To a 2.5 M aqueous solution of the D,L-amino acid at pH = 10 was added, under vigorous stirring, phenylacetyl chloride (1.1 equiv) during 15 min at 0–5 °C, maintaining the pH at approximately 10 by the simultaneous addition of 50 wt.% aqueous NaOH solution. Subsequently, the solution was stirred for another 2 h at ambient temperature and acidified to pH = 1 using 32 wt.% aqueous HCl solution, which resulted in precipitation of the N-phenylacetylated D,L-amino acids. The precipitates were isolated by filtration, recrystallized

from water/ethanol and dried under reduced pressure. *N*-Phenylacetyl-D,L-Gln, *N*-phenylacetyl-D,L-Glu, *N*-phenylacetyl-D,L-Asn, *N*-phenylacetyl-D,L-Asp, and *N*-phenylacetyl-D,L-Ser were obtained in 72%, 75%, 68%, 61%, and 49% yield, respectively. ^{1}H NMR (DMSO-d_6), δ (ppm): *N*-phenylacetyl-D,L-Gln: 1.77 (1H, m, $-CH_2$–CHNH), 1.96 (1H, m, $-CH_2$–CHNH), 2.13 (2H, t, $-CH_2$–$CONH_2$), 3.48 (2H, s, CH_2Ph), 4.15 (1H, dd, CH), 6.77 (1H, s, $CONH_2$), 7.28 (5H, m, Ph), 7.37 (1H, s, $CONH_2$), 8.39 (1H, s, NHCO). *N*-Phenylacetyl-D,L-Glu: 1.60 (1H, m, $-CH_2$–CHNH), 1.95 (1H, m, $-CH_2$–CHNH), 2.38 (2H, t, CH_2COOH), 3.50 (2H, s, CH_2Ph), 4.15 (1H, dd, CH), 7.28 (5H, m, Ph), 7.70 (1H, s, NHCO). *N*-Phenylacetyl-D,L-Asn: 2.52 (2H, m, $-CH_2$–CON), 3.48 (2H, s, CH_2Ph), 4.50 (1H, dd, CH), 6.90 (1H, s, $CONH_2$), 7.27 (5H, m, Ph), 7.37 (1H, s, $CONH_2$), 8.30 (1H, s, NHCO). *N*-Phenylacetyl-D,L-Asp: 2.72 (2H, m, CH_2CO_2H), 3.44 (2H, s, CH_2Ph), 4.40 (1H, dd, CH), 7.27 (5H, m, Ph), 8.20 (1H, s, NHCO), 11.0 (COOH). *N*-Phenylacetyl-D,L-Ser: 2.10 (1H, br s, OH), 3.44 (2H, s, CH_2Ph), 4.00 (2H, d, CH_2OH), 4.50 (1H, dd, CH), 7.06–7.14 (5H, m, Ph), 8.20 (1H, s, NHCO).

4.4. HPLC analysis 1: determination of the ee of amino acid substrates

An HPLC column (size 150 mm × 4.0 mm) consisting of Crownpak Cr(+) from Daicel Technologies was used, which was operated at 5 °C, except for the phenacetylation of D,L-Ser where it was operated at 0 °C. Aqueous $HClO_4$ (with concentrations as indicated in Table 3) was used as the eluent, the injection volume was 20 μL and the flow 0.5 mL/min except for the phenylacetylation of D,L-Ser where the flow was 0.4 mL/min. The compounds were detected by fluorescence at 338 nm after post-column derivatization of the amino group consisting of a reaction with *o*-phthalaldehyde and 2-mercapto-ethanol at pH = 10 at ambient temperature. Under these conditions the D- and L-enantiomers of the various amino acids had the retention times as shown in Table 3.

4.5. HPLC analysis 2: determination of the ee of *N*-phenylacetylated amino acid products

For *N*-phenylacetylated Gln, Asn, Asp, and Ser, a Chirobiotic T HPLC column (Astec, size 250 mm × 4.0 mm) was used in series with an Inertsil ODS-3 HPLC column (150 mm × 4.6 mm, particle size 5 μm). Both columns were operated at ambient temperature. The mobile phase was a gradient of eluent A (80 vol % 15 mM ammonium acetate in water at pH = 4.1 and 20 vol % methanol) and eluent B (20 vol % 15 mM ammonium acetate in water at pH = 4.1 and 80 vol % methanol) with the following programme: 0–13 min (0% B), 13–13.1 min (0→100% B), 13.1–18 min (100% B), 18–18.1 min (100→0% B) and 18.1–25 min (0% B). The injection volume was 10 μL and the flow 1.0 mL/min. The compounds were detected by UV (215 nm). Under these conditions the D- and L-enantiomers of the various *N*-phenylacetylated amino acids had the retention times as shown in Table 4.

For *N*-phenylacetyl-Glu, a Chiralcel OJ HPLC column (Chiral Technologies Inc., size 250 mm × 4.6 mm, particle size 10 μm) was used in series with a Lichrospher diol (Alltech) HPLC column (150 mm × 4.6 mm, 5 μm), both operated at ambient temperature. The mobile phase was *n*-hexane/*t*-butanol/trifluoroacetic acid (80/20/0.1 v/v/v). The injection volume was 20 μL and the flow rate 1.0 mL/min. The compounds were detected by UV (210 nm). Under these conditions, D- and L-*N*-phenylacetyl-Glu had a retention time of 22.5 and 26.5 min, respectively.

4.6. Computer simulations

The kinetic model follows similar principles as the model described by Youshko et al.[17,18] for the acylation of β-lactam compounds, but incorporates two enantiomers of the nucleophile (Fig. 3). The model consists of differential equations (dA/dt, dP_D/dt, dP_R/dt, dE/dt), which were derived from the scheme shown in Figure 3 and describe the change of the concentrations of all substrates and enzyme in time. The microscopic rate

Table 3. HPLC eluents and retention times for the amino acid substrate enantiomers

Amino acid	Eluent	pH	Retention time of D-enantiomer (min)	Retention time of L-enantiomer (min)
Gln	10 mM $HClO_4$	2	4.50	7.45
Glu	10 mM $HClO_4$	2	4.90	11.45
Asn	100 mM $HClO_4$	1	4.60	5.45
Asp	50 mM $HClO_4$	1.3	5.20	7.10
Ser	100 mM $HClO_4$	1	5.85	7.35

Table 4. Retention time of the enantiomers of the N-phenylacetylated amino acids

N-Phenylacetylated amino acid	Retention time of L-enantiomer (min)	Retention time of D-enantiomer (min)
N-PhAc-Gln	8.40	11.50
N-PhAc-Asn	6.50	7.65
N-PhAc-Asp	9.40	11.10
N-PhAc-Ser	7.90	9.10

constants and equilibrium constants in the equations for substrate conversion were replaced by three lumped steady-state kinetic parameters called α, β_0 and γ, for each enantiomer. The parameters α_L and α_D describe the competition between acyl donor (phenylacetic acid methylester, AD in Fig. 3) and product (phenylacetylated amino acid, P_L or P_D) for the free enzyme and equal the ratios between the k_{cat}/K_m values of the enzyme for substrate and product. Parameter β_{0D} equals $k_{tD}/k_h \cdot K_{N_D}$ (and a similar relation exists for the L-enantiomer) and describes the concentration dependence of the reactivity (relative to water, mM^{-1}) of the nucleophilic amino acid (N_D, N_L) that is converted by reacting with the acylenzyme. Parameter γ (k_{tD}/k_{hD}) is the inverse of the maximum nucleophile reactivity (relative to water). The value of β_{0L}/β_{0D} corresponds to the apparent *E* value of the enzyme for the chiral amino acids, defined as $V_{N_L}/V_{N_D} = E*[N_L]/[N_D]$. The lumped parameters can be determined experimentally in separate steady-state measurements, for example, by measuring the initial rate of product formation with the pure enantiomers, as will be described in detail elsewhere. After replacing the constants given in Figure 3 by these lumped parameters α, β, and γ, the equations were numerically integrated using the programme Mathcad 12 (Mathsoft) to obtain substrate and product concentrations in time, from which the ee and conversion in time were calculated. Furthermore, a Monte Carlo routine implemented in Matlab 7 (Mathworks) was used for fitting the differential equations to the experimental data (Fig. 2). This gave values for the lumped kinetic parameters that provided a good match of the simulations with the experimental data (Fig. 2). The initial substrate concentrations that were used in these simulations were in accordance with the experiments (1.3 M phenylacetic acid methylester, 1 M racemic amino acid). Starting values for microscopic kinetic constants and equilibrium constants were adopted from Youshko et al.[19] and converted to lumped parameters, that were used as starting values for the simulations, which yielded the results shown in Table 2. All conversions were treated as closed systems, without removal of products. The obtained parameters were used in simulations, from which ee and *c* were calculated and compared to the experimental data (Fig. 2).

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