Glutaryl Acylases: One-Reaction Enzymes or Versatile Enantioselective Biocatalysts?

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Abstract: A significant broad substrate specificity, that crosses over the usual β-lactam derivatives, has been observed with an industrial glutaryl-7-aminocephalosporanic acid acylase (GA). This enzyme possesses significant enantioselective amidase and even esterase activity, with a stereopreference for the *S*-enantiomer. The easy separation of products from unreacted reagents, possessing different physical-chemical properties, is achieved by solvent extraction, avoiding chromatography or distillation during reaction work-up.

Keywords: biocatalysis; biotransformations; enantioselectivity; enzymatic kinetic resolution; glutaryl acylase; hydrolases

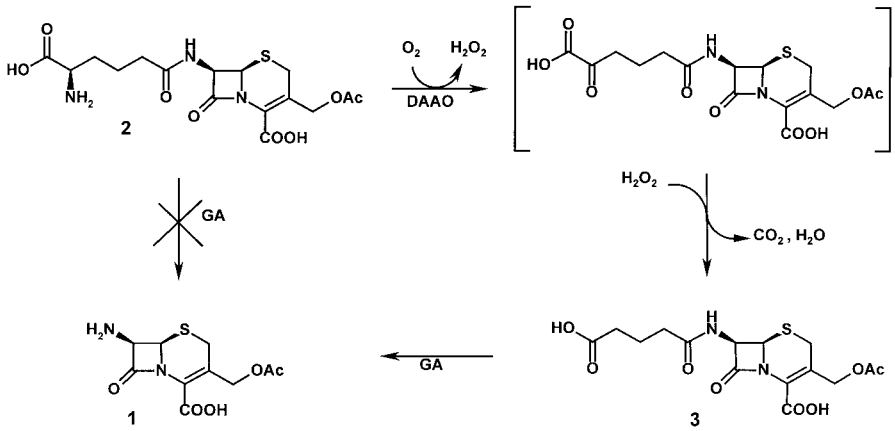
Introduction

7-Aminocephalosporanic acid (7-ACA, **1**) is an important industrial intermediate for the production of semisynthetic cephalosporins. Chemical deacylation of cephalosporin C (CefC, **2**)[1] is still the primary method used to produce **1** industrially, even if this process has several drawbacks related to the operative reaction conditions and to the environmental impact.

Great efforts have been made to develop a bioconversion process that could overcome these problems by converting CefC into 7-ACA under mild reaction conditions. The efficient one-step biotransformation of **2** into **1** is still an unachieved target, but a two-step enzymatic protocol has been developed and is presently being operated on an industrial scale (Scheme 1).[2] This route includes the oxidative deamination of **2** to glutaryl-7-aminocephalosporanic acid (glutaryl-7-ACA, **3**) catalyzed by a D-amino acid oxidase (DAAO), and the subsequent deacylation of **3** to **1** catalyzed by a glutaryl-7-ACA-acylase (GA). The present report will briefly cover the literature reports on the latter group of enzymes and will present our recent results on the investigation of substrate specificity of an industrial GA preparation.

Glutaryl Acylases: Biochemical Properties

Glutaryl acylases (GAs) belong to the N-terminal nucleophile (Ntn) hydrolases superfamily,[3] and are a subgroup of the so-called "cephalosporin acylase (CA)



Scheme 1. Enzymatic synthesis of 7-aminocephalosporanic acid (7-ACA, **1**) from cephalosporin C (CefC, **2**).

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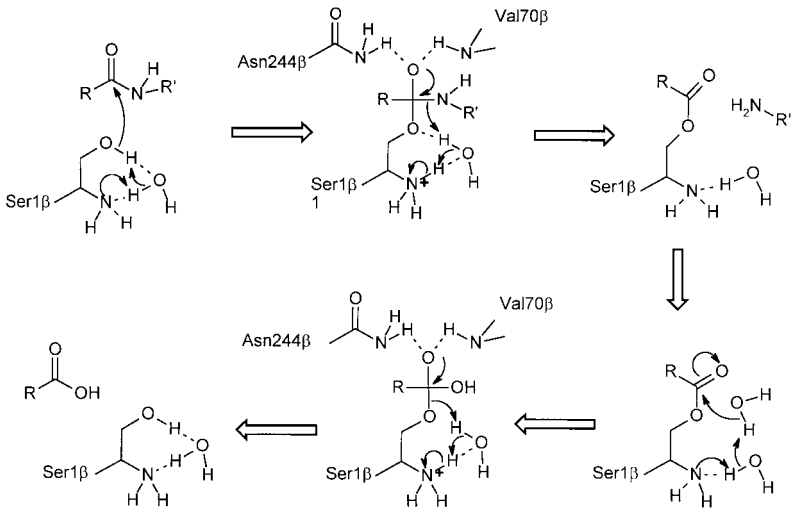


Scheme 2. Catalytic mechanism of a GA from *Pseudomonas diminuta* (see Ref.[8]).

family". In fact, in a first proposed classification these enzymes were divided in two groups depending either on their ability to show a measurable, albeit low, hydrolytic activity towards CefC (**2**) – the "cephalosporin acylases" – or on their inability to perform such a reaction – the "glutaryl acylases" (Scheme 1). A more systematic classification, based on their genetic sequence, molecular weight and enzymatic properties has been recently proposed,[4] dividing the CAs into five groups. The enzymes belonging to the first four groups consist of two subunits (α and β) folded in heterodimers $(\alpha\beta)$,[5] tetramers $(\alpha\beta)_2$,[6] or, less frequently, octamers $(\alpha\beta)_4$.[4] The two subunits are coded by a single gene which is expressed as an inactive precursor polypeptide that, during post-translational processing, is converted into the two subunits.[5]

As shown in Figure 1, the CA's ORF usually starts with a signal peptide, followed by the α-subunit, a spacer and the β-subunit. For instance, in the case of the GA from *Pseudomonas* sp. strain GK16 (belonging to group I), it has been shown that the resulting *N*-terminal serine of the β-subunit (54 kDa) is responsible for the intramolecular cleavage of the precursor polypeptide (74 kDa). The same Ser199 of the β-subunit cuts the residual progenitor peptide giving origin to the α-subunit (16 kDa), and the two mature subunits fold in the active $(\alpha\beta)_2$ heterotetramer.[6b,7] This mechanism of activation is typical of the N-terminal nucleophile (Ntn) hydrolases, which have been shown to possess an N-terminal residue essential not only for the production of the mature enzyme but also for the catalytic activity. For instance, this residue is threonine in aspartylglucosaminidases, cysteine in glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferases, and serine in penicillin acylases and CAs. In the case of the GA from *Pseudomonas* sp. strain GK16, it has been demonstrated that the *N*-terminal Ser199 of the β-subunit is the

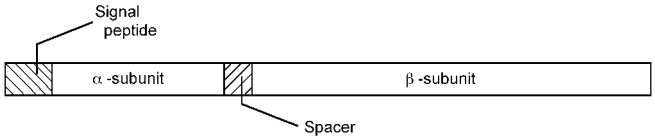
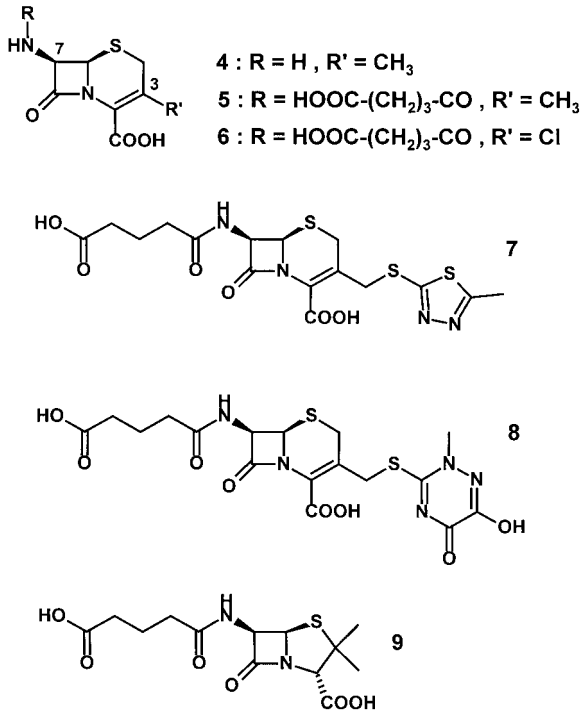


Figure 1. Schematic picture of the precursor peptide of the monomeric form of a generic GA.

catalytic residue acting both as a nucleophile (the side chain OH) and a base (the terminal NH_2), in a way similar to serine and histidine residues in the active sites of serine proteases.[6b,7] This catalytic mechanism has been confirmed in a study on the 3D-structure (solved at 2.0 Å resolution) of a GA from *Pseudomonas diminuta* KAC-1, an αβ heterodimer that consists of an α-subunit with 169 residue and a β-subunit with 520 residue.[8] The catalytic residue is the terminal Ser1β and, additionally, two nearby nitrogens of the amino acids Val70β and Asn244β form an oxyanion hole that stabilizes the tetrahedral intermediate (Scheme 2).

Cephalosporin and Glutaryl Acylases: Substrate Specificity

Very few reports are available on the hydrolytic activity of CAs and GAs towards non-β-lactam substrates. Apparently these enzymes possess a very strict substrate specificity: literature reports on a limited number of cephalosporin derivatives, carrying either a side chain linked at N-7 (R, Scheme 3) different from glutarate or a methyl group instead of an acetate in C-3 (derivatives of 7-ADCA, **4**),[9] which are still substrates for these enzymes. Additionally, it is worth noting that CefC is a poor substrate for all the CAs isolated up to now: these enzymes, in contrast to their name, show a hydrolytic



Scheme 3. Glutarylamides of cephalosporin and penicillin derivatives.

activity towards **2** that, at the best, is not higher than 4% of the activity displayed towards **3**.

A more recent paper has described the structure of the active site of the GA from *Pseudomonas diminuta* in complex with **3**.[9a] X-ray analysis of the crystallized protein identified three substrate moieties that are specifically recognized by the enzyme: the glutaric chain, the β-lactam nucleus and the substituent at C-3 (an acetate in **3**).

Results and Discussion

As a development of our investigation on the performance of an industrial GA,[10] in the present work we studied the substrate specificity of this enzyme in more detail. Initially, several amides of 7-ACA and 7-ADCA were prepared by reaction of **2** or **4** with a suitable anhydride. We found that the glutarate moiety was by far the preferred one and, additionally, no conversion was observed with substrates carrying a neutral aliphatic side chain, like a butanoate or a pentanoate.[11]

On the other hand, the enzyme was much less sensitive to the substituent at the C-3 position and glutaryl derivatives of 7-ACA carrying different substituents at C-3 were all efficiently hydrolyzed. We also found that the presence of the cephalosporanic skeleton was not a strict requirement, as glutaryl-6-aminopenicillanic acid (**9**) was also an excellent substrate for the enzyme (Table 1).

X-ray analysis showed that the third interaction of **2** with the GA from *Pseudomonas diminuta* was due to the β-lactam ring,[9a] therefore a logical further step for our investigation was to carry out the modification of this part of the molecule and to check the performance of our GA with these new substrates. Accordingly, we synthesized a series of *N*-glutarylamino acids and of their methyl esters and we found that all of them were substrates for GA, showing that the presence of a β-lactam moiety is not essential for enzyme activity. The ability of the same GA to hydrolyze *N*-glutarylamino acids has been confirmed in a very recent paper by Gardossi and coworkers, that describes the performance of GA in organic solvents and in heterogeneous substrate mixtures (in this paper, the rate of hydrolysis

Table 1. Relative initial rates of hydrolysis of compounds **3**, **5**–**9**.

Compound	Relative rate[a]
3	100
5	111.1 ± 1.8
6	104.4 ± 0.5
7	97.2 ± 0.2
8	87.5 ± 0.9
9	118.5 ± 0.1

[a] The initial rate of hydrolysis of compound **3** (4.4 μmol/min using 0.22 U/mL GA) was taken as 100. Experiments were repeated in duplicate at least. For details, see Experimental Section

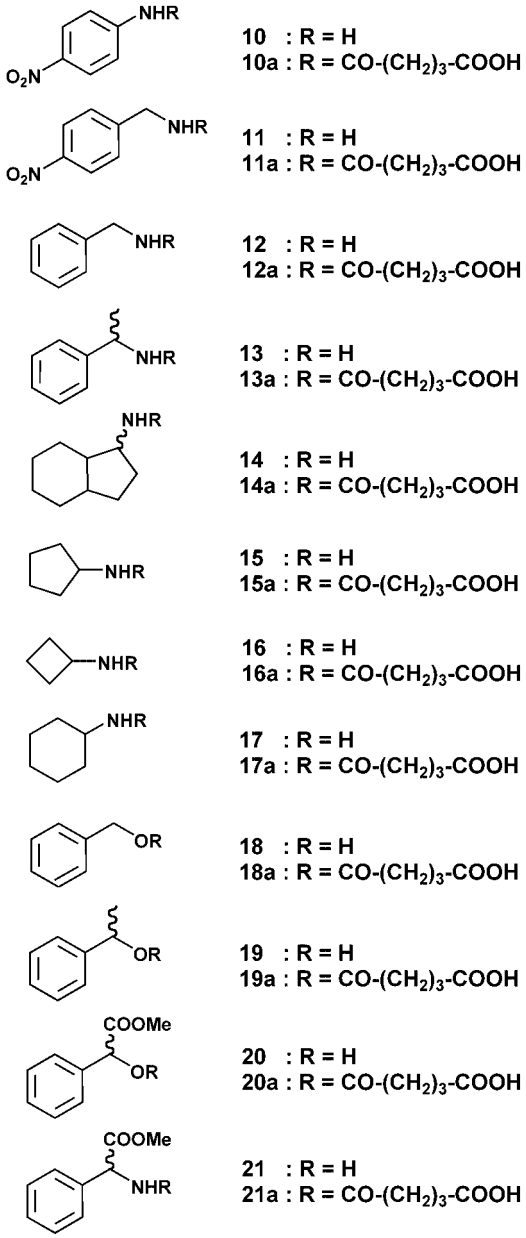
Table 2. Relative initial rates of hydrolysis of *N*-glutarylamino acids.

Compound	Relative rate[a]
N-Glutarylglycine methyl ester	9.7 ± 0.2
N-Glutaryl-β-alanine methyl ester	2.9 ± 0.1
N-Glutaryl-L-alanine methyl ester	25.0 ± 0.6
N-Glutaryl-D-alanine methyl ester	2.2 ± 0.2
N-Glutaryl-L-alanine	2.7 ± 0.2
N-Glutaryl-D-alanine	< 0.1
N-Glutaryl-L-phenylalanine methyl ester	15.7 ± 2.0
N-Glutaryl-D-phenylalanine methyl ester	4.5 ± 0.2
N-Glutaryl-L-phenylalanine	2.5 ± 0.1
N-Glutaryl-D-phenylalanine	2.0 ± 0.4
N-Glutaryl-L-phenylglycine methyl ester	59.3 ± 1.0
N-Glutaryl-D-phenylglycine methyl ester	0.23 ± 0.01
N-Glutaryl-L-phenylglycine	7.5 ± 0.8
N-Glutaryl-D-phenylglycine	1.1 ± 0.2
N-Glutaryl-L-leucine methyl ester	38.7 ± 0.6
N-Glutaryl-D-leucine methyl ester	2.7 ± 0.1
N-Glutaryl-L-tryptophan methyl ester	17.7 ± 0.9
N-Glutaryl-D-tryptophan methyl ester	3.0 ± 0.2

[a] The initial rate of hydrolysis of compound **3** (4.4 μmol/min using 0.22 U/mL GA) was taken as 100. Experiments were repeated in duplicate at least. For details, see Experimental Section.

of *N*-glutaryl-L-phenylglycine methyl ester and of *N*-glutaryl-L-tyrosine methyl ester have also been reported).[12] In addition, the relative rate of hydrolysis of these compounds, reported in Table 2, allowed us to conclude that *N*-glutarylamino acids methyl esters were better substrates than the corresponding free acid derivatives and, above all, that GA displayed a significant enantiopreference for the glutarylamides of L-amino acids (similarly to the enantioselectivity displayed by penicillin acylases).[13]

Stimulated by these significant results on GA enantioselectivity, substrate modification was pushed further and glutarates of racemic amines (i.e., **13a**) and even of racemic alcohols (i.e., **19a**) were considered (Scheme 4).



Scheme 4. Racemic glutarylamides and esters.

Table 3. Relative initial rates of hydrolysis of the glutarylamides **10a – 17a**.

Compound	Relative rate[a]
10a	8.36 ± 0.19
11a	1.00 ± 0.02
12a	0.16 ± 0.01
13a[b]	0.24 ± 0.02
14a[b]	< 0.08
15a	< 0.08
16a	< 0.08
17a	< 0.08

[a] The initial rate of hydrolysis of compound **3** (4.4 μmol/min using 0.22 U/mL GA) was taken as 100. Experiments were repeated in duplicate at least. For details, see Experimental Section.
[b] Racemic mixture.

Table 4. Enantioselectivity of GA towards glutarylamides and esters.

Compound	Conversion [%]	Product ee [%]	E[a]
21a	47	95	104.4
13a	43	78	14.6
20a	50	62	7.9
19a	48	58	6.3

[a] Calculated as described in Ref.[14]

We found that both these classes of compounds were hydrolyzed by GA, thus showing for the first time that this enzyme also possesses a significant esterase activity (for instance, the relative rate of hydrolysis of **18a** compared to **3** was 25%) with an appreciable preference for the *S*-enantiomers. Table 3 reports the data for a series of glutarylamides (**10a – 17a**), while Table 4 shows the enantiomeric values (E)[11,14] obtained with a homogeneous set of racemic compounds (glutarylamides or esters).

A few years ago we described the kinetic resolutions of racemic carboxylates by lipase-catalyzed transesterifications with amino alcohols in organic solvents,[15] separating the products from the unreacted substrates simply by washing the organic phase with a mildly acidic water solution. The results described in the present report expand this methodology to racemic amines and alcohols. In fact, the chemical-physical difference between the substrates **10a – 20a** (water-soluble carboxylates) and the products (the amines **10 – 17** and the alcohols **18 – 20**), allows their separation either by direct extraction (alcohols) or by selective extraction following pH-adjustment (amines). In this way it is possible to overcome one of the practical drawbacks of enzymatic kinetic resolutions: the similar chemical-physical properties of reagents and products make their separation possible only by chromatography or careful fractional

distillation, procedures that can be a serious obstacle to the scaling up of these biotransformations.

Reactions with compounds **21a**, **13a**, **18a** and **19a** were performed on a preparative scale and the corresponding pure (*S*)-amines and (*S*)-alcohols were isolated by selective extraction (see Experimental Section). GA was also used immobilized on a solid support[10] and was recycled 4 times without showing appreciable loss of activity or of enantioselectivity.

Conclusions

The unexpectedly wide substrate specificity of the industrial GA used in the present investigation, accompanied by its significant enantioselectivity, makes this acylase an interesting enzyme for synthetic applications.

It should be emphasized that the GA used in the present report is an industrial enzyme that has been developed to provide the best performances for a very specific biotransformation, that is the tons-scale hydrolysis of **3** to **2**. GAs from different sources are becoming available[9,16] and their performances can be tuned by side-directed and/or random mutagenesis.[16a,17] Therefore it is reasonable to expect that these new enzymes might match further synthetic requests in terms of improved stability and selectivity.

Experimental Section

Materials

Glutaryl acylase (both as an enzyme solution and as an immobilized preparation) and authentic samples of compounds **3** and **5** were a generous gift from Recordati S.p.A. (Opera, MI, Italy), while the precursors of compounds **7** and **8** were an appreciated gift from Dobfar S.p.A. (Tribiano, MI, Italy). All the others reagents and solvents were from Aldrich.

Hydrolytic reactions were monitored at 25 °C using a 718 STAT Titrino automatic titrator (Metrohm Ltd.). HPLC analyses were performed using a Jasco 880/PU instrument equipped with a Jasco 875 UV/VIS detector. Enantiomeric excesses were evaluated using a suitable chiral column (Chiralcel OD or Chiralcel OJ, Daicel Ltd.; Chirobiotic T, Astec Inc.).

Synthesis of Glutarylamides and Glutaryl Esters

*a) Typical procedure for the synthesis of β-lactam glutarylamides (Compounds **3**, **5 – 9**):* 7-ACA (3.5 mmol) was dissolved in 20 mL of 1 M $NaHCO_3$. Glutaric anhydride (1 equiv.) was dissolved in 5 mL acetone and the two solutions were mixed and left to react for 3 h at 20 °C (TLC: *n*-BuOH-AcOH-H_2O, 6:2:2). Acetone was evaporated, the water solution was acidified to pH 1.5 with 1 M HCl and extracted three times with 100 mL AcOEt. The organic layer was evaporated and the solid residue was washed on a Buchner funnel with 20 mL AcOEt and dried.

*b) Typical procedure for the synthesis of glutaryl amides and esters (Compounds of Table 2 and **10a – 21a**):* Compound **20** or compound **21** (5 mmol) was dissolved in 30 mL dioxane. Glutaric anhydride (1 equiv.) was dissolved in 5 mL dioxane and the two solutions were mixed and left to react for 8 h at 50 °C (TLC: *n*-BuOH-AcOH-H_2O, 6:2:2). Dioxane was evaporated, the residue was dissolved in 20 mL H_2O. In the case of **21** (and of the other amines), the solution was acidified to pH 3.0 with 1 M HCl and extracted three times with 100 mL AcOEt; the organic layer was evaporated and the solid residue was used as it was. In the case of **20** (and of the other alcohols), the solution was brought to pH 8.0 and extracted twice with 50 mL AcOEt to remove the unreacted alcohol, then the solution was acidified to pH 3.0 with 1 M HCl and extracted three times with 100 mL AcOEt; the organic layer was evaporated and the solid residue was used as it was. Product structures were confirmed by ^{1}H-NMR.

The possible presence of small amounts of contaminating glutaric acid in some of our model substrates was irrelevant, as we found that GA did not show any appreciable product inhibition even in the presence of 100 mM glutaric acid.

Glutaric anhydride: ^{1}H NMR (200 MHz, DMSO-d_6): δ = 2.73 (4H, t, J = 6.8 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 1.90 (2H, br quint, J = 6.8 Hz, CH_2-$\underline{CH_2}$-CH_2).

3: ^{1}H NMR: δ = 8.82 (1H, d, J = 8.1 Hz, CONH), 5.68 (1H, dd, J_1 = 8.2 Hz, J_2 = 4.8 Hz, H-7), 5.11 (1H, d, J = 4.8 Hz, H-6), 5.02 + 4.71 (1H each, d each, J = 12.8 Hz, CH_2OAc), 3.65 + 3.49 (1H each, d each, J = 18.0 Hz, H-2a + H-2b), 2.28 (4H, br t, J = 7.2 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 2.05 (3H, s, CH_3CO), 1.75 (2H, br quint, J = 7.2 Hz, CH_2-$\underline{CH_2}$-CH_2).

5: ^{1}H NMR: δ = 8.78 (1H, d, J = 8.2 Hz, CONH), 5.57 (1H, dd, J_1 = 8.1 Hz, J_2 = 4.6 Hz, H-7), 5.04 (1H, d, J = 4.6 Hz, H-6), 3.58 + 3.36 (1H each, d each, J = 18.7 Hz, H-2a + H-2b), 2.24 (4H, br t, J = 7.3 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 2.04 (3H, s, CH_3), 1.75 (2H, br quint, J = 7.2 Hz, CH_2-$\underline{CH_2}$-CH_2).

6: ^{1}H NMR: δ = 8.89 (1H, d, J = 8.1 Hz, CONH), 5.68 (1H, dd, J_1 = 8.1 Hz, J_2 = 4.6 Hz, H-7), 5.20 (1H, d, J = 4.6 Hz, H-6), 3.99 + 3.69 (1H each, d each, J = 18.1 Hz, H-2a + H-2b), 2.26 (4H, m, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 1.71 (2H, m, CH_2-$\underline{CH_2}$-CH_2).

7: ^{1}H NMR: δ = 8.82 (1H, d, J = 8.2 Hz, CONH), 5.66 (1H, dd, J_1 = 8.2 Hz, J_2 = 4.7 Hz, H-7), 5.09 (1H, d, J = 4.8 Hz, H-6), 4.51 + 4.23 (1H each, d each, J = 13.2 Hz, CH_2SR), 3.79 + 3.59 (1H each, d each, J = 18.0 Hz, H-2a + H-2b), 2.70 (3H, s, CH_3), 2.26 (4H, br t, J = 7.2 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 1.72 (2H, br quint, J = 7.2 Hz, CH_2-$\underline{CH_2}$-CH_2).

8: ^{1}H NMR: δ = 8.81 (1H, d, J = 8.1 Hz, CONH), 5.67 (1H, dd, J_1 = 8.1 Hz, J_2 = 4.5 Hz, H-7), 5.09 (1H, d, J = 4.9 Hz, H-6), 4.39 + 4.10 (1H each, d each, J = 13.5 Hz, CH_2SR), 3.70 + 3.59 (1H each, d each, J = 18.2 Hz, H-2a + H-2b), 3.61 (3H, s, N-CH_3), 2.25 (4H, br t, J = 7.2 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 1.73 (2H, m, CH_2-$\underline{CH_2}$-CH_2).

9: ^{1}H NMR: δ = 5.33 (2H, br s, H-5 and H-6), 3.87 (1H, s, H-3), 2.21 + 2.17 (2H each, br t each, J = 7.4 Hz, 2 CH_2CO), 1.70 (2H, br quint, J = 7.4 Hz, CH_2-$\underline{CH_2}$-CH_2), 1.57 + 1.46 (3H each, s each, CH_3-2).

10a: ^{1}H NMR: δ = 10.5 (1H, s, NH), 8.23 + 7.86 (2H each, d each, J = 9.2 Hz, aromatic CH), 2.45 + 2.30 (2H each, t each, J = 7.4 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 1.84 (2H, quint., J = 7.3 Hz, CH_2-$\underline{CH_2}$-CH_2).

11a: ^{1}H NMR: δ = 8.48 (1H, t, *J* = 5.7 Hz, NH), 8.20 + 7.52 (2H each, d each, *J* = 8.8 Hz, aromatic CH), 4.40 (2H, d, *J* = 5.8 Hz, $\underline{CH_2}$NH), 2.25 + 2.23 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 1.77 (2H, quint., *J* = 7.3 Hz, CH_2-$\underline{CH_2}$-CH_2).

12a: ^{1}H NMR: δ = 8.30 (1H, t, *J* = 5.7 Hz, NH), 7.28 (5H, m, aromatic CH), 4.26 (2H, d, *J* = 5.7 Hz, $\underline{CH_2}$NH), 2.24 + 2.19 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 1.76 (2H, quint., *J* = 7.3 Hz, CH_2-$\underline{CH_2}$-CH_2).

13a: ^{1}H NMR: δ = 8.22 (1H, d, *J* = 7.5, NH), 7.30 (5H, m, Ar), 4.93 (1H, quint, J = 7.3, $\underline{CH}$NH), 2.21 + 2.17 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.73 (2H, br quint, *J* = 7.2 Hz, CH_2-$\underline{CH_2}$-CH_2), 1.34 (3H, d, *J* = 7.2 Hz, CH_3).

14a: ^{1}H NMR: δ = 8.15 (1H, d, *J* = 8.1 Hz, NH), 7.20 (4H, m, aromatic CH), 5.29 (1H, br q, *J* = 7.9 Hz, $\underline{CH}$NH), 2.84 (2H, m, $\underline{CH_2}CH_2$CHNH), 2.38 + 1.73 (1H each , m each, $CH_2\underline{CH_2}$CHNH), 2.26 (4H, br t, *J* = 7.4 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$, *J* = 7.3 Hz, CH_2-$\underline{CH_2}$-CH_2).

15a: ^{1}H NMR: δ = 7.72 (1H, d, *J* = 6.6 Hz, NH), 3.98 (1H, br sextet, *J* = 6.6 Hz, $\underline{CH}$NH), 2.25 (4H, br t, *J* = 7.4 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 1.76 (2H, br quint., *J* = 7.3 Hz, CH_2-$\underline{CH_2}$-CH_2).

16a: ^{1}H NMR: δ = 8.01 (1H, d, *J* = 7.9 Hz, NH), 4.20 (1H, br sextet, *J* = 7.9 Hz, $\underline{CH}$NHCO), 2.19 + 1.72 (12H total, m, aliphatic CH_2 and glutaric CH_2).

17a: ^{1}H NMR: δ = 7.61 (1H, d, *J* = 7.7 Hz, NH), 3.56 (1H, m, $\underline{CH}$NHCO), 2.30 – 1.10 (16H, several m, aliphatic CH_2 and glutaric CH_2).

18a: ^{1}H NMR: δ = 7.37 (5H, m, ArH), 5.11 (2H, s, CH_2O), 2.41 + 2.28 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}CH_2\underline{CH_2}$CO), 1.78 (2H, quint., *J* = 7.3 Hz, CH_2-$\underline{CH_2}$-CH_2).

19a: ^{1}H NMR: δ = 7.31 (5H, m, Ar), 5.78 (1H, q, *J* = 7.3 Hz, CH), 2.68 + 2.35 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.84 (2H, quint, *J* = 7.4 Hz, CH_2-$\underline{CH_2}$-CH_2), 1.43 (3H, d, *J* = 7.3 Hz, CH_3).

20a: ^{1}H NMR: δ = 7.45 (5H, m, Ar), 5.99 (1H, s, CH), 3.67 (3H, s, OCH_3), 2.50 + 2.33 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.82 (2H, quint, *J* = 7.4 Hz, CH_2-$\underline{CH_2}$-CH_2).

21a: ^{1}H NMR: δ = 8.67 (1H, d, *J* = 7.1 Hz, NH), 7.39 (5H, m, Ar), 5.42 (1H, d, *J* = 7.1 Hz, $\underline{CH}$NH), 3.64 (3H, s, OCH_3), 2.24 (4H, br t, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.74 (2H, br quint, *J* = 7.4 Hz, CH_2-$\underline{CH_2}$-CH_2).

Glutarylamino Acids of Table 2

***N*-Glutarylglycine methyl ester:** ^{1}H NMR: δ = 8.24 (1H, t, *J* = 6.0 Hz, NH), 3.82 (2H, d, *J* = 6.0 Hz, $\underline{CH_2}$NH), 3.64 (3H, s, OCH_3), 2.25 (4H, br t, *J* = 7.4 Hz, $COCH_2$), 1.73 (2H, br quint, *J* = 7.4 Hz, $CH_2\underline{CH_2}CH_2$).

***N*-Glutaryl-β-alanine methyl ester:** ^{1}H NMR: δ = 7.88 (1H, br t, NH), 3.61 (3H, s, OCH_3), 3.31 (2H, m, $\underline{CH_2}$NH), 2.46 (2H, t, *J* = 6.9 Hz, $\underline{CH_2}CH_2$NH), 2.21 + 2.09 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.73 (2H, br quint, *J* = 7.4 Hz, $CH_2\underline{CH_2}CH_2$).

L- or D-*N*-glutarylalanine methyl ester: ^{1}H NMR: δ = 8.22 (1H, d, *J* = 7.0 Hz, NH), 4.24 (1H, dq, *J* = 7.3 Hz, $\underline{CH}$NH), 3.63 (3H, s, OCH_3), 2.26 + 2.15 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.72 (2H, br quint, *J* = 7.4 Hz, $CH_2\underline{CH_2}CH_2$), 1.27 (3H, d, *J* = 7.3 Hz, CH_3CH).

L- or D-*N*-glutarylalanine: ^{1}H NMR: δ = 8.09 (1H, d, *J* = 7.3 Hz, NH), 4.19 (1H, dq, *J* = 7.3 Hz, $\underline{CH}$NH), 2.23 + 2.15 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.72 (2H, br quint, *J* = 7.4 Hz, $CH_2\underline{CH_2}CH_2$), 1.26 (3H, d, *J* = 7.3 Hz, CH_3CH).

L- or D-*N*-glutarylphenylalanine methyl ester: ^{1}H NMR: δ = 8.26 (1H, d, *J* = 7.8 Hz, NH), 7.24 (5H, m, Ar), 4.47 (1H, m, $\underline{CH}$NH), 3.61 (3H, s, OCH_3), 3.04 (1H, dd, J_1 = 5.5 Hz, J_2 = 13.7 Hz, CH_2(a)), 2.88 (1H, dd, J_1 = 9.4 Hz, J_2 = 13.8 Hz, CH_2(b)), 2.11 (4H, t, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.72 (2H, br quint, *J* = 7.4 Hz, $CH_2\underline{CH_2}CH_2$).

L- or D-*N*-glutarylphenylalanine: ^{1}H NMR: δ = 8.11 (1H, d, *J* = 7.8 Hz, NH), 7.25 (5H, m, Ar), 4.44 (1H, m, $\underline{CH}$NH), 3.09 (1H, dd, J_1 = 4.9 Hz, J_2 = 13.8 Hz, CH_2(a)), 2.83 (1H, dd, J_1 = 9.7 Hz, J_2 = 13.8 Hz, CH_2(b)), 2.12 + 2.09 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.72 (2H, br quint, *J* = 7.4 Hz, $CH_2\underline{CH_2}CH_2$).

L- or D-*N*-glutarylphenylglycine: ^{1}H NMR: δ = 8.54 (1H, d, *J* = 7.4, NH), 7.39 (5H, m, Ar), 5.34 (1H, d, *J* = 7.4, $\underline{CH}$NH), 2.22 + 2.20 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.73 (2H, br quint, *J* = 7.4 Hz, CH_2-$\underline{CH_2}$-CH_2).

L- or D-*N*-glutarylleucine methyl ester: ^{1}H NMR: δ = 8.17 (1H, d, *J* = 7.6 Hz, NH), 4.28 (1H, m, $\underline{CH}$NH), 3.63 (3H, s, OCH_3), 2.27 + 2.17 (2H each, t each, *J* = 7.4 Hz, CO$\underline{CH_2}$), 1.72 (2H, br quint, *J* = 7.4 Hz, $CH_2\underline{CH_2}CH_2$), 1.70 – 1.40 (3H, m, $(CH_3)_2CH\underline{CH_2}$-), 0.91 + 0.86 (3H each, d each, *J* = 6.3 Hz, $(\underline{CH_3})_2$CH).

L- or D-*N*-glutaryltryptophan methyl ester: ^{1}H NMR: δ = 8.24 (1H, d, *J* = 7.5 Hz, NH), 7.51 + 7.35 (1H each, br d each, *J* = 7.3 Hz), 7.15 (1H, br s), 7.09 + 7.00 (1H each, br t each, *J* = 7.3 Hz, aromatic protons), 4.51 (1H, br q, *J* = 7.8 Hz, $\underline{CH}$NH), 3.52 (3H, s, OCH_3), 3.15 (1H, dd, J_1 = 5.9 Hz, J_2 = 14.5 Hz, CH_2(a)), 2.99 (1H, dd, J_1 = 8.2 Hz, J_2 = 14.5 Hz, CH_2(b)), 2.17 (4H, m, CO$\underline{CH_2}$), 1.68 (2H, br quint, *J* = 7.4 Hz, $CH_2\underline{CH_2}CH_2$).

Relative Rate of Hydrolysis of Compounds in Tables 1 – 3

Table 1: Total volume, 20 mL: 50 mM substrate in H_2O, 0.22 U/mL GA. Reaction solutions were stirred at 25 °C in the automatic titrator vessel maintaining a constant pH value (8.0) by adding 0.1 M NaOH. The rate of hydrolysis of compound **3** (4.4 μmol/min) was taken as 100. Spontaneous hydrolytic rates were evaluated by leaving the reaction solutions in the pH-Stat for 10 minutes before adding the enzyme. The blank relative hydrolytic rates were < 0.1% in all cases, with the exception of compound **20a** which showed a 1.6% relative rate.

Table 2: Total volume, 20 mL: 50 mM substrate in H_2O, 0.22 U/mL GA. Reaction solutions were stirred at 25 °C in the automatic titrator vessel maintaining a constant pH value (8.0) by adding 0.1 M NaOH (same reaction conditions but 0.44 U/mL GA and 0.025 M NaOH were used with *N*-glutaryl-D-alanine (entry 5) and with *N*-glutaryl-D-phenylglycine methyl ester (entry 11). The rate of hydrolysis of compound **3** (4.4 μmol/min) was taken as 100.

Table 3: Total volume, 20 mL: 50 mM substrate in H_2O, 0.55 U/mL GA. Reaction solutions were stirred at 25 °C in the automatic titrator vessel maintaining a constant pH value (8.0) by adding 0.01 M NaOH. The rate of hydrolysis of compound **10a** (0.92 μmol/min) was taken as 100.

Enantioselectivity of GA towards Glutarylamides and Esters (Table 4)

Reactions conditions: 50 mM substrate in H_2O, 2 U/mL GA. Reaction solutions were stirred at 25 °C in the automatic

titrator vessel maintaining a constant pH value (7.0) by adding 0.1 M NaOH. Conversion and product ee were evaluated on a chiral column by HPLC ($\lambda = 254$ nm).

Compound 21a, conversion: Chirobiotic T column, eluent MeOH-TEAA 20:80 (TEAA = H_2O-Et_3N-AcOH, 100:0.1:0.1, pH 4.1), flow rate 0.9 mL/min; product ee: Chiralcel OD column, eluent hexane-*i*-PrOH-Et_2NH, 95:5:0.1, flow rate 0.5 mL/min.

Compound 13a, conversion: Chirobiotic T column, eluent MeOH-TEAA, 10:90, flow rate 1 mL/min; product ee: Chiralcel OD column, eluent hexane-*i*-PrOH-Et_2NH, 95:5:0.1, flow rate 1 mL/min.

Compound 20a, conversion and product ee: Chiralcel OJ column, eluent hexane-*i*-PrOH-HCOOH, 9:1:0.1, flow rate 0.8 mL/min.

Compound 19a, conversion and product ee: Chiralcel OD column, eluent hexane-*i*-PrOH-CF_3COOH, 98:2:0.1, flow rate 0.75 mL/min.

Preparative-Scale Kinetic Resolution of Racemic Glutarates

In a typical experiment 1.5 g (5 mmol) of **21a** were dissolved in 100 mL H_2O. The pH was adjusted to 7.0 and the reaction was started by adding 200 U of GA and monitored by chiral column HPLC (see above), keeping the pH constant by adding 0.1 M NaOH *via* the automatic titrator. The reaction was stopped at 47% conversion (approximately 2 h) by adjusting the pH to 3.0 with AcOH. The solution was extracted with 50 mL AcOEt (3 times) to remove the unreacted **21a** and glutaric acid. The water phase was adjusted to pH 7.0 and lyophilized to give pure L-phenylglycine methyl ester with 95% ee.

References

[1] a) B. Fetching, H. Peter, H. Bickel, E. Visher, *Helv. Chim. Acta* **1968**, *51*, 1109 – 1120; b) R. B. Morin, B. G. Jackson, E. H. Flynn, R. W. Roeske, *J. Am. Chem. Soc.* **1969**, *91*, 1396 – 1400.

[2] a) S. Cambiaghi, S. Tomaselli, R. Verga, *EP Patent* 469,993, **1992**; *Chem. Abstr.* **1992**, *117*, 232217p; b) W. Cabri, R. Verga, S. Cambiaghi, E. Bernasconi, *La Chimica e l'Industria* **1999**, *81*, 461 – 464.

[3] J. A. Brannigan, G. Dodson, H. J. Duggleby, P. C. E. Moody, J. L. Smith, D. R. Tomchick, A. G. Murzin, *Nature* **1995**. *378*, 416 – 419.

[4] Y. Li, J. Chen, W. Jiang, X. Mao, G. Zhao, E. Wang, *Eur. J. Biochem.* **1999**, *262*, 713 – 719.

[5] A. Matsuda, K. Komatsu, *J. Bacteriol.* **1985**, *163*, 1222 – 1228.

[6] a) E. Batistel, D. Bianchi, R. Bortolo, L. Bonoldi, *Appl. Biochem. Biotech.* **1998**, *69*, 53 – 67; b) Y. S. Lee, H. W. Kim, S. S. Park, *J. Biol. Chem.* **2000**, *275*, 39200 – 39206.

[7] Y. S. Lee, S. S. Park, *J. Bacteriol.* **1998**, #180#*17*, 4576 – 4582.

[8] Y. Kim, K.-H. Yoon, Y. Khang, S. Turley, W. G. J. Hol, *Structure* **2000**, *8*, 1059 – 1068.

[9] a) Y. Kim, W. G. J. Hol, *Chem. Biol.* **2001**, *8*, 1253 – 1264; b) Shibuya, K. Matsumoto, T. Fujii, *Agric. Biol. Chem.* **1981**, *45*, 1561 – 1567.

[10] D. Monti, G. Carrea, S. Riva, E. Baldaro, G. Frare, *Biotechnol. Bioeng.* **2000**, *70*, 239 – 244.

[11] S. Raimondi, L. Forti, D. Monti, S. Riva, *Tetrahedron Asymmetry* **2003**, *14*, 1091 – 1094.

[12] S. Biffi, L. De Martin, C. Ebert, L. Gardossi, P. Linda, *J. Mol. Cat. B: Enzymatic* **2002**, *19 – 20*, 135 – 141.

[13] D. T. Guranda, L. M. van Langen, F. van Rantwijk, R. A. Sheldon, V. K. Svedas, *Tetrahedron Asymmetry* **2001**, *12*, 1645 – 1650, and references cited therein.

[14] C.-S. Chen, Y. Fujimoto, G. Girdaukas, C. J. Sih, *J. Am. Chem. Soc.* **1982**, *104*, 7294 – 7299.

[15] a) F. Cantele, A. Restelli, S. Riva, S. Tentorio, M. Villa, *Adv. Synth. Catal.* **2001**, *343*, 721 – 725; b) M. Villa, D. Tentorio, A. Restelli, S. Riva, (Zambon Group SpA), *WO Patent* 00/17384, **2000**; *Chem. Abstr.* **2000**, *132*, 235972b.

[16] a) Y. Ishii, Y. Saito, T. Fujimura, H. Sasari, Y. Noguchi, H. Yamada, M. Niwa, K. Shimomura, *Eur. J. Biochem.* **1995**, *230*, 773 – 778; b) I. Aramori, M. Fukagawa, M. Tsumura, M. Iwami, H. Ono, H. Kojo, M. Kohsaka, Y. Ueda, H. Imanaka, *J. Bacteriol.* **1991**, *173*, 7848 – 7855; c) A. Matsuda, K. Matsuyama, K. Yamamoto, S. Ichikawa, K.-I. Komatsu, *J. Bacteriol.* **1987**, *169*, 5815 – 5820.

[17] K. Fritz-Wolf, K. P. Koller, G. Lange, A. Liesum, K. Sauber, H. Schreuder, W. Aretz, W. Kabsch, *Protein Sci.* **2002**, *11*, 92 – 103.

