One-Pot Chemoenzymatic Synthesis of 3′-Functionalized Cephalosporines (Cefazolin) by Three Consecutive Biotransformations in Fully Aqueous Medium

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We illustrate a new chemoenzymatic synthesis of cefazolin from cephalosporin C, involving three consecutive biotransformations in full aqueous medium. This *one-pot* three-step synthesis includes the D-amino acid oxidase catalyzed oxidative deamination of the cephalosporin C side chain, hydrolysis of the resulting glutaryl derivative catalyzed by glutaryl acylase, and the final penicillin G acylase (PGA)-catalyzed acylation of 7-aminocephalosporanic acid (**1**, 7-ACA). The product, 7-[(1*H*-tetrazol-1-yl)acetamido]-3-(acetoxymethyl)-Δ^3-cephem-4-carboxylic acid (**5**), was used as an intermediate for cefazolin synthesis by 3′-acetoxy group displacement with 2-mercapto-5-methyl-1,3,4-thiadiazole. Very high yields have been achieved with all the enzymatic reactions performed; high product concentrations were obtained in short reaction times. This synthetic approach presents several advantages when compared with the conventional chemical processes. The use of the toxic reagents and chlorinated solvents is avoided, while the substrate specificity and chemoselectivity of the enzymes makes reactive group protection and intermediate purification unnecessary. The enzymatic deacylation of cephalosporin C was performed by the simultaneous use of D-amino acid oxidase and glutaryl acylase. The substrate specificity of PGA allowed the acylation of 7-ACA (**1**) to be performed without purification from the glutaric acid produced during the enzymatic deacylation. These results were achieved by optimization and *correct assembly* of the different biotransformations involved. Special attention has been applied to the kinetically controlled acylation reaction. High yields were obtained through a careful selection of the enzyme catalyst, experimental conditions, and synthetic strategy.

Introduction

Among the β-lactam antibiotics, the second-generation cephalosporins present a substantially enhanced antibacterial activity and spectrum.[1] This class of antibiotics includes a number of derivatives where the 3′-functionalization improves the pharmacokinetic profile.[2] Among them, cefazolin **3** is a drug of clinical relevance[3] currently synthesized[4] (Scheme 1) from cephalosporin C by chemical cleavage of the 7-side chain, displacement of the 3′-acetoxy function with the appropriate S-nucleophile to give 7-amino-3-[[(5-methyl-1,3,4-thiadiazol-2-yl)thio]-methyl]-3-cephem-4-carboxylic acid **2** (7-ZACA), and finally chemical acylation of the 7-amino group with (1*H*-tetrazol-1-yl)-2-acetic acid methyl ester (**4**, TZAM).[4a]

This synthetic strategy presents some drawbacks. The drastic reaction conditions required in the 3′-acetoxy displacement by 2-mercapto-5-methyl-1,3,4-thiadiazole (MMTD) cause partial hydrolysis of β-lactam ring and reduce the product yield. According to Scheme 1, two intermediate purification stages are required to separate the 7-ACA (**1**) and the derived β-lactam nucleus **2** from byproducts formed during the chemical deacylation of cephalosporin C and the 3′-acetoxy displacement. On account of the high sensitivity of the β-lactam ring to acid and alkaline media, easily removable protecting groups and very low reaction temperature during the acylation reaction (−40 °C) are required. Moreover, the use of toxic reagents for the activation of the carboxyl group in the acyl donor and the use of chlorinated solvents pose problems of environmental impact for this synthetic procedure.

To avoid the above-mentioned problems, the use of enzyme catalysts such as D-amino acid oxidase (DAO) and glutaryl acylase (GA)[5] or cephalosporin C acylase[6] for cephalosporin C hydrolysis and penicillin G acylase (PGA),[7] for β-lactam nucleus acylation could be considered of great interest. The high selectivity, specificity,

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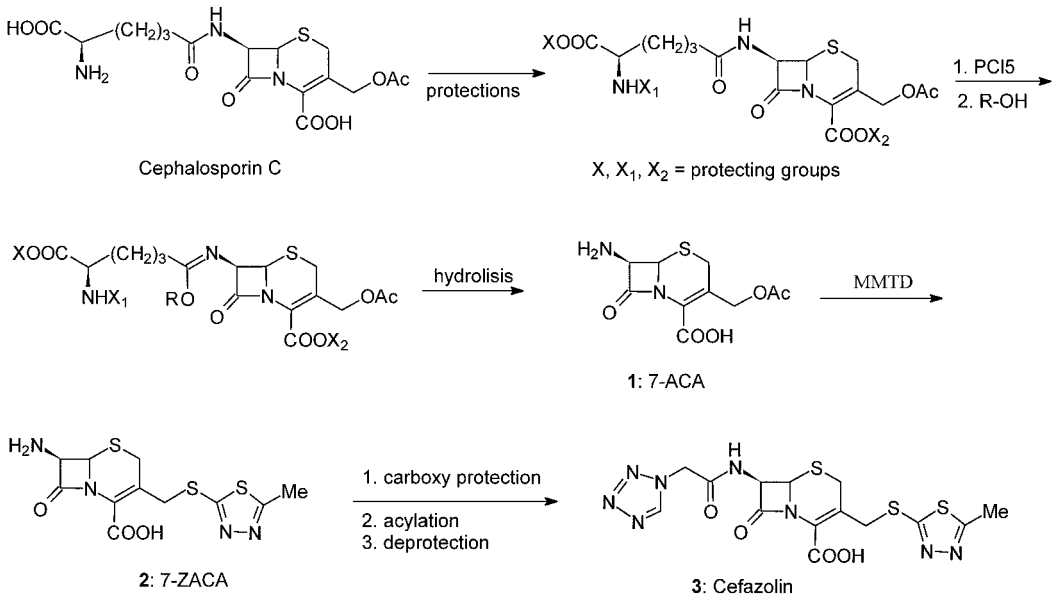
Scheme 1



and activity of these enzymes under mild reaction conditions (aqueous medium, neutral pH, and moderate temperature) avoid the use of organic solvents as well as the reactive group protection/deprotection sequences.

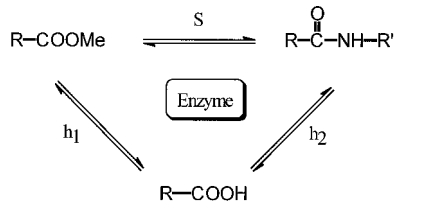
The use of PGA as catalyst makes the utilization of low temperature and the use of the toxic reagents and chlorinated solvents, currently employed in the chemical acylation, unnecessary, while the chemical activation of the carboxy group in the acyl donor may not be required. However, the requirement for a large excess of acylating agent, the low yields achieved, and the complexity of the reaction mixture obtained are the most serious shortcomings of PGA-catalyzed processes.

This enzyme is currently used in the hydrolysis of penicillin G to 6-aminopenicillanic acid and cephalosporin G to 7-aminodesacetoxycephalosporanic acid.[7,8] PGA can be also utilized in the semisynthesis of β-lactam antibiotics following two different strategies:[9] the thermodynamically controlled (equilibrium controlled) and the kinetically controlled synthesis.

The first[10] is the direct condensation of β-lactam nuclei with free acids as acyl donors. This strategy has the clear advantage that prior substrate modifications are not required. However, the high concentrations of organic solvents necessary to shift the equilibrium[10] makes this synthetic approach less attractive.[11]

In the kinetically controlled synthesis, the use of esters or amides as activated acyl donors becomes necessary. The yields obtained depend on the balance of three different reactions catalyzed by the same enzyme[9] (Figure 1). These processes are the synthesis of the β-lactam compound (S), the hydrolysis of the activated acyl donor (h_1), and the hydrolysis of the synthesized antibiotic (h_2). The maximum yields achieved are transient and depend on the saturation degree of the enzymatic active center by the nucleophile, on the ratio antibiotic synthesis/ester hydrolysis (V_S/V_{h_1}) and on the ratio antibiotic synthesis/hydrolysis (V_S/V_{h_2}). The results reported in a previous paper[12] indicate that increasing the V_S/V_{h_2} ratio is the main way to improve the yields while the V_S/V_{h_1} ratio defines the maximum theoretical yield in absence of hydrolysis of the reaction product.

Although this process is more complex, the milder experimental conditions required and, consequently, the possibility of using enzymes without stabilization or immobilization, make the kinetically controlled acylation the most useful for enzymatic synthesis of β-lactam antibiotics.[13] The most extensively utilized and studied PGAs[14] are isolated from *Escherichia coli* ATCC 9637 and ATCC 11105. However, previous works[10,12,15] report the study of an industrial PGA (Antibioticos S.A. Leon, Spain), produced from *E. coli* ATCC 11105 submitted to



S= synthetic reaction
h_1= ester hydrolysis reaction
h_2= β-lactamic compound hydrolysis reaction

Figure 1. Reactions involved in a kinetically controlled synthesis of β-lactamic antibiotics.

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conventional chemical mutagenesis, to obtain a hyperproducer strain and stabilized by covalent multiple-point attachment on activated agarose beads.[16]

Previous papers also report the PGA-catalyzed acylation of 7-ZACA (**2**) with different (1*H*-tetrazol-1-yl)-2-acetic esters[17] but the yields were not quantitative even in the presence of very large excesses of acylating agents. We have investigated a chemoenzymatic process for the synthesis of cefazolin (**3**) by using three different enzymes in full aqueous medium: DAO, GA, and PGA.[18] In this process, the kinetically controlled PGA-catalyzed acylation may be considered the key pathway and the most complex enzymatic reaction to be optimized in order to obtain the final product in high yields. A careful selection of the enzymatic catalyst, experimental conditions, and synthetic strategy has been performed.

The optimization of the PGA-catalyzed acylations with TZAM (**4**) is discussed, and the results obtained with the enzyme derivatives prepared from the mutated strain of *E. coli* ATCC 11105 (*E. coli* A)[16] have been compared with those achieved with the commercially available PGA isolated from *E. coli* ATCC 11105 and immobilized on Eupergit C (*E. coli* B).

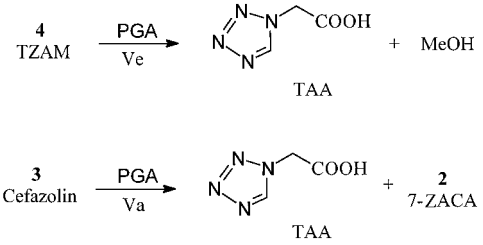
The possibility to perform the 3′-functionalization before or after the PGA-catalyzed acylation could be critical in order to obtain high yields, considering the different structures of 7-ACA (**1**) and 7-ZACA (**2**) and their possible influence on the catalytic properties of PGA. Moreover, the possibility to perform the 3′-functionalization before the acylation[19] could permit the synthesis of cefazolin (**3**) through the direct acylation of the crude 7-ACA (**1**) without purification from the glutaric acid produced during the enzymatic deacylation of cephalosporin C. To choose the best synthetic strategy, 7-ACA (**1**) and 7-ZACA (**2**) have been compared as nucleophiles.

The different biocatalytic reactions have been studied separately, and the final *correct assembly* of the enzymatic deacylation of cephalosporin C and the PGA-catalyzed acylation of 7-ACA (**1**) with the 3′-acetoxy displacement reaction, has been investigated in order to obtain cefazolin (**3**) in high overall yield.

Results and Discussion

Optimization of PGA-Catalyzed Acylation of 7-ZACA. To perform the enzymatic synthesis of cefazolin (**3**) we have first studied the PGA-catalyzed acylation of the β-lactam nucleus 7-ZACA (**2**) with ester **4**, according to the reaction reported in Scheme 3. In kinetically controlled acylations many factors could affect the obtainable yields. The most important are the reaction conditions (e.g., pH and temperature) and the characteristics of the catalyst used, as well as its selectivity and specificity.

Scheme 2



The V_S/V_{h_2} ratio is the most important parameter to define the obtainable yields in the synthesis of β-lactam antibiotics catalyzed by PGA.[12] We have supposed that the esterase activity versus amidase activity (V_e/V_a) of the enzymatic catalyst was a parameter strictly related to this ratio. The hydrolytic activity toward the ester **4** (V_e: esterasic activity) could even be considered a measure of the enzyme capability to give the acyl−enzyme complex, the first step in the synthetic reaction. On the other hand, the capacity of the catalyst to hydrolyze the cefazolin (**3**) (V_a: amidasic activity) is a direct measure of the enzyme capability to hydrolyze the reaction product. Under these hypotheses, the easy and rapid experimental determination of V_e/V_a could be useful in determining the reaction conditions required to obtain the highest V_S/V_{h_2} value and, consequently, the highest yield in cefazolin (**3**) synthesis.

The esterase and amidase activities have been evaluated by measuring the initial hydrolysis rate of the (tetrazol-1-yl)acetic acid methyl ester (**4**) and cefazolin (**3**), respectively (Scheme 2) to give (tetrazol-1-yl)acetic acid (TAA), as reported in the Experimental Section. The results obtained by using PGA from *E. coli* A immobilized on agarose beads indicate the temperature as the most important factor affecting V_e/V_a. A remarkable increase of V_e/V_a was in fact observed upon decreasing the temperature at all the pH values tested, whereas the influence of pH became more important at low temperature (Figure 2A).

At 4 °C, an effective V_e/V_a increase was observed by reducing the pH: the maximum was at pH 6.5 (V_e/V_a = 101), while lower values were obtained by further decreasing the pH (Figure 2B). These results indicated 4 °C and pH 6.5 as the optimum reaction conditions, whereas the higher temperatures and pH values elsewhere reported for cefazolin synthesis[17] give a V_e/V_a almost 10-fold lower.

The enzyme derivative obtained by immobilization on agarose gel of the PGA isolated from *E. coli* A has been compared with the commercially available PGA isolated from *E. coli* B and immobilized on Eupergit C. The obtained results show the same behavior for the two enzymes. The conditions to obtain the best V_e/V_a were

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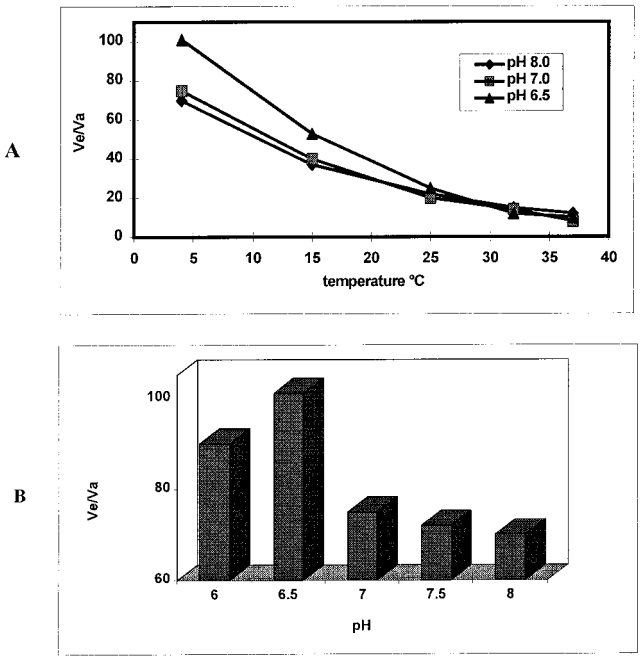


Figure 2. Influence of reaction conditions on the V_e/V_a ratio of the enzyme derivative obtained from *E. coli* A (agarose). A: Effect of temperature; substrate concentrations 10 mM. B: Effect of pH; substrate concentrations 10 mM, temperature 4 °C.

Table 1. Comparison of the Enzyme Derivatives Obtained by Immobilization of the PGA Isolated from *E. coli* A and *E. coli* B. Esterasic Versus the Amidasic Activity (V_e/V_a) Obtained under Different Conditions of pH and Temperature

enzyme	pH	T (°C)	V_e[a]	V_e/V_a
coli A	8.0	37	74	12
coli B	8.0	37	69	7
coli A	6.5	4	8.8	101
coli B	6.5	4	7.1	56

[a] The esterasic activity (V_e) is expressed as IU (μmol/min × mL of enzyme derivative). Substrate concentrations = 10 mM; number of replicates = 4; enzyme derivatives from *E. coli* A (agarose) and *E. coli* B (Eupergit C). V_e = hydrolysis rate of ester **4**; V_a = hydrolysis rate of cefazolin (**3**).

very different from the pH and temperature in which the enzymatic catalyst shows the highest activity: higher V_e/V_a were observed at 4 °C and pH 6.5, while higher temperature and pH gave an increased esterasic activities but a 10-fold lower V_e/V_a with both the enzymes (Table 1). The catalyst from *E. coli* A seems to possess better characteristics, resulting from the higher V_e/V_a showed at acidic pH and low temperature. Substantial variations have not been observed by immobilizing this enzyme either on agarose or Eupergit C, suggesting that the differences observed between the two enzymes arise from small differences in the native proteins and not from the different immobilization supports used.

Comparing the two different catalysts in the enzymatic acylation of the β-lactam nucleus **2** (Scheme 3), higher yields (28%) were obtained with the PGA from *E. coli* A while only 22% yield was achieved using PGA from *E. coli* B. These results agree with the higher V_e/V_a exhibited by the former enzyme. These syntheses were carried out in alkaline medium (pH 7.5) because of the low solubility of the β-lactam nucleus **2** at pH 6.5. When the enzyme isolated from *E. coli* A was used even in the presence of a high concentration of nucleus **2** (50 mM) and an excess of ester **4** (150 mM), the yields were not quantitative, probably as a consequence of the high h_2 activity shown by the enzyme under these reaction conditions.

Study of Different β-Lactam Nuclei in the PGA-Catalyzed Acylation. 7-ACA (**1**) and 7-ZACA (**2**) were compared as nucleophiles in the PGA-catalyzed acylation with methyl ester **4** (Scheme 4). 7-ACA (**1**) could be considered a more suitable nucleophile than compound **2** considering its higher solubility at low pH and temperature and the possibility of using its acylation products as intermediates in the synthesis of 3′-functionalized cephalosporins.[19] Moreover, the substrate specificity of PGA suggests the possibility to develop a multienzymatic process for cefazolin (**3**) synthesis, directly from the natural cephalosporin C and without any intermediate purification stage.

To compare these β-lactam nuclei in the PGA-catalyzed acylations, the reactions were carried out in the presence of high nucleophile excess and in alkaline medium (pH 7.5) to ensure the complete solubility of 7-ZACA (**2**). Some kinetic parameters have been calculated in order to investigate how the different chemical structures of the two β-lactam nuclei may influence the catalytic behavior of the enzyme. The V_S and V_{h_1} were evaluated by measuring the rate of acylation product and TAA formations (Scheme 4) during the first reaction period (see the Experimental Section).

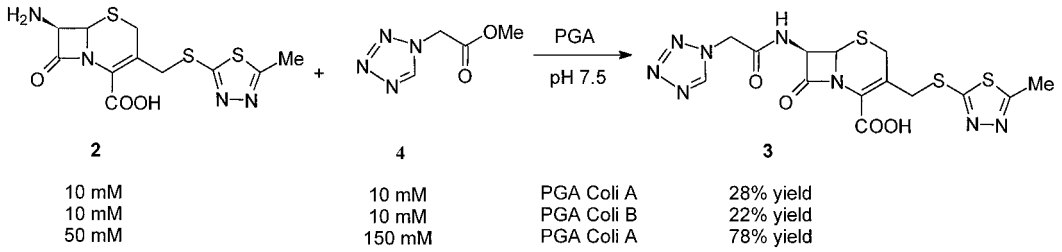
Higher global enzymatic activity (V_S plus V_{h_1}) was observed in the acylation of the 3′-functionalized β-lactam nucleus **2** (Table 2). However, the higher theoretical yield, V_S/V_{h_1} ratio, and V_S obtained with 7-ACA (**1**) suggest a better interaction of this β-lactamic nucleus in the PGA active site. When the acylation of 7-ACA (**1**) was carried out at lower pH (6.5) a further increase of V_S and V_S/V_{h_1} values was observed despite a slight inhibition of the total enzymatic activity, probably as consequence of the enhanced affinity of this β-lactam nucleus toward the enzymatic active site.

These results demonstrate that the pH may strongly influence the catalytic behavior of PGA from *E. coli*, as V_S/V_{h_1} and the V_S obtained at pH 6.5 were much better than those obtained at pH 7.5. This feature, together with the higher V_e/V_a obtained under the same conditions, further indicate pH 6.5 as the best for the PGA catalyzed acylation, despite the lower activity showed by the enzymatic catalyst in acidic medium. Moreover, the very low solubility and the poorer affinity of nucleus **2** toward the PGA active site, as well as the better kinetic parameters obtained with 7-ACA (**1**), make this latter nucleus a more interesting nucleophile in the PGA-catalyzed acylation. We therefore decided to optimize the acylation of 7-ACA (**1**) with TZAM (**4**) to develop a multienzymatic *one-pot synthesis* of cefazolin (**3**) from cephalosporin C (Scheme 5).

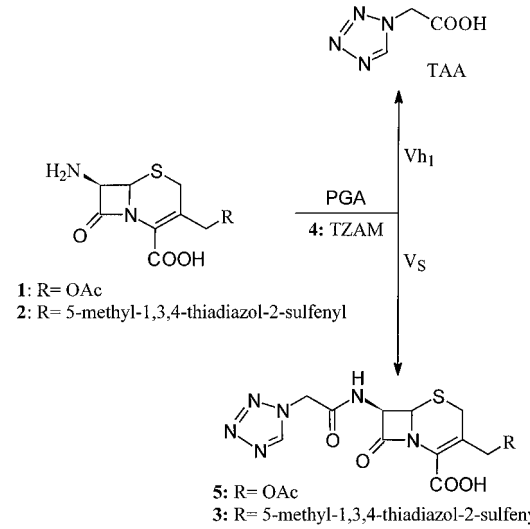
Optimization of PGA-Catalyzed Acylation of 7-ACA. Figure 3 reports the results obtained by studying the effect of different factors on the acylation of 7-ACA (**1**) with TZAM (**4**). The amount of acylating agent required to obtain quantitative yields has been studied under the optimal reaction conditions previously determined (Figure 3A). As expected, the synthetic rate and yields obtained improved when an excess of ester **4** was used; high yields (98%) were achieved by using a 3:1 molar ratio.

Figure 3B reports the results obtained using different catalysts and reaction conditions. A quantitative yield

Scheme 3



Scheme 4



Scheme 5

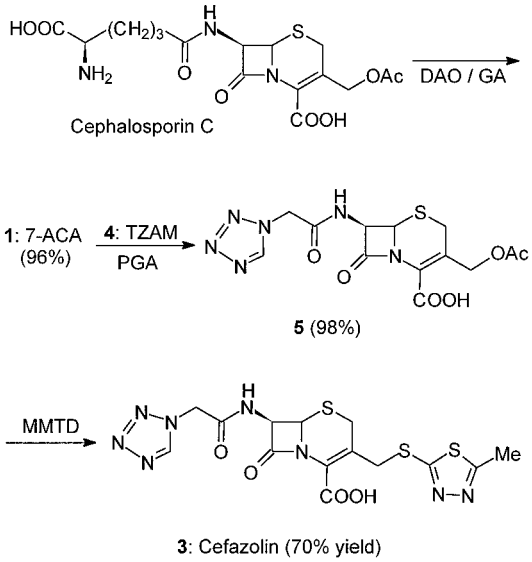


Table 2. Comparison of 7-ACA (1) or 7-ZACA (2) as Nucleophiles in the PGA-Catalyzed Acylation Reaction with (1-Tetrazolyl)acetic Methyl Ester (4)

nucleophyl	pH	total activity[a,b]	V_S[a]	V_S/V_{h_1}	theoretical yield (%)
7-ZACA	7.5	28.5	3.5	0.14	12
7-ACA	7.5	12.4	4.1	0.33	25
7-ACA	6.5	7.96	7.0	7.30	88

[a] The activities (V_S) were expressed as IU (μmol/min × mL of enzyme derivative). [b] The total enzymatic activity was calculated as sum of the rates of acid formation (V_{h_1}) and acylation (V_S). Ester concentration = 10 mM; β-lactamic nucleus concentrations = 50 mM; temperature = 4 °C; enzyme derivative from *E. coli* A (agarose).

was obtained by using the PGA from *E. coli* A, whereas the use of the commercially available PGA (*E. coli* B) gave lower yields. When the synthesis of product **5** was performed at higher temperature and pH values, a dramatic increase of the hydrolysis of the product (h_2 activity) and a concomitant decrease in the yields were observed.

In this context, the V_e/V_a evaluations seems a rapid and appropriate approach to find the best reaction conditions and enzymatic catalyst for the optimization of kinetically controlled synthesis of β-lactam antibiotics. Under the reaction conditions that give the highest V_e/V_a (pH 6.5 and 4 °C) hydrolysis of the reaction product was not observed, while a higher amidase activity could be detected when the synthetic reaction was conducted at higher temperature and pH. Quantitative acylation of 7-ACA (**1**) has been obtained under the reaction conditions where the enzymatic catalyst shows the lowest amidase activity. This result confirms the importance of the V_S/V_{h_2} ratio in determining the maximum yields acheived.[12]

Chemoenzymatic Synthesis of Cefazolin from Cephalosporin C. To develop a multienzymatic *one-pot synthesis* of cefazolin (**3**) from cephalosporin C, through the direct enzymatic acylation of the crude 7-ACA (**1**) (Scheme 5), it is necessary that the acylase not recognize the glutaric acid produced during the enzymatic deacylation of cephalosporin C as a substrate or inhibitor. To study the possible influence on the synthesis of compound **5**, the reaction was performed in the presence of high concentrations (50 mM) of this dicarboxylic acid. As expected, only a slight inhibition of the synthetic activity was observed, and changes in the yields obtained were not detected.

Several studies are in progress to investigate the use of cephalosporin C acylases as catalyst in cephalosporin C deacylation.[6] This reaction gives the α-aminoadipic acid as a secondary product, and a study on the effect caused by this amino acid on the PGA catalytic properties could be of interest. As previously observed with glutaric acid, relevant changes in the reaction rate and in the yields obtained were not detected when the acylation of 7-ACA (**1**) was carried out in the presence of high concentrations of α-aminoadipic acid.

These results demonstrate the possibility to perform the PGA-catalyzed acylation on the crude 7-ACA (**1**) obtained by either oxidative or hydrolytic deacylation of the natural cephalosporin C, the acylation yields obtained always being higher than 95%. In both cases, the specificity of PGA renders the purification from secondary products unnecessary, making suitable the proposed *one-pot* synthesis of cefazolin (**3**).

We have performed the enzymatic deacylation of cephalosporin C in one batch by the simultaneous use of DAO from *Trigonopsis variabilis*, GA from *Acetobacter*

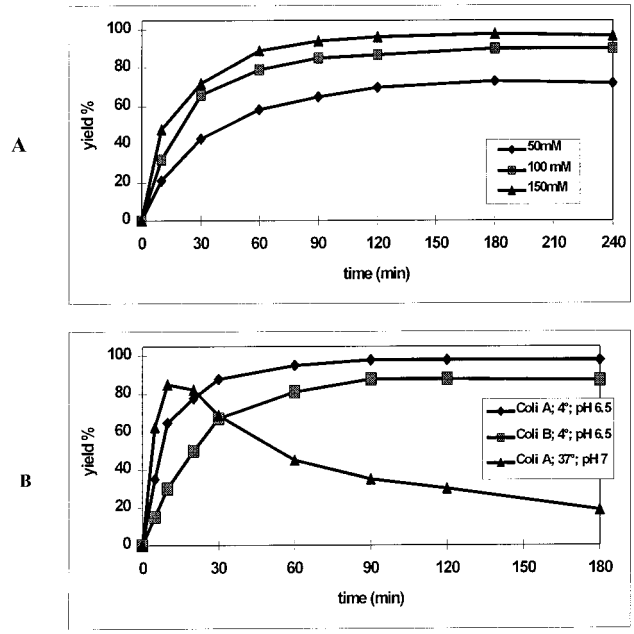


Figure 3. Acylation of 7-ACA (**1**) (50 mM) with 1-tetrazolylacetic acid methyl ester (**4**). A: Effect of ester concentration (50, 100, 150 mM); enzyme derivative from *E. coli* A (agarose); 4 °C; pH 6.5. B: Effect of the enzyme derivative and reaction conditions used; ester **4** concentration 150 mM.

sp., and a continuous flow of O_2 (Scheme 5). DAO is known[5] to catalyze the oxidative deamination of the α-aminoadipic side chain of cephalosporin C to give the α-ketoadipic derivative. Its decarboxylation and further oxidation gives the glutaryl analogue, which is then deacylated by GA to 7-ACA (**1**). The high activity of these enzyme derivatives allowed the complete transformation of high concentrations of cephalosporin C (50 mM) into 7-ACA (**1**) in only 2.5 h (96% yield). Furthermore, the simultaneous use of these stabilized enzymes avoids intermediate product purification. The results obtained in the DAO and GA immobilization and stabilization studies, as well as in the cephalosporin C enzymatic hydrolysis optimization,[20] will be published elsewhere.

The subsequent acylation was performed directly on the solution of the crude 7-ACA (**1**) after filtration, using the immobilized PGA isolated from *E. coli* A and a 3:1 molar excess of ester **4**. A high yield (98%) was obtained at pH 6.5 and 4 °C.

Final displacement of the 3-acetoxy group with MMTD was performed directly by heating to 65 °C the aqueous solution of crude **5** obtained by filtration of the enzyme derivative of PGA. This step was not optimized, and cefazolin (**3**) was isolated in 70% yield.

Conclusions

In this work, the *one-pot synthesis* of cefazolin (**3**) from cephalosporin C, by using three different enzymes in fully aqueous medium, has been described. The three-step chemoenzymatic synthesis of this 3′-functionalized cephalosporin has been accomplished through a PGA-catalyzed acylation, performed directly on the crude 7-ACA (**1**) obtained by enzymatic cleavage of cephalosporin C catalyzed by the simultaneous use of DAO and GA. All the enzymatic reactions performed gave quantitative or very high yields, and compound **5** was then used, without purification, for the 3′-acetoxy group displacement with MMTD to afford cefazolin (**3**) in 70% yields.

This synthetic approach presents several practical advantages if compared with the conventional chemical processes. The use of the toxic reagents and chlorinated solvents is avoided, while the substrate specificity and chemoselectivity of the used enzymes makes reactive group protections unnecessary and reduces the purification stages required. The performances obtained with the bienzymatic system utilized in the cephalosporin C hydrolysis allows high-yield transformation of high substrate concentration, avoiding the intermediate purification of the glutaryl derivative before GA-catalyzed hydrolysis. Similarly, the substrate specificity of PGA allowed the acylation of 7-ACA (**1**) with TZAM (**4**) to be performed, avoiding the purification from the glutaric acid produced during deacylation of cephalosporin C.

To obtain high concentrations of final product in high yields and very short reaction times, a careful optimization and a *correct assembly* of the different biotransformations involved were necessary. The kinetically controlled PGA-catalyzed acylation of 7-ACA (**1**) was the most complex enzymatic reaction to be optimized, and a careful selection of the enzymatic catalyst and experimental conditions were necessary. Some general comments can be made about this reaction:

The optimal conditions for the synthesis of compound **5** (pH 6.5 and 4 °C) were very different from those reported in the literature in which the enzymatic catalyst show the highest activity for 7-ZACA (**1**) acylation.[17] However, the possibility of using very active enzyme derivatives allows a quantitative yield and high product concentrations to be obtained in a short reaction time.

The results obtained in this work with the PGA isolated from *E. coli* A demonstrate the importance of the microbial source of the enzymatic catalyst. Small differences in the native enzyme used may strongly influence the yields obtained in the kinetically controlled acylation of β-lactam nuclei. The search of new microbial sources, or the study of mutated strains, could be a key approach to obtain new PGAs with better catalytic properties.

The strategy utilized to synthesize cefazolin (**3**) allows the use of 7-ACA (**1**) as nucleophile in the acylation reaction. The high affinity toward the PGA active site and the high solubility of this β-lactam nucleus in the optimum reaction conditions (4 °C, pH 6.5) were very important to achieve quantitative yields and high product concentrations. In contrast, the very low solubility of cefazolin nucleus **2** under the same conditions and its poorer affinity toward the enzymatic active site make this compound less suitable as nucleophile for PGA-catalyzed acylation.

The results obtained in this work indicate the proposed chemoenzymatic synthesis to be a promising approach for the synthesis of 3′-functionalized cephalosporins, especially for large-scale production where processes with low environmental impact are more and more required. In this context, optimization studies are in progress in order to obtain higher concentrations of final product and to reduce the acylating ester excess required for quantitative yields in the PGA-catalyzed acylation of 7-ACA (**1**).

(20) Guisán, J. M.; Rodriguez, V.; Fernández-Lafuente, R.; Bastida, A.; Ceinos, P. Spanish Patent 9500369, 1995.

Experimental Section

General Methods. Commercially available reagent-grade solvents were used without purification. Semipurified extracts of DAO from *Trigonopsis variabilis*, GA from *Acetobacter* sp., and PGA from *E. coli* A were from Antibioticos S.A. (Leon, Spain), while PGA from *E. coli* B immobilized on Eupergit C was commercially available (Recordati, Milano, Italy). Crosslinked 6% agarose beads were donated by Hispanagar (Burgos, Spain), while Eupergit C was from Röhm Pharma (Darmstadt, Germany). Melting points were measured on a Kofler hot-stage apparatus and were not corrected. The elemental analysis was performed on a Carlo Erba 1106 elemental analyzer. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AC 250 spectrometer, and chemical shifts are reported in ppm. For NMR analysis, β-lactam compounds were dissolved in D_2O/sodium bicarbonate, while NMR spectra of the ester **4** were recorded using DMSO-d_6 as solvent. GC−MS in EI analysis of compound **4** was performed with a Finnigan ITD instrument. The GC oven was fitted with a Mega (Legnano, Italy) SE 52 column (15 m × 0.25 mm i.d). The oven was programmed as follows: starting with 80 °C (for 2 min) and then increased to 270 °C at 10 °C/min. The injector was in the splitless mode at 220 °C. The detector temperature was held at 280 °C, and the electron energy was at 70 eV. HPLC analyses were run on a Merk-Hitachi L-7100 equipped with UV detector L-7400. The column was a LiChroCART 250-4 RP select-B (Merck, Darmstadt, Germany) and analyses were run at 25 °C by using a L-7300 column oven. The LC conditions are described for each compound. The pH of the solutions during the enzymatic hydrolysis and synthesis reactions were kept constant by using an automatic titrator 718 Stat Tritino from Metrohm (Herisau, Switzerland).

(1*H*-Tetrazol-1-yl)-2-acetic Acid Methyl Ester (4). TZAM (**4**) was prepared from (1*H*-tetrazol-1-yl)-2-acetic acid which, in turn, was prepared by reaction of glycine with sodium azide and ethyl orthoformate in acetic acid.[21] Esterification was accomplished with diazomethane.

(1*H*-Tetrazol-1-yl)-2-acetic acid (TAA): mp 124−126 °C (2-propanol); ^{1}H NMR (DMSO-d_6) 5.45 (s, 2H), 9.29 (s, 1H). Anal. Calcd for $C_3H_4N_4O_2$: C, 28.13; H, 3.15; N, 43.74. Found: C, 28.27; H, 3.39; N, 43.27. HPLC analysis: 10% acetonitrile, phosphate buffer 10 mM, pH 3.2, flow 1.0 mL/min, UV detector 215 nm, t_R = 2.99 min.

(1*H*-Tetrazol-1-yl)-2-acetic Acid Methyl Ester (4). The acid (1.5 g) was dissolved in dry methanol (50 mL) and then reacted with diazomethane to obtain, after solvent evaporation, pure methyl ester **4** (1.63 g) in 73% overall yield: mp 50−52 °C; ^{1}H NMR (DMSO-d_6) 3.81 (s, 3H), 5.66 (s, 2H), 9.48 (s, 1H); MS *m*/*z* (relative abundance) 142 (M^+, 84), 87 (61), 61 (12), 55 (100). Anal. Calcd for $C_4H_6N_4O_2$: C, 33.81; H, 4.26; N, 39.42. Found: C, 33.87; H, 4.38; N, 39.35. GC analysis: t_R = 14.03 min. HPLC analysis: 10% acetonitrile, phosphate buffer 10 mM, pH 3.2, flow 1.5 mL/min, UV detector 215 nm, t_R = 4.13 min.

Preparation of the Enzyme Derivatives. Immobilization of PGA from *E. coli* A on Agarose Beads. Activation of agarose gel by etherification with glycidol and oxidation with periodate, and the further control of the PGA (amine)−agarose (aldheyde) multiple-point attachment, were performed as previously described[16] by reaction at pH 10 and final reduction of the imino double bonds with sodium borohydride.

Immobilization of PGA from *E. coli* A on Eupergit C. Wet Eupergit C (2 g) was suspended under mechanic stirring into 90 mL of phosphate buffer (1 M) at room temperature and pH 8. A solution (10 mL) of PGA (200 IU/mL) was then added to the suspension, and the reaction mixture was stirred for 24 h. After filtration, the immobilized enzyme was washed with distilled water and resuspended into 100 mL of a solution of ethanolamine (0.1 M) in phosphate buffer (0.1 M) at pH 8 and room temperature. After 2 h under mechanical stirring, the suspension was filtered and the enzyme derivative washed with distilled water.

(21) Takshi, K.; Yoshihisa, S. U.S. Patent 3,767,667, 1973.

Immobilization of the DAO and GA on Agarose Beads. Activation of agarose gel by reaction with ethylenediamine was carried out as previously described.[22] The aminated gel was then reacted with 10% glutaraldehyde solution for 12 h at pH 7 and room temperature. The activated gel was incubated under mechanic stirring with DAO and GA at pH 6 and room temperature as previously reported[18] to obtain the enzyme derivatives of DAO (100 IU/mL) and GA (250 IU/mL).

Determination of the Esterase and Amidase Activity of the PGA (Scheme 2). The esterase and amidase activities have been evaluated by measuring the initial hydrolysis rate of the (tetrazol-1-yl)acetic acid methyl ester (**4**) and cefazolin (**3**), respectively. The enzyme derivative of PGA (100 IU) was added to 20 mL of substrate solution (10 mM) in phosphate buffer 10 mM under magnetic stirring (200 rpm) and at different pH and temperature. During the reaction the pH was kept constant by automatic titration and the reaction rate (μmol/min × mL of enzyme derivative) was calculated from the NaOH consumption.

Enzymatic Synthesis of Cefazolin (3) (Scheme 3). Following a general procedure, the ester **4** was dissolved into a solution (20 mL in phosphate buffer 10 mM) of 7-ZACA (**2**). The mixture was cooled to 4 °C and pH adjusted at 7.5. The enzyme derivative of PGA from *E. coli* A or *E. coli* B (100 IU) was then added to the solution under magnetic stirring (200 rpm), and during the reaction course the pH was kept constant by automatic titration. The reaction was monitored by HPLC analysis (10% acetonitrile, phosphate buffer 10 mM, pH 3.2, flow 1 mL/min; UV detector 274 nm). When the maximum yield was achieved, the reaction mixture was filtered and the acylation product **3** (t_R = 10.6 min) was purified from the unreacted β-lactam nucleus **2** on a Dowex 50 column eluted with water. After lyophilization, compound **3** was identified by NMR analysis. The HPLC and ^{1}H and ^{13}C NMR analytical data of the synthesized cefazolin (**3**) were in agreement with those of an authentic sample.

Determination of V_S and V_{h_1} in the Acylation Reaction of Different β-Lactamic Nuclei (Scheme 4). The V_S/V_{h_2} ratios in the PGA-catalyzed acylation of 7-ACA (**1**) and 7-ZACA (**2**) have been evaluated by measuring the initial rates of synthesis (V_S) and TZAM (**4**) hydrolysis (V_{h_2}), respectively. Following a general procedure, the ester **4** (0.0321 g) was dissolved into a solution of the appropriate β-lactam nucleus (50 mM) in 20 mL in phosphate buffer 10 mM. The enzyme derivative of PGA (100 IU) was added into the substrate solution at 4 °C under magnetic stirring (200 rpm). During the reaction, the desired pH was kept constant by automatic titration, and the reaction rates (μmol/min × mL of enzyme derivative) were calculated from the NaOH consumption, the V_{h_1}, while the formation of the acylation products was evaluated by HPLC analysis: 2.5% acetonitrile phosphate buffer 10 mM, pH 3.2, flow 1.5 mL/min; UV detector 220 nm; tetrazol-1-ylacetic acid t_R = 1.64; TZAM (**4**), t_R = 4.47; cefazolin (**3**), t_R = 25.12; compound **5**, t_R = 9.99.

Enzymatic Deacylation of Cephalosporin C to 7-Aminocephalosporanic Acid (1) (Scheme 5). To a solution (50 mM) of cephalosporin C (0.415 g; 1 mmol) in 50 mL of phosphate buffer 10 mM at pH 8 and 25 °C were added 0.2 mL of the enzyme derivative of DAO (20 IU) and 0.15 mL of the enzyme derivative of GA (40 IU) 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 (2% acetonitrile, ammonium acetate buffer 20 mM, pH 3.2, flow 1.5 mL/min; UV detector 274 nm). After 2 h, the complete deacylation of cephalosporin C to 7-ACA (**1**) (t_R = 3.24 min) was achieved, and the reaction mixture was filtered. The solution of crude 7-ACA (**1**) was used for the next reaction without purification. For identification product **1** was isolated by acidification to pH 4.5 and filtration of the white crystals obtained. The HPLC and ^{1}H and ^{13}C NMR analytical data of the synthesized 7-ACA (**1**) corresponded with those of an authentic sample.

(22) Fernández-Lafuente, R.; Rossel, C. M.; Rodriguez, V.; Santana, C.; Soler, G.; Bastida, A.; Guisán, J. M. *Enzyme Microb. Technol.* **1993**, *15*, 546.

Enzymatic Acylation of 7-ACA to 7-[(1*H*-Tetrazol-1-yl)acetamido]-3-(acetoxymethyl)-Δ^3-cephem-4-carboxylic acid (5) (Scheme 5). The ester **4** (0.427 g, 3 mmol) was directly dissolved into 20 mL of 7-ACA (**1**) crude solution (50 mM) obtained by the enzymatic procedure described above. The mixture was cooled to 4 °C and pH adjusted at 6.5. The enzyme derivative (PGA from *E. coli* A; 100 IU) was then added to the solution under magnetic stirring (200 rpm), and the pH was kept constant by automatic titration. The reaction was monitored by HPLC analysis. When the maximum yield was achieved (98%) the reaction mixture was filtered and the solution of the crude acylation product **5** was used without purification for the next reaction. For identification, compound **5** was purified in a Dowex 50 column eluted with water. After lyophilization, compound **5** was identified by NMR analysis: 10% acetonitrile, phosphate buffer 10 mM, pH 3.2, flow 1 mL/min, UV detector 274 nM, t_R = 9.97 min; ^{1}H NMR (D_2O/carbonate) δ 2.04 (s, 3H,), 3.47 (q, 2H, ^2J = 18.0 Hz), 4.74 (q, 2H, ^2J = 12.0 Hz), 5.10 (d, 1H, ^3J = 4.5 Hz), 5.49 (s, 2H), 5.68 (d, 1H, ^3J = 4.5 Hz), 9.11 (s, 1H); ^{13}C NMR (D_2O/carbonate) δ 21, 26, 52, 58, 60, 65, 117, 132, 146, 165, 168, 169, 174. Anal. Calcd for $C_4H_6N_4O_2$: C, 38.91; H, 3.82; N, 22.69. Found: C, 38.56; H, 3.91; N, 22.72.

Synthesis of Cefazolin (3) (Scheme 5). (2-Mercapto-5-methyl)-1,3,4-tiadiazole (0.264 g, 2 mmol) was directly dissolved into 20 mL of compound **5** solution (50 mM) obtained by the above-described PGA-catalyzed acylation of 7-ACA (**1**). The pH of the mixture was adjusted at 6.5, and the solution was heated to 65 °C. The reaction was monitored by HPLC analysis (10% acetonitrile, phosphate buffer 10 mM, pH 3.2, flow 1 mL/min; UV detector 274 nM). After 3 h, the displacement of the 3-acetoxy group to obtain cefazolin (**3**) (t_R = 10.6 min) was complete, and the reaction mixture was cooled to 4 °C and acidified to pH 5 to precipitate the excess of MMTD. After filtration, the solution was further acidified to pH 3.5 to give, after filtration, 0.26 g of pure cefazolin (**3**) as a white solid (68% yield). The HPLC and ^{1}H and ^{13}C NMR analytical data of the synthesized compound were in agreement with those of an authentic sample.

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