Influence of Substrate Structure on PGA-Catalyzed Acylations. Evaluation of Different Approaches for the Enzymatic Synthesis of Cefonicid

Marco Terreni,[a,*] Joseph Gapesie Tchamkam,[a] Umberto Sarnataro,[b,d] Silvia Rocchietti,[b] Roberto Fernández-Lafuente,[c] José M. Guisán[c]

[a] Pharmaceutical Biocatalysis Laboratories, Dipartimento di Chimica Farmaceutica, Via Taramelli 12, Università degli Studi, 27100 Pavia, Italy
Phone: (+39)-382-987265, fax: (+39)-382-987589, e-mail: markt@pbl.unipv.it
[b] Innovate Biotechnology s.r.l., Parco Scientifico Tecnologico, Strada Savonesa 9, 15050 Rivalta Scrivia (AL), Italy
[c] Departamento de Biocatálisis, Instituto de Catálisis-CSIC Campus UAM, Cantoblanco, 28049 Madrid, Spain
[d] Present address: Merck Sharp & Dohme Italia, Via Emilia 21, 27100 Pavia, Italy

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Abstract: The influence of the substrate structure on the catalytic properties of penicillin G acylase (PGA) from *Escherichia coli* in kinetically controlled acylations has been studied. In particular, the affinity of different β-lactam nuclei towards the active site has been evaluated considering the ratio between the rate of synthesis (v_s) and the rate of hydrolysis of the acylating ester (v_{h1}). 7-Aminocephalosporanic acid (7-ACA) and 7-amino-3-(1-sulfomethyl-1,2,3,4-tetrazol-5-yl)thiomethyl-3-cephem-4-carboxylic acid (7-SACA) showed a good affinity for the active centre of PGA. The enzymatic acylation of these nuclei with *R*-methyl mandelate has been studied in order to evaluate different approaches for the enzymatic synthesis of cefonicid. The best results have been obtained in the acylation of 7-SACA. Cefonicid (**8**) was recovered from the reaction mixture as the disodium salt in 65% yield and about 95% of purity. Furthermore, through acylation of 7-ACA, a "one-pot" chemo-enzymatic synthesis was carried out starting from cephalosporin C using three enzymes in sequence: D-amino acid oxidase (DAO), glutaryl acylase (GA) and PGA. Cefonicid disodium salt was obtained in three steps, avoiding any intermediate purification, in 35% overall yield and about 94% purity. This approach presents several advantages compared with the classical chemical processes.

Keywords: D-amino acid oxidase; biotransformations; cefonicid; enzyme catalysis; glutaryl acylase; penicillin G acylase

Introduction

Cefazolin, cefamandole and cefonicid are cephalosporins of clinical relevance.[1] These compounds are currently synthesized[2–4] through the cleavage of cephalosporin C to give 7-aminocephalosporanic acid (7-ACA) that is then reacted with the appropriate S-nucleophile affording 7-amino-3-(1-methyl-1,2,3,4-tetrazol-5-yl)thiomethyl-3-cephem-4-carboxylic acid (7-TACA), 7-amino-3-(1-sulfomethyl-1,2,3,4-tetrazol-5-yl)thiomethyl-3-cephem-4-carboxylic acid (7-SACA) or 7-amino-3-(5-methyl-1,3,4-thiadiazol-2-yl)thiomethyl-3-cephem-4-carboxylic acid (7-ZACA). Finally, the chemical acylation of the C-7 amino group gives, respectively, cefamandole, cefonicid or cefazolin (Scheme 1). However, this multi-step process requires the use of toxic reagents and solvents, the purification of the intermediates and very low reaction temperatures ($-40\,^{\circ}C$). To partially avoid these drawbacks, the use of enzymatic catalysts such as penicillin G acylase (PGA) from *Escherichia coli* has been considered by many authors. For example, the enzymatic *N*-acylations of 7-TACA with *R*-methyl mandelate (MAME, **1**)[5] and of 7-ZACA with tetrazol-1-yl-2-acetic acid methyl ester (TAME, **2**)[6] catalyzed by this enzyme have been reported for the syntheses of cefamandole and cefazolin, respectively. However, this approach only solves the problems relevant to the *N*-acylation of the 3′-functionalized nuclei and, besides, the yields are often not competitive with the chemical process. To the best of our knowledge, no reports have been published so far describing the synthesis of cefonicid by enzymatic *N*-acylation of 7-SACA.

The catalytic properties of the PGA from *E. coli* strongly depend on changes induced by the adsorption of the acyl donor, as suggested by crystallographic analyses,[7] as well as by the different behaviour of phenylacetic acid analogues used in the acylation reactions.[8,9] Besides, also the β-lactam nucleus used as substrate can

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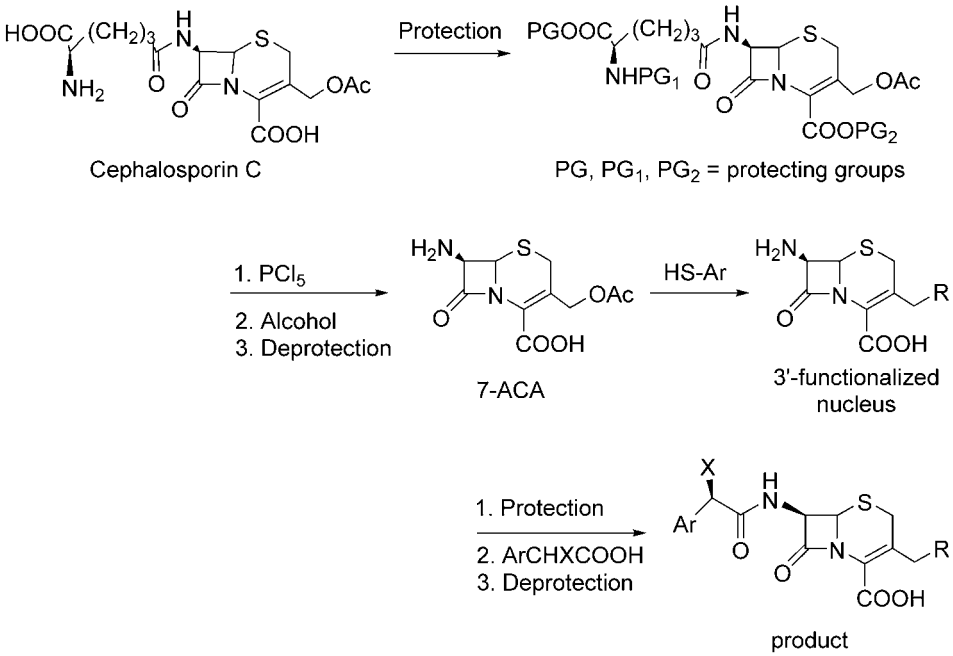
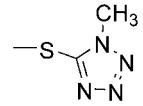
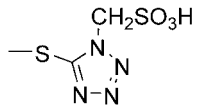
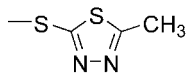
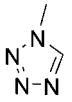


3'-functionalized nucleus	R	Ar	X	product
7-TACA		Ph	OH	Cefamandole
7-SACA		Ph	OH	Cefonicid
7-ZACA			H	Cefazolin

Scheme 1.

affect the catalytic properties of this enzyme, for example, 7-ACA has been reported to be a better substrate than 6-aminopenicillanic acid[10] or 7-ZACA.[11]

Considering the better affinity of 7-ACA for the PGA active site, as well as its higher water solubility compared with 7-ZACA (the cefazolin nucleus), a new approach has been proposed for the synthesis of cefazolin. 7-ACA was prepared by enzymatic cleavage of cephalosporin C, catalyzed by D-amino acid oxidase (DAO) and glutaryl acylase (GA) followed by PGA-catalyzed *N*-acylation of this nucleus with TAME (**2**) to obtain cefazolin.[11] This approach presents several advantages compared with the classical chemical synthesis. The substrate specificity of the enzymes allowed us to prepare cefazolin without any intermediate isolation or purification step. All reactions were carried out in fully aqueous medium and mild conditions. Similarly, cefamandole was synthesized by *N*-acylation of 7-ACA with MAME (**1**).[12]

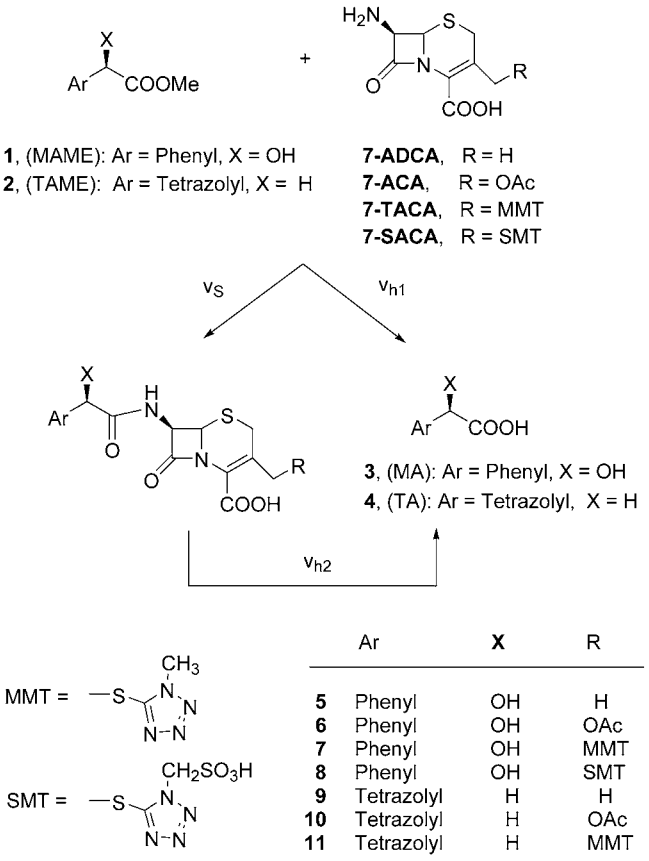
In this work, the properties of PGA from *E. coli* have been investigated in the kinetically controlled *N*-acylation of different cephalosporanic nuclei with MAME (**1**) and TAME (**2**). The influence of the substrate structure on the enzymatic properties has been evaluated. In particular, the enzymatic *N*-acylations of 7-SACA and 7-ACA with **1** have been studied to exploit different approaches for the synthesis of cefonicid. In fact, the enzymatic synthesis of this cephalosporin has been studied considering both the *N*-acylation directly performed on the isolated pure 7-SACA and the synthesis performed *via* PGA-catalyzed *N*-acylation of 7-ACA according to the "multienzymatic approach" previously developed for cefazolin and cefamandole.[11,12]

Results and Discussion

Influence of the Substrate Structures on the Catalytic Properties of PGA

In the kinetically controlled *N*-acylation, the yields depend on the balance of three different reactions catalyzed by the same enzyme:[5,13] the synthesis (s), the hydrolysis of the activated acyl donor (h_1) and the hydrolysis of the synthesized antibiotic (h_2). To obtain high yields, the values of the ratio between the rate of synthesis and the rates of product (v_s/v_{h2}) and ester (v_s/v_{h1}) hydrolyses, should be as high as possible. Particularly, the v_s/v_{h1} ratio is strictly related to the affinity of the enzyme active centre for the β-lactam nucleus and it defines the yield that could be achieved when the hydrolysis of the reaction product is negligible.[5] This value decreases during the reaction course as far as the nucleus is consumed. At the beginning, the concentration of the acylation product is very low and its hydrolysis can be discarded. Thus, from the initial v_s/v_{h1} ratio, the percentage of activated acyl donor transformed into the acylation product can be evaluated (synthesis %).

Different cephalosporanic nuclei have been studied (Scheme 2) in order to evaluate the influence that the nature of the substituent in the 3′-position exerts on the substrate's affinity towards the PGA active site.



	Ar	X	R
5	Phenyl	OH	H
6	Phenyl	OH	OAc
7	Phenyl	OH	MMT
8	Phenyl	OH	SMT
9	Tetrazolyl	H	H
10	Tetrazolyl	H	OAc
11	Tetrazolyl	H	MMT

Scheme 2.

Table 1. Evaluation of synthetase/esterase activities ratio (v_s/v_{h1}): influence of the β-lactam and acyl donor structures.

Acyl donor	Nucleus	v_s[a]	v_s/v_{h1}	Product
1	7-ADCA	0.45	>20	5
1	7-ACA	16	8.2	6
1	7-TACA	19	3.4	7
1	7-SACA	15	11.7	8
2	7-ADCA	0.2	12.5	9
2	7-ACA	7	7.3	10
2	7-TACA	8	2.1	11

Reaction conditions: $T = 4\,^{\circ}C$; pH 6.5; [β-lactam nucleus]: 50 mM; [acyl donor]: 10 mM.
[a] v_s is expressed as μmol/min/g (of enzyme derivative)

Using esters **1** and **2** (10 mM) for the *N*-acylation of 7-aminodeacetoxycephalosporanic acid (7-ADCA, R = H), 7-ACA (R = OAc), 7-TACA (R = 1-methyl-1,2,3,4-tetrazol-5-mercaptyl) and 7-SACA (R = 1-sulfomethyl-1,2,3,4-tetrazol-5-mercaptyl), compounds **5**–**11** have been synthesized according to Scheme 2. We evaluated the initial v_s/v_{h1} ratio monitoring the acids (**3** and **4**) and the products formation at the beginning of the reaction, as reported in the Experimental Section. In the *N*-acylation of 7-ADCA (50 mM), a very high v_s/v_{h1} value (>20) was obtained in spite of the very low synthetic activity observed with this nucleus (Table 1); the hydrolysis of the acylating ester **1** was, in fact, negligible.

With 7-ACA, 7-TACA and 7-SACA, higher synthetic activities but lower initial v_s/v_{h1} values were observed in comparison with 7-ADCA. For example, the v_s/v_{h1} for 7-ACA was 8.2 and 3.4 for 7-TACA, although with both nuclei the synthetic activity was remarkable (15–20 μmol min^{-1}/g of enzyme derivative). A similar trend was observed in the *N*-acylations performed with ester **2** (Table 1). In particular, the initial v_s/v_{h1} ratios observed with the different substrates decreased from 12.5 for 7-ADCA to 7.3 and 2.1 for 7-ACA and 7-TACA, respectively.

In Figure 1 is shown the percentage of synthesis (considered as percentage of ester transformed into the acylation product at the beginning of the reaction) calculated at different concentrations of nucleus, from the initial v_s/v_{h1} values[5] obtained in the *N*-acylation of 7-ADCA, 7-ACA and 7-TACA with ester **1** (10 mM). The results obtained confirm that modifications of the 3′-substituent sensitively affect the affinity of the β-lactam nucleus towards the enzyme active site. The best curve (Figure 1) was obtained with 7-ADCA (having the less hindered substituent, R = H) in the synthesis of compound **5** (complete saturation at 20 mM of nucleus concentration). Similarly, 7-ACA (R = OAc) proved to be a better nucleophile than the 3′-functionalized nucleus (7-TACA).

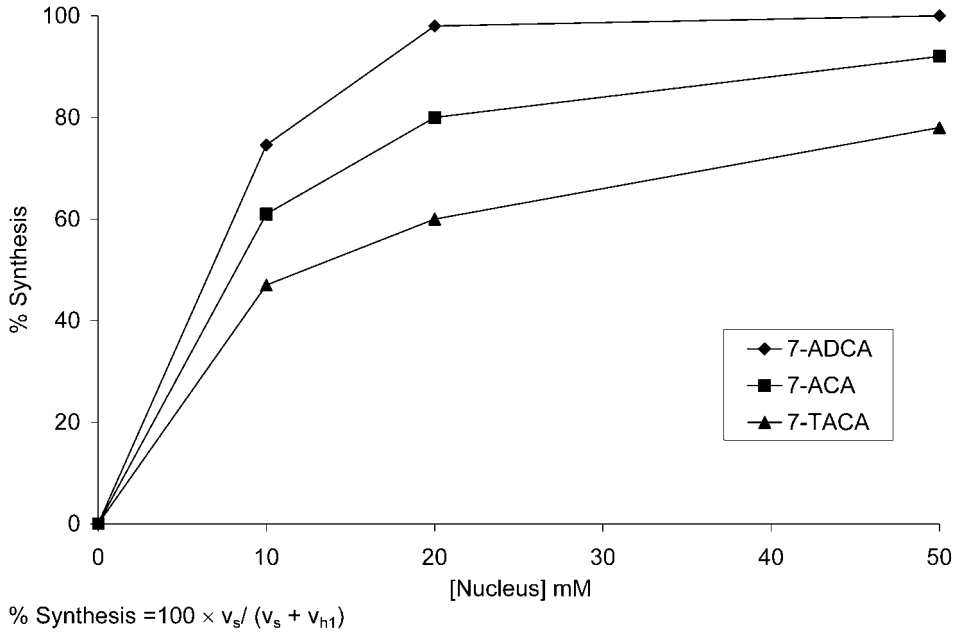


Figure 1. Acylation of 7-ADCA, 7-ACA and 7-TACA at different concentrations (Scheme 2) with **1** (MAME, 10 mM).

When 7-SACA was tested in the *N*-acylation with ester **1** (Table 1) to cefonicid (**8**), the initial v_s/v_{h1} was 11.7, much higher than that resulting with 7-ACA or 7-TACA. The relevant difference of the values observed with 7-TACA and 7-SACA is surprising as these 3′-nuclei differ only in the sulfonic group on the tetrazole ring. Thus, these results seem to support that the changes in the affinity observed cannot be ascribed only to the steric hindrance of the substituent in the 3-position. This conclusion appears to be confirmed by the crystallographic analysis performed on the complex between PGA and penicillin G sulfoxide (PGSO),[14] a non-hydrolyzable substrate analogue.[15] The structure of this complex shows that PGSO is adsorbed in the active site of the enzyme through the β-lactam ring and the carboxylic group of the thiazole ring. The two methyl groups of the thiazole are instead placed on the open side of the active site and seem to have a poor interaction with the protein structure (Figure 2).

The position of these two groups in the penicillanic structure could be considered quite indicative of the spatial disposition of the substituent in the 3 position of simple cephalosporanic substrates such as 7-ADCA. However, considering more complex substrates like the 3′-functionalized cephalosporanic nuclei, further studies should be performed in order to understand the chemical-physical parameters crucial in determining the affinity of these substrates for the PGA active site.

In contrast, the acylating agent (**1** or **2**) seems not to influence the affinity of the β-lactam nucleus for the PGA active centre. Comparing the curves obtained in the *N*-acylation of 7-ACA using esters **1** and **2** (Figure 3), the results were in fact very similar and, in both cases, a complete saturation at the active centre ($v_s/v_{h1} > 20$) was achieved at 100 mM concentration of the nucleus.

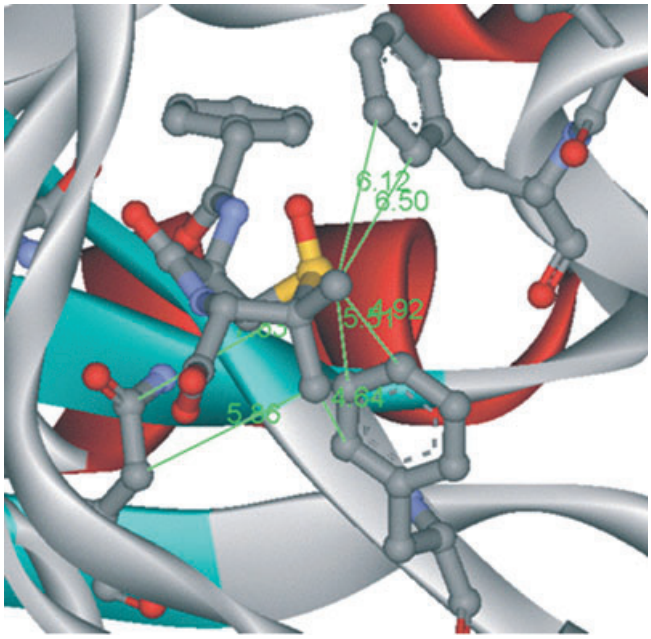


Figure 2. Structure of the complex between PGA and penicillin G sulfoxide (PGSO) obtained using the PDB file 1gm9.[14]

Study of *N*-Acylations for the Enzymatic Synthesis of Cefonicid (8)

Several reactions have been studied using an excess of ester in an attempt to obtain the quantitative transfor-

Table 2. PGA-catalyzed acylation of different β-lactam nuclei.

Nucleus	Ester	Product	pH	MeOH [% v/v]	Conversion [%]
7-ACA	**1**	**6**	6.5	–	71
7-ACA	**2**	**10**	6.5	–	95
7-SACA	**1**	**8**	6.5	–	75
7-ACA	**1**	**6**	6.5	20	80
7-SACA	**1**	**8**	6.5	20	84
7-SACA	**1**	**8**	6.0	30	86
7-SACA	**1**	**8**	5.5	30	88
7-SACA[a]	**1**	**8**	5.5	30	92

Reaction conditions: $T = 4\,^{\circ}C$; [β-lactam nucleus]: 50 mM; [**1**] or [**2**]: 150 mM.
[a] [7-SACA]: 100 mM; [**1**]: 300 mM

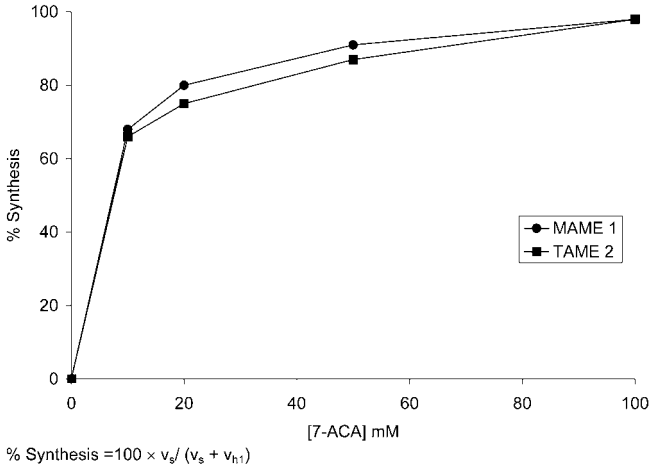


Figure 3. Acylation of 7-ACA at different concentrations with **1** (MAME) and **2** (TAME).

mation of the different nuclei into the corresponding acylated compounds. *N*-Acylation of 7-ACA (50 mM) with esters **1** and **2** (150 mM) afforded different results (Table 2). In the synthesis of **6**, yields were not quantitative because of the high hydrolytic activity shown by the enzyme towards this compound. In fact, when the maximum conversion was achieved (about 70% in 2 hours), the concentration of **6** rapidly decreased (results not shown). On the contrary, the acylation of 7-ACA with **2** gave almost a quantitative conversion (about 95% in 2 hours) as a consequence of the very low hydrolytic activity shown by PGA towards the product **9** (the concentration of this compound being constant after the maximum yield was achieved).

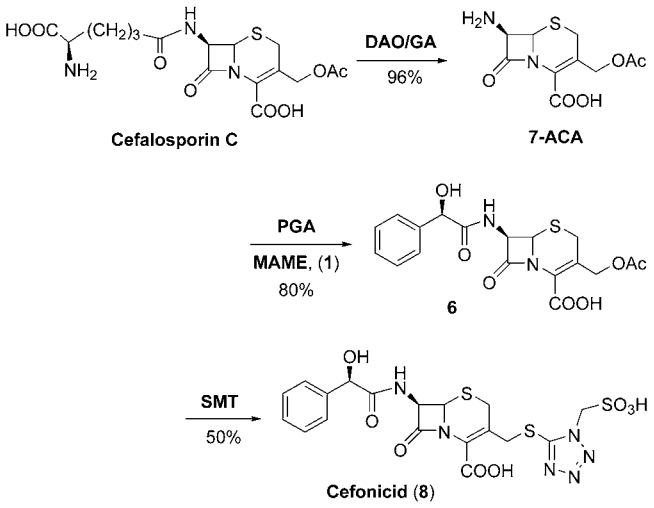
Thus, in the *N*-acylation with **2**, high yields can be achieved, as indicated by the v_s/vh_1 obtained in the conditions used for this synthesis ($v_s/v_{h1} = 5.0$), while in the acylation with **1**, the high hydrolytic activity shown by PGA towards the product makes it very difficult to reach yields as high as those indicated by the v_s/v_{h1} value ($v_s/v_{h1} = 5.5$). For the same reason, also in the reaction of 7-SACA (Table 2) to synthesize **8** (cefonicid), the conversion was not quantitative (76%) although being higher than that obtained with 7-ACA, according to the better affinity of this nucleus toward the active site of PGA.

The *N*-acylations of 7-ACA and 7-SACA have been further studied in order to evaluate different approaches for the enzymatic synthesis of cefonicid (**8**). This compound can be directly obtained by *N*-acylation of 7-SACA (Scheme 2) or, alternatively, using **6** obtained from 7-ACA (Scheme 3) as precursor. Different conditions have been considered to optimize the final yield and the use of PGA covalently immobilized on agarose beads by multipoint attachment ensured the complete stability of the catalyst in a wide range of conditions, in agreement with the results previously reported.[16]

In both cases, the conversion degree improved when methanol was used as co-solvent, according to the effect exerted by this solvent on the catalytic properties of PGA from *E. coli*.[10,17] Thus, in the presence of methanol (20% v/v), the conversion increased from 71% to 79% in the *N*-acylation of 7-ACA, according to the v_s/v_{h1} ratio (from 5.5 to 7.5). Similarly, in the case of 7-SACA, yields improved from 75% to 84% (Table 2).

The high water solubility of 7-SACA allowed us to test more "drastic" reaction conditions than those used with other nuclei in an attempt to optimize the enzymatic *N*-acylation. In this case, on decreasing the pH from 6.5 to 5.5 and increasing the methanol concentration up to 30% (Table 2), the conversion was 88%. On increasing also the concentration of 7-SACA (from 50 mM to 100 mM) the conversion degree further improved up to 92%. In a preparative experiment, cefonicid (**8**) was recovered from the reaction mixture as its disodium salt in 65% yield and about 95% of purity (see Experimental Section).

According to Scheme 3, the "one-pot" synthesis of cefonicid (**8**) was instead performed starting from cephalosporin C by the simultaneous use of immobilized DAO and GA to obtain 7-ACA (96% yield). The enzymatic acylation of this nucleus, catalyzed by PGA, was then performed directly on the crude solution obtained after filtration of DAO and GA using methanol (20%) and a 3:1 molar excess of **1** (150 mM); this reaction afforded **6** in 80% yield. After filtration of the immobilized PGA, the final displacement of the 3-acetoxy group with 1-sulfomethyl 5-mercap-



Scheme 3.

totetrazole (SMT) was directly performed by heating the aqueous solution of **6** at 65 °C (50% of conversion). In a preparative experiment, the final product was isolated from the reaction mixture as its disodium salt (as reported in the Experimental Section) in 35% overall yield and about 93–94% purity. The main impurity in the isolated product was the residual SMT (about 2%).

Conclusion

The results reported in the present work indicate that the nature of the substituent at the C-3′ position of the cephalosporanic nucleus may strongly influence the performance of PGA as catalyst in kinetically controlled acylations. The changes in the affinity observed for the PGA active site towards the different cephalosporanic nuclei seem to be not related to the steric hindrance of the substituent at 3′ but they might be probably ascribed to electronic effects and to the hydrophobicity of the moiety. For not functionalized substrates, such as 7-ADCA, this conclusion can be supported by a qualitative comparison with the structure of the PGA-PGSO complex obtained from crystallographic data.[14] For more complex substrates like the 3′-functionalized cephalosporanic nuclei considered in this work, further studies should be performed in order to understand the chemical-physical parameters that can play a pivotal role in determining their affinity for the PGA active site. In this context, further studies are in progress, including the docking of these nuclei in the PGA active site.

Considering the good affinity of 7-SACA and 7-ACA towards the PGA active site, two approaches have been proposed for the enzymatic synthesis of cefonicid. The first approach was the PGA catalyzed acylation of the 3′-functionalized nucleus that gave the product in good yield and purity.

The second strategy was a "one-pot" synthesis starting from cephalosporin C performed using three different enzymes (DAO, GA and PGA). This synthesis has been accomplished through a PGA-catalyzed acylation directly performed on the crude 7-ACA obtained by enzymatic cleavage of cephalosporin C. In spite of the yield obtained, this process seems very advantageous if compared with the synthesis performed by chemical or enzymatic *N*-acylation of the 3′-functionalized nucleus 7-SACA. In particular, all reactions were performed in aqueous medium, avoiding any intermediate purification and with a 35% overall yield. With the aim to improve the results of the enzymatic acylation step, different approaches can be taken into account:

a) Selection of acylases with better catalytic properties;
b) Improvement of the catalytic properties of the acylase selected by design of suitable immobilization strategies;
c) Improvement of the performances of the processes by reaction medium design.

These studies are in progress and the results will be presented in forthcoming publications.

Experimental Section

General

Commercially available reagent grade solvents were used without purification. Immobilized DAO and GA and the crude extract of PGA from *E. coli* were from Recordati S.p.a. (Opera, Milano, Italy). Cross-linked 6% agarose beads (Sepharose 6B-CL) were from Pharmacia Biotech AB (Uppsala, Sweden); MAME (**1**), 7-ACA and 7-ADCA were purchased from Aldrich (Milwaukee, WI, USA), whereas the other β-lactam nuclei and 1-*H*-tetrazol-1-ylacetic acid (TA) were from Farmabios S.r.l (Gropello Cairoli, Pavia, Italy). The immobilization of acylase on agarose beads by multiple-point attachment was performed following the general procedure previously reported.[16]

The pH of the solutions during the enzymatic hydrolyses and syntheses reactions were kept constant by automatic titration using a 718 Stat Tritino from Metrohm (Herisau, Switzerland). HPLC analyses were run on a Merck-Hitachi L-7100 equipped with UV detector L-7400. The column was a LiChroCART 250-4 RP select-B (Merck, Darmstadt, Germany) kept at 25 °C using an L-7300 column oven.

Mass spectra were recorded with a Finnigan (San Jose, CA, USA) LCQ ion trap mass spectrometer. HPLC elution was introduced into the electrospray source at 300 $\mu L \cdot min^{-1}$. The ESI source was operated at 4.5 kV, the capillary temperature was set at 250 °C and its voltage at 10 eV; the experiment was performed in the negative ion mode. MS/MS: the ion of interest was isolated in the ion trap and collisionally activated with 25% ejection amplitude at standard He pressure. ^1H NMR spectra were recorded with a Bruker 400 MHz AMX spectrometer (chemical shifts are reported in ppm).

Synthesis of (1*H*-Tetrazol-1-yl)-2-acetic Acid Methyl Ester (TAME, 2)

Tetrazol-1-ylacetic acid (TA, **4**, 1.5 g) was refluxed in dry methanol (50 mL) containing 96% H_2SO_4 (150 μL). After 6 hours, the solvent was evaporated and the oil obtained was dissolved in ethyl acetate (50 mL). The resulting solution was washed (2×20 mL) with $NaHCO_3$ solution. The organic phases were dried over anhydrous Na_2SO_4, filtered and the solvent was removed under vacuum affording pure **2**; yield: 1.14 g (69%); mp 50–52 °C; ^{1}H NMR (DMSO-d_6): $\delta = 3.81$ (s, 3H), 5.66 (s, 2H), 9.48 (s, 1H); HPLC analysis (210 nm): 2.5% acetonitrile in 10 mM phosphate buffer, pH 3.2, flow 1.5 mL/min: TA (**4**) Rt = 1.64 min; TAME (**2**) Rt = 4.47 min (purity > 98%).

Determination of the Esterasic and Amidasic Activities (v_e/v_a)

The esterasic and amidasic activities of PGA have been evaluated by measuring the initial hydrolysis rate of esters **1** or **2** and of the corresponding cephalosporin. As a general procedure, the enzyme derivative (20 UI) was added to the substrate solution (20 mL, 10 mM) under magnetic stirring (200 rpm). During the hydrolysis the pH was kept constant by automatic titration and the activities were evaluated from the sodium hydroxide (100 mM) consumption (μmol of substrate transformed/min/g of enzyme derivative).

Determination of v_s and v_{h1} in the *N*-Acylation of β-Lactam Nuclei and the Saturation Curves

The v_s/v_{h1} ratios in the PGA-catalyzed acylations of the different β-lactam nuclei have been evaluated by reaction with ester **1** or **2** at 10 mM concentration. The reactions were performed at the desired concentration of the β-lactam nucleus (4 °C, pH 6.5). Following a general procedure, the ester was dissolved into a solution of the β-lactam nucleus (20 mL in 10 mM phosphate buffer) at the desired concentration. The enzyme derivative of PGA (20 UI) was then added to the solution at 4 °C under magnetic stirring (200 rpm). During the reaction, the pH was kept constant by automatic titration. The v_s/v_{h1} ratio was evaluated by measuring the initial rates of the syntheses (v_s) and ester hydrolyses (v_{h1}) before 20% of the initial amount of ester had been consumed. The acids and products formation was monitored by HPLC analysis at 210 nm. The curves obtained with the different β-lactam nuclei (Figures 1 and 3) were plotted by performing the acylation reactions using a constant concentration (10 mM) of ester, while increasing the concentration of the nucleus and recording the % of synthesis ($100 \times v_s/v_s/v_{h1}$) versus the nucleus concentration. In the acylation with MAME (**1**), the analytical conditions were 20% acetonitrile in 10 mM phosphate buffer, pH 3.2, flow 1.0 mL/min: MA (**3**), Rt = 3.45 min; **5**, Rt = 8.2 min; **6**, Rt = 9.40 min; **7**, Rt = 19 min; **1**, Rt = 12.6 min. For compound **8**, obtained by acylation of 7-SACA, the HPLC analysis was performed with 12.5% acetonitrile (**8**, Rt = 6.7 min). In the acylation with TAME (**2**), the conditions used were 2.5% acetonitrile in 10 mM phosphate buffer, pH 3.2, flow 1.5 mL/min: TA (**4**) Rt = 1.64 min; **2**, Rt = 4.47 min; **9**, Rt = 8.0 min; **10**, Rt = 10.0 min; **11**, Rt = 13.0 min.

The acylation products **5**–**7** and **9**–**11** were identified by HPLC-mass spectrometry considering their molecular mass: **5** = 346.0; **6** = 407.0; **7** = 460.1; **9** = 323.1; **10** = 381.1; **11** = 437.0. Cefonicid (**8**) was identified by HPLC analysis by comparison with a sample of "*Cefonicid bisodic salt – USP Working standard*".

Study of the Enzymatic *N*-Acylations

The study of the acylation of 7-ACA and 7-SACA (50 mM) has been performed under different reaction conditions, using 150 mM of ester **1**. The nucleus was first dissolved in 25 mM phosphate buffer (20 mL) and then **1** (0.498 g) was added to the resultant solution. When the reaction was performed in presence of methanol, the ester was added as a solution in the amount of methanol necessary for the reaction. After the complete dissolution of the substrates, the mixture was cooled (4 °C) and the pH adjusted at the desired value. A suitable amount of immobilized PGA from *E. coli* was then added (0.5–1 g) under magnetic stirring (200 rpm) keeping the pH constant by automatic titration. The reactions were monitored by HPLC analysis at 274 nm using the analytical conditions reported above for compounds **6** and **8**.

Enzymatic Synthesis of Cefonicid (8) by Enzymatic *N*-Acylation of 7-SACA +

In a general procedure, 7-SACA (4.08 g) was dissolved in 25 mM phosphate buffer (160 mL) at pH 7. Ester **1** (4.98 g), dissolved in methanol (60 mL, 30% of the total volume) was then added and the pH was adjusted to 5.5. Immobilized PGA (10 g) was added to the reaction mixture under mechanical stirring. The reaction was carried out as reported above and monitored by HPLC analysis at 274 nm using the analytical conditions reported above for **8**. When the maximum conversion of 7-SACA into cefonicid (**8**) had been achieved, the enzymatic catalyst was recovered by filtration. The crude solution was concentrated under vacuum and acidified to pH 2.5 using 3 N HCl, maintaining the mechanical stirring and the temperature at 4 °C. The acidic solution was extracted with ethyl acetate (3×150 mL). The aqueous solution was further acidified (pH 1.5) and saturated with NaCl (80 g). The mixture was then extracted with THF (3×80 mL). The organic extracts were collected and washed twice with brine (50 mL). The organic phases were evaporated under reduced pressure keeping the temperature below 32 °C. The resultant oil was dissolved in ethanol (50 mL) and further concentrated under vacuum. Finally, the raw product was dissolved in a mixture of acetone/ethanol (2:1, 90 mL) and added dropwise to a solution containing sodium 2-ethylhexanoate (2.5 g in 45 mL of acetone/ethanol 2:1) at 4 °C. The white precipitate obtained was filtered and dried under vacuum. Cefonicid bisodium salt was obtained in 65% yield (3.8 g). The product was analyzed by HPLC at 220 and 254 nm using the analytical conditions above reported for compound **8**. Identification was performed by comparing the isolated product with "*Cefonicid bisodic salt – USP Working standard*". The purity of the isolated **8** as evaluated by HPLC analysis was 94% at 220 nm and 96% at 254 nm.

Multienzymatic Synthesis of Cefonicid (8)

To a solution of cephalosporin C (4.15 g) in 25 mM phosphate buffer pH 8 (160 mL) at 25 °C the enzyme derivatives of DAO (300 UI) and GA (500 UI) were added under magnetic stirring (200 rpm). During the reaction, a continuous flow of O_2 was maintained and the pH was kept constant by automatic titration. The reaction was monitored by HPLC (3% acetonitrile, 20 mM ammonium acetate buffer, pH 3.2, flow 1.5 mL/min; λ 274 nm). After 3 hours, the complete deacylation of cephalosporin C into 7-ACA (Rt = 3.24 min) was achieved and the reaction mixture was filtered. The crude solution of 7-ACA was used for the next reaction by addition of **1** (4.98 g) dissolved in methanol (40 mL). The mixture was cooled to 4 °C and the pH adjusted at 6.5. Immobilized PGA (10 g) was then added under magnetic stirring (200 rpm) keeping the pH constant by automatic titration. The reactions were monitored by HPLC analysis at 274 nm using the analytical conditions reported above for **6**. When the maximum yield had been achieved, the reaction mixture was filtered. The solution of the crude product **6**, obtained after filtration of the immobilized PGA, was used without purification for 3′-functionalization to obtain cefonicid (**8**). In this reaction, SMT (3.92 g) was dissolved in the crude solution of **6** obtained after enzymatic acylation. The mixture was heated at 65 °C at pH 6.5 for 4 hours affording **8** in 50% yield as evaluated by HPLC analysis performed as reported above at 274 nm and using an external standard of "*Cefonicid bisodic salt – USP Working standard*".

From the aqueous solution obtained after the last chemical step, cefonicid was isolated as its disodium salt prepared with sodium 2-ethylhexanoate in organic medium following the procedure reported above but performing the extraction with a larger volume of THF (6 × 80 mL). After concentration of the THF extracts, the sodium salt was prepared dissolving the oil obtained in 90 mL of a mixture of acetone/ethanol (2:1) and dropping this solution into a solution of 2.5 g of sodium 2-ethylhexanoate in 45 mL of acetone/ethanol (2:1) cooled at 4 °C. The white precipitate obtained was filtered and dried under vacuum. Cefonicid bisodium salt was obtained in 35% yield (2.0 g). The product was analyzed by HPLC as reported above for the enzymatic synthesis. The purity of the isolated cefonicid (**8**) as evaluated by HPLC analysis was 92% at 220 nm and 94% at 254 nm.

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