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0273-2289/92/3303—0177$02.00

New Tetrazole-1-Acetic Acid Esters for Enzymatic Synthesis of Cefazolin

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Received June 17, 1991; Accepted February 11, 1992

ABSTRACT

The enzymatic synthesis of cefazolin (CEZ) using esters of tetrazole-1-acetic acid (TzAA esters) with saturated lower alcohols is reported. The optimum ratios of acyl-donor:acyl-acceptor in the enzymatic synthesis were determined. It is shown that a threefold molar excess of acyl-donor for about 165 min, a conversion rate of about 55% is obtained with these TzAA esters. The syntheses were carried out with commercial immobilized penicillinamidase/E.C.3.5.1.11/ (Eupergit PcA) in a batch-type reactor. After 40 batches, the enzyme activity loss was 5–7% toward the initial one.

Index Entries: Enzymatic synthesis; penicillinacylase; cephalosporins; cefazolin; tetrazole-1-acetic acid.

INTRODUCTION

Many researchers report about the enzymatic synthesis of semisynthetic penicillins and cephalosporins by penicillinamidase/penicillin amidohydrolase, E.C.3.5.1.11 (PA) (*1–3*). In most of the reports, however, they speak about the enzymatic *N*-acylation of 6-aminopenicillanic acid (6-APA) and 7-aminocephem compounds with esters of α-phenylglycine and their derivatives, wherein the chemical methods provide high yields

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and the enzymatic method cannot be a competitive one. The enzymatic acylation with other than α-phenylglycine acylating agents for the synthesis of cefamandole, cefazolin, cephoperazone, and other semisynthetic cephalosporins evokes bigger interest since the shortcomings of the chemical methods are well known. This paper presents a further study on the enzymatic synthesis of cefazolin based on the data already published by us (*4*). The results from the investigation of a suitable TzAA ester as well as the possibility for continuous synthesis are summarized.

MATERIALS AND METHODS

Materials

The 7-Aminocephalosporanic acid (7-ACA), 3-[5-methyl-1,3,4-thiadiazol-2-yl]-7-ACA (M-7-ACA), and cefazolin (CEZ) were supplied by Antibiotic Ltd. (Bulgaria). The immobilized penicillin amidase was a commercial product (Eupergit PcA, Röhm Pharma GmbH, Germany) with an activity of 109 U/g (substrate:benzylpenicillin) (*5*). All other chemicals except the TzAA esters were of analytical grade. The TzAA esters were obtained by esterification of the acid with the corresponding alcohol by a conventional method (*6*). In this way, methyl- (TzAA-OMe), ethyl-(TzAA-OEt), propyl- (TzAA-OPr), isopropyl- (TzAA-OiPr), butyl- (TzAA-OBu), and octyl-esters (TzAA-OOc) of TzAA were obtained. The ester of TzAA with 5-methyl-1,3,4-thiadiazol-2-thiol was obtained by esterification with *N,N'*-dicyclohexyl-carbodimide (*7*). All esters were of higher than 92% purity according to high-pressure liquid chromatography (HPLC) analysis (column: LiChrosorb RP-8, Merck Co., Germany); eluent: 75% (v/v) methanol in 0.03*M* phosphate buffer and UV = 254 nm).

Enzymatic Synthesis of CEZ

A solution of M-7-ACA (10.0 mg/mL) in water is mixed with such a quantity of Eupergit PcA that ensures enzyme activity of about 10 U/mL of reaction medium. The calculated amount of TzAA ester is added, and by continuous addition of 0.1*M* sodium hydroxide, the pH is kept at 6.8. Suitable equipment for carrying out this reaction is the automatic titration system Radiometer RTS 822 (Radiometer Co., Denmark).

Assay of CEZ and Related Compounds

The rate of enzymatic synthesis was followed by the quantity of CEZ formed during the reaction. It was assayed by HPLC analysis according to USP XXI (*8*) with a separating column (LiChrosorb RP-8, Merck).

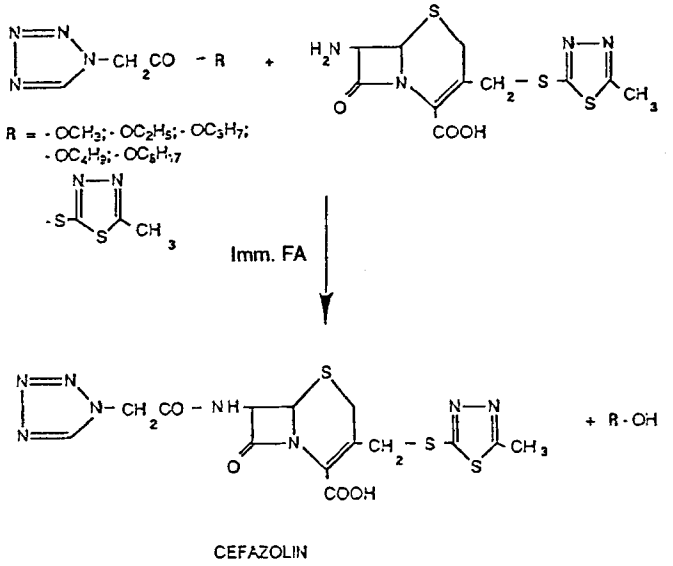

Fig. 1. Enzymatic synthesis of cefazolin by immobilized PA.

RESULTS AND DISCUSSION

Choice of Acyl-Donor for CEZ Synthesis

The scheme of the enzymatic synthesis of CEZ by different TzAA esters is shown in Fig. 1.

In a previous paper (*4*), we reported a temperature of 35°C and a pH of 6.8 as optimum conditions for the encymatic synthesis. These conditions, with negligible deviations, were also the optima for CEZ synthesis using the other TzAA esters. From the kinetic point of view, the conversion rate of the synthesis reaction depends on the deacylating action of the nucleophiles (in this case M-7-ACA and water) on the enzyme–acyl complex (immobilized PA–TzAA ester) and the reaction conditions (*9,10*). Therefore, the rate of synthetic reaction depends on the type and concentration of the acyl-acceptor because the deacylating action of the water is constant for a given acyl-donor at given reaction conditions. In the case of CEZ synthesis, a conversion rate of 55% was reported (*11*) at an acyl-donor:acyl-acceptor ratio of 4.78:1. The authors used as an acyl-donor the ester of TzAA with pentaethyleneglycol-methyl ether/TzAA-O($CH_2CH_2O)_5CH_3$/ and claimed that the TzAA esters with polyethyleneglycol (PEG) and derivatives are better for enzymatic synthesis than those with saturated lower alcohols because of better solubility in water of the former. In our experiments, similar and even higher

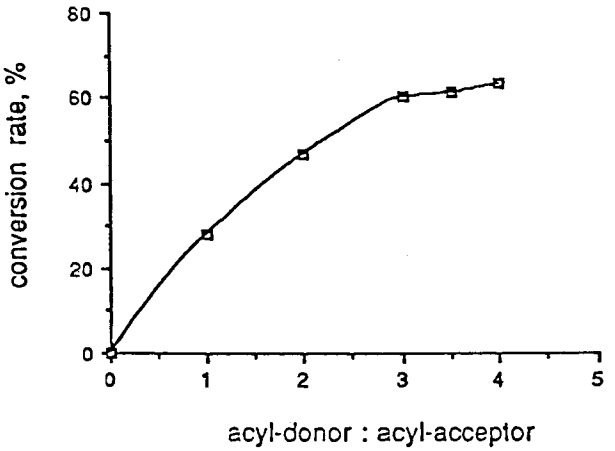

Fig. 2. Effect of the acyl-donor:acyl-acceptor concentrations ratio on CEZ synthesis by eupergit PcA. The reaction mixture containing 5.0–17.5 mg/mL of acyl-donor (TzAA-OMe), 10.0 mg/mL of acyl-acceptor (M-7-ACA), and 110 U enzyme activity was incubated at 35°C and pH 6.8 for 165 min.

conversion rates were reached with TzAA-OMe, TzAA-OEt, TzAA-OPr, TzAA-OBu, and TzAA-OOc at an acyl-donor:acyl-acceptor ratio of 3.0–3.5:1 (Fig. 2).

It has been mentioned (*11*) that the authors used approx a fivefold molar excess of acyl-donor (TzAA ester) to obtain a CEZ conversion rate of 55%. Obviously, for an economic process, a recovery of the excess acyl-donor is required. In the cited reference, recovery is not reported, but our investigations showed that after 250–300 min of synthetic reaction, more than 80% of the initial TzAA ester is hydrolyzed to TzAA and the corresponding PEG. Similar results were obtained with all TzAA esters used in this work. At the same time, the concentration of TzAA at the end of the reaction is so low that extraction and precipitation methods practically are not applicable for its recovery. Thus, the higher the conversion rate, the larger amount of TzAA ester is used and the higher are the loses of TzAA. Therefore, it is essential to find the optimum ratio between the reagents for every particular TzAA ester. The achieved maximum relative conversion rates to CEZ with different TzAA esters under the same reaction conditions are presented on Table 1.

Comparing our results to those reported by Mineyuki, Mitsugi, and Kondo (*11*) we found out that in the enzymatic synthesis of CEZ, the higher solubility of TzAA esters on the basis of PEG and PEG-monomethyl ether is not an advantage. Despite low solubility of TzAA esters in water with lower saturated alcohols, a satisfactory conversion rate was obtained at a given duration of the synthetic reaction [165 min in our case compared to 225 min in (*11*)]. We considered the possibility that the ester solubilities are influenced by the presence of some quantity of alcohol (as a product of the reaction) and the carrier for enzyme immobilization, but these effects were negligible.

Table 1
Effect of the Type of Acyl-Donor on the Enzymatic Synthesis of CEZ

Acyl-donor/acceptor, mol/mol	Relative conversion rate, %						
	TzAA-OMe	TzAA-OEt	TzAA-OPr	TzAA-OiPr	TzAA-OBu	TzAA-OOc	TzAA-S-MTD
1.0	21.3	26.3	18.7	17.2	31.4	30.0	27.5
2.0	43.3	49.2	30.2	24.4	48.1	43.8	38.9
3.0	46.8	53.1	45.9	34.7	56.2	54.7	46.8
4.0	53.9	56.1	51.5	40.6	61.7	57.7	47.0
5.0	58.1	58.8	54.8	47.3	63.8	60.3	—

Note: The abbreviations TzAA-OMe, TzAA-OEt, TzAA-OPr, and so on are described in Materials and Methods.

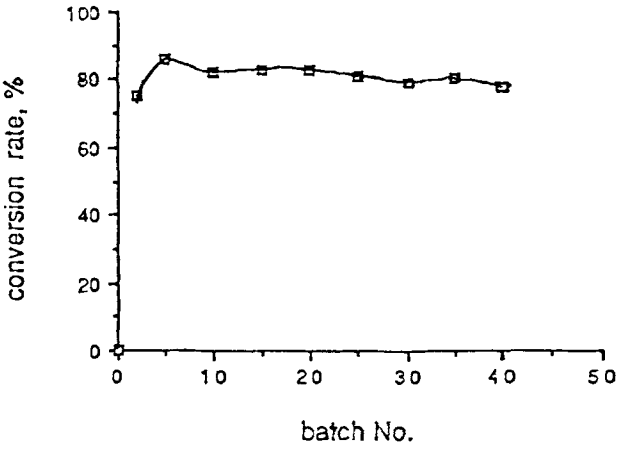

Fig. 3. Continuous synthesis of CEZ with eupergit PcA. For the conditions, *see* the text above.

Enzymatic Synthesis of CEZ

The experiments with continuous CEZ synthesis were carried out in a batch-reactor with a jacket and seeve bottom. The immobilized PA (Eupergit PcA) in the reactor is in a quantity to provide about 10 U of enzyme activity per mL of reaction medium. The solution, containing 10.0 mg/mL of M-7-ACA and 17.0 mg/mL of TzAA-OBu, is heated up to 35°C and poured into the reactor. By continuous stirring, a solution of 2*M* sodium hydroxide is added to maintain a pH of 6.8. The end of the reaction is determined by HPLC analysis (*see* Materials and Methods). After removal of the immobilized enzyme by filtration, the filtrate containing 7.06 mg/mL of CEZ (56.2% conversion rate at 95.2% purity of M-7-ACA) is treated for isolation of the product. In this way, more than 40 batches with the same sample of Eupergit PcA were carried out. The conversion rate in a given reaction batch remained approx the same (Fig. 3). The enzymatic activity assay of Eupergit PcA after 40 batches showed a 5–7% decrease in activity.

SUMMARY

Tetrazole-1-acetic acid esters with lower saturated straight chain alcohols are better for enzymatic synthesis of CEZ than those with branched chain alcohols or thioalcohols. Despite the lower water solubility of the TzAA esters containing lower saturated alcohol than those with polyethyleneglycol (*11*), good conversion rates were obtained with lower quantities of the esters and a shorter time. Multiple batch use of Eupergit PcA for enzymatic synthesis of CEZ with a negligible loss of enzyme activity was observed.

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