Pergamon

Tetrahedron: *Asymmetry* 11 (2000) 1789–1796

TETRAHEDRON: *ASYMMETRY*

D-Phenylglycine and D-4-hydroxyphenylglycine methyl esters via penicillin G acylase catalysed resolution in organic solvents

Alessandra Basso, Paolo Braiuca, Luigi De Martin, Cynthia Ebert, Lucia Gardossi* and Paolo Linda

Dipartimento di Scienze Farmaceutiche, Università di Trieste, Piazzale Europa 1, 34127 Trieste, Italy

Received 18 February 2000; accepted 30 March 2000

Abstract

Penicillin G acylase in organic solvents catalyses specifically the acylation of the L-enantiomers of methyl esters of phenylglycine and 4-hydroxyphenylglycine. Hydrolytic reactions are prevented by controlling the water activity of the system and no excess of acylating agent is required. The process leads to the facile isolation of the enantiomerically pure D-enantiomer, which is of practical use for the preparation of β-lactam antibiotics. Electrospray mass spectroscopy has been applied to the study of the enantioselectivity of the enzyme.

1. Introduction

Optically active D-amino acids are widely used in the pharmaceutical industry as intermediates for the production of semisynthetic antibiotics, pesticides and new drugs.[1–5] Activated forms of D-4-hydroxyphenylglycine and D-phenylglycine are employed in the total enzymatic synthesis of β-lactams following a kinetically controlled approach.[4,6,7]

Penicillin G acylase (PGA) has been largely used in aqueous solutions to obtain optically active compounds by means of phenylacetyl derivative hydrolysis of interesting compounds.[8–10] Moreover, Cole demonstrated, already in 1969, that the enzyme can be employed also in the synthetic direction[11a,b] and Zmijewski was the first to use PGA for catalysing acylations in the resolution of racemates.[12] However, acylation in aqueous media requires excess of the acyl donor component or extracting/precipitating the product[2,12–15] to reverse the equilibrium towards synthesis. Recently Rosell et al. resolved racemic mixtures of phenylglycine using the synthetic route in water by means of a stabilised derivative of PGA.[2] The application of the synthetic route to the resolution of phenylglycine methyl ester (PhGlyOMe) leads to the major advantage that

* Corresponding author. Fax: +39-040-52572; e-mail: gardossi@univ.trieste.it

0957-4166/00/$ - see front matter
PII: S0957-4166(00)00129-4

the useful D-enantiomer can be recovered unreacted, since PGA acylates preferentially the L-isomer. On the contrary, the hydrolytic route in aqueous solution requires the preventive chemical derivatisation of the racemate which, however, gives the L-enantiomer upon enzymatic hydrolysis.[13,14]

In principle, partially replacing water with organic solvents should be beneficial for acylation reactions and also would translate into a higher solubility of phenylacetic derivatives.[16] Unfortunately, water/solvent mixtures are not optimal media for PGA since high percentages of co-solvents reduce the activity of the enzyme dramatically.[17]

Water is essential for preserving the activity of PGA, nevertheless we have demonstrated recently that native and immobilised PGA are active in different pure organic solvents[16,18,19] only when the hydration of the enzyme is controlled. This can be achieved by maintaining the thermodynamic parameter of water activity (a_w)[20] of the reaction system in the range 0.45–0.85.[16] The use of PGA in organic media at controlled a_w allows the acylation to be carried out while avoiding competing hydrolytic reactions and improving the solubility of hydrophobic substrates.[16]

Now we present the first study concerning the enantioselectivity of PGA in pure organic solvent. Two different preparations of PGA were employed for the resolution of PhGlyOMe and 4-hydroxyphenylglycine methyl ester (4-HO-PhGlyOMe) using the synthetic route in organic media, avoiding any excess of acylating agent.[19] This allows the facile production and isolation of the enantiomerically pure D-enantiomer. Electrospray mass spectroscopy (ES-MS) has been used to confirm the enantioselectivity of the method.

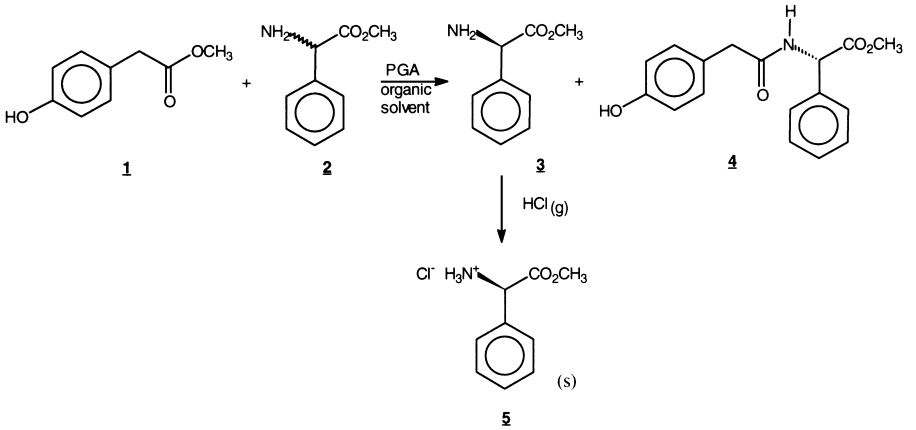
2. Results

Two preparations of PGA were demonstrated to be enantioselective towards the L-enantiomer of PhGlyOMe. A racemic mixture of D,L-PhGlyOMe was firstly resolved by acylation catalysed by a covalently immobilised form of penicillin G acylase (PGA-450 from Boehringer). PGA-450 (original water content = 63% w/w) was previously partially dehydrated by suspending the enzyme in petroleum ether and adding dry Celite® R-640 rods.[18] After this treatment the immobilised catalyst suspended in toluene gave a_w values between 0.73 and 0.77.

Two equivalents of D,L-PhGlyOMe **1** reacted with an equivalent of methyl 4-hydroxyphenylacetate **2** giving, within 24 h, exclusively the corresponding L-amide **4** which precipitated upon formation. At the end of the reaction the amide and the enzyme were removed by filtration and the unreacted D-PhGlyOMe **3** was easily recovered from the organic solution by converting it to the corresponding hydrochloride salt **5**, that precipitated in the organic solvent (Scheme 1). The enantioselectivity of the enzymatic process was determinated by polarimetric analysis of the unreacted D-PhGlyOMe as hydrochloride **5** (ee% > 98).

D,L-PhGlyOMe was also resolved using the native PGA adsorbed on Celite® R-640 rods,[21] working in dichloromethane at $a_w = 0.85$ and employing the same acylating agent **2**. The unreacted D-PhGlyOMe was recovered by precipitating the corresponding hydrochloride salt **5**. The use of the pure hydrophobic organic solvent instead of aqueous mixtures makes the leakage of the enzyme negligible so that the biocatalyst is suitable to be recycled.

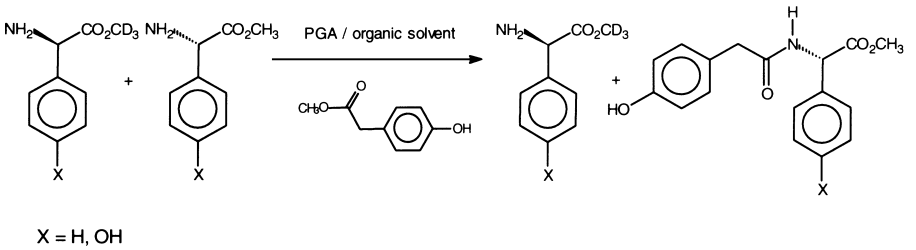
Amide **4** is more soluble in dichloromethane than in toluene and does not precipitate during the process. However, dichloromethane led to unsatisfactory results in reactions catalysed by PGA-450, since the catalyst floats on the surface of the solvent, thus limiting the mass transfer. On the other hand, toluene proved to be unsuitable for carrying out reactions catalysed by PGA/Celite®,



Scheme 1.

because the amide formed during the reaction precipitated and adsorbed on the Celite® rods, causing severe diffusion limitations, which translated into a dramatic decrease of the reaction rate and eventually into a loss of selectivity of the process (ee% = 75 by polarimetric analysis).

The enantioselectivity of the two enzymatic processes described here was confirmed by exploiting the high sensitivity of ES-MS. Namely, the d_3-methyl ester of the D-enantiomer was synthesised and the enzymatic acylation was carried out on an equimolar mixture of L-PhGlyOMe and D-PhGlyOCD$_3$ (Scheme 2).



Scheme 2.

As shown in Fig. 1a, there is a signal corresponding to the amide **4** with an m/z = 300 $(M+1)^+$ whereas there is no signal corresponding to the deuterated amide (m/z = 303). It must be noted that we have verified that ES-MS allows to detect the deuterated amide at concentrations lower than 1%, and therefore it represents a very sensitive technique for analysing samples having high enantiomeric excess.

ES-MS was also used to study the enantioselectivity of the PGA-450 catalysed resolution of 4-HO-PhGlyOMe performed in toluene. Also in this case, ES-MS analysis of the crude reaction mixture confirmed the enantioselectivity of PGA towards the L-enantiomer (Fig. 1b). Despite the fact that the poor solubility of substrates has often seemed as a restriction for enzymatic reactions in organic solvents, we have been able to carry out the synthesis in hydrophobic solvent employing a poorly soluble amine which was present in the reaction medium mainly as a suspension.

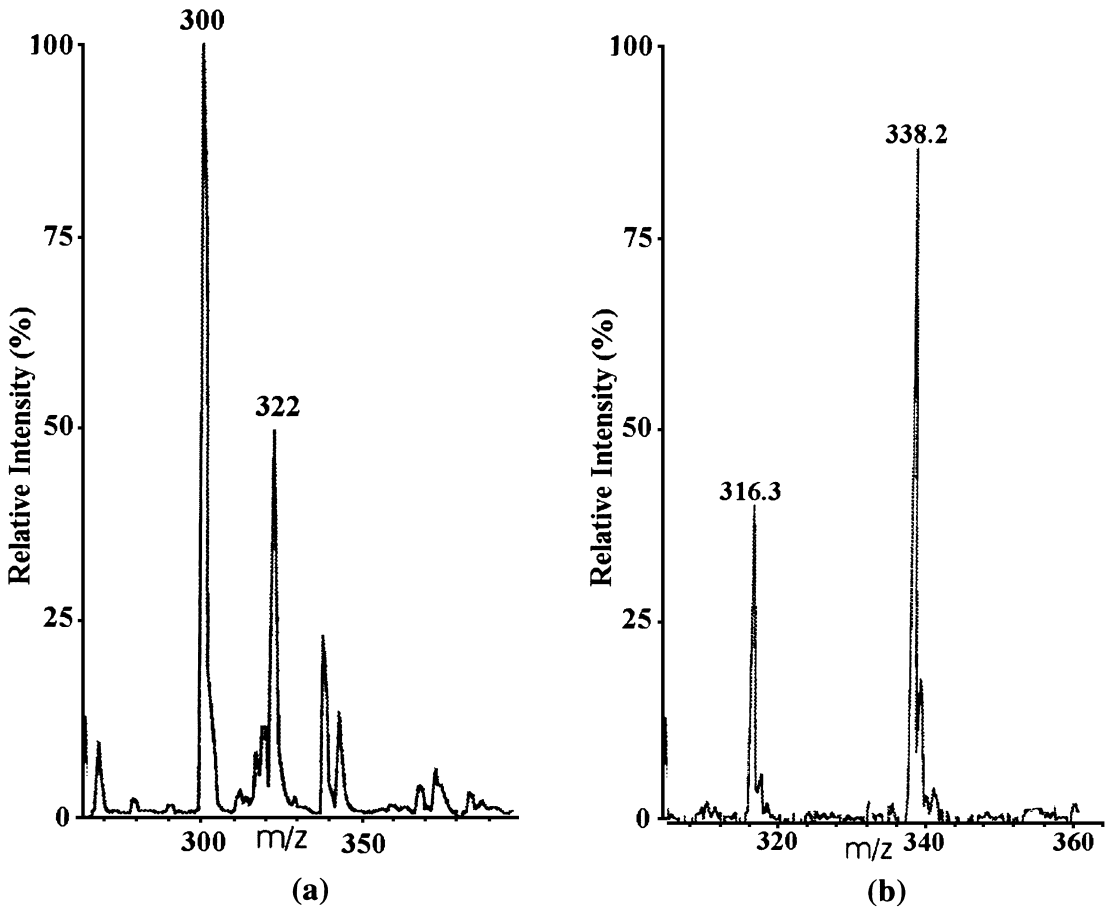


Figure 1. ES-MS of the amides enzymatically synthesised using ester **2** as acyl donor. (a) Product obtained starting from L-PhGlyOMe and D-PhGlyOCD$_3$: m/z = 300 $(M+1)^+$; m/z = 322 $(M+Na)^+$. (b) Product obtained from L-4-HO-PhGlyOMe and D-4-HO-PhGlyOCD$_3$: m/z = 316 $(M+1)^+$; m/z = 338 (M+Na)

A further proof of the selectivity of the enzyme came from the kinetic study of the acylation of each of the enantiomer of the amines taken separately. As shown in Table 1, L-PhGlyOMe was acylated with an initial rate 18 times higher than the D-enantiomer, whereas, in the case of the 4-HO-PhGlyOMe, no reaction at all was observed after 48 h using the D-enantiomer.

3. Conclusions

PGA in toluene and in dichloromethane catalyses the specific acylation of the L-enantiomers of the methyl esters of phenylglycine and 4-hydroxyphenylglycine, leading to the facile recovery of the D-enantiomer which is used in the preparation of β-lactam antibiotics. Electrospray mass spectroscopy has proved to be a valuable tool to confirm the enantioselectivity of the reactions.

This study demonstrates for the first time, to the best of our knowledge, the enantioselectivity of PGA in pure organic media and opens new perspectives for practical applications of this enzyme to asymmetric synthesis of valuable compounds.

Table 1
Initial rates (v_0) of acylation catalysed by PGA-450 in toluene

Substrate	v_0 ($\mu mol \cdot h^{-1} \cdot U^{-1}$)[a]
L-PhGlyOMe	4.58
D-PhGlyOMe	0.25
L-4-HO-PhGlyOMe	3.06
D-4-HO-PhGlyOMe	no reaction

[a] v_0 are normalised on the basis of the enzymatic activity assayed in aqueous buffer (see Experimental and ref. 19)

4. Experimental

4.1. Materials

PGA-450 was a generous gift of Boehringer-Mannheim. It consists of penicillin G acylase from *E. coli* covalently immobilised on a polymer the chemical nature of which is not disclosed by the producer. PGA-450 has a water content of 62.3% and it was partially dehydrated before use with the aid of Celite® R-640 according to the following procedure:[17] 2 g of enzyme were added to 100 mL of petroleum ether containing 4 g of dry Celite® R-640. After 15 days at 4°C the Celite® was removed and PGA was stored in the same solvent at 4°C. The final water content of the enzyme was 27% (determined by Karl Fischer titration–Mettler) and its activity was 401 U/g. Batches were prepared on a gram scale and there was no decrease in activity over at least four months. As required, enzymatic samples were withdrawn and the volatile organic solvent used for the storage was removed from the enzymatic sample at room temperature and atmospheric pressure without causing any detrimental effect to the catalyst.

Penicillin G acylase from *E. coli* (EC 3.5.1.11) was purchased from Fluka as an aqueous suspension that was stored as lyophilised powder (12.3 U/mg).

Celite® R-640 was from Fluka. Toluene and dichloromethane used in enzymatic reactions were dried over molecular sieves (4 Å).

Methyl 4-hydroxyphenylacetate, D- and L-phenylglycine methyl ester hydrochloride and cinnamyl alcohol were from Aldrich. D- and L-4-Hydroxyphenylglycine (4-HO-PhGlyOH) and D-phenylglycine were from Fluka. Deuterated methanol (CD_3OD) was from Trimital.

4.1.1. Preparation of D- and L-PhGlyOMe free bases

Hydrochloride salt (20 mmol) was suspended in 20 mL of dry dichloromethane together with 30 mmol of $Na_2CO_3 \cdot 10H_2O$. After 15 min of magnetic stirring the organic solution was filtered and dried over anhydrous sodium sulphate. Finally, the solvent was removed under vacuum to obtain the free amines (yields: 71 and 73%, respectively) that were characterised by ^1H NMR.

D- and L-PhGlyOMe: ^1H NMR (200 MHz, $CDCl_3$): 2.05 (s, 2H, NH_2); 3.51 (s, 3H, CH_3O); 4.58 (s, 1H, CHPh); 7.31 (m, 5H, **Ph**).

4.1.2. Synthesis of D- and L-4-HO-PhGlyOMe, D-4-HO-PhGlyOCD$_3$ and D-PhGlyOCD$_3$

10 mmol of D- or L-4-HO-PhGlyOH, 100 mmol of dry methanol and 0.5 mL of concentrated sulphuric acid were refluxed for 3 h until the suspension converted to a clear solution that was concentrated under vacuum to remove the methanol in excess. The residue was dissolved in 10 mL of water and the pH was adjusted to 8 using a NaOH solution (0.1N), obtaining the ester as a white precipitate that was filtered, rinsed with dichloromethane and characterised by ^{1}H and ^{13}C NMR.

D-4-HO-PhGlyOCD$_3$ and D-PhGlyOCD$_3$ were synthesised according to the same procedure but employing deuterated methanol. In the case of D-PhGlyOCD$_3$ the product was extracted from the basic solution (pH = 8) with dichloromethane, dehydrated with Na_2SO_4 and concentrated to obtain a yellow oil.

D- and L-4-HO-PhGlyOMe: ^{1}H NMR (200 MHz, DMSO): 2.17 (s, 2H, N**H**$_2$); 3.52 (s, 3H, C**H**$_3$O); 4.35 (s, 1H, C**H**Ph); 6.65 (d, 2H, **H**$_3$ **H**$_5$-Ph, J = 6.0 Hz,); 7.10 (d, 2H, **H**$_2$ **H**$_6$-Ph, J = 8.0 Hz). ^{13}C NMR (200 MHz, DMSO): 52.40; 58.36; 115.71; 128.56; 132.00; 157.35; 175.51.

D-4-HO-PhGlyOCD$_3$: m/z = 185 $(M+1)^+$.

D-PhGlyOCD$_3$: ^{1}H NMR (200 MHz, $CDCl_3$): 2.05 (s, 2H, N**H**$_2$); 4.58 (s, 1H, C**H**Ph); 7.31 (m, 5H, **Ph**). ^{13}C NMR (200 MHz, $CDCl_3$): 58.80; 126.86; 128.08; 128.83; 140.20; 174.39.

4.2. Polarimetry

A JASCO DIP-1000 polarimeter was used. The specific rotation of a chemical standard of commercial (Aldrich) D-PhGlyOMe·HCl was measured: $[\alpha]_D = -117$ ($c = 1$, H_2O) (lit. –118).[21] Specific rotation results are reported in Sections 4.5.1 and 4.5.2.

4.3. Determination of water activity

Water activity was measured using a hygrometer DARAI-Trieste, Italy. Measurements were carried out by sealing the sensor into the open end of 5 mL glass vials, thermostatted, until constant reading.[19] Samples were equilibrated for at least 24 h in an air-bath type thermostatted orbital shaker (DARAI-Trieste, Italy).

4.4. Assay of PGA activity in water

Enzymatic activity of the immobilised PGA was assayed in phosphate buffer by automated titration (TTT80 Radiometer, Denmark) of the phenylacetic acid formed during the hydrolysis of benzylpenicillin (Aldrich). One enzymatic unit corresponds to the amount of enzyme that hydrolyses 1 μmol of benzylpenicillin for 1 min at pH 8.0 at 37°C.[19]

*4.5. Enzymatic resolution of **1***

4.5.1. Reaction catalysed by PGA-450

Reaction was carried out taking samples of the enzyme stored in petroleum ether. After the fast evaporation of the solvent 1 g of PGA-450 was added to 10 mL of dry toluene and the system was equilibrated for 24 h ($a_w = 0.73$–0.77). Reaction was started by adding 2 mmol of D,L-PhGlyOMe **1** and 1 mmol of methyl 4-hydroxyphenylacetate **2** and stopped when all the ester **2** was consumed. The PGA-450 was removed by filtration and the organic solution was cooled down in

an ice-bath and a flow of gaseous HCl was bubbled into the solution leading to the precipitation of the D-PhGlyOMe hydrochloride salt **5**. The white solid was filtered and rinsed with dichloromethane (60% isolated yield).

D-PhGlyOMe·HCl: $[\alpha]_D = -115$ ($c = 1$, H_2O). Enantiomeric excess = 98.3%. Mp = 204°C. ^1H NMR (200 MHz, D_2O): 3.51 (s, 3H, C**H**$_3$O); 5.08 (s, 1H, C**H**Ph); 7.31 (m, 5H, **Ph**).

4.5.2. Reaction catalysed by PGA/Celite®

Native PGA (50 mg) dissolved in 350 μL of water were added to 1 g of Celite® R-640 rods in 10 mL of dry toluene contained in a 20 mL glass vial.[22] Due to the presence of the hydrophobic solvent, a uniform coating of the aqueous phase formed around the Celite® rods and the enzymatic solution was adsorbed on the Celite® within 24 h (30°C in an orbital shaker). The toluene was removed from the vessel and the Celite® rods rinsed with 2 mL of dry dichloromethane. Dry dichloromethane (10 mL) was then added together with the reactants (2 mmol of D,L-PhGlyOMe **1** and 1 mmol of methyl 4-hydroxyphenylacetate **2**). During the course of the reaction a_w was 0.85. The reaction was stopped when all the ester was consumed. The PGA/Celite® was removed by filtration and the D-PhGlyOMe hydrochloride salt **5** was precipitated and purified as previously described (isolated yield = 65%).

D-PhGlyOMe·HCl: $[\alpha]_D = -88$ ($c = 1$, H_2O). Enantiomeric excess = 75%. Mp = 204°C. ^1H NMR (200 MHz, D_2O): 3.51 (s, 3H, C**H**$_3$O); 5.08 (s, 1H, C**H**Ph); 7.31 (m, 5H, **Ph**).

4.6. Electrospray mass spectroscopy

An API I (Perkin–Elmer SCIEX) electrospray mass spectrometer was used in a positive mode analysis, 5500 V. Positive ion detection was selected since low molecular weight polar compounds form single charged ions by gaining a proton, thus leading to a peak at m/z = M+1.

Enzymatic reactions analysed by mass spectrometry were carried out in 1 mL of toluene using either 62.5 mg of PGA-450 or 5 mg of native PGA adsorbed on 100 mg of Celite® R-640 rods and employing 100 μmol of ester **2** and 100 μmol each of the deuterated and not deuterated forms.

It was verified that there is no appreciable variation between the rates of acylation of D-PhGlyOMe and D-PhGlyOCD_3.

4.7. Determination of the initial rates of the acylation of the single enantiomers

80 μmol of each amine in the pure enantiomeric form and 100 μmol of the ester **2** were added to 1 mL of dry toluene together with 62.5 mg of PGA-450 and incubated in a thermostatted orbital shaker at 30°C. After complete conversion amides were isolated simply by evaporating the solvents, since no side product is formed during the reactions. The reaction course was followed withdrawing samples from the supernatant phase and analysing them by RP HPLC (Pharmacia) using a C-18 Chrompack column. Isocratic elution: acetonitrile:water = 70:30 and 0.025% of TFA, flow = 1 mL/min. Detector: UV–vis spectrophotometer at a $\lambda_{max} = 260$ nm.

Conversions were evaluated on the basis of the ratio of the areas of the ester **2** and an internal standard (cinnamyl alcohol). First 15% conversion data were utilised to determine initial rates as usual in enzymatic reactions.[23]

Amides were characterised by ^1H and ^{13}C NMR. Compound **4**: ^1H NMR (200 MHz, $CDCl_3$): 3.52 (s, 2H, PhC**H**$_2$CO); 3.67 (s, 3H, C**H**$_3$O); 5.54 (d, 1H, C**H**PhGly, $J_1 = 7.0$ Hz); 6.48 (d, 1H, N**H**, $J_1 = 6.2$ Hz); 6.73 (d, 2H, **H**$_3$ **H**$_5$ C_6H_4OH); 7.07 (d, 2H, **H**$_2$ **H**$_6$ C_6H_4OH); 7.20–7.35 (m, 5H,

Ph). ^{13}C NMR (200 MHz, $CDCl_3$): 42.64; 53.01; 56.68; 116.00; 125.86; 127.17; 128.64; 129.03; 130.63; 136.62; 155.35; 170.54; 171.17.

In the case of 4-HO-PhGlyOMe only the L-enantiomer was acylated giving the corresponding amide that was characterised by ^{1}H NMR and ^{13}C NMR: ^{1}H NMR (200 MHz, DMSO): 3.34 (s, 2H, PhC**H**$_2$CO); 3.54 (s, 3H C**H**$_3$O); 5.16 (d, 1H, C**H**Ph, J = 6.0 Hz); 6.62 (d, 2H, **H**$_2$ **H**$_6$ HOPhGly, J = 8.0 Hz); 6.72 (d, 2H, **H**$_2$ **H**$_6$ C_6H_4OH, J = 8.0 Hz); 7.00 (d, 2H, **H**$_3$ **H**$_5$ HOPhGly, J = 8.0 Hz); 7.13 (d, 2H, **H**$_3$ **H**$_5$ C_6H_4OH, J = 8.0 Hz); 8.68 (d, 1H, N**H**, J = 6.0 Hz). ^{13}C NMR (200 MHz, DMSO): 41.38; 52.74; 56.64; 115.62; 116.09; 126.77; 126.90; 129.68; 130.59; 156.56; 158.23; 171.30; 172.16.

Acknowledgements

We are grateful to Dr. Fabio Hollan for ES-MS analysis and to Boehringer-Mannheim for the generous gift of PGA-450. Thanks are due to C.N.R. and M.U.R.S.T. (Roma) for financial support to P.L.

References

1. Kim, M. G.; Lee, S. B. *J. Mol. Catal. B: Enzymatic* **1996**, *1*, 71–80.
2. Rosell, C. M.; Fernández-Lafuente, R.; Guisán, J. M. *J. Mol. Catal.* **1993**, *84*, 365–371.
3. Rosell, C. M.; Fernández-Lafuente, R.; Guisán, J. M. *Ann. N. Y. Acad. Sci.* **1995**, *750*, 425–428.
4. van Langen, L. M.; de Vroom, E.; van Rantwijk, F.; Sheldon, R. *FEBS Lett*. **1999**, *456*, 89–92.
5. Bossi, A.; Cretich, M.; Righetti, P. G. *Biotechnol. Bioeng*. **1998**, *60*, 454–461.
6. Boccù, E.; Ebert, C.; Gardossi, L.; Gianferrara, T.; Zacchigna, M.; Linda, P. *Il Farmaco* **1991**, *46*, 565–577.
7. Jústiz, O. H.; Terreni, M.; Pagani, G.; García, J. L.; Guisán, J. M.; Fernández-Lafuente, R. *Enzyme Microb. Technol.* **1999**, *25*, 336–343.
8. Cardillo, G.; Tolomelli, A.; Tomasini, C. *J. Org. Chem.* **1996**, *61*, 8651–8654.
9. Baldaro, E.; D'Arrigo, P.; Pedrocchi-Fantoni, G.; Rosell, C. M.; Servi, S.; Tagliani, A.; Terreni, M. *Tetrahedron: Asymmetry* **1993**, *4*, 1031–1034.
10. Fuganti, C.; Grasselli, P.; Servi, S.; Lazzarini, A.; Casati, P. *Tetrahedron* **1988**, *9*, 2575–2582.
11. (a) Cole, M. *Biochem. J*. **1969**, *115*, 747–756. (b) Cole, M. *Biochem. J*. **1969**, *115*, 757–764.
12. Zmijewski, M.; Briggs, B. S.; Thompson, A. R.; Wright, I. G. *Tetrahedron Lett*. **1991**, *32*, 1621–1622.
13. Kim, M. G.; Lee, S. B. *J. Mol. Catal. B: Enzymatic* **1996**, *1*, 181–190.
14. Didžiapetris, R.; Drabnig, B.; Schellenberger, V.; Jakubke, H.-D.; Švedas, V. *FEBS* **1991**, *287*, 31–33.
15. Pessina, A.; Lüthi, P.; Luisi, P. L. *Helv. Chim. Acta* **1988**, *71*, 631–641.
16. Basso, A.; De Martin, L.; Ebert, C.; Gardossi, L; Linda, P. *J. Mol. Catal. B: Enzymatic*, in press.
17. Pohl, T.; Waldmann, H. *Tetrahedron Lett*. **1995**, *36*, 2963–2966.
18. Basso, A.; De Martin, L.; Ebert, C.; Gardossi, L.; Linda, P.; Zlatev, V. *J. Mol. Catal. B: Enzymatic*, in press.
19. De Martin, L.; Ebert, C.; Garau, G.; Gardossi, L.; Linda, P. *J. Mol. Catal. B: Enzymatic* **1999**, *6*, 437–445.
20. Halling, P. J. *Enzyme Microb. Technol.* **1994**, *16*, 178–206.
21. Clark, J. C.; Phillipps, G. H.; Steer, M. R.; Cooksey, A. R.; Stephenson, L. *J. Chem. Soc., Perkin Trans. 1* **1976**, 471–474.
22. Basso, A.; De Martin, L.; Ebert, C.; Gardossi, L.; Linda, P. *Chem. Commun*. **2000**, 467–468.
23. Ebert, C.; Gardossi, L.; Linda, P. *Tetrahedron Lett*. **1996**, *37*, 9377–9380.