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Enantioselective esterase activity of an industrial glutaryl acylase

Sara Adani,[a,b] Stefano Raimondi,[b] Luca Forti,[b] Daniela Monti[a] and Sergio Riva[a,*]

[a]*Istituto di Chimica del Riconoscimento Molecolare (ICRM), C.N.R., Via Mario Bianco 9, 20131 Milano, Italy*
[b]*Dipartimento di Chimica, Università di Modena & Reggio Emilia, Via Campi 183, 41100 Modena, Italy*

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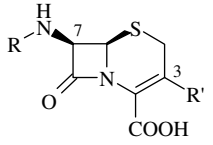
Abstract—The unexpected esterase activity of an industrial glutaryl acylase was investigated. Glutaryl esters of a series of primary and secondary alcohols as well as of phenols were all efficiently hydrolyzed, the only exception being the sterically hindered glutarate of thymol. The enantioselectivities of the acylase, which were evaluated with three of these substrates, were quite low (*E* values ranging between 1.9 and 7.2), but were significantly improved by substrate and/or solvent engineering. Enantiomerically enriched hydrolyzed alcohols and unreacted glutarates can be easily separated by selective extraction, thus avoiding chromatographic steps.

1. Introduction

Glutaryl acylases (GAs) are enzymes industrially exploited for the two-step biocatalyzed production of 7-aminocephalosporanic acid **1** (7-ACA, Scheme 1) from cephalosporin C **2** via glutaryl-7-ACA **3** (Glu-7-ACA).[1] These proteins have been optimized in order to efficiently hydrolyze **3**,[1,2] but it has been shown that they also possess a broad substrate tolerance. Our data[3] along with a few other reports[4] have demonstrated that GAs do require an amide carrying a carboxylated side chain (glutarates are the best substrates, succinates and adipates can also be efficiently hydrolyzed) but are also quite flexible concerning the amine substituent, which can be significantly different from a β-lactam skeleton. As a matter of fact, not only was a series of glutarylated amino acids and glutarylated amines quite efficiently hydrolyzed by an industrial glutaryl acylase commercialized by Recordati SpA (GAR), but this enzyme also showed a significant enantiopreference for the respective L-enantiomers.[3]



1 : R = H ; R' = CH_2OAc (7-ACA)
2 : R = HOOC-$CHNH_2$-$(CH_2)_3$-CO ; R' = CH_2OAc
3 : R = HOOC-$(CH_2)_3$-CO ; R' = CH_2OAc
4 : R = HOOC-CH_2-O-CH_2-CO ; R' = CH_2OAc
5 : R = H ; R' = CH_3 (7-ADCA)
6 : R = HOOC-CH_2-O-CH_2-CO ; R' = CH_3

Scheme 1. Structures of cephalosporinic amides.

Preliminary experiments indicated that GAR was also able to catalyze the enantioselective hydrolysis of glutaryl esters.[3b] Herein, we report the results of a much more detailed investigation on the esterase activity of this enzyme and on the parameters that can influence its enantioselectivity.

2. Results and discussion

A recent paper has described the structure of the active site of a GA from *Pseudomonas diminuta* KAC-1 complexed with **3**.[5] X-ray analysis of the crystallized protein identified three substrate moieties that were specifically recognized by this enzyme: the glutaric chain, the β-lactam nucleus and the acetate substituent in C-3. We have previously shown that only the glutarate side chain (or a similar substituent derived by the condensation of **1** or **5** with the anhydride of a dicarboxylic acid) is really needed by GAR, and we were particularly surprised to measure significant esterase activity on the model glutaryl ester **8a**.[3]

* Corresponding author. Tel.: +39 02 2850 0032; fax: +39 02 2890 1239; e-mail: sergio.riva@icrm.cnr.it

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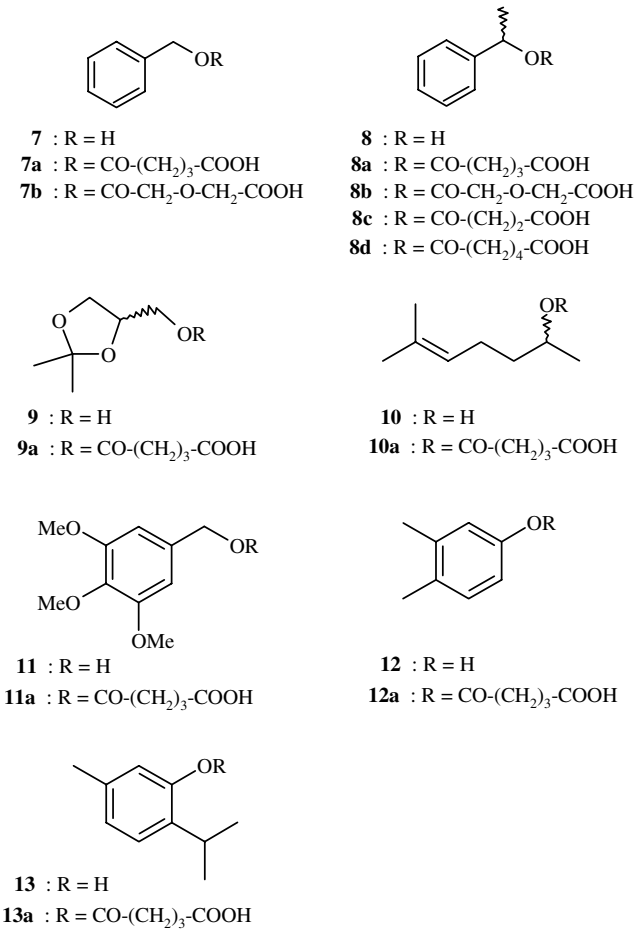
In order to obtain a better understanding of this new GAR hydrolytic activity, a series of glutaryl esters **7a**–**13a** (Scheme 2) was synthesized. The benzylic alcohol derivative **7a** was chosen as a reference compound, its initial rate of hydrolysis being 15% that of the 'natural' cephalosporinic substrate **3**. Table 1 reports the relative initial reaction rates of compounds **7a**–**13a**: glutaryl esters of primary **7a**, **9a**, **11a** and secondary alcohols **8a** and **10a** as well as of phenols **12a** were all efficiently hydrolyzed, the only exception being the sterically hindered glutarate of thymol **13a**.



Scheme 2. Structures of compounds **7**–**13**.

Table 1. GAR-catalyzed hydrolysis of glutaryl, diglycoyl, succinoyl, or adipoyl esters

Compound	Relative rate[a]	*E*-values[b]
7a	100	
8a	33	7.2
9a	112	3.2
10a	16	1.9
11a	243	
12a	262	
13a	0	
7b	83	
8b	33	5.4
8c	3	18.5
8d	10	12.4

[a] Reactions conditions: see Experimental part.

[b] See Ref. 6. Reactions were monitored by chiral HPLC (compounds **8a**–**d**) or chiral GC (compounds **9a** and **10a**) in order to evaluate the respective ee and degrees of conversion (*c*) values (for details see Experimental).

Three of these substrates **8a**–**10a** were racemates and therefore GAR enantioselectivity was also evaluated, although the obtained *E*-values[6] were quite low (between 1.9 and 7.2, Table 1).

Several methods are currently available to improve the enantioselectivity of a hydrolytic enzyme, most of them exploiting molecular biology and, specifically, random mutagenesis techniques.[7] Another approach is based on the optimization of the reaction conditions via the so-called 'substrate engineering'[8] or 'medium engineering'.[8,9] Considering the 'substrate engineering' first, alcohol **8** was condensed with three different dicarboxylic anhydrides (diglycolic,[10] succinic and adipic[11] anhydrides) and the corresponding esters **8b**–**d** submitted to GAR action. As shown in Table 1, the hydrolysis rates of these compounds were lower in comparison with **8a**, but the *E*-values did improve, particularly with succinyl derivative **8c**.[†]

In the next series of experiments, water miscible organic cosolvents were added to the reaction mixtures ('solvent engineering'), as in the past it had been demonstrated that they can significantly influence the enantioselectivity of hydrolytic enzymes.[8,12] Since, to the best of our knowledge, no data are available on the effect of organic cosolvents on GAs' activity, initially the hydrolysis of the glutaryl ester of benzylic alcohol **7a** was studied in the presence of various amounts (v/v) of different cosolvents. As shown in the first two columns of Table 2, with the exception of acetonitrile all the solvents were well-tolerated when used at 20% v/v, whereas GAR performances were strongly affected by the presence of higher amounts (40% v/v) of organic modifiers, the activity being reduced to 25% in the best case. The kinetic resolution of **8a** was then performed in the presence of 20% v/v of the best cosolvents, and, as shown in the last column of Table 2, a significant positive effect on the enantioselectivity of the enzyme was observed.

In the last experiment, the hydrolysis of the best substrate (the succinyl derivative **8c**, $E = 18.5$, Table 1) was performed in the presence of 20% v/v MeOH (the best cosolvent for the hydrolysis of **8a**). However, it was found that the positive effect of these two

[†] As a diglycolic anhydride has never been used before for these kind of reactions, we decided to gain some additional information on the derivative of this dicarboxylic anhydride. Acylation of 7-ACA **1** and 7-ADCA **5** was quite neat with the corresponding amide derivatives **4** and **6** (Scheme 1) hydrolyzed by GAR with similar relative rates, 7.2% and 7.7%, respectively, in comparison with **3**. As diglycolic anhydride is a quite reactive acylating agent, it might become a useful alternative to glutaric anhydride for the GAR-catalyzed kinetic resolutions of racemic amides.

Table 2. GAR-catalyzed hydrolysis of glutaryl ester **7a** and **8a** in the presence of organic cosolvents

Cosolvent	**7a**, Relative rates[a]		**8a**, Relative rates[a]	*E*-values[b]
	20% v/v	40% v/v	20% v/v	
—	100	100	100	7.2
MeOH	89	17	84	15.8
i-PrOH	71	5	58	7.6
Acetone	76	7	80	11.6
Dioxane	41	n.d.	61	5.9
DMSO	93	10	61	11.6
DMF	106	26	62	12.5
Acetonitrile	3	n.d.	n.d.	n.d.

[a] Reactions conditions: see Experimental.
[b] See Ref. 6. Reactions were monitored by chiral HPLC.

parameters was not additive, as the measured *E* value was only 9.8.

Reactions with compounds **8a** (in the presence of 20% v/v MeOH), **8c** and **8d** were scaled up (Table 3). The transformations of **8a** and **8d** were found to be even more enantioselective than in the small scale experiments, while the data obtained with **8c** were worse than expected. A possible explanation might be related to the longer reaction times needed with this substrate, which in the meantime might suffer concomitant aspecific spontaneous chemical hydrolysis. In all cases, the alcohol product and the residual unreacted esters could be separated by selective extraction from the aqueous reaction solutions, avoiding chromatographic or distillation steps during work-up.

Table 3. Preparative scale GAR-catalyzed hydrolysis of **8a**, **8c**, and **8d**[a]

Substrate	Cosolvent	Reaction time (h)	Conv.	ee_P	ee_S	*E*-values[b]
8a	20% v/v MeOH	8	48.7	79.5	75.6	19.8
8c	—	71	50.9	65.2	67.7	9.4
8d	—	23	39.8	80.4	53.1	15.5

[a] Reactions conditions: see Experimental.
[b] See Ref. 6. Reactions were monitored by chiral HPLC.

3. Conclusion

GAR is an industrial enzyme that has been developed for the efficient hydrolysis of a specific substrate, namely Glu-7-ACA **3**. Due to practical and scientific interests in this group of enzymes, an increasing number of GAs have become available[13] and, moreover, wild-type enzymes have been modified by protein engineering (site-directed and/or random mutagenesis) in order to better understand their catalytic mechanism and to alter their substrate specificity.[14] Herein, we have shown that an industrial GA possesses a significant esterase activity and that its enantioselectivity can be modulated by substrate and solvent engineering. All this information allowed us to conclude that GAs might be ideal targets for further studies, combining random mutagenesis techniques and solvent and/or substrate optimization to significantly improve the performances of a biocatalyst in terms of enantioselectivity.

4. Experimental

4.1. Materials and methods

Glutaryl Acylase (GAR) and a sample of glutaryl-7-ACA **3** were a gift from Recordati S.p.A. (Opera, MI, Italy). All other reagents and solvents were from Aldrich. TLC: precoated silica gel 60 F_{254} plates (Merck). Flash chromatography: silica gel 60 (70–230 mesh, Merck).

Hydrolytic reactions were monitored at 25 °C using a 718 STAT Titrino automatic titrator (Metrohm Ltd). HPLC analyses: Jasco HPLC instrument (model 880-PU pump, model 870-UV/vis detector, λ: 200 nm) and a Lichrospher 100 RP-18 (5 μm, Merck) reverse phase analytical column or a Chiralcel OD column. GC analyses: Hewlett Packard 5890 series II instrument and a capillary chiral column (DMePentilBETACDX column, 25 m × 0.25 mm ID × 0.15 μm film thickness, MEGA). ^{1}H spectra at 200 MHz were recorded on a Bruker DPX-200.

4.2. Synthesis of glutaryl and diglycoyl amides 4 and 6

7-ACA or 7-ADCA (5 mmol) was dissolved in 20 mL of 1 M $NaHCO_3$. The anhydride (1 equiv) was dissolved in 5 mL of acetone and the two solutions mixed and left to react for 3 h (TLC: *n*-BuOH–AcOH–H_2O = 6:2:2). Acetone was evaporated, the water solution acidified to pH 1.5 with 1 M HCl and extracted three times with 100 mL of EtOAc. The organic layer was evaporated and the solid residue washed on a Buchner funnel with 20 mL of EtOAc and then dried. Products structures were confirmed by ^{1}H NMR. Compound **4** (200 MHz, DMSO-d_6) δ: 8.72 (1H, d, NH); 5.71 (1H, dd, $J_1 = 8.4$ Hz, $J_2 = 5.0$ Hz, NH–*CH*); 5.11 (1H, d, $J = 5.0$ Hz, *CH*–S); 4.99 and 4.68 (1H each, d each, $J = 18.0$ Hz, CH_2–O); 4.17 and 4.12 and 4.09 (2H each, s each, CH_2COOH, CH_2CONH, CH_2O); 2.07 (3H, s, CH_3). Compound **6** (200 MHz, DMSO-d_6) δ: 8.63 (1H, d, NH); 5.60 (1H, dd, $J_1 = 8.6$ Hz, $J_2 = 4.6$ Hz, NH–*CH*); 5.05 (1H, d, $J = 4.6$ Hz, *CH*–S); 4.12 and 4.09 (2H each, s each, CH_2COOH, CH_2CONH); 3.54 and 3.36 (1H each, d each, $J = 18.1$ Hz, CH_2–S); 2.02 (3H, s, CH_3).

4.3. Synthesis of glutaryl, diglycoyl, succinyl, and adipoyl esters 7a–14a, 8b–d

The respective alcohol **7**–**14** (5 mmol) was dissolved in 10 mL dioxane. Glutaryl anhydride (1 equiv) was dissolved in 10 mL of dioxane and the two solutions then mixed and allowed to react either at 70 °C or by microwave irradiation (TLC: appropriate mixture of hexane–EtOAc–MeOH). The solvent was evaporated, the residue redissolved in EtOAc and the products extracted with a 5% w/v $NaHCO_3$ solution. The water solution was acidified to pH 1.5 with 2 M HCl and extracted

three times with 50 mL EtOAc. The organic layer was evaporated and the residue either used as it was or, in case of contamination with unreacted glutaryl anhydride, purified by flash chromatography. Product structures were confirmed by ^{1}H NMR (200 MHz, DMSO-d_6): **7a**, δ: 7.45 (5H, m, ArH); 5.09 (2H, s, CH_2O); 2.39 (2H, t, $J = 6.9$ Hz, ROOC-CH_2); 2.25 (2H, t, $J = 6.9$ Hz, CH_2COOH); 1.76 (2H, m, $J = 6.9$ Hz, $CH_2CH_2CH_2$). Compound **8a**, δ: 7.35 (5H, m, ArH); 5.86 (1H, q, $J = 6.7$ Hz, CH); 2.36 (2H, t, $J = 7.2$ Hz, ROOC-CH_2); 2.23 (2H, t, $J = 7.2$ Hz, CH_2-COOH); 1.73 (2H, quintet, $J = 7.2$ Hz, $CH_2CH_2CH_2$); 1.45 (3H, d, $J = 6.7$ Hz, CH_3). Compound **9a**, δ: 4.22 (1H, dq, $J_1 = 6.0$ Hz, $J_2 = 4.2$ Hz, H-4); 4.10 (1H, dd, $J_1 = 11.4$ Hz, $J_2 = 4.1$ Hz, CH_aO); 4.00 (1H, dd, $J_1 = 8.4$ Hz, $J_2 = 6.5$ Hz, H-5a); 3.99 (1H, dd, $J_1 = 11.4$ Hz, $J_2 = 6.0$ Hz, CH_bO); 3.65 (1H, dd, $J_1 = 8.4$ Hz, $J_2 = 6.1$ Hz, H-5b); 2.35 (2H, t, $J = 7.5$, ROOC-CH_2); 2.25 (2H, t, $J = 7.4$, CH_2COOH); 1.74 (2H, m, CH_2CH_2-CH_2); 1.26 e 1.31 (3H each, s each, CH_3). Compound **10a**, δ: 5.07 (1H, m, H-5); 4.77 (1H, m, H-2); 2.27 (2H, t, $J = 7.2$ Hz, ROOC-CH_2); 2.24 (2H, t, $J = 7.2$ Hz, CH_2COOH); 1.95 and 1.48 (2H each, m each, CH_2-3 and CH_2-4); 1.72 (2H, m, $CH_2CH_2CH_2$); 1.64 and 1.55 (3H each, br s each, CH_3-7 and CH_3-8); 1.14 (3H, d, $J = 6.3$ Hz, CH_3-1). Compound **11a**, δ: 6.67 (2H, s, ArH); 5.00 (2H, s, CH_2O); 3.76 (6H, s, *m*-(CH_3O)); 3.64 (3H, s, *p*-CH_3O); 2.40 (2H, t, $J = 7.4$ Hz, ROOCCH_2); 2.26 (2H, t, $J = 7.4$ Hz, CH_2COOH); 1.76 (2H, m, $J = 7.4$ Hz, $CH_2CH_2CH_2$). Compound **12a**, δ: 7.13 (1H, d, $J = 8.2$ Hz, H-5); 6.89 (1H, d, $J = 2.3$ Hz, H-2); 6.82 (1H, dd, $J_1 = 8.2$ Hz, $J_2 = 2.3$ Hz, H-6); 2.58 (2H, t, $J = 7.3$ Hz, ROOCCH_2); 2.33 (2H, t, $J = 7.3$ Hz, CH_2COOH); 2.20 (6H, br s, ArCH_3); 1.84 (2H, m, $J = 7.3$ Hz, $CH_2CH_2CH_2$). Compound **13a**, δ: 7.22 (1H, d, $J = 8.6$ Hz, H-3); 7.12 (1H, br d, $J = 8.6$ Hz, H-4); 6.83 (1H, br s, H-6); 2.89 (1H, m, $CH(CH_3)_2$); 2.64 (2H, t, $J = 7.4$ Hz, ROOC-CH_2); 2.34 (2H, t, $J = 7.4$ Hz, CH_2COOH); 2.25 (3H, s, CH_3-5); 1.86 (2H, m, $J = 7.4$ Hz, $CH_2CH_2CH_2$); 1.11 (6H, d, $J = 7.4$ Hz, $CH(CH_3)_2$).

The esters **8b–d** were prepared with similar protocols. Compound **8b**, δ: 7.37 (5H, m, ArH); 5.87 (1H, q, $J = 5.7$ Hz, CH); 4.23 (2H, s, ROOC-CH_2O); 4.08 (2H, s, OCH_2COOH); 1.49 (3H, d, $J = 5.7$ Hz, CH_3). Compound **8c**, δ: 7.32 (5 H, m, ArH); 5.79 (1H, q, $J = 7.0$, CH); 2.51 (4H, m, CH_2–COOR); 1.44 (3H, d, $J = 7.0$, CH_3); Compound **8d**, δ: 7.30 (5H, m, ArH); 5.89 (1H, q, $J = 6.5$, CH); 2.34 (4H, m, CH_2COOR); 1.68 (4H, m, CH_2CH_2), 1.52 (3H, d, $J = 6.5$ Hz, CH_3).

4.4. Relative rates of hydrolysis of 3, 4, 6 and of the compounds in Table 1

Compounds **3**, **4**, and **6**: Total volume, 10 mL: 50 mM substrate in H_2O, 1 U/mL GAR (1 Unit is defined as the amount of GAR that hydrolyzes 1 μmol of **3** per minute at pH 8.0 and at 25 °C. The specific activity of the GAR sample used herein was 2.3 U/mg). Reaction solutions were stirred at 25 °C in an automatic titrator maintaining a constant pH value (8.0) by adding 0.1 M NaOH. Experiments were repeated in duplicate at least. The initial rates of hydrolysis were calculated from the amount of NaOH solution added in the time unit. The initial rate of hydrolysis of compound **3** (10.0 μmol/min) was taken as 100.

Table 1: Total volume, 10 mL: 50 mM substrate in H_2O, 1 U/mL GAR. Reaction solutions were stirred at 25 °C in an automatic titrator maintaining a constant pH value (7.0) by adding 0.1 M NaOH. The initial rates of hydrolysis were calculated from the amount of NaOH solution added in the time unit. Experiments were repeated in duplicate at least. The initial rate of hydrolysis of compound **7a** (1.5 μmol/min) was taken as 100.

Table 2: Total volume, 10 mL; organic cosolvent 20% or 40% v/v; 50 mM substrate, 1 U/mL GAR. Reaction solutions were stirred at 25 °C in an automatic titrator maintaining a constant pH value (7.0) by adding 0.1 M NaOH. The initial rates of hydrolysis were calculated from the amount of NaOH solution added in the time unit. Experiments were repeated in duplicate at least. The initial rates of hydrolysis of compounds **7a** (1.5 μmol/min) and **8a** (0.6 μmol/min) were taken as 100%, respectively.

4.5. Enantioselectivity of GA towards racemic esters (Tables 2 and 3)

Conversion degrees and ee of the hydrolysis of compounds **8a–d** were evaluated by chiral column HPLC (λ 254 nm) using a Chiralcel OD column, eluent: hexane–*i*PrOH–CF_3COOH 98:2:0.1, 0.75 mL/min (**8b–d**). Retention times (min) at 0.75 mL/min flow rate: (***R***)-**8**: 25.08, (***S***)-**8**: 33.42; (***S***)-**8a**: 37.17, (***R***)-**8a**: 33.42. Retention times (min) at 0.75 mL/min; flow rate: (***R***)-**8**: 17.08, (***S***)-**8**: 22.08; (***S***)-**8b**: 16.58, (***R***)-**8b**: 21.46; (***R***)-**8c**: 26.42, (***S***)-**8c**: 33.08; (***R***)-**8d**: 25.96, (***S***)-**8d**: 34.21.

The ee_{Prod} of compounds **9** and **10** was evaluated by chiral GC (DMePentil-BETACDX column). Compound **9** (previously acetylated): init. *T*: 60 °C; init. time: 30 min; rate: 0.5 °C/min; final *T*: 120 °C; ret. times: 25.3 and 28.6 min. Compound **10**: init. *T*: 50 °C; init. time: 20 min; rate: 1 °C/min; final *T*: 200 °C; ret. times: 20.6 and 21.9 min.

4.6. Preparative-scale kinetic resolution of racemic glutarates (Table 3)

In a typical experiment 800 mg (3.4 mmol) of **8a** was dissolved in 40 mL of a 4:1 mixture of H_2O–MeOH. The pH was adjusted to 7.0 and the reaction was started by adding 100 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 48.7% conversion (approximately 8 h). The solvent was partially evaporated to eliminate most of the MeOH and the remaining solution was extracted with 50 mL EtOAc (two times) to remove the product **8** (79.5 ee, 25% yields). The water phase was adjusted to pH 3.0 with 2 M HCl and extracted with 50 mL EtOAc (three times) to recover unreacted **8a** (75.6 ee, 43% yields).

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