AQUEOUS ACETYLATION OF DESACETYL GLUTARYL 7-AMINO-CEPHALOSPORANIC ACID (7ACA) AND SPECULATION ON THE ORIGIN OF DESACETYL CEPHALOSPORIN C IN FERMENTATION BROTH†

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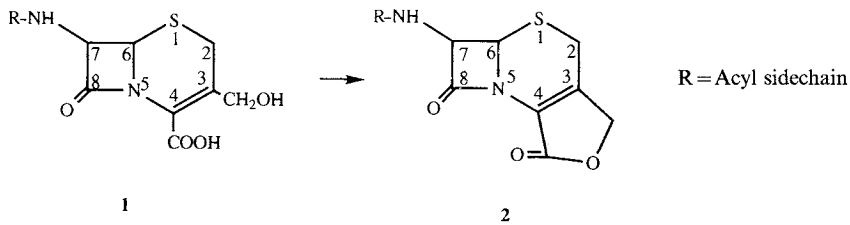
Acetylation of desacetyl glutaryl 7-aminocephalosporanic acid (7ACA), an intermediate in the two-stage enzymatic cleavage of cephalosporin C, has been accomplished in aqueous media with acetic anhydride. Because the reaction is done in water, conversion of desacetyl glutaryl 7ACA to glutaryl 7ACA can be accomplished at an early stage in the purification process. Evidence is presented that desacetyl cephalosporin C in cephalosporin C broth is formed from the mycelial catalyzed hydrolysis of cephalosporin C.

The presence of desacetyl cephalosporin C (**6**) in cephalosporin C (**3**) broth has been a constant thorn in the side of the cephalosporin C purification process. Desacetyl cephalosporin C is always present in cephalosporin C fermentation broth and special or added steps are frequently required to remove or separate it. We had observed that with a large excess of acetic anhydride the 3′-hydroxy of desacetyl cephalosporin C could be acetylated in aqueous solutions; however, as expected, acetylation of the side chain amino occurs as well so that *N*-acetyl cephalosporin C is formed. We proposed that the two-step enzymatic cleavage route to 7-aminocephalosporanic acid (7ACA) (**5**) could offer a solution since desacetyl glutaryl 7ACA (**7**), the desacetyl intermediate formed from desacetyl cephalosporin C, no longer contains an amino group and could be reacetylated so as to obtain its cephalosporin C analog, glutaryl 7ACA (**4**).

Prior efforts at reacetylation of 3′-hydroxy unprotected cephalosporins in aqueous solutions were generally unsuccessful. VAN HEYNIGEN[1] showed that the 3′ acetylation of desacetyl cephalosporins (**1**) in aqueous media was not easily accomplished because of the ease with which the 3-hydroxymethyl lactonized with the 4-carboxyl to form the lactone (**2**) (Fig. 1). Only aromatic acid chlorides were successfully used to esterify the 3′-hydroxyl of desacetyl cephalosporins. SOMERFIELD *et al.*[2] succeeded in acylating the 3-hydroxymethyl group by first forming an ester at the 4-carboxy position to prevent lactone formation.

Fig. 1. Conversion of desacetyl cephalosporins (**1**) to cephalosporin lactones (**2**).



1 **2**

† An account of this work was presented at the 205th American Chemical Sciety National Meeting, Denver, March 28~April 2, 1993.

Their reactions were performed in anhydrous solvents using an organic base, although aqueous acetone was also used with the protected 4-carboxyester derivatives. TSUSHIMA *et al.*[3,4] were able to acetylate desacetyl cephalosporins in non-aqueous solvents such as dimethylformamide. They formed other 3′ ester derivatives as well. In their reactions an organic base such as triethylamine was required.

Chemistry

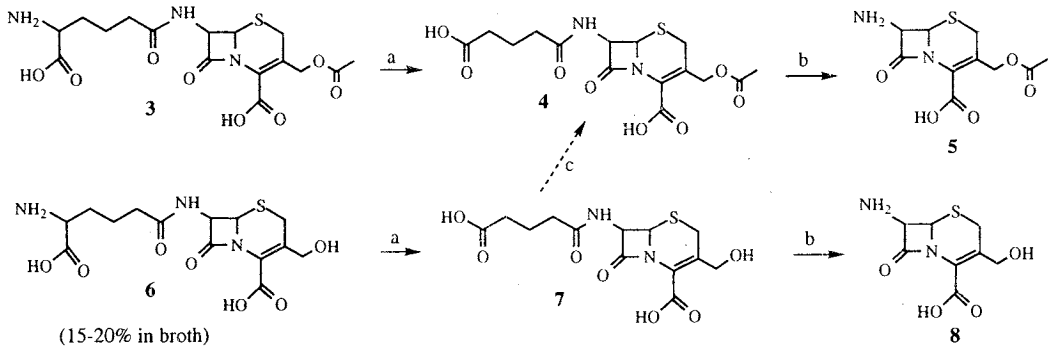
Removal of the α-aminoadipyl side chain of cephalosporin C to obtain 7ACA, a key intermediate in the synthesis of final cephalosporins, has been carried out on a commercial scale on cephalosporin C or derivatives of cephalosporin C by chemical cleavage, most generally using PCl_5[5]. The discovery that enzymatic removal of the glutaryl side chain from cephalosporins was possible has led to a two step process where the first step converts the α-aminoadipyl side chain of cephalosporin C to the glutaryl derivative either by a chemical transamination followed by oxidation with hydrogen peroxide[6] or by enzymatic oxidative deamination[7,8]. In the second step the glutaryl side chain is cleaved by a cephalosporin acylase enzyme to give 7ACA[9~12] (Scheme 1).

Enzymatic cleavage of the α-aminoadipyl group on a commercial scale offers both ecological and health advantages over the chemical cleavage since corrosive reagents such as PCl_5, dimethylaniline or triethylamine, silyl halides, *etc.* are not required. Additionally, the use of toxic solvents such as methylene chloride and tetrahydrofuran is avoided, and the recovery or disposal of these reagents and solvents is not an issue.

The two step enzymatic cleavage process affords an opportunity for additional benefits if the substantial amount of desacetyl cephalosporin C found in fermentation broth can ultimately be utilized. Since the α-aminoadipyl side chain of desacetyl cephalosporin C undergoes the deamination step just as it does in cephalosporin C, the desacetyl analog (desacetyl glutaryl 7ACA) is produced. We have succeeded in converting desacetyl glutaryl 7ACA to glutaryl 7ACA by reacting aqueous solutions containing desacetyl glutaryl 7ACA with acetic anhydride. Scheme 1 illustrates the scenario for the proposed route, with desacetyl glutaryl 7ACA short-circuited back to the mainstream by its conversion to glutaryl 7ACA. This also avoids formation of desacetyl 7ACA (**8**).

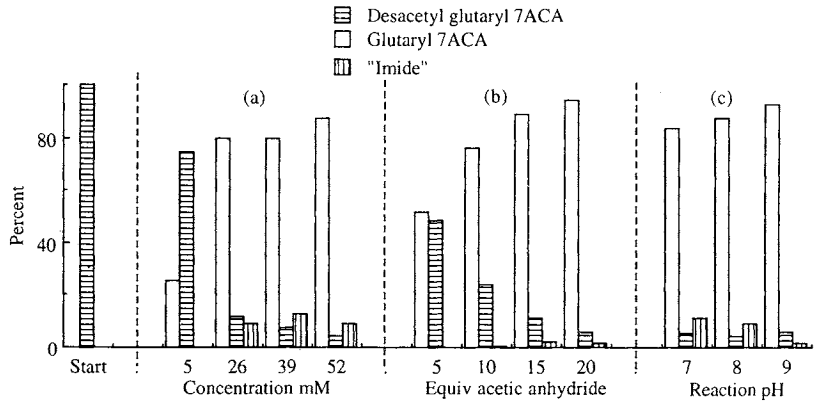
After the acetic anhydride reaction subsequent steps for the purification and isolation of glutaryl

Scheme 1. Two step enzymatic cleavage of cephalosporin C (**3**) and desacetyl cephalosporin C (**6**) showing 3′-acetylation of desacetyl glutaryl 7-aminocephalosporanic acid (7ACA) (**7**) to glutaryl 7ACA (**4**).



a) cephalosporin oxidase, b) cephalosporin acylase, c) acetic anhydride

Fig. 2. Effect of desacetyl glutaryl 7ACA concentration, equivalents acetic anhydride, and pH on acetylation reaction in water.



(a) Reactions at pH 8 with 20 equiv acetic anhydride, (b) reactions at pH 9 with 52 mM desacetyl glutaryl 7ACA, (c) reactions with 52 mM desacetyl glutaryl 7ACA and 20 equiv acetic anhydride.

7ACA or 7ACA can be carried out in a normal manner except that titers will have increased to reflect the conversion of the desacetyl moiety to the desired 3′-acetylated derivative.

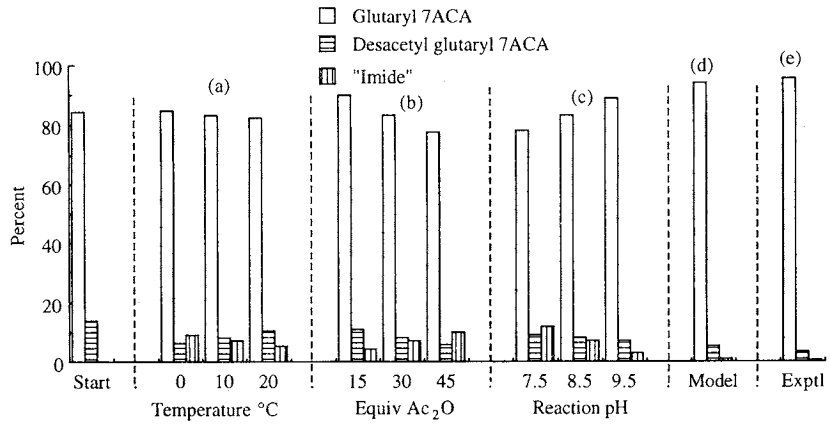
Our preliminary studies with purified desacetyl glutaryl 7ACA (Fig. 2) showed that almost quantitative conversion of desacetyl glutaryl 7ACA to glutaryl 7ACA could be attained if a large excess of acetic anhydride were employed. In addition, the reaction worked best at high concentrations of desacetyl glutaryl 7ACA—presumably to minimize the competing reaction between acetic anhydride and water—and conversion efficiency was also improved at alkaline pH. (Also indicated on the graphs is the synthesis of a byproduct, suspected to be the glutarimidocephalosporanic acid derivative ("imide"). Although not purified, preliminary NMR analysis showed the absence of the amido nitrogen. This product is also formed when glutaryl 7ACA is reacted with acetic anhydride.)

Product identity, in addition to the HPLC profile, was confirmed by comparing the NMR and IR spectra of the reaction product (isolated as the calcium salt) with the calcium salt of glutaryl 7ACA synthesized from 7ACA and glutaric anhydride, as well as with previously published spectra[8].

Initial attempts at controlling the reaction pH with sodium hydroxide proved difficult, with wide variability noted; automatic pH control proved crucial to achieving a relatively steady pH profile. Although it was much easier to control the pH at 9~10 when sodium or potassium carbonate was substituted for sodium hydroxide, this proved to be of little value since virtually no 3′ acetylation occurred when sodium or potassium carbonate was used for pH control.

For broth or eluate solutions, which contain approximately 15% desacetyl glutaryl 7ACA, a factorial design experiment was generated to help establish the best conditions for the acetylation reaction. The results surprisingly showed that an excessive amount of acetic anhydride, although enhancing the 3′-acetylation reaction with desacetyl glutaryl 7ACA, nevertheless gave a reduced level of glutaryl 7ACA. This is a result of the competing formation of "imide", which becomes more significant when the initial concentration of glutaryl 7ACA is high. This is shown in Fig. 3 (a), (b) and (c), where the reaction statistical model is used to illustrate the effect of temperature, acetic anhydride equivalents, and pH on the product profile.

Fig. 3. Relationship of temperature, acetic anhydride equivalents, and pH on acetylation reaction with oxidase treated eluate calculated from factorial design generated statistical model.



(a) Reaction with 30 equiv acetic anhydride, pH 8.5, (b) reaction at 10°C, pH 8.5, (c) reaction at 10°C, with 30 equiv acetic anhydride, (d) predicted and (e) experimental results at 5°C, pH 10.5, with 30 equiv acetic anhydride.

Table 1. Conversion of desacetyl glutaryl 7-aminocephalosporanic acid (7ACA) (**7**) to glutaryl 7ACA (**4**) in oxidase-treated anion exchange resin eluate with 30 equivalents acetic anhydride at pH 10.5 and 0~5°C.

	4	**7**	"Imide"
Starting eluate	53.4 mmol	8.1 mmol	0 mmol
Eluate after acetic anhydride	59.0 mmol	2.1 mmol	0.5 mmol
Net	+5.6 mmol	−6.0 mmol	+0.5 mmol
Yield	+10.5%	−74.1%	

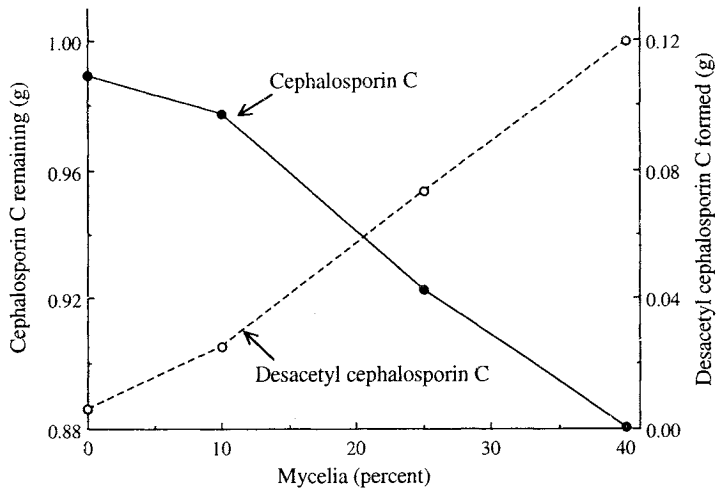
Extrapolating beyond the limits of the factorial experiment, the model projected that conversion yields could be improved and the competing side reaction minimized at pH 10.5 with 30 equivalents of acetic anhydride. (The reaction was run at low temperature because of concern for possible degradation of the β-lactam ring at pH 10.5 and, since the factorial study also showed that the rate of acetic anhydride addition had no significant effect, to keep the time at pH 10.5 to a minimum the reaction time was kept at about 30 minutes.) As is seen in Fig. 3 (d) and (e), the experimental results closely correlated with the model. The net effect, summarized in Table 1, was to increase the amount of glutaryl 7ACA by 10.5%.

The two-step enzymatic cleavage route then would afford the opportunity to realize an additional 10 to 15% more 7ACA by incorporating the acetic anhydride conversion step into the process. This approach would represent a more useful alternative to that of the current practice of discarding desacetyl cephalosporin C at an early or late stage in the cephalosporin C purification process[13]. In addition, if present, the more acid labile desacetyl cephalosporin derivatives rarely survive the chemical cleavage process, but with the enzymatic cleavage route desacetyl 7ACA would remain as an impurity. By combining reacetylation of the 3'-desacetyl group with the enzymatic cleavage process many of these difficulties could be avoided.

Origin of Desacetyl Cephalosporin C

We have also attempted to resolve the controversy regarding the origin of desacetyl cephalosporin C

Fig. 4. Effect of cephalosporin C mycelia on disappearance of cephalosporin C and formation of desacetyl cephalosporin C after 3 hours incubation at 30°C.



in cephalosporin C fermentation broth. Although originally thought to arise principally from chemical degradation[14], the desacetyl cephalosporin C in cephalosporin C broth (usually present at the end of the cephalosporin C fermentation cycle in an amount equivalent to 15~20 mole-percent relative to cephalosporin C), was considered by subsequent investigators to be present in an amount greater than could be explained from chemical degradation alone[15,16]. We have investigated the effect of *Cephalosporium acremonium* mycelia on desacetyl cephalosporin C formation from cephalosporin C.

Table 2. Effect of phosphorous acid and phosphite inhibitors on disappearance of cephalosporin C (**3**) and formation of desacetyl cephalosporin C (**6**) catalyzed by 40% (w/v) *Cephalosporium acremonium* mycelia after 4 hours incubation at 30°C.

Inhibitor added[a]	**3** remaining[b] (g)	**6** formed[b] (g)
None	0.75	0.20
Phosphorous acid	0.89	0.09
Dimethyl phosphite	0.85	0.04
Trimethyl phosphite	0.89	0.05

[a] Inhibitor added at 10 mg/ml to incubation mixture.
[b] Based on 1.0 g **3** initially present.

In our experiments we added washed cephalosporin C mycelia to a buffered solution of cephalosporin C and monitored for the disappearance of cephalosporin C and the appearance of desacetyl cephalosporin C. As is illustrated in Fig. 4, although some desacetyl cephalosporin C is formed in its absence, the disappearance of cephalosporin C and the formation of desacetyl cephalosporin C is markedly enhanced by the addition of mycelia to buffered cephalosporin C solutions.

Where the non-enzymatic rate for cephalosporin C degradation at 25°C in these experiments is 0.35% $hour^{-1}$, the effect of mycelia is to increase the degradation rate by an average of 0.07% $hour^{-1}$ per weight percent wet mycelia added. Presumably an esterase present in *C. acremonium* catalyzes the hydrolysis of the 3'-acetyl. Evidence for an enzymatic hydrolysis is further bolstered by demonstrating the *in vitro* inhibition of cephalosporin C to desacetyl cephalosporin C degradation by the addition of enzyme inhibitors which had been demonstrated previously to be effective for reducing the amount of desacetyl cephalosporin C produced in the cephalosporin C fermentation process[16] (Table 2).

Experimental

Chromatography

The acetylation reaction with acetic anhydride was followed by HPLC analysis using a 15 cm Zorbax C8 column and monitored at 254 nm. Mobile phase: CH_3CN - 0.005 M tetrabutyl ammonium hydroxide (pH 5.0 with AcOH) (30 : 70). Flow rate: 1.5 ml/minute.

The reaction of cephalosporin C with mycelia was monitored by HPLC using a 15 cm Zorbax C8 column and a 260 nm detector. Mobile phase: MeOH - 0.02 M tetrabutyl ammonium hydroxide, 0.035 M AcOH (25 : 75). Flow rate: 1.5 ml/minute.

Preparation of Desacetyl Glutaryl 7ACA (7)

Glutaryl 7ACA (**4**) (2.0 g, 5.2 mmol) obtained synthetically from 7ACA (**5**) and glutaric anhydride as described by SHIBUYA *et al*[10] was slurried in 45 ml water and the pH was adjusted to 6.0 with 5 N NaOH to dissolve **4**, then diluted to 100 ml with water. To that solution was added 0.3 g of crude yeast cephalosporin acetylesterase[17]; the solution was stirred at 22°C ~ 25°C for 4 hours while maintaining the pH at 5.9 ~ 6.1 with 5 N NaOH. The yield by HPLC to **7** was 98% with < 1% **4** remaining. The resulting solution was filtered with filter aid to remove the microbial enzyme and aliquots were used for subsequent chemical conversions of **7** to **4**. The solution may be stored frozen.

Reaction of Acetic Anhydride with 7

The initial screening experiments, except for the very dilute solution, were carried out on 4 ml aliquots (0.21 mmol) of **7** diluted with water to concentrations shown in Fig. 2. To these solutions in a 30 ml magnetically stirred conical titrator cup set in an ice bath was added dropwise 0.4 ml (4.2 mmol) acetic anhydride in 0.1 ml aliquots; the pH was maintained at 7, 8, or 9 by the automatic addition of 5 N NaOH from a Masterflex pump activated by a Cole Palmer L-05652 pH controller. After the addition of each 0.1 ml aliquot of acetic anhydride a 0.1 ml or 0.2 ml aliquot of the reaction solution was withdrawn and diluted to 10 ml with water for HPLC analysis.

Crystallization of 4 Calcium Salt

Compound **4** (5.2 mmol) was slurried in 30 ml water in a beaker and dissolved by adding 1 ml 5 N NaOH. The pH rose from 2.2 to 3.7. To the stirred solution was added 1.1 g $CaCl_2$ (10 mmol) and another 1 ml 5 N NaOH (final pH was 5.4). The solution was diluted to 40 ml with water. To a 10 ml aliquot was then added with swirling 50 ml EtOH. The precipitate of **4** calcium salt was filtered on a 4.25 cm Büchner with Whatman 1, and the cake was washed on the filter with 25 ml EtOH to give 0.52 g of dry product. This was used as a control for NMR and IR.

^1H NMR (D_2O) δ 5.53 (1H, d, J=4.7 Hz, 7-H), 5.03 (1H, d, J=4.7 Hz, 6-H), 3.32 and 3.58 (2H, ABq, J_{AB}=17.9 Hz, S–CH_2), 4.64 and 4.80 (2H, ABq, J_{AB}=12.4 Hz, CH_2–OAc), 2.28 (2H, t, J=7.5 Hz, CH_2–CO), 2.15 (2H, t, J=7.6 Hz, CH_2–CO), 1.79 (2H, m, J=7.6 Hz, –CH_2–), and 2.02 (3H, s, Ac).

IR ν_{max} (KBr) cm^{-1}: 3386, 1761, 1558, 1417, 1237, 1118, 1073, 1039.

Crystallization of 7 Calcium Salt

An aqueous solution of **4** was prepared by slurrying 4.0 g (10.4 mmol) in 60 ml water and adjusting the pH to approx 4.0 with 5 N NaOH. To the solution was added 2.2 g $CaCl_2$ (20 mmol) and the pH was adjusted to approx 6.0 with 5 N NaOH. Crude yeast cephalosporin acetylesterase (0.4 g) was added and the solution was stirred at 22°C ~ 25°C for 2.5 hours while the pH was maintained at 5.9 ~ 6.1 with 5 N NaOH. The yield by HPLC to **7** was 97% with < 1% **4** remaining. The resulting solution was filtered with filter aid to remove the enzyme and was diluted with water to 90 ml. The product was precipitated by the addition of 450 ml EtOH. The crystals were filtered, washed with EtOH, and dried. The yield was 83%.

^1H NMR (D_2O) δ 5.53 (1H, d, J=4.6 Hz, 7-H), 5.03 (1H, d, J=4.7 Hz, 6-H), 3.37 and 3.56 (2H, ABq, J_{AB}=17.8 Hz, S–CH_2), and 4.17 and 4.20 (2H, ABq, J_{AB}=12.9 Hz, CH_2–OH), 2.27 (2H, t, J=7.5 Hz, CH_2–CO), 2.14 (2H, t, J=7.5 Hz, CH_2–CO), and 1.77 (2H, m, J=7.6 Hz, –CH_2–).

IR ν_{max} (KBr) cm^{-1}: 3379, 1757, 1557, 1416, 1114, 1071, 1037.

Preparation of **4** Calcium Salt from **7** Calcium Salt

An aqueous solution of **7** calcium salt prepared by dissolving 2.0 mmol in approx 15 ml water was chilled in an ice bath. To it was added 1.9 ml acetic anhydride (20 mmol) at 0.0475 ml/minute and 10 N NaOH at a rate to maintain the reaction pH at 10.0±1. By HPLC the percent of **4** after all the acetic anhydride was added was 97%. The pH was allowed to drift down to 9.1 and 3.3 g $CaCl_2$ (30 mmol) was added which lowered the pH to 7.2. One drop of 6 N HCl was added to drop the pH to 6.4 and the reaction solution was diluted with water to 25 ml. The *in situ* yield to **4** by HPLC was 90%. The calcium salt of **4** was precipitated by adding 10 volumes (250 ml) of EtOH to the reaction solution. A gelatinous precipitate of $Ca(OAc)_2$ coprecipitated with the product. The precipitate was filtered and washed with 50 ml EtOH, then was dried at 50°C in the vacuum oven. The resulting dry powder was pulverized and reslurried in 250 ml MeOH to dissolve $Ca(OAc)_2$. The product was filtered and washed with 50 ml MeOH. Crystallization yield to **4** was 93%. HPLC retention time, NMR and IR analysis of this product matched that of **4** calcium salt derived from the reaction of **5** with glutaric anhydride.

Fractional Factorial Study with **4** Eluate

To a 40 ml aliquot of *Triginopsis variabilis* oxidase treated cephalosporin C (**3**) ion exchange resin eluate[18] containing 4.1 mmol **4** and 0.75 mmol **7** was added 1, 2, or 3 ml acetic anhydride (10.6, 21.2, and 31.7 mmol, respectively) with a syringe pump set to deliver at 1.7 ml/hour, 4.1 ml/hour, or 6 ml/hour. The pH was maintained at 7.5, 8.5, or 9.5 by the automatic addition of 10 N NaOH from a Masterflex pump activated by a Cole Palmer auto pH controller. The reaction flask was set in a bath cooled to 0°C, 10°C, or 20°C with ice and water. At the end of the addition and after the pH had stabilized a 0.4 ml aliquot was withdrawn and diluted to 10 ml with 0.005 M tetrabutyl ammonium acetate, pH 5, for HPLC analysis.

Optimized Eluate Acetylation

To a 1-liter round bottom flask was added 500 ml oxidase treated **3** eluate containing 53.4 mmol **4** and 8.1 mmol **7**. The flask was set in an ice bath and the pH adjusted to 10.5 with 10 N NaOH. With stirring, 25 ml acetic anhydride (265 mmol) was added at 0.78 ml/minute. The pH was controlled at 10.5±0.5 by the automatic addition of 10 N NaOH from a Masterflex pump activated by a Cole Palmer auto pH controller. A total of 50 ml 10 N NaOH (500 mmol) was used for pH control and the temperature went to 7°C during the acetic anhydride addition. The resulting eluate now contained 59.0 mmol **4** and 2.1 mmol **7**. By HPLC analysis the amount of "imide" produced was approx 1%.

Mycelial Esterase Experiments

Mycelia from **3** fermentation with *C. acremonium* was obtained by filtration on Whatman 1 filter paper (no additives or pH adjustment prior to filtration). The filter cake was washed with 1/10 the original volume deionized water. The moist filter cake could be stored in the freezer.

To 500-ml Erlenmeyer flasks containing 100 ml of a solution of **3** (0.024 M) in 0.2 M potassium phosphate buffer, pH 7 was added varying amounts of moist **3** mycelia. The flasks were set on a rotary shaker at 30°C. A reagent blank consisting of the **3**-buffer mixture without mycelia and a mycelial blank consisting of mycelia in buffer without **3** were also prepared.

For the phosphite inhibition studies 1 g respectively of phosphorous acid, trimethyl phosphite, and dimethyl phosphite were added to the flasks prior to 40% (w/v) mycelia addition.

To test for desacetyl cephalosporin C (**6**) formation approximately 20 ml aliquots were withdrawn periodically and gravity filtered through Whatman 1 filter paper. The filtrate was diluted and analyzed by HPLC.

Acknowledgments

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