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Dynamic kinetic resolution of phenylglycine esters via lipase-catalysed ammonolysis

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Abstract

Ammonolysis of D,L-phenylglycine methyl ester catalysed by Novozym 435 at 40°C in *tert*-butyl alcohol gave D-phenylglycine amide in 78% *ee* at 46% conversion, corresponding to an enantiomeric ratio (*E*) of 16. Lowering the temperature improved the enantioselectivity (*E*=52 at −20°C). Combination of ammonolysis with pyridoxal-catalysed in situ racemisation of the unconverted ester (dynamic kinetic resolution), at −20°C, gave D-phenylglycine amide with 88% *ee* at 85% conversion. The amide racemised much slower than the ester at this low temperature.

1. Introduction

As a result of recent developments in the manufacture of penicillin and cephalosporin antibiotics,[1] multi-step chemical procedures for the coupling of the D-phenylglycine and D-(4-hydroxyphenyl)glycine side-chains with the β-lactam nuclei are being replaced by enzymatic alternatives (Scheme 1).

In the chemical procedure, D-phenylglycine and its 4-hydroxy derivative are key intermediates. Two processes are used for the commercial production of these amino acids: classical resolution via diastereomeric salt crystallisation[2] or enantioselective hydrolysis of the corresponding hydantoin.[3,4] In the former, the L-isomer has to be racemised while the latter process is a dynamic kinetic resolution and produces the D-isomer as the sole product. The enzymatic coupling (Scheme 1) is successful only with an activated side-chain, e.g. an amide or an ester.[5] Hence, when the side-chain is produced as the free acid (see above) additional steps are required to convert it to the corresponding ester or amide. Consequently, an efficient enzymatic coupling process requires a direct method for the synthesis of the D-ester or D-amide.

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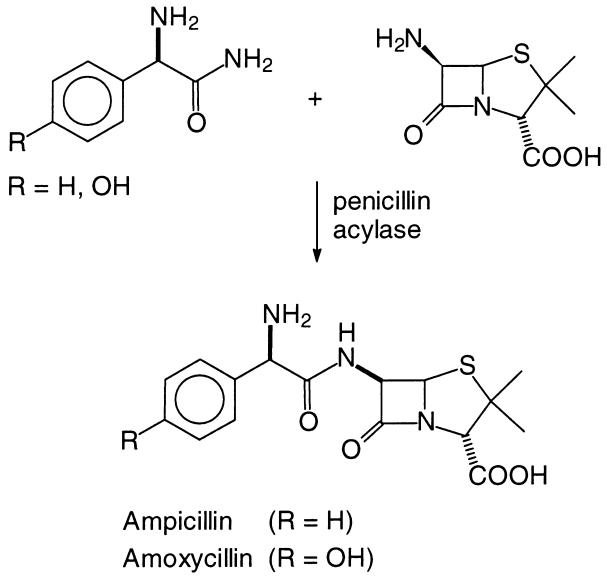
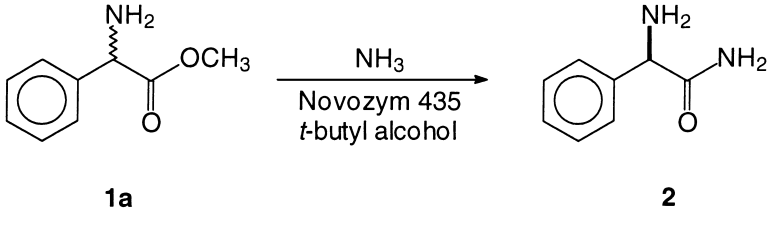


Scheme 1. Enzyme-catalysed coupling of D-phenylglycine amide and 6-aminopenicillic acid

An enzymatic route to D-phenylglycine amide has been developed and commercialised by DSM.[6] This process involves a kinetic resolution of the racemic amide by an L-specific aminopeptidase and, consequently, suffers from the inherent disadvantage of producing L-phenylglycine as the coproduct. The latter needs to be racemised and converted, via the ester, to the D,L-amide substrate. A direct method for the enantioselective conversion of racemic ester to D-amide would have obvious advantages, especially if it could be performed with in situ racemisation, i.e. as a dynamic kinetic resolution.

We recently showed[7] that the lipase (Novozym 435) catalysed ammonolysis of racemic phenylglycine methyl ester **1a** affords D-phenylglycine amide **2** (Scheme 2). This method would meet the criteria outlined above if the slow racemisation of the ester, which we observed[7] (5% over 24 h) in the course of the reaction, could be sufficiently accelerated to allow for a dynamic kinetic resolution. Obviously, for such a scheme to be effective the product amide should racemise much slower than the ester substrate.



Scheme 2. Enantioselective ammonolysis of phenylglycine esters

The racemisation of amino acid esters is known to be effficiently catalysed by aromatic aldehydes such as 2-hydroxybenzaldehyde (salicylaldehyde) and 3-hydroxy-2-methyl-5-hydroxymethyl-pyridine-4-carboxaldehyde (pyridoxal) under basic conditions.[8,9] The use of aldehyde-based racemisation catalysts under ammonolytic conditions would seem questionable, however, because reaction of the aldehyde group with ammonia[8,9] might be expected to interfere. The problem of undesired racemisation of D-phenylglycine amide **2** would seem more manageable; as the amide **2** is racemised much slower and at high concentrations, it precipitates and is thus effectively removed from the reaction mixture.

Indeed we found previously[10] that pyridoxal and salicylaldehyde readily racemise phenylglycine

methyl ester **1a** in ammonia-saturated *tert*-butyl alcohol, thus providing the basis for a dynamic kinetic resolution process, which we have now investigated in more detail.

2. Results and discussion

2.1. Ammonolysis of phenylglycine esters

Because *Candida antarctica* lipase B (Novozym 435) emerged from our initial study[7] as the catalyst of choice, it was used throughout the present work. Initially we investigated the influence of the chain length of the ester group on the course of the reaction. The data in Table 1 show that the butyl ester **1c** reacted at one third of the rate of the methyl ester **1a**. The effect on the enantioselectivity ratio E,[11] which varied between 16 and 19 was negligible.[†,‡] In view of these results all further experiments were performed with **1a**.

Table 1
Effect of the chain length of the ester in the ammonolysis of phenylglycine esters **1**[a]

Ester	Conversion (%)	$ee_{\text{L-}\mathbf{1}}$ (%)	$ee_{\text{D-}\mathbf{2}}$ (%)	$E_{\mathbf{1}}$	$E_{\mathbf{2}}$
Methyl **1a**	47	69	78	16	17
Ethyl **1b**	36	47	84	18	18
Butyl **1c**	16	17	89	19	20

[a] Reaction conditions: D,L-phenylglycine ester **1** (1 mmol) was shaken with Novozym 435 (50 mg) in ammonia-saturated *tert*-butyl alcohol (5 ml, 12.5 mmol NH_3) at 40 °C for 4 h.

The concentration of D,L-methyl ester **1a** was varied between 50 and 800 mM. The initial reaction rate of L-**1a** was linearly proportional to the concentration over the whole range measured, whereas D-**1a** followed Michaelis–Menten kinetics (Fig. 1). The apparent Michaelis constant K_m, which is approximately 830 mM for D-**1a**, was deduced by fitting the experimental data to the Michaelis–Menten rate equation.

Due to the deviation of D-**1a** from first order kinetics, an increased starting concentration of D,L-**1a** resulted in longer reaction times and a slight decrease in E. The concentration of ammonia had no influence on either E or the reaction rate between 1.7 and 2.5 M (data not shown).

† The enantiomeric ratios for the ester (E_e) and the amide (E_a) were calculated from the conversion (c) and the enantiomeric excesses of the ester (ee_e) and the amide (ee_a) as follows:
For the ester:

$$E_e = \frac{\ln[(1-c)(1-ee_e)]}{\ln[(1-c)(1+ee_e)]}$$

For the amide:

$$E_a = \frac{\ln[1-c(1+ee_a)]}{\ln[1-c(1-ee_a)]}.$$

‡ Experimental values for E were lower than those found previously[7] which can be attributed to improvements in the analytical procedure.

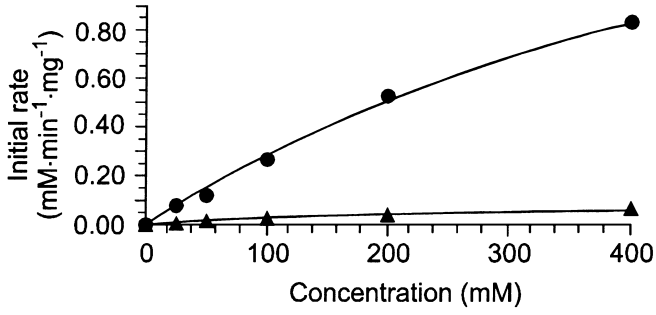


Figure 1. Initial reaction rate as function of the concentration of D-**1a** (●) and L-**1a** (▲)

The *E*-value of 16 that we consistently observed in the ammonolysis of **1a** in *tert*-butyl alcohol is rather low for an efficient kinetic resolution. We note, however, that in an ideal dynamic kinetic resolution this would result in product *ee* of 88%.[§] Nevertheless, there is an obvious incentive to improve the enantiomeric ratio.

Since it is known that the reaction medium can dramatically influence enantiopreferences of enzymes,[12–14] the effect of solvent on the ammonolysis of **1a** was studied. However, little variation in the *E*-value was observed (Table 2). The reaction rate, however, is strongly dependent on the reaction medium, but there was no apparent correlation with any solvent parameter such as hydrophobicity (log P), dielectric constant or dipole moment.

Table 2
Ammonolysis of **1a** in various solvents[a]

Solvent	t (h)	Conversion (%)	$ee_{D\text{-}\mathbf{2}}$ (%)	*E*	Initial rate ($mM \cdot min^{-1} \cdot mg^{-1}$)
tert-Butyl alcohol	4	46	78	16	16.7
tert-Amyl alcohol[b]	3	48	76	17	14.2
1,2-Dimethoxyethane	6	40	85	21	8.80
tert-Butyl methyl ether[b]	5	43	73	12	7.66
Dioxane	21	43	83	20	3.66
Acetonitrile	66	40	82	18	1.96
Hexane[b]	15	<5	-	-	-

[a] Reaction conditions: D,L-phenylglycine ester **1a** (1 mmol) was shaken with Novozym 435 (50 mg) in ammonia saturated solvent (5 ml) at 40 °C.

[b] Novo SP 611, *Candida Antarctica* lipase B on Accurel EP 100, (50 mg) was used instead of Novozym 435 to minimise hydrolysis of **1a** due to traces of water in the carrier material.

§ In an ideal dynamic kinetic resolution the racemisation of the reactant is fast compared with its transformation into product, whereas the product racemisation is negligible.

The reaction temperature is also known to affect *E*.[15,16] Since the reaction rate decreases with temperature, only solvents with a sufficiently high initial rate at 40°C were used in the low-temperature reactions (e.g. *tert*-butyl alcohol, *tert*-amyl alcohol and *tert*-butyl methyl ether). From the solvents tested, the upward trend in *E* was most pronounced in *tert*-butyl alcohol (Table 3), resulting in an *E*-value up to 52 at −20°C. Measurements at even lower temperatures were impractical owing to the low reaction rates.

Table 3
Ammonolysis of **1a** at low temperature[a]

Solvent	T (°C)	t (h)	Lipase (mg)	Conversion (%)	$ee_{D\text{-}\mathbf{2}}$ (%)	*E*
tert-Amyl alcohol	40	3	50	48	76	17
	4	9	50	31	88	25
	-10	23	100	22	91	28
tert-Butyl methyl ether	40	5	50	43	73	12
	4	9	250	27	90	26
	-10	9	600	22	90	24
tert-Butyl alcohol	40	4	50	46	78	16
	4	11	100	39	89	30
	-10[b]	29	500	42	90	39
	-20[b]	24	1000	33	94	52

[a] Reaction conditions: D,L-phenylglycine ester **1a** (1 mmol) was shaken with SP 611 in ammonia-saturated solvent (5 ml) the selected temperature.
[b] *tert*-Butyl methyl ether was added as cosolvent (30% *v*/*v*) to avoid freezing of *tert*-butyl alcohol saturated with NH_3

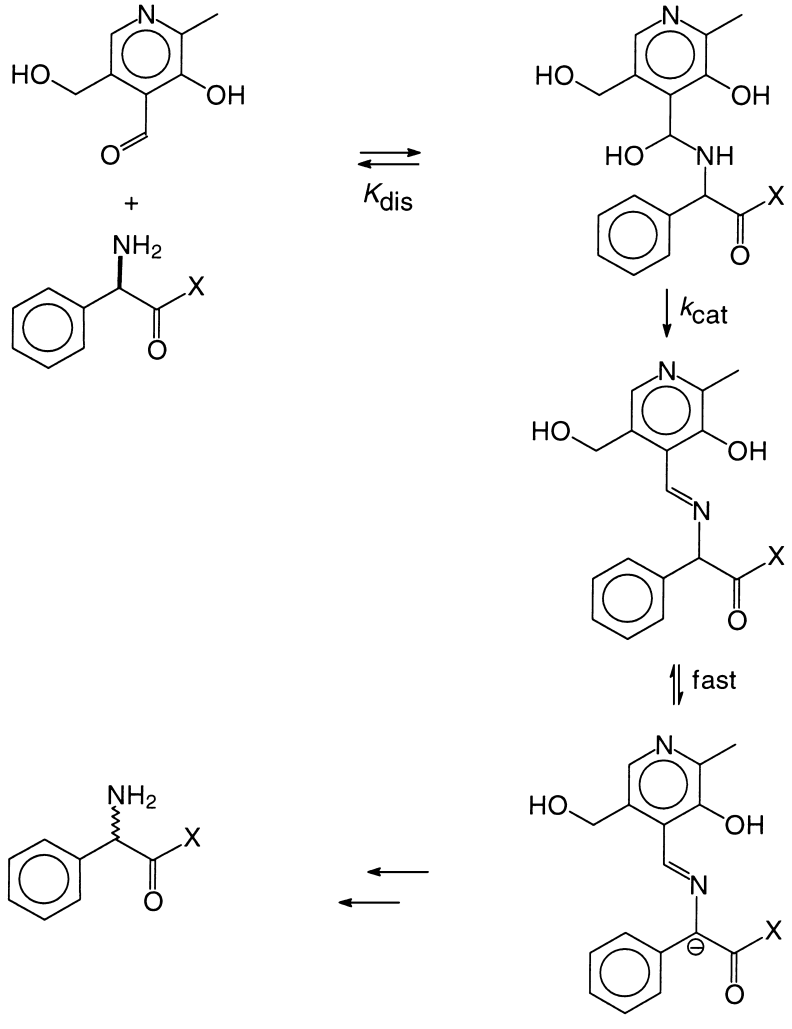
2.2. Racemisation via Schiff base intermediates

D-Methyl ester **1a** and D-amide **2** were subjected to pyridoxal or salicylaldehyde catalysed racemisation under ammonolysis conditions (*tert*-butyl alcohol, 2.5 M NH_3 at 40°C). In all cases the *ee* decreased to 0% according to first order kinetics. The initial racemisation rate obeyed Michaelis–Menten kinetics according to:

$$v = \frac{k_{rac} \cdot [\text{D-PGX}]}{K_{dis} + [\text{D-PGX}]}[\text{rac. cat.}] \quad (1)$$

It has generally been assumed that racemisation takes place via Schiff base formation.[8,9,17] We found, however, saturation kinetics, which imply a fast pre-equilibrium with dissociation constant K_{dis}. We propose that racemisation takes place via a hemiaminal which undergoes slow dehydration with catalytic rate constant k_{cat} followed by fast racemisation of the Schiff base (Scheme 3). The kinetic parameters were determined from Lineweaver–Burk plots (Table 4). It should be noted that the measured

racemisation rate constant is half the kinetic rate constant k_{cat}. (The hemiaminal or Schiff base can revert equally well to either D- or L-isomer.)



Scheme 3. Racemisation via Schiff base intermediates

The catalytic rate constants show that both catalysts racemise **1a** 30 times as fast as **2** under V_{max} conditions. At lower concentrations this rate advantage of **1a** will be less, as can be deduced from the k_{cat}/K_{dis} values. Moreover, due to its lower K_{dis} value, **2** would be expected to compete effectively for the racemisation catalyst, whose effect would be less pronounced for salicylaldehyde (Table 4). We tentatively concluded that both racemisation catalysts appear to be suitable for in situ racemisation.

2.3. Ammonolysis with in situ racemisation

We next combined ammonolysis and racemisation of **1a** in *tert*-butyl alcohol (2.5 M NH_3 at 40°C). From the time–course and *ee* of reactant and product (Fig. 2) we conclude that L-**1a** is efficiently racemised by pyridoxal. Its *ee* at 50% conversion is just over 40%, compared with 70% in the absence of racemisation catalyst. The racemisation catalyst also accelerated the reaction from a 50% conversion in 4 h to 60%, because it counteracts the depletion of fast-reacting D-**1a**. On the other hand, the downward

Table 4
Kinetic data of the racemisation of **1a** and **2**

Rac. cat.	X	K_{dis} (mM)	k_{cat} (10^{-3} s^{-1})	V_{max} ($\mu M \cdot s^{-1}$)	k_{cat}/K_{dis} ($M^{-1} \cdot s^{-1}$)
Pyridoxal	OCH_3	180	20.8	13.5	0.116
	NH_2	45	0.74	0.48	0.016
Salicylaldehyde	OCH_3	44	7.56	5.36	0.172
	NH_2	28	0.25	0.18	0.009

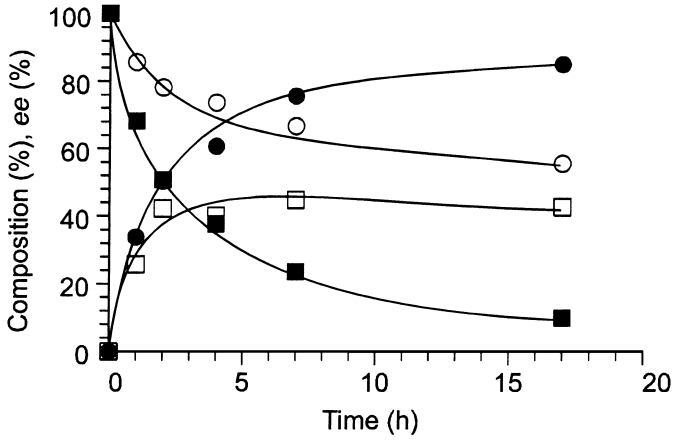


Figure 2. Composition and *ee*s of ester **1a** and amide **2** as function of time: (□) *ee* of ester **1a**, (■) amount of ester **1a**, (○) *ee* of amide **2**, (●) amount of amide **2**. Reaction conditions: D,L-phenylglycine methyl ester **1a** (200 mM) was shaken with Novozym 435 (50 mg) and pyridoxal (2 mM) in ammonia-saturated *tert*-butyl alcohol (5 ml, 12.5 mmol NH_3) at 40°C

trend of the *ee* of **2** in the course of the reaction shows that pyridoxal-catalysed racemisation of **2** is quite significant.

In order to establish the optimum conditions for a dynamic kinetic resolution, we varied the concentration of pyridoxal between 40 μM and 2 mM. Similar experiments were performed with salicylaldehyde (1 to 4 mM) as racemisation catalyst. From the results (Table 5) no clear optimum with regard to concentration and *ee* is apparent. Racemisation catalyst concentrations exceeding 1 mM mainly affect the conversion rate. Pyridoxal and salicylaldehyde at 1 and 2 mM perform similarly with regard to *ee*, but in the case of pyridoxal a somewhat higher conversion is reached.

In order to gain more insight into the effect of the racemisation catalysts, reactions were followed over time and the *ee* values were plotted against the conversion (Figs. 3 and 4). Up to 50% conversion, the results appeared to be independent of the concentration of the racemisation catalyst. Even when no racemisation catalyst was added, the same result was obtained. For conversions higher than 50% we observed that at low pyridoxal concentrations (40 to 200 μM, see Fig. 3) the racemisation of **1a** was too slow to be effective. When the pyridoxal concentration was increased to 400 μM the results improved slightly, but a further increase had no effect. For salicylaldehyde a very similar pattern was observed (Fig. 4). It would seem that, once the racemisation catalyst concentration exceeds a critical value, higher concentrations affect **1a** and **2** equally. Consequently, the reaction proceeds faster due

Table 5
Ammonolysis with in situ racemisation[a]

concentration	pyridoxal		salicylaldehyde	
rac.cat (mM)	conversion (%)	*e.e.* (%)	conversion (%)	*e.e.* (%)
4.0			91.2	53.6
2.0	90.0	55.6	86.3	55.9
1.0	86.8	60.7	78.5	60.9
0.4	81.5	57.1		
0.2	75.1	50.5		
0.04	62.1	54.7		
1.0[b]	83.1	60.6		

[a] Reaction conditions: D,L-phenylglycine methyl ester **1a** (1 mmol, 200 mM) was shaken with Novozym 435 (50 mg) and pyridoxal or salicylaldehyde in ammonia-saturated *tert*-butyl alcohol (5 ml, 12.5 mmol NH_3) at 40 °C for 17 hours.

[b] D,L-phenylglycine methyl ester **1a** (4 mmol, 800 mM) was shaken with Novozym 435 (200 mg) and pyridoxal (0.02 mmol, 4 mM) in ammonia-saturated *tert*-butyl alcohol (5 ml, 12.5 mmol NH_3) at 40 °C for 17 hours.

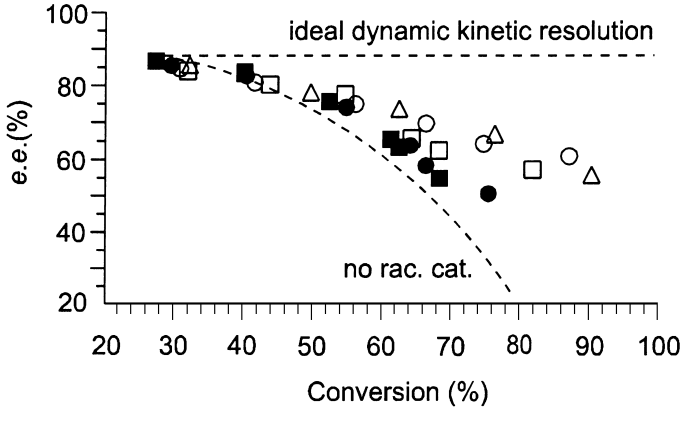


Figure 3. Enantiomeric excess of amide **2** as a function of the conversion at different pyridoxal concentrations (mM): (Δ) 2, (○) 1, (□) 0.4, (●) 0.2, (■) 0.04

to faster racemisation of L-**1a**, but the racemisation of D-**2** is also accelerated and hence, no optimum becomes apparent.

Because **2** would be expected to precipitate and hence to be effectively withdrawn from the reaction mixture, we would expect that a high concentration of **1a** would accelerate its racemisation relative to **2**. However, when we increased the concentration of **1a** and pyridoxal fourfold (to 800 and 4 mM, respectively) no effect on *ee* vs concentration became apparent (Table 5). This is probably due to the

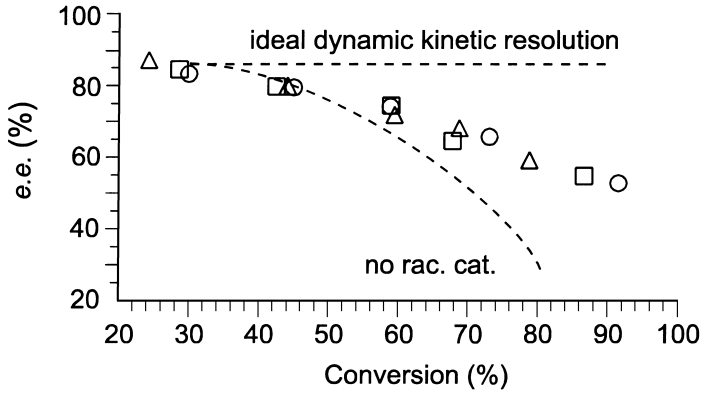


Figure 4. Enantiomeric excess of amide **2** as a function of the conversion at different salicylaldehyde concentrations (mM): (○) 4, (□) 2, (Δ) 1

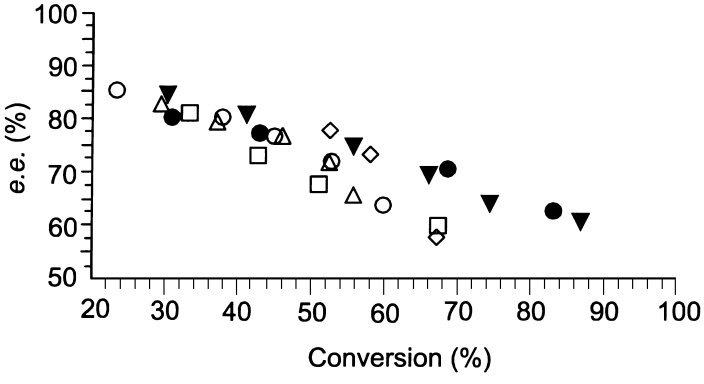


Figure 5. Enantiomeric excess of amide **2** as a function of the conversion in different organic solvents: (▼) *tert*-amyl alcohol, (●) *tert*-butyl alcohol, (◇) dioxane, (○) 1,2-dimethoxy ethane, (Δ) acetonitrile, (□) *tert*-butyl methyl ether

lower *E*-value at high concentrations of **1a** (see Fig. 1). Rapid exchange of precipitated D-**2** with the product in solution may also contribute to this unsatisfactory result.

To improve the results of the dynamic kinetic resolution, the racemisation rate of **1a** should be increased with respect to **2**. We assumed that the racemisation rate could be influenced by the reaction medium. Hence, we studied ammonolysis with in situ racemisation in various solvents at 40°C. Experiments were carried out with pyridoxal (1 mM) as racemisation catalyst. As can be seen from Fig. 5, the substrate is most efficiently racemised in *tert*-butyl alcohol and *tert*-amyl alcohol. In all other — non-protic — solvents, the racemisation of the substrate was so slow that L-**1a** accumulated, which caused low yields with low *ee*.

Encouraged by the good results at low temperature we next combined the ammonolysis of **1a** with in situ racemisation at −20°C. *tert*-Butyl alcohol:*tert*-butyl methyl ether (70:30% v/v) was used as solvent and pyridoxal (4 mM) as racemisation catalyst. The reaction was followed over time and after 66 h we obtained 85% yield with 88% *ee*. This result shows that at low temperature the substrate is indeed racemised much faster than the product and a dynamic kinetic resolution is feasible whereby the product is obtained in good yield and high *ee*.

3. Conclusion

Pyridoxal or salicylaldehyde catalysed racemisation of phenylglycine methyl ester **1a** is compatible with lipase-catalysed enantioselective ammonolysis. The catalyst became more enantioselective at low temperature. At −20°C D-phenylglycine amide **2** was obtained with 88% *ee* at 85% conversion.

4. Experimental

4.1. General

The reaction mixtures of phenylglycine methyl ester were analysed by chiral HPLC on a Daicel Chemical Industries Ltd, 4.6×150 mm 5 μ Crownpak CR (+) column using a Waters 625 LC pump and a Waters 486 UV detector. The eluent was aqueous $HClO_4$, pH 1.5 at a flow of 0.6 ml/min, the column temperature was 18°C. The reaction mixtures of the ethyl and butyl esters of phenylglycine were analysed by chiral HPLC on a 4.6×250 mm 10 μ Chiracel OD column with a Waters 510 pump, and a Shimadzu SPD-6a UV detector. Hexane:*iso*-propyl alcohol:dimethyl amine (90:10:0.1, v/v) at 0.5 ml/min was used as eluent. ^{1}H and ^{13}C NMR spectra were recorded using a 400 MHz Varian-VXR 400S spectrometer.

Immobilised *Candida antarctica* lipase B, Novozym 435, was a gift from Novo Nordisk A/S (Bagsværd, Denmark). Immobilised *Candida Antarctica* lipase B on Accurel EP 100, SP 611 (ex Novo Nordisk A/S) was a gift from Uniqema (Gouda, The Netherlands). Salicylaldehyde was purchased from Janssen Chimica, pyridoxal hydrochloride from Aldrich. Racemic phenylglycine was obtained from Acros. Enantiomerically pure phenylglycine, phenylglycine methyl ester and phenylglycine amide were kindly donated by DSM (Geleen, The Netherlands). Solvents were dried on Zeolite CaA (Uetikon, activated at 400°C for 24 h before use). The methyl, ethyl, and butyl esters of phenylglycine were synthesised from the amino acid according to the literature.[18] Phenylglycine methyl ester was used as free base, obtained by bulb to bulb distillation of a mixture of phenylglycine methyl ester hydrochloric acid salt and sodium methoxide (30% solution in methanol). The free base distilled at 100°C (1 mbar) as a colourless liquid, which solidified by standing at room temperature.

4.2. Ammonolysis of phenylglycine esters

A mixture of 1.0 mmol phenylglycine ester (165 mg methyl ester **1a**, 216 mg ethyl ester hydrochloric salt **1b**, or 232 mg butyl ester hydrochloric salt **1c**) and *Candida antarctica* lipase B in 5.0 ml ammonia saturated solvent was shaken in a 40 ml reaction vessel at the selected temperature (see Tables 1–3 for further details). Reactions were monitored over time. The partial pressure of ammonia was maintained at 1 atmosphere in all reactions, which guarantees a constant thermodynamic activity of ammonia. The reactions were stopped by adding concentrated formic acid and the reaction mixture was dissolved by adding water. When *tert*-amyl alcohol or *tert*-butyl methyl ether was used as the solvent methanol was also added; when hexane was used as solvent, ethanol was added to dissolve the reaction mixture. A sample was taken for HPLC analysis.

4.3. Racemisation of phenylglycine methyl ester and phenylglycine amide

All kinetic measurements were made in 40 ml reaction vessels at 40°C; ammonia saturated *tert*-butyl alcohol was used as solvent. When pyridoxal was used as racemisation catalyst the concentration of **1a** was varied between 50 and 400 mM, with salicylaldehyde between 30 and 300 mM. The concentration of phenylglycine amide **2** was varied between 40 and 160 mM in both cases. The concentration of pyridoxal as well as salicylaldehyde was kept constant at 1.3 mM. Samples were periodically withdrawn and analysed by HPLC. The kinetic constants were determined by fitting the experimental data in a Lineweaver–Burk plot.[19]

4.4. Ammonolysis with in situ racemisation

Compound **1a** (1.0 mmol), racemisation catalyst and *Candida antarctica* lipase B in 5.0 ml solvent saturated with ammonia were shaken in 40 ml reaction vessels at the selected temperature (see Table 5 for further details). The reactions were carried out under 1 atmosphere of ammonia by bubbling dry ammonia gas through the reaction mixtures. The concentration of the racemisation catalyst was varied between 0.04 and 4 mM for pyridoxal and between 1 and 4 mM for salicylaldehyde. The reactions were followed over time. The reactions were stopped by adding formic acid and the reaction mixture was dissolved by adding water. When *tert*-amyl alcohol or *tert*-butyl methyl ether was used as solvent, methanol was also added. A sample was taken for HPLC analysis.

Acknowledgements

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