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One-Pot, Regioselective Synthesis of Substituted Arylglycines for Kinetic Resolution by Penicillin G Acylase

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Dedicated to Chi-Huey Wong on the occasion of his 60th birthday

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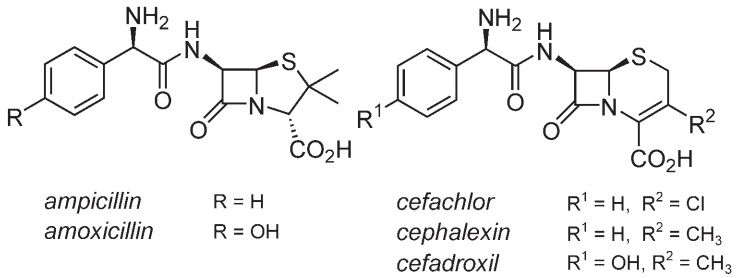
Abstract: Amido-alkylation of electron-rich arenes with phenylacetamide and glyoxylic acid offers an inexpensive route to a large variety of *N*-phenylacetylated arylglycines that are suited for immediate enzymatic resolution by penicillin G acylase. When performed under mild conditions at 5 °C in acetic acid/HCl, this simple one-pot operation resulted in the formation of single regioisomers only (≥98%). Subsequent kinetic resolution of the amino acid derivatives by penicillin G acylase at pH 8.0 occurred generally with *E* values >100 and thus furnished free (*S*)-configurated arylglycines with high enantiomeric purity. The corresponding enantiopure (*R*)-substrates, easily separable by a phase-selective extraction process, provided the corresponding (*R*)-enantiomers upon conventional hydrolysis. This one-pot, two-step procedure for arylglycine synthesis, resolution and work-up requires a minimum of equipment and grants rapid access to both enantiopure (*S*)- and (*R*)-antipodes of non-natural α-amino acids in small- to large-scale quantities.

Keywords: amino acids; antibiotics; enzyme catalysis; *p*-hydroxyphenylglycine; kinetic resolution; penicillin G acylase

Introduction

β-Lactam antibiotics, following the discovery of penicillin by Fleming in 1929,[1] have revolutionised chemotherapy against pathogenic micro-organisms. The rapid spread of bacterial resistance to natural antibiotics accessible by fermentation has stimulated the development of semisynthetic β-lactam derivatives, which offer a broader spectrum of activity for the treatment of a wider range of infections. Arylglycines are important intermediates in the commercial production of semisynthetic β-lactam antibiotics, the (*R*)-configurated D-enantiomers of phenylglycine (ampicillin, cefachlor) and *p*-hydroxyphenylglycine (**1a**) (amoxicillin, cefadroxil) being the predominant representatives (Figure 1). According to WHO data, in 2000 ampicillin and amoxicillin together accounted for almost half of the 45,000 metric tons of β-lactam antibiotics produced globally.[2,3] Apart from a few isolated examples, all clinically important semisynthetic



ampicillin	R = H	*cefachlor*	R^1 = H, R^2 = Cl
amoxicillin	R = OH	*cephalexin*	R^1 = H, R^2 = CH_3
		cefadroxil	R^1 = OH, R^2 = CH_3

Figure 1. Examples of the most important semisynthetic β-lactam antibiotics.

antibiotics containing optically active side chains are derived from arylglycine (e.g., the ureidopenicillins).[2]

While D-phenylglycine is available on a technical scale from benzaldehyde by conventional Strecker synthesis, resolution with camphorsulphonic acid and subsequent racemisation of the distomer,[4] (*R*)-*p*-hydroxyphenylglycine [(*R*)-**1a**] cannot be prepared by this route in a cost-competitive way, because *p*-hydroxybenzaldehyde is too expensive.

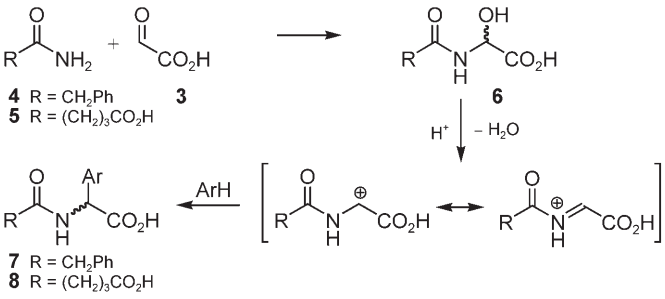
More elegantly, (*R*)-**1a** is produced by an enzyme-mediated, dynamic kinetic resolution of 5-(4-hydroxyphenyl)-hydantoin (*p*-HPH) formed by Mannich-type condensation of phenol (**2a**), glyoxylic acid (**3**) and urea.[4] Although a variety of processes (Kaneka, Recordati, etc.) have been patented, the fundamental benefits and drawbacks are the same: Assisted by spontaneous racemisation of the substrate *p*-HPH, two consecutive biocatalytic steps involving (*R*)-selective hydantoinases and *N*-carbamoylases furnish the enantiopure aminoacid (*R*)-**1a** in nearly quantitative yield;[5] the major drawbacks are due to an incomplete selectivity in the formation of *p*-HPH by amido-alkylation of phenol where a significant amount of regioisomeric 5-(2-hydroxyphenyl)-hydantoin (*o*-HPH) is also produced and large amounts of mineral acid are needed.[6,7] The moderate regioselectivity of the arene substitution results from the elevated temperatures applied to maximise the space-time yield. The synthesis of enantiopure arylglycines is currently further limited, particularly on a smaller than industrial scale, by the lack of suitable (*S*)-selective hydantoinases and *N*-carbamoylases towards the complementary enantiomeric (*S*)-arylglycines, or by the need to introduce additional protecting groups to ease kinetic resolution or subsequent coupling steps.

For an alternative approach, aminopeptidases and aminoamidases are most prominent amongst the enzymes applied for the kinetic resolution of amino acids.[9] Industrial penicillin G acylase (PGA; EC 3.5.1.11), which is used for the production of β-lactam intermediates, has a rather narrow specificity for the phenylacetyl moiety, but accepts a broad spectrum of phenylacylated substrates and therefore has been explored extensively in organic synthesis. PGA has been investigated, e.g., for kinetic resolutions of alcohols[10] and amines,[11] including α-amino acid,[12] β-amino acid,[13] and γ-amino acid derivatives,[14] stereospecific peptide synthesis,[15] or protecting group operations.[16] Less attention has so far been paid to PGA's capability to enantioselectively hydrolyse *N*-phenylacetamides of free α-amino acids.[17] For applications of the related industrial glutaryl acylase (GA; EC 3.5.1.93), there are few studies only; the enzyme has recently been found to hydrolyse a variety of amides and esters enantioselectively,[18] but data concerning the stereoselectivity towards *N*-acylated amino acids are scarce.[19] Representatives for both type of enzymes have been well characterised by X-ray crystal structure determination, including those of substrate-liganded complexes.[20]

Stimulated by a program to investigate the synthetic potential of GA and PGA for asymmetric synthesis and protecting group chemistry,[8] we were interested to study the potential of PGA and GA for the resolution of phenylacetamide and glutaric monoamide derivatives of arylglycines and have investigated an opportunity to prepare suitable amino acid derivatives by direct amido-alkylation of arenes. Here we report that enantiopure arylglycines can be produced from readily available bulk chemicals in a simple one-pot operation with high regioselectivity, and resolved into both (*S*)- and (*R*)-antipodes of high enantiomeric purity by highly efficient enzyme-mediated kinetic resolution.

Results and Discussion

In the industrial hydantoinase process phenol is transformed by amido-alkylation with **3** and urea to give racemic arylhydantoins for subsequent resolution to arylglycines. By analogy, the amido-alkylation of (hetero)arenes using a combination of **3** and simple carboxylic acid amides under acidic conditions has been shown to yield *N*-acylated aromatic α-amino acids (Scheme 1).[21] In the course of the reaction, *N*-acyl



Scheme 1. Formation of *N*-acylated arylglycines by amido-alkylation of arenes.

hemiaminals are formed which, upon protonation and loss of water, give rise to resonance-stabilised carbocations that will react with electron-rich arene nucleophiles. By this sequence, *N*-protected arylglycines become accessible in a facile one-pot/two-step synthesis. Research activities so far mainly focussed on the amides of acetic, chloroacetic and acrylic acid, as well as on ethyl carbamate.[22] Usually, product mixtures are obtained with good regioselectivity.[21,22]

In essence, the choice of the acyl moiety in the starting amide determines the type of N-protection in the arylglycine product, and thus the type of appropriate amidase biocatalysts for potential racemate reso-

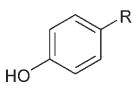
lution, including their respective configurational selectivity.[23] Using phenylacetamide (**4**) or glutaric acid monoamide (**5**) as starting materials for the formation of the respective acyl hemiaminals (**6**), this procedure should yield immediate substrates for biocatalytic resolution by PGA or GA. In addition, the nature of the acyl substituent will contribute to the efficacy of work-up conditions. It was anticipated that for both reactants the acylated starting material and hydrolysed product should be readily separable by pH-selective phase-switching separation, and that the aromatic nature of the phenylacetyl, or the polar carboxylate of the glutaryl residues should also facilitate product purification by crystallisation.

The *N*-acyl hemiaminals were prepared by reacting the hydrate of **3** with the respective amide in acetic acid at 50 °C. Although phenylacetamide (**4**) is available commercially, it is easily prepared by partial hydrolysis of inexpensive benzyl cyanide.[24] The monoamide of glutaric acid (**5**) was obtained by ammonolysis of glutaric anhydride. In this study, only a range of electron-rich arenes were included as benzene and less reactive derivatives require more drastic conditions that cause a low regioselectivity.[21,22] The catalyst of choice proved to be gaseous hydrogen chloride as it can easily be removed after the reaction by applying reduced pressure.

From phenylacetamide, the respective arylglycines (**7a**–**h**) were obtained in moderate to excellent yields (Table 1). Highly oxygenated components (e.g., **2e**/**g**) gave dark reaction mixtures with loss of material, probably from oxidative side reactions. Initially, reactions were run at room temperature, and products were isolated after solvent removal by simple washing the solid remainder with water and ether, except for thymol (**2f**) where unreacted arene had to be removed by thorough extraction. The regioselectivity of the aromatic substitution (**2a–h**) could be further enhanced by reducing the temperature to 5 °C. At even lower temperatures the reaction mixture becomes highly viscous and finally solidifies, prohibiting the homogeneous dispersion of the acid catalyst. Keeping the temperature of the reaction mixture close to 5 °C resulted in most cases in the formation of a single regioisomer only (>99%), as confirmed by HPLC analysis of the crude reaction mixtures and by NMR analysis of the isolated solids. Only **2a** yielded detectable amounts of the *ortho*-adduct (*o*-HPG, ~2%), which is easily removed during work-up as it forms a less polar lactone.

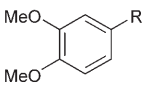
In comparison, the industrially operated hydantoinase process results in moderate regioselectivity only with *p*-HPH/*o*-HPH ratios not better than 7.2,[7] which is due to the need for elevated reaction temperatures (>40 °C). The unwanted isomer has to be removed in an additional step by selective crystallisation of *p*-HPH. Other preparative methods leading to 5-arylhydantoins, like the Bucherer–Bergs synthesis[25]

Table 1. Preparation of *N*-acylated arylglycines by amido-alkylation of arenes.[a]

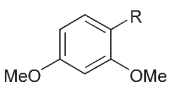


2a R = H
7a R = Pag

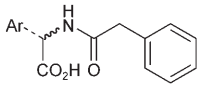
2b R = H
7b R = Pag



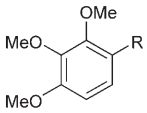
2c R = H
7c R = Pag
8c R = Gag



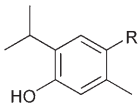
2d R = H
7d R = Pag



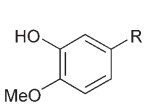
Pag



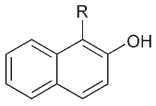
2e R = H
7e R = Pag



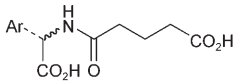
2f R = H
7f R = Pag



2g R = H
7g R = Pag



2h R = H
7h R = Pag



Gag

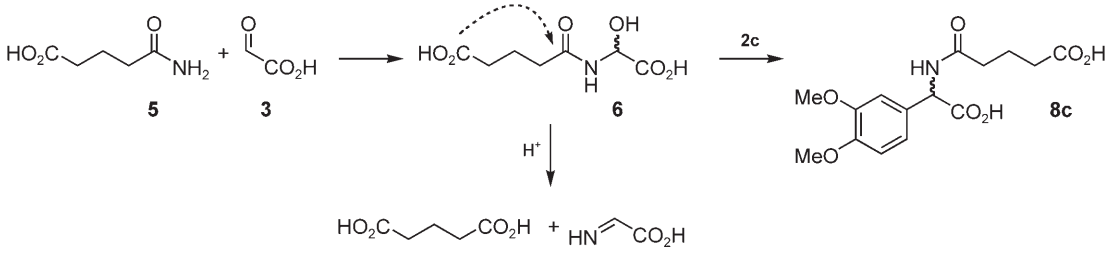
Entry	Acylamide	Arene	Arylglycine	Regioselectivity	Yield [%]
1	phenylacetamide (**4**)	**2a**	**7a**	98%[b]	63
2	**4**	**2b**	**7b**	>99%	39
3	**4**	**2c**	**7c**	>99%	86
4	**4**	**2d**	**7d**	>99%	61
5	**4**	**2e**	**7e**	>99%	36
6	**4**	**2f**	**7f**	>99%	73
7	**4**	**2g**	**7g**	>99%	32
8	**4**	**2h**	**7h**	>99%	88
9	glutarylmonoamide (**5**)	**2c**	**8c**	>99%	9

[a] One-pot, two-step reactions were performed, first by condensation of acylamide with glyoxylic acid in acetic acid at 50 °C, then arenes were reacted at 5 °C using HCl gas as catalyst.
[b] 2% *ortho*-isomer

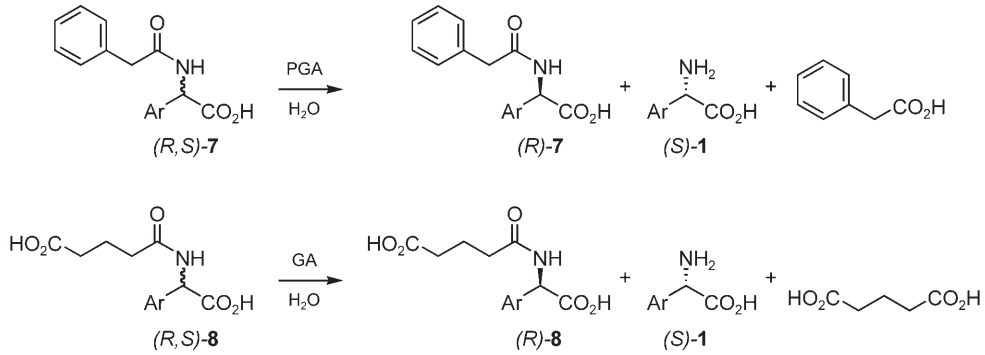
or amido-alkylation with 5-bromohydantoin,[6] are hampered by requiring expensive or highly toxic starting materials (e.g., substituted carbaldehydes, HCN or bromine).

From a corresponding series of reactions with reagent **5** expected to yield *N*-glutaryl-arylglycines, only the 3,4-dimethoxyphenyl derivative **8c** could be isolated from the reaction mixture, however, in a disappointingly low yield (Scheme 2). Presumably, this inefficiency is due to the instability of the *N*-glutaryl-amidoglycolic acid (**6**) intermediate under the acidic reaction conditions, as the amide bond may be sensitive to intramolecular attack by the terminal carboxylate group. No further efforts were invested for optimisation.

For a determination of the acylase enantioselectivity in the racemate resolution of selected *N*-acylated arylglycines (Scheme 3, Table 2) the influence of the reaction medium was also investigated as, like for many enzymes, PGA acitivity and selectivity have been shown to be remarkably influenced by the addition of small amounts of organic cosolvent.[26] The influence of organic cosolvents on the enantioselectivity of enzymes is thought to originate mainly from two



Scheme 2. Inefficient formation of *N*-glutaryl-arylglycines.



Scheme 3. Kinetic resolution of *N*-acylated arylglycines.

Table 2. PGA mediated hydrolysis with/without cosolvent.[a]

Compound	Enzyme	Medium	Conversion [%]	ee_S [%]	*E*
7a	PGA	H_2O	49.6 ± 0.3	95.5 ± 0.1	> 100 (256)
		5% MeOH	50.9 ± 0.3	95.1 ± 0.1	87
7b	PGA	H_2O	50.7 ± 0.3	96.1 ± 0.1	> 100 (117)
		5% MeOH	50.2 ± 0.3	96.1 ± 0.1	> 100 (166)
7c	PGA	H_2O	50.4 ± 0.2	97.1 ± 0.1	> 100 (187)
		5% MeOH	50.5 ± 0.2	97.8 ± 0.1	> 100 (214)
7d	PGA	H_2O	50.3 ± 0.2	98.2 ± 0.1	> 100 (313)
		5% MeOH	49.8 ± 0.2	97.6 ± 0.1	> 100 (547)
8c	GA	phosphate buffer	50.6 ± 0.2	90.3 ± 0.1	49
		buffer + 5% MeOH	48.4 ± 0.2	84.0 ± 0.1	48

[a] Data based on HPLC analysis; average values from three independent determinations each. PGA reactions performed at room temperature, GA reaction at 37 °C using purified soluble enzymes. Absolute configurations of the products are related to an authentic sample of *(S)*-**1a**.

distinct effects.[27] Firstly, water acts as a molecular lubricant allowing for a high degree of conformational flexibility; addition of cosolvent rigidifies the enzyme structure by conserving hydrogen bonds. This reduces the ability of complex formation between enzyme and the less favoured enantiomer, resulting in an improved enantioselectivity. The second effect concerns the desolvation when a substrate traverses from hydrophilic reaction medium to the more hydrophobic active site; a less polar medium lowers the barrier between bulk phase and the enzyme's catalytic centre, resulting in a higher contribution of the velocity of enzyme-substrate complex formation relative to the total reaction velocity.

For PGA reactions with **7a–d**, water and addition of 5% MeOH were chosen, while the pH was kept constant at the enzyme's optimum of pH 8.0 by automated titration with 0.1 M Na_2CO_3. Due to the limited quantity of **8c** and the small scale, hydrolytic reactions with GA were carried out in 0.1 M sodium phosphate buffer for convenience and compared to solvent including 5% MeOH. For PGA-mediated hydrolysis, conversion and enantiomeric excess were determined simultaneously by HPLC analysis on lyophilised samples by relating the peaks obtained for phenylacetic acid and for both enantiomers of the respective *N*-phenylacetyl-arylglycine to calibration charts recorded independently. For the GA-mediated hydrolysis of **8c** conversion and *ee* were determined from separate analyses. The enantiomer selectivity *E* was calculated from the conversion (*c*) and *ee* values of the substrate as determined by HPLC analysis.[28] *E* is defined as

$$E = \frac{\ln[(1-c)(1-ee_S)]}{\ln[(1-c)<(1+ee_S)]}.$$

All PGA resolutions gave high enantioselectivity ratios that allow an efficient access to both enantiopure antipodes of aryl glycines. PGA enantioselectivity clearly increases with the steric bulk of the aryl substituent, i.e., 2,4-dimethoxyphenyl > 3,4-dimethoxyphenyl > thienyl. Remarkably, the uncharged arylglycines (**1b–d**) were resolved with higher *E* values with 5% MeOH as a cosolvent, in good accordance with prior observations on amine resolutions.[26] Resolution of the (*p*-hydroxyphenyl)-glycine derivative (**7a**) using PGA proved to be more efficient without cosolvent, which may be due to the fact that the phenolic OH group is deprotonated at pH 8.0, the molecule thus bearing an additional negative charge. Compounds **7e–h** have not been tested in this study but can be assumed to be amenable to kinetic differentiation with similar efficiency.

In a preparative experiment performed on a gram-scale, enzymatic hydrolysis of **7a** was stopped by acidification, after which unreacted **7a** and phenylacetic acid were removed by selective extraction with EtOAc. Enantiopure free (*S*)-**1a** was recovered from the aqueous phase in high yield by evaporation, followed by extraction of the solid remainder using boiling EtOH and recrystallisation. The enantiopure free (*R*)-**1a** was obtained from the EtOAc extraction phase by acidic hydrolysis with 1 M HCl and subsequent recrystallisation from 1-propanol.

Due to the unsatisfactory results in the amido-alkylation with **5**, an investigation of the GA's resolving capability was only of general interest. The effect of organic cosolvents on GA enantioselectivity has so far only been investigated for the hydrolysis of ester, but not amide functionalities.[29] We found that, in contrast to PGA, addition of small concentration of organic cosolvent to the reaction medium had no significant effect on the kinetic enantiomer differentiation of **8c** by GA. The enantioselectivity found is acceptable, but clearly inferior to that of PGA, for the generation of optically pure amino acids.

Conclusions

The amido-alkylation of aromatic hydrocarbons with phenylacetamide and glyoxylic acid offers a facile and inexpensive route to a large variety of arylglycines. Low temperatures yield almost exclusively one regioisomer and work-up procedures are in most cases reduced to a simple washing protocol. Kinetic resolution of the free amino acids with PGA, a readily available, robust biocatalyst, furnishes the phenylacetylated (*R*)-**7a–d** and free (*S*)-**1a–d** amino acids, respectively, with high enantiomeric purity; substrate and product can easily be separated by a phase-selective extraction process. This result is of special interest with respect to the fact that the synthesis of (*S*)-arylglycines by the hydantoinase process has not yet been implemented on an industrial scale. By adding a suitable *N*-acylamino acid racemase[9c,30] to the resolution process even quantitative yields would be feasible by dynamic kinetic resolution. The corresponding (*R*)-enantiomers can easily be obtained by subsequent acidic or enzymatic hydrolysis of the corresponding phenylacetylamides. Our method of arylglycine synthesis, resolution and work-up procedure requires a minimum of equipment and therefore grants rapid access to enantiopure non-natural α-amino acids in small- to large-scale quantities.

The corresponding reaction pathway using glutaric monoamide as starting material is apparently hampered by the intermediate's instability and resulted in low yields. Also, GA seems to be a less powerful catalyst for the resolution of arylglycines than PGA.

Experimental Section

General Procedure for the Synthesis of *N*-Phenylacetyl-arylglycines

A 50% aqueous solution of glyoxylic acid (**3**, 37.00 g, 0.25 mol) was concentrated to 80% acid content under reduced pressure. The syrup was dissolved in acetic acid (60 mL), and phenylacetamide (**4**; 33.80 g, 0.25 mol) was added to the solution. The mixture was kept at 50 °C for 5 h, then cooled to 5 °C and the aromatic component added. A gentle stream of dry hydrogen chloride was fed into the solution with stirring for 1 h, making sure that the temperature did not exceed 10 °C. The mixture was kept at 5 °C for 16 h, then the volatile compounds were removed under reduced pressure by gradually rising the temperature to 70 °C. The remainder was triturated in cold water. The solid was collected by filtration and thoroughly washed with cold water, Et_2O and dried under vacuum.

Determination of Conversion and Enantioselectivity

Hydrolytic reactions were performed at room temperature using a Titroline Alpha automatic titrator (Schott) in the pH-Stat mode. Spontaneous chemical hydrolysis was evaluated by monitoring the pH of the reaction solutions 10 min prior to enzyme addition and was found to be negligible.

PGA catalysis: the substrate (200 mg) was dissolved in H_2O (or in an aqueous solution containing 5% of MeOH; total volume 20 mL), and reactions were started by addition of 25 μL (1140 U/mL) of PGA suspension. Reaction solutions were gently stirred at room temperature in an open flask maintaining the pH constant at 8.0 by automated addition of 0.1 M Na_2CO_3. Samples of 200 μL were withdrawn after 30 min, mixed with acetonitrile (400 μL) to stop the enzymatic hydrolysis, and the solution was lyophilised immediately.

GA catalysis: the substrate (20 mg) was dissolved in 0.1 M sodium phosphate buffer (with/without 5% MeOH; total volume 2 mL), and reactions were started by addition of 10 μL (368 U/mL) of GA suspension. Reaction solutions were shaken at 600 rpm and 37 °C in an Eppendorf Thermomixer. Samples (200 μL) were withdrawn after 15 min, mixed with acetonitrile (400 μL), and the solution was lyophilised immediately.

HPLC analysis: lyophilised samples were dissolved by addition of EtOH (100 μL), TFA (2 μL), $CHCl_3$ (200 μL) and hexane (400 μL), centrifuged at 12,000 × *g* for 1 min and submitted to HPLC analysis. For the *N*-phenylacetyl-arylglycines the conversion and *ee* were determined simultaneously by using a Chiracel IB column (250 × 4.6 mm), eluent hexane-$CHCl_3$-EtOH-TFA = 66:30:4:0.1, flow rate 1.0 mL min^{-1}, λ = 265 nm [t_R: **7a** = 19.8 (*R*)-enantiomer, 21.5 (*S*)-enantiomer; *o*-hydroxyphenylglycine = 6.3, 6.6; **7b** = 6.7, 7.2; **7c** = 8.9, 9.5; **7d** = 6.5, 7.8; phenylacetic acid = 4.9 min]. The degree of conversion was determined by comparing the ratio of the peaks obtained for phenylacetic acid to the sum of both enantiomers of *N*-phenylacetylarylglycines **7a–d** with the aid of calibration charts.

Conversions of the *N*-glutarylacylated arylglycine was determined by using a thermostated (30 °C) Partisil 5C8 column (250 × 4.6 mm), eluent 0.1 M NH_4OAc (pH 5.2)-MeCN = 95:5, flow rate 1.2 mL min^{-1}, λ = 254 nm (t_R: **1c** = 3.4 min; **8c** = 4.8 min). The *ee* was determined on a Chiracel IB column as described for the *N*-phenylacetyl arylglycines (t_R: **8c** = 19.7, 21.8 min).

Preparative Scale Kinetic Resolution of Racemic *N*-Phenylacetyl-(4-hydroxyphenyl)-glycine

Racemic **7a** (1.00 g, 3.5 mmol) was dissolved in H_2O (20 mL), the pH adjusted to 8.0 and the reaction started by addition of PGA (200 μL, 228 U). The pH was kept constant by automatic titration of 0.1 M Na_2CO_3. After 20 min the pH was adjusted to 3.0 with 2.0 M HCl, the denatured enzyme removed by filtration and the total volume adjusted to 50 mL with H_2O. The solution was extracted with EtOAc (3 × 50 mL), the organic phases combined, dried over Na_2SO_4, and the solvent removed under reduced pressure. The composition of the obtained solid (0.71 g) was examined by HPLC and found to be phenylacetic acid and pure (*R*)-*N*-phenylacetyl-D-(4-hydroxyphenyl)-glycine *(R)*-**7a**. The free *(R)*-amino acid was obtained by hydrolysing the solid with 1 M HCl (50 mL) at 50 °C for 10 h. The solution was evaporated to dryness and the remainder recrystallised from 1-propanol to afford enantiopure *(R)*-**1a**; yield: 0.27 g (93%).

Free *(S)*-**1a** was recovered from aqueous phase after the extractions by evaporation, and extraction of the solid remainder with EtOH under reflux (3 × 25 mL). The liquid extracts were combined and evaporated to yield 0.26 g (*S*)-**1a** with 91% *ee* (89%). Recrystallisation from 1-propanol yielded enantiopure (*S*)-**1a**.

Supporting Information

Full details of methods and materials as well as the characterisation data for new compounds are available as Supporting Information.

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