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Selectivity of penicillin G acylase towards phenylacetic acid derivatives in amide bond synthesis in toluene

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Abstract

Penicillin G acylase (PGA) accepts in toluene several analogues of phenylacetic acid as acyl donors. The 4-hydroxyphenylacetic group is preferentially accepted, and steric factors, such as the substitution at the aromatic ring or at the methylene group, cause a remarkable reduction in the initial rates. Results indicate that the selectivity follows a similar trend both in toluene and in aqueous media and that the synthetic potential of PGA can be fully exploited also in non-aqueous media with the consequent advantages, such as the absence of competitive hydrolytic reactions, higher solubility of phenylacetic derivatives and the possibility of working with equimolar amounts of reactants.

Keywords: Penicillin G acylase; Phenylacetic analogues; Acylation; Organic solvent; Water activity

1. Introduction

Penicillin G acylase (PGA, E.C. 3.5.1.11) is a hydrolytic enzyme industrially employed in the preparation of 6-aminopenicillanic acid (6-APA) via regioselective hydrolysis of penicillin G [1]. The selectivity of PGA towards other substrates than penicillin G has attracted the interest of researchers since 1969, when Cole described the selectivity properties of the cell-bound penicillin acylase of *Escherichia coli*, showing through semiquantitative studies that the enzyme accepts preferentially the phenylacetic group and its 4-hydroxy derivative in both hydrolytic [2,3] and synthetic [4,5] reactions in water. Later kinetic studies demonstrated that this enzyme is able to hydrolyse a large variety of amides and esters containing several aromatic acyl moieties [6–11].

Similar results were obtained in studies concerning the synthesis in aqueous media of β-lactam antibiotics [12–14], the protection [15–18] and the resolution [19,20] of amino acids and amines. The selectivity of PGA towards various acyl moieties has been studied in the acylation of a *cis*-racemic β-lactam intermediate in the synthesis of Loracarbef in aqueous media [21].

However, the efficiency of PGA in the synthetic route in aqueous media is often associated with many drawbacks, such as the low solubility of hydrophobic substrates and the hydrolysis of the products formed. One way to overcome the above-mentioned problems is to replace the aqueous medium with an organic solvent to improve the solubility of phenylacetic derivatives and to shift the thermodynamic equilibrium of the reaction towards synthesis [22–24].

We have demonstrated that PGA is active in pure organic solvent only when the hydration of the enzyme is controlled [25–31]. More recently, PGA was successfully employed in toluene and dichloromethane for the enantiospecific acylation of L-phenylglycine and

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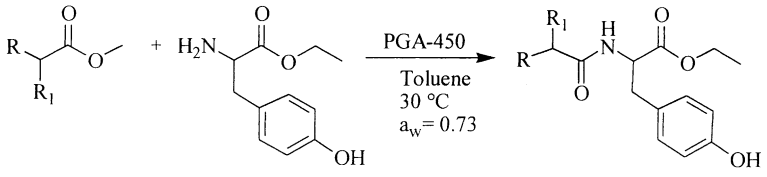


2a-i **1** **3a-i**

R= Phenyl-, phenoxy-, 4-hydroxyphenyl-, 3-hydroxyphenyl-, 2-hydroxyphenyl-, 4-nitrophenyl-; R_1= H-

R= Phenyl-; R_1= CH_3O-, H_2N-

Scheme 1.

L-4-hydroxyphenylglycine methyl esters using methyl 4-hydroxyphenylacetate as acylating agent [30].

It has been reported that the selectivity of an enzyme can change by moving from water to organic solvent [32–34]. In this paper, we report the first quantitative study on the selectivity of PGA towards various phenylacetic derivatives (**2a**–**i**) in the acylation of L-Tyrosine ethyl ester (L-TyrOEt: **1**) in toluene (Scheme 1) in order to provide guidelines for the choice of proper acyl donors in synthetic reactions in organic media, such as the protection of amino groups or enantioselective acylations.

2. Experimental

2.1. Materials

PGA-450 was a generous gift of Roche. 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. L-TyrOEt (**1**) was purchased from Sigma. Methyl 4-hydroxyphenylacetate (**2a**), 2-hydroxyphenylacetic acid (**2b**), 3-hydroxyphenylacetic acid (**2c**), 4-nitrophenylacetic acid (**2e**), methyl phenoxyacetate (**2f**), D- and L-phenylglycine methyl ester hydrochloride (**2h**) and phenylacetic acid (**2i**) were obtained from Aldrich. Methyl phenylacetate (**2d**) and *S*-(+)-α-methoxyphenylacetic acid (**2g**) were purchased from Fluka. All the substrates were used without further purification. Toluene used in enzymatic reactions was dried over molecular sieves (4 Å). The free amines of D- and L-phenylglycine methyl esters (**2h**) were obtained according to a procedure previously described [30] and characterised by ^{1}H NMR ($CDCl_3$).

2.1.1. Free amines of D- and L-phenylglycine methyl esters (2h)

^{1}H NMR ($CDCl_3$) (ppm): δ 2.05 (s, 2H, N**H**$_2$), 3.51 (s, 3H, C**H**$_3$O), 4.58 (s, 1H, C**H**Ph), 7.31 (m, 5H, **H**Ph).

2.2. 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. All samples were previously equilibrated for at least 24 h in an air-bath type thermostatted orbital shaker (DARAI, Trieste, Italy).

2.3. Assay of PGA activity in water

Enzymatic activity of PGA-450 was assayed in sodium phosphate buffer, 0.05 M, by automated titration (TTT80 Radiometer, Denmark) of the phenylacetic acid formed during the hydrolysis of benzylpenicillin potassium salt (Fluka). One enzymatic unit corresponds to the amount of enzyme that hydrolyses 1 μmol of benzylpenicillin in 1 min at pH 8.0 at 37°C [29].

2.4. General procedure for dehydration of PGA-450

PGA-450 has a water content of 62.3% and it was partially dehydrated before use by using Celite R-640® (Fluka) according to the following procedure:

2 g of the wet enzyme were added to 100 ml of petroleum ether containing 4 g of dry Celite R-640®. After 15 days at 4°C, the large rods of Celite R-640® were removed by easy separation with a strainer and PGA-450 was stored in the same solvent at 4°C. The final water content of the enzyme was 27% (determined using a Karl Fischer titrator Mettler). The hydrolytic activity of the dry preparation, measured in water, was 401 U/g and comparable to that of the commercial preparation (490 U/g) [31]. There was no decrement of activity for at least 4 months. As required, enzymatic samples were withdrawn and the 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.

2.5. Chemical synthesis of esters **2*b***, **2*c***, **2*e***, *and* **2*g***

Methyl 3- and 2-hydroxyphenylacetic acids (**2b** and **2c**) were prepared by esterification of the corresponding acids with methanol in the presence of H_2SO_4 as catalyst [35]. Methyl 4-nitrophenylacetate (**2e**) and methyl *S*-(+)-α-methoxyphenylacetate (**2g**) were synthesised using 2,2-dimethoxypropane and the corresponding free carboxylic acids in the presence of concentrated HCl [36]. All the esters chemically synthesised were purified by silica gel chromatography and characterised by ^{1}H NMR and ^{13}C NMR.

2.5.1. Methyl 3-hydroxyphenylacetic acid (**2*b***)

^{1}H NMR ($CDCl_3$) (ppm): δ 3.67 (s, 2H, C**H**$_2$Ph), 3.73 (s, 3H, C**H**$_3$O), 6.6–6.8 (m, 2H, **H**Ph), 7.0–7.15 (m, 2H, **H**Ph). ^{13}C NMR ($CDCl_3$) (ppm): δ 37.76, 52.85, 117.6, 120.59, 120.95, 129.25, 131.04, 155.11, 174.31.

2.5.2. Methyl 2-hydroxyphenylacetic acid (**2*c***)

^{1}H NMR ($CDCl_3$) (ppm): δ 3.56 (s, 2H, C**H**$_2$Ph), 3.69 (s, 3H, C**H**$_3$O), 6.7–7.3 (m, 4H, **H**Ph). ^{13}C NMR ($CDCl_3$) (ppm): δ 41.18, 52.39, 114.3, 116.2, 121.52, 129.84, 135.33, 155.97, 172.55.

2.5.3. Methyl 4-nitrophenylacetate (**2*e***)

^{1}H NMR ($CDCl_3$) (ppm): δ 3.73 (s, 2H, C**H**$_2$Ph), 3.75 (s, 3H, C**H**$_3$O), 7.46 (d, J = 8.0 Hz, 2H, **H**Ph), 8.20 (d, J = 8.0 Hz, 2H, **H**Ph). ^{13}C NMR ($CDCl_3$) (ppm): δ 41.86, 53.75, 123.19, 130.25, 141.90, 147.13, 171.78.

2.5.4. Methyl S-(+)-α-methoxyphenylacetate (**2*g***)

^{1}H NMR ($CDCl_3$) (ppm): δ 3.40 (s, 3H, C**H**$_3$O), 3.71 (s, 3H, C**H**$_3$OCO), 4.77 (s, 1H, C**H**OCH$_3$), 7.33–7.46 (m, 5H, **H**Ph). ^{13}C NMR ($CDCl_3$) (ppm): δ 53.67, 56.65, 82.19, 126.88, 128.34, 128.52, 134.48, 172.34.

2.6. Chemical synthesis of the amides **3*e***, **3*f***, *and* **3*g*** *used for calibration curves*

An amount of 50 mmol of the corresponding acid was added to 15 ml of dry CH_2Cl_2 together with 75 mmol of dicyclohexylcarbodiimide and the mixture was cooled in an ice bath and maintained under vigorous stirring. After 30 min, the mixture was taken to room temperature and filtered to remove the 1,3-dicyclohexylurea. An amount of 50 mmol of L-TyrOEt (**1**) was added to the organic solution and the reaction was kept under stirring for 3–6 h. The solvent was removed under vacuum and the product purified by means of silica gel chromatography using hexane/ethyl acetate as eluents. Products were characterised by means of ^{1}H NMR and ^{13}C NMR.

2.6.1. Amide **3*e***

^{1}H NMR ($CDCl_3$) (ppm): δ 1.28 (t, J = 7.1 Hz, 3H, CH$_2$C**H**$_3$), 2.96 (dd, J_1 = 6.2 Hz, J_2 = 12.0 Hz, 1H, C**H**HTyr), 3.05 (dd, J_1 = 5.7 Hz, J_2 = 12.0 Hz, 1H, CH**H**Tyr), 3.63 (s, 2H, C**H**$_2$Ph), 4.20 (q, J = 7.0 Hz, 2H, C**H**$_2$CH$_3$), 4.84 (m, 1H, C**H**Tyr), 5.72 (s, 1H, O**H**), 5.94 (d, J = 10.7 Hz, 1H, N**H**), 6.60 (d, 2H, **H**PhTyr), 6.80 (d, J = 8 Hz, 2H, **H**PhTyr), 7.34 (d, J = 8 Hz, 2H, **H**Ph), 8.14 (d, J = 9 Hz, 2H, **H**Ph). ^{13}C NMR ($CDCl_3$) (ppm): δ 14.27, 36.98, 43.06, 53.44, 62.04, 115.55, 123.91, 126.70, 130.25, 130.5, 141.90, 147.13, 155.57, 169.46, 171.78.

2.6.2. Amide **3*f***

^{1}H NMR ($CDCl_3$) (ppm): δ 1.25 (t, J = 6.1 Hz, 3H, CH$_2$C**H**$_3$), 3.05 (d, J = 6.8 Hz, 2H, C**H**$_2$Tyr), 4.18 (q, J = 6.0 Hz, 2H, C**H**$_2$CH$_3$), 4.49 (s, 2H, C**H**$_2$OPh), 4.90 (m, 1H, C**H**Tyr), 5.07 (m, 1H, N**H**), 6.72 (d, J = 10.5 Hz, 2H, **H**PhTyr), 6.85–6.94 (m, 4H, **H**Ph + **H**PhTyr), 7.32 (t, J = 9 Hz, 2H, **H**Ph),

7.04 (t, J = 9 Hz, 1H, **H**Ph). ^{13}C NMR ($CDCl_3$) (ppm): δ 14.27, 37.46, 52.92, 61.81, 67.26, 114.82, 115.58, 122.24, 127.17, 129.80, 130.42, 155.18, 157.14, 168.28, 171.18.

2.6.3. Amide ***3g***

^{1}H NMR ($CDCl_3$) (ppm): δ 1.28 (t, 3H, $CH_2C\mathbf{H}_3$), 2.94 (dd, J_1 = 6.2 Hz, J_2 = 12.0 Hz, 1H, C**H**HTyr), 3.03 (dd, J_1 = 5.7 Hz, J_2 = 12.0 Hz, 1H, C**H**HTyr), 3.33 (s, 3H, $C\mathbf{H}_3O$), 4.22 (q, J = 6.0 Hz, 2H, $C\mathbf{H}_2CH_3$), 4.59 (s, 1H, C**H**OCH_3), 4.83 (m, 1H, C**H**Tyr), 6.56 (d, J = 9 Hz, 2H, **H**PhTyr), 6.77 (d, J = 9 Hz, 2H, **H**PhTyr), 7.20–7.35 (m, 5H, **H**Ph). ^{13}C NMR ($CDCl_3$) (ppm): δ 14.29, 37.22, 52.73, 61.75, 83.79, 115.44, 126.98, 127.12, 128.45, 128.52, 130.41, 136.66, 155.13, 170.48, 171.61.

2.7. Reactions catalysed by PGA-450 in toluene

Reactions were carried out taking the required amount of the enzyme dehydrated and stored in petroleum ether. After the evaporation of the solvent, PGA-450 was added to 1 ml of dry toluene and the system equilibrated for 24 h and the measured a_w of the system was between 0.73 and 0.77.

A typical enzymatic reaction, for the evaluation of initial rates, was performed adding 62.5 mg (25 U) of PGA-450 to the dry toluene containing 80 μmol of L-TyrOEt (**1**) and 100 μmol of the acyl donor (**2a–i**) (reaction volume = 1 ml). Reactions were incubated at 30°C. The reaction course was followed withdrawing samples from the supernatant phase and analysing them by RP HPLC (Pharmacia) using a C-18 Chrompack column, with a flow rate of 1 ml/min, isocratic elution: acetonitrile:water = 70:30 and 0.025% of trifluoroacetic acid. Detector: UV–VIS spectrophotometer at a λ_{max} = 260 nm. Initial rates (v_0) were evaluated following the first 10% of conversion.

In order to calculate conversions at 24 h and to characterise the enzymatically synthesised products, reactions were carried out on a larger scale (2 ml of toluene) using equimolar amounts of the reactants (200 μmol) and 200 mg of PGA-450 (80 U). After the acylation went to completion, the organic phase was removed and the enzyme was rinsed twice with 2 ml of methanol. Products were isolated simply by evaporating the solvents, since no side-products formed during the reactions. No reaction was detected in the absence of the enzyme. Products were characterised by means of ^{1}H NMR and ^{13}C NMR.

2.7.1. Amide ***3a***

^{1}H NMR (CD_3OD) (ppm): δ 1.19 (t, J = 8.0 Hz, 3H, $CH_2C\mathbf{H}_3$), 2.83 (dd, J_1 = 6.2 Hz, J_2 = 12.0 Hz, 1H, C**H**HTyr), 2.97 (dd, J_1 = 5.7 Hz, J_2 = 12.0 Hz, 1H, C**H**HTyr), 3.30 (s, 2H, $C\mathbf{H}_2$Ph), 4.15 (q, J = 8.0 Hz, 2H, $C\mathbf{H}_2CH_3$), 4.56 (m, 1H, C**H**Tyr), 6.6–6.8 (m, 4H, **H**PhTyr), 6.8–7.0 (m, 4H, **H**Ph). ^{13}C NMR (CD_3OD) (ppm): δ 12.62, 35.64, 40.83, 53.63, 60.54, 114.39, 114.48, 125.24, 126.58, 129.21, 129.33, 155.36, 155.42, 171.17, 172.45.

2.7.2. Amide ***3b***

^{1}H NMR ($CDCl_3$) (ppm): δ 1.22 (t, J = 7.0 Hz, 3H, $CH_2C\mathbf{H}_3$), 2.95 (m, 2H, $C\mathbf{H}_2CH$), 3.51 (s, 2H, $C\mathbf{H}_2$Ph), 4.13 (q, 2H, J = 7.0 Hz, $C\mathbf{H}_2CH_3$), 4.73 (m, 1H, C**H**Tyr), 6.62 (d, J = 10 Hz, 2H, **H**PhTyr), 6.73 (d, J = 10.1 Hz, 2H, **H**PheTyr), 6.8–6.95 (m, 2H, **H**Ph-2-OH), 7.0 (d, J = 9 Hz, 1H, **H**Ph-2-OH), 7.14 (t, J = 9 Hz, 1H, **H**Ph-2-OH). ^{13}C NMR ($CDCl_3$) (ppm): δ 14.24, 36.84, 40.02, 53.72, 62.02, 115.59, 117.25, 120.61, 121.25, 126.84, 129.17, 130.42, 130.87, 155.16, 155.31, 171.58, 172.77.

2.7.3. Amide ***3c***

^{1}H NMR ($CDCl_3$) (ppm): δ 1.24 (t, J = 7.7 Hz, 3H, $CH_2C\mathbf{H}_3$), 2.84 (dd, J_1 = 7.4 Hz, J_2 = 14.0 Hz, 1H, CH**H**Tyr), 3.00 (dd, J_1 = 6.5 Hz, J_2 = 14.0 Hz, 1H, C**H**HTyr), 3.44 (s, 2H, $C\mathbf{H}_2$Ph), 4.16 (q, J = 7.5 Hz, 2H, $C\mathbf{H}_2CH_3$), 4.77 (m, 1H, C**H**Tyr), 5.96 (d, J = 9 Hz, 1H, N**H**), 6.50–6.80 (m, 8H, **H**Ph). ^{13}C NMR ($CDCl_3$) (ppm): δ 14.31, 39.55, 41.19, 52.35, 61.50, 114.45, 115.80, 116.44, 121.21, 127.65, 129.78, 130.43, 135.30, 155.31, 156.32, 174.65, 177.00.

2.7.4. Amide ***3d*** *(=**3i**)*

^{1}H NMR ($CDCl_3$) (ppm): δ 1.23 (t, J = 7.1 Hz, 3H, $CH_2C\mathbf{H}_3$), 2.90 (dd, J_1 = 5.9 Hz, J_2 = 13.9 Hz, C**H**HTyr), 3.00 (dd, J_1 = 5.7 Hz, J_2 = 14.0 Hz, CH**H**Tyr), 3.55 (s, 2H, $C\mathbf{H}_2$Ph), 4.14 (q, J = 7.0 Hz, 2H, $C\mathbf{H}_2CH_3$), 4.81 (m, 1H, C**H**Tyr), 5.68 (s, 1H, O**H**), 5.85 (d, J = 7.8 Hz, 1H, N**H**), 6.61 (d, J = 4.2 Hz, 2H, **H**PhTyr), 6.75 (d, J = 4.2 Hz, 2H, **H**PhTyr), 7.16–7.20 (m, 2H, **H**Ph), 7.28–7.36 (m, 3H, **H**Ph). ^{13}C NMR ($CDCl_3$) (ppm): δ 14.08, 36.91,

43.47, 53.31, 61.69, 115.56, 126.50, 127.50, 129.04, 129.42, 130.17, 134.05, 155.61, 171.36, 171.60.

For **3e**, **3f**, and **3g** see Section 2.6.

2.8. Quantitative analysis

The formation of the amides **3a**, **3b**, and **3c** was evaluated by means of the ratio of the areas of the acyl donor and an internal standard (cinnamyl alcohol). The synthesis of amides **3d** and **3i** was followed by comparing areas of the amide and the L-TyrOEt (**1**) and conversion was calculated using the extinction coefficients of substrates and products calculated from the corresponding calibration curves.

The formation of the amides **3e**, **3f**, and **3g** was evaluated on the basis of calibration curves obtained for each product.

2.9. Reactions catalysed by PGA-450 in aqueous buffer

An amount of 62.5 mg (25 U) of PGA-450 treated as above was added to 1 ml of sodium phosphate buffer, 0.05 M, pH 7.00, containing 100 μmol of L-TyrOEt (**1**) and 100 μmol of the acyl donors methyl 4-hydroxyphenylacetate (**2a**) or methyl *S*-(+)-α-methoxyphenylacetate (**2g**). The two reactions were incubated at 30°C. Reaction courses were followed withdrawing samples from the supernatant and analysing them by the same procedure as above.

3. Results and discussion

A preparation of covalently immobilised enzyme (PGA-450) was employed in the study. Such enzymatic preparation is highly hydrated (62.3%, w/w), and once suspended in hydrophobic solvents, it forms clusters which entrap water, leading to a_w values above 0.90. The formation of aggregates in organic solvent causes diffusion limitation from the bulk organic phase to the enzyme, while the high value of a_w leads to competitive hydrolytic reactions. For these reasons, the biocatalyst was partially dehydrated as previously described (see Section 2 and [31]). The final water content of the enzyme after this treatment was 27% (w/w), corresponding to a_w values between 0.73 and 0.77, measured suspending the preparation in toluene for 24 h. At this value of a_w, PGA-450 had previously shown an optimal activity in synthetic reactions in toluene [31], so that the same reaction conditions were chosen to study the acylation of L-TyrOEt (**1**) with different analogues of phenylacetic acid (**2a**–**i**) to give the corresponding amides (**3a**–**i**) (Scheme 1). Initial reaction rates (v_0) are reported in Table 1.

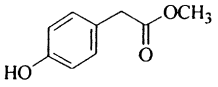
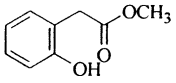
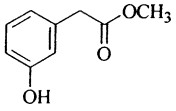
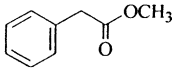
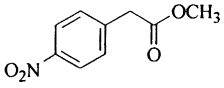
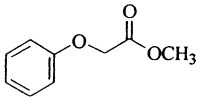
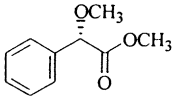
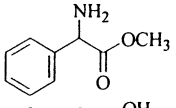
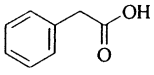
All the esters as well as phenylacetic acid used in this study are soluble in toluene at the concentrations used. In the experimental conditions used, the methyl 4-hydroxyphenylacetate (**2a**) gave the highest acylation rate of L-TyrOEt (**1**), thus resulting the best acyl donor in toluene, followed by methyl phenylacetate. The presence of a hydroxy group in 2- or 3-position (**2b** and **2c**) caused an appreciable reduction of v_0 if compared with the 4-hydroxy derivative (**2a**). When the 4-nitro-(**2e**) and the α-methoxy-(**2g**) derivatives were used, a larger reduction of v_0 was observed. It is quite remarkable that rates measured with methyl phenoxyacetate (**2f**) and the reference substrate methyl phenylacetate (**2d**) differed only for one order of magnitude.

Results in Table 1 indicate also that the acylation is complete within 1 day working with all the acyl donors, except for α-methoxy-analogue (**2g**) which led to 20% of conversion after 24 h. This low conversion is still a better result if compared to the same reaction performed in aqueous buffer. As a matter of fact, less than 5% of L-TyrOEt (**1**) was acylated in the presence of methyl *S*-(+)-α-methoxyphenylacetate (**2g**) in aqueous buffer, while only 50% of conversion was achieved when methyl 4-hydroxyphenylacetate (**2a**) was used.

All attempts of using L- and D-phenylglycine methyl ester (**2h**) as acyl donors were unsuccessful. Preliminary molecular modelling studies indicate that this lack of activity might be ascribed to unfavourable interactions between the polar amino group of phenylglycine and the aromatic side-chains lining the PGA hydrophobic pocket hosting the phenylacetic moiety (unpublished results). On the other hand, we have recently demonstrated that both L- and D-phenylglycine methyl esters are accepted as nucleophiles by PGA in organic solvent [30].

Results obtained in the present work show that the behaviour of PGA towards the different phenylacetic analogues follow a similar trend both in toluene and aqueous solution [4,5,16,21], and that PGA accepts

Table 1
Initial rates (v_0) and conversions at 24 h in the PGA-450[a]-catalysed acylation of L-TyrOEt (**1**) using several phenylacetic acid analogues

Acyl donor		v_0[b] (μmol/h U)	Conversion[c]		Products
			%	Time (h)	
	**2a**	14.0	>98	0.5	**3a**
	**2b**	5.6	>98	2	**3b**
	**2c**	2.7	>98	2	**3c**
	**2d**	6.3	>98	24	**3d**
	**2e**	7.2×10^{-2}	>98	24	**3e**
	**2f**	0.6	>98	24	**3f**
	**2g** (*S*-)	8.7×10^{-3}	20	24	**3g**
	**2h** (D- or L-)	$<10^{-5}$[d]	<0.1	>48 h	**3h**
	**2I**	1.4	>98	24	**3i** (=**3d**)

[a] Enzymatic activity assayed in aqueous buffer was 401 U/g (see Section 2 and [31]).
[b] Initial rates were determined in the following conditions: 1 ml of toluene, 62.5 mg of PGA-450, **1** = 80 μmol, **2a–i** = 100 μmol.
[c] Determined in the following conditions: 2 ml of toluene, 200 mg of PGA-450, **1** = 200 μmol, **2a–i** = 200 μmol.
[d] Detection limit in these analysing conditions.

in toluene a wide variety of structural analogues of phenylacetic acid although with considerable differences in the measured reaction rates (up to three orders of magnitude).

It has been previously reported that the active site of PGA is relatively small, so that maximum three water molecules can fit the hydrophobic binding pocket positioned in the inner part of the active site [37]. There is some evidence that when the phenylacetic derivatives enter the pocket, the water molecules are readily replaced [38], so that we can assume that neither toluene, which is a much larger molecule than water, nor water itself are present in the pocket during the reaction. This would explain the experimental observation that the selectivity of PGA towards phenylacetic analogues does not change moving from water to toluene. Other factors, such as solvation of substrates and products, desolvation energies, ionisation state of the protein and of reactants, might influence the selectivity of enzymatic reactions changing the medium. However, the evaluation of such effects would require further investigations that go beyond the aim of this paper.

It must be pointed out that the time required to achieve complete conversion was shorter for those

reactions leading to products which precipitated upon formation. For instance, in the case of methyl 4-hydroxyphenylacetate (**2a**), the final conversion was achieved in only 30 min. The same effect was observed also with 2- and 3-hydroxyphenylacetic acids methyl esters (**2b** and **2c**).

It is quite remarkable that also free phenylacetic acid (**2i**) was accepted as acyl donor in toluene in the thermodynamically controlled synthesis of the amide **3i**. The use of the carboxylic acid would lead to a progressive increase of the water activity of the reaction system during the amide synthesis due to the release of water. For this reason, 200 mg (six rods) of Celite R-640® were added to the reaction system to adsorb the released water and to maintain a_w sufficiently low ($a_w < 0.8$) so that hydrolytic reactions were avoided.

4. Conclusions

Unlike some other enzymes, which modify their selectivity in organic solvent [39], PGA in synthetic reactions in toluene maintains a selectivity comparable to that displayed in water. Besides, in organic solvent, PGA catalyses the complete acylation of L-TyrOEt (**1**) working with equimolar amounts of the substrates due to the absence of hydrolytic reactions so that products are isolated simply by solvent evaporation, and no further purification steps are required.

These features make PGA an enzyme of large and practical use in the protection/deprotection of amine groups and in their enantioselective acylation in organic solvent.

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