Isolation and Identification of a New Cephem Compound from *Penicillium chrysogenum* Strains Expressing Deacetoxycephalosporin C Synthase Activity

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During the course of our effort to identify compounds present in a recombinant strain of *Penicillium chrysogenum* expressing deacetoxycephalosporin C synthase activity, we have identified a new cephem compound 16-hydroxyadipoyl-7-ADCA (**2**) along with deacetoxycephalosporin C (**1**).

The biosynthesis of penicillins and cephalosporins share the first two steps in common. First, L-α-aminoadipic acid, L-cysteine and L-valine are condensed to form LLD-α-aminoadipyl-cysteinyl-valine, which then cyclize to form isopenicillin N. In organisms that produce cephem compounds, the isopenicillin N is epimerized to penicillin N by the action of a specific epimerase. The enzyme expandase then uses penicillin N as a substrate to form deacetoxycephalosporin C (**1**). Expandase has no activity with isopenicillin N[1].

On the other hand, penicillin producing organisms, such as *P. chrysogenum*, do not epimerize isopenicillin N, and instead produce different penicillins through the action of IPNS:acyl-coenzyme A acyltransferase. This enzyme catalyzes the replacement of the L-α-aminoadipic acid side chain of isopenicillin N with other carboxylic acids that are present in the cell as their coenzyme A derivatives, including hexanoyl-CoA, hexenoyl-CoA and octanoyl-CoA. By feeding specific carboxylic acids such as phenylacetic acid to cultures, certain penicillins such as penicillin G are produced.

It has been shown that feeding adipate to penicillin-producing strains *P. chrysogenum* expressing *S. clavuligerus* expandase activity leads to production of adipyl-7-ADCA, providing an efficient process for producing the cephalosprin intermediate, 7-ADCA[2]. When these same strains were cultured without addition of adipate, two components were observed that had a UV spectrum characteristic of cephem compounds. Here we describe the isolation and characterization of these two components, deacetoxycephalosporin C (**1**) and the new cephem 16-hydroxyadipoyl-7-ADCA (**2**).

Creation of the recombinant *P. chrysogenum* strain used in this study (PC100) was previously described[2]. The strain expresses the *S. clavuligerus* expandase gene to high levels through its fusion to the isopenicillin N synthetase gene promoter. The selectable marker is a gene expressing a phleomycin binding protein with no known enzymatic activity. Thus, the only additional enzyme activity in this strain is that of expandase. This strain was cultured in production media containing no side chain precursor.

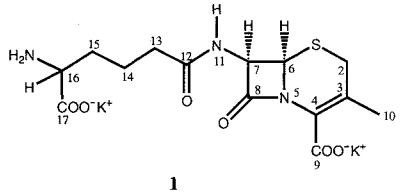
Results

Isolation

The metabolites were isolated from the fermentation broth by a series of chromatographic procedures. The filtered broth was first applied to an HP-20 resin column and the components sequentially eluted with H_2O, 50% aqueous acetone and finally with 100% acetone. The metabolites of interest was concentrated in the 50% aqueous acetone and 100% acetone fractions. Final purification of **1** and **2** were achieved by repeated semi-preparative HPLC of the 100% acetone fraction on tandem Nova-pak C_{18} cartridges with 10 mM phosphate buffer and MeOH. The HLPC fractions having the diagnostic UV absorption maxima at 260 nm[3] were collected and identified as cephems **1** and **2**.

Structure Determination

The characteristic features in the ^1H NMR spectrum of these compounds are two pairs of an AB quartet originating from the vicinally coupled β-lactam protons ($J=4.38$ Hz, consistent with a *cis* relationship between these two protons) and the geminally coupled 2-methylene protons ($J=17.98$ Hz). The unusually large geminal coupling between the C-2 protons can be attributed to the presence of the double bond between the C-3 and C-4 positions (proximity of the p orbital causing the greater geminal coupling). This large coupling is diagnostic of the rigid dihydrothiazine ring geometry in 3-cephems, and the chromophore was further substantiated by a UV spectrum with an absorption



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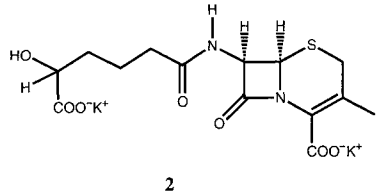

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Table 1. ^{1}H NMR data of compound **1** and **2**.

Proton	Chemical shift (multiplicity)	
	1	**2**
2	3.36 (d, J=17.98 Hz)	3.37 (d, J=17.98 Hz)
2′	3.05 (d, J=17.98 Hz)	3.04 (d, J=17.98 Hz)
6	4.86 (d, J=4.38 Hz)	4.87 (d, J=4.29 Hz)
7	5.33 (d, J=4.38 Hz)	5.33 (d, J=4.29 Hz)
10	1.75 (s)	1.73 (s)
13	2.19 (m)	2.16 (m)
14	1.51 (m)	1.50 (m)
15	1.67 (m)	1.50 (m)
16	3.59 (m)	4.02 (m)

Table 2. ^{13}C NMR data of compound **1** and **2**.

Carbon	Chemical shift	
	1	**2**
2	29.4	29.3
3	126.3	125.6
4	126.3	126.3
6	57.6	57.6
7	59.4	59.4
8	165.5	165.5
9	170.0	170.1
10	19.4	19.3
12	177.4	178.0
13	35.3	35.6
14	21.6	21.8
15	30.4	33.6
16	57.6	71.1
17	174.9	179.6

maxima at 260 nm.

The compound **1** is a colorless powder. The spectroscopic data of compound **1** matched with that reported for deacetoxycephalosporin C[4]. The ion-spray mass spectra afforded ($M^+ + K$) and ($M^+ - H + 2K$) at m/z 396 and 434, respectively in positive-ion mode and 356 ($M^+ - H$) in negative-ion mode. Thus compound **1** was identified as the potassium salt of deacetoxycephalosporin C.

Compound **2** is also a colorless powder. When ^{1}H and ^{13}C NMR data were compared with other related 3-cephems, such as deacetoxycephalosporin C (**1**)[4], similarities were observed (Tables 1 and 2). However, significant differences were observed for the C-15, C-16 and C-17 resonances; down field shifts of $\Delta\delta$ +3.2, +13.5 and +4.7, respectively, were found comparing the signals of **2** with the respective signals of **1**. A similar trend was observed in the ^{1}H NMR spectrum of **2**. The C-16 resonance of **2** was shifted 0.42 ppm down field from that in **1** suggesting the replacement of an amino group on C-16 with a hydroxyl group[5]. Mass spectra of **2** were obtained by ion spray mass spectrometry, which gave peaks at m/z 397 ($M^+ + K$) and at m/z 435 ($M^+ - H + 2K$) in positive-ion mode and at m/z 357 ($M^+ - H$) in the negative-ion mode, indicating a molecular weight of 358 and 434 for the free acid and potassium salt, respectively. Thus compound **2** was identified as the potassium salt of 16-hydroxyadipoyl-7-ADCA.

The production of **1** by these strains suggests that *P. chrysogenum* contains some IPNS epimerase activity. This activity is probably masked by the high activities of the penicillin biosynthetic enzymes during precursor feeding. It would be interesting to determine whether this weak epimerase activity is catalyzed by an enzyme with any relationship to the epimerase found in cephem biosynthetic gene clusters, or whether the activity is a side reaction of an amino acid racemase. The production of 16-hydroxyadipyl-7-ADCA (**2**) can be explained in a number of ways. Perhaps α-hydroxyadipate is present in the cells and is accepted by acyltransferase to yield α-hydroxyadipyl-6-APA. α-Hydroxyadipate could be an intermediate in an unknown anabolic pathway to α-aminoadipate, or an unknown catabolic pathway from α-aminoadipate. Alternatively, the amino function of isopencillin N or pencillin N could be converted to the hydroxy function by an unknown pathway. All of the above explanations require that α-hydroxyadipyl-6-APA is a substrate for the heterologous expandase activity, which has not been established experimentally.

Experimental

General

^{1}H NMR and ^{13}C NMR spectra were recorded at on a Bruker 300 AC/AM spectrometer operating at 300 MHz and 100 MHz, respectively. The D_2O signal was used as a reference for the ^{1}H NMR spectra and ^{13}C NMR data are referenced internally to 1,4-dioxane (δ 67.3 ppm). Individual ^{13}C NMR peaks assignments follow the literature assignments for **1** and are also based on chemical shift arguments, but they have not been confirmed by 2D NMR experiments. Mass spectra were obtained on a PE Sciex API III triple-quadrupole mass spectrometer interfaced *via* a Sciex ion spray probe. HPLC chromatography was performed on a Waters Delta Prep 4000 equipped with a Water's 4000 System Controller and a Water's photodiode array detector. Separation was achieved using a linear gradient with 10 mM phosphate buffer, pH 3.5 and MeOH on two 25 × 100 mm, C_{18}-Nova-pak cartridges at ambient temperature with flow rate of 10 ml/minute.

Physico-chemical Properties

Deacetoxycephalosporin C (**1**) Colorless powder; UV

λ_{max} 260 nm; Ion-Spray-MS (positive ion) *m/z* 396 ($M^+ + K$), 434 ($M^+ - H + 2K$) and Ion-Spray-MS (negative ion) 356 ($M^+ - H$); ^1H NMR (see Table 1); ^{13}C NMR (see Table 2).

16-Hydroxyadipoyl-7-ADCA (**2**) Colorless powder; UV λmax 260 nm; Ion-Spray-MS (positive ion) *m/z* 397 ($M^+ + K$), 435 ($M^+ - H + 2K$) and Ion-Spray-MS (negative ion) 357 ($M^+ - H$); ^1H NMR (see Table 1); ^{13}C NMR (see Table 2).

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