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ADIPOYL-6-AMINOPENICILLANIC ACID IS A SUBSTRATE FOR DEACETOXYCEPHALOSPORIN C SYNTHASE (DAOCS)

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Abstract: Adipoyl-6-aminopenicillanic acid **5** was incubated with recombinant DAOCS and the resulting cephalosporin isolated. Adipoyl-6-aminopenicillanic acid **5** is approximately 735 times less efficient as a substrate for DAOCS *in vitro* (as judged by k_{cat}/K_m) than penicillin N **1**.

The first committed step in the biosynthesis of all cephalosporins is the expansion of penicillin N **1** to deacetoxycephalosporin C (DAOC, **2**). This is followed by hydroxylation of the latter to give deacetylcephalosporin C (DAC, **3**) (Figure 1). In prokaryotes, such as *Streptomyces clavuligerus*, these steps are catalysed by two separate enzymes, deacetoxycephalosporin C synthase (DAOCS) and deacetylcephalosporin C synthase (DACS), whilst in eukaryotes, such as *Cephalosporium acremonium*, they are catalysed by a single, bifunctional enzyme (DAOC/DAC synthase). In *C. acremonium* DAC **3** is converted by acetylation into cephalosporin C **4**. In prokaryotes alternative pathways also exist to give other cephems, *e.g.* in *S. clavuligerus* DAC **3** is converted *via* several steps into cephamycin C.[1]

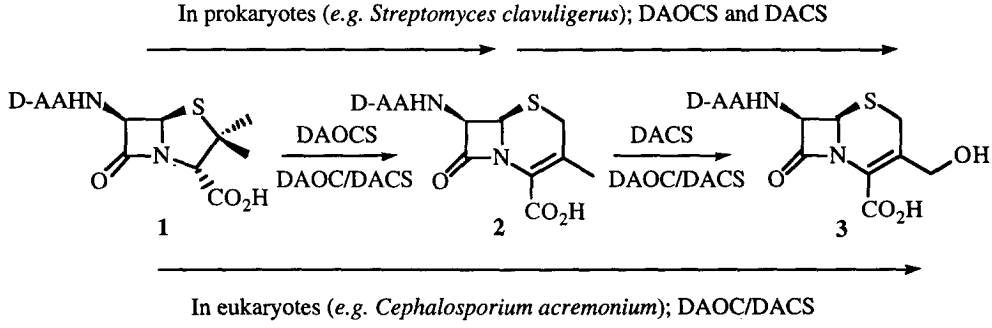


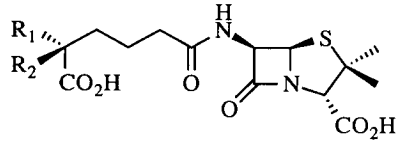
Figure 1: Initial steps in the biosynthesis of the cephalosporin antibiotics D-AA = *D*-δ-(α-aminoadipoyl)

Adipoyl-6-aminopenicillanic acid (adipoyl-6-APA, **5**), the analogue of penicillin N **1** lacking the side chain amino group, has been previously reported to be a substrate for native DAOC/DAC synthase from *C. acremonium*.[2b, 2c] However, the expansion of adipoyl-6-APA **5** to adipoyl-7-aminodeacetoxycephalosporanic acid (adipoyl-7-ADCA, **6**) by DAOCS *in vitro* has not been observed despite studies by two different groups.[3,4] Indeed, Maeda *et al.* recently reported a study in which 18 penicillins were examined as substrates of crude

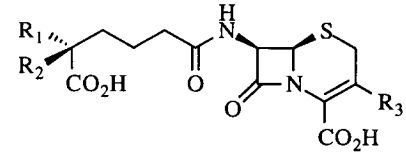
preparations of DAOCS from *S. clavuligerus*. The presence of cephalosporins was assayed for by the holed-plate assay method using *Escherichia coli* Ess as the indicator organism.[4] From their preliminary studies using crude preparations of DAOCS they concluded that "adipoyl-6-APA **5**, *m*-carboxyphenylacetyl-6-APA and D-glutamyl-6-APA were inactive in the present work" and that the "substrate specificity of DAOCS from *S. clavuligerus* is extremely narrow".[4] These results were somewhat surprising in the light of previous studies in which we were able to isolate the anticipated expanded products from incubations of **5** and *m*-carboxyphenylacetyl-6-APA with partially purified DAOCS/DACS from *C. acremonium*. We concluded that DAOCS/DACS has a similar requirement to isopenicillin N synthase (IPNS), in that for efficient conversion substrates require a 6-carbon side chain terminating in a carboxylic acid.[2] However, IPNS will accept substrates with both the *L*- and *D*-α-aminoadipoyl side chains[2a], whilst DAOC/DAC synthase will apparently only accept penicillins with the *D*-α-aminoadipoyl side chain, and not those with the *L*-α-aminoadipoyl side chain, *e.g.* isopenicillin N **7**.[5a] Sequence comparisons between the enzymes of cephalosporin biosynthesis and IPNS in the light of the recently reported crystal structure of the latter[6] suggest that they possess closely related structures.

Crawford *et al.* have recently reported a bioprocess for the production of 7-aminodeacetylcephalosporanic acid (7-ACA **8**) or 7-aminodeacetoxycephalosporanic acid (7-ADCA **6**).[7] Recombinant *Penicillium chrysogenum* was transformed[8] with the genes for either DAOCS or DAOC/DACS and fermented in the presence of added adipic acid. Recombinant organisms containing the DAOCS gene produced adipoyl-7-ADCA **6**, whilst those containing the DAOC/DACS gene produced adipoyl-7-ADCA **6** and adipoyl-7-ACA **8**.[7] The adipoyl side chains can be cleaved *in vitro* by an amidase catalysed reaction. It was speculated that exchange of the *L*-α-aminoadipoyl side chain of isopenicillin N **7** occurred *in vivo* to give **5** which is then ring expanded, *i.e.* that both DAOCS and DAOCS/DACS are capable of expanding adipoyl-6-APA **5** to adipoyl-7-ADCA **6**.

Previously **5** has been prepared[2a,9b] using protected 6-APA and protected adipic acid. The disodium salt of adipoyl-6-APA **5** may be more conveniently prepared by direct reaction[9a] of adipoyl anhydride with 6-APA in the presence of $NaHCO_3$ (2 equiv.) in acetone, water [1:1(v/v)].[9b] Incubations of adipoyl-6-APA **5** were carried out using recombinant DAOCS isolated from *E. coli* BL21 (DE3)/pML1 to > 95% purity by SDS-PAGE analysis.[10] Incubation of **5** with DAOCS in the presence of the appropriate cofactors and cosubstrates gave a new cephalosporin product which was detected by ^1H NMR analysis (500 MHz)[11] of the crude incubation mixture. Purification *via* reverse phase HPLC (ODS, 25 mM NH_4HCO_3, 5% methanol) yielded, adipoyl-7-ADCA **6** (Figure 2) [electrospray ionisation mass spectrometry ($M-H^+ = 341$)].[11]



adipoyl-6-APA **5**, $R_1 = R_2 = H$
isopenicillin N **7**, $R_1 = H$, $R_2 = NH_2$



cephalosporin C **4**, $R_1 = NH_2$, $R_2 = H$, $R_3 = OAc$
adipoyl-7-ADCA **6**, R_1, R_2, $R_3 = H$
adipoyl-7-ACA **8**, R_1, $R_2 = H$, $R_3 = OH$

Figure 2: Structures of **4**, **5**, **6**, **7** and **8**

Kinetic studies to assess the efficiency of adipoyl-6-APA **5** as a substrate for DAOCS used an anion-

exchange HPLC assay.[12,13b] Apparent kinetic parameters for adipoyl-6-APA **5** were determined: K_m = 1.22 ± 0.15 mM, k_{cat} = 0.071 ± 0.004 S^{-1}. Using the same preparation of DAOCS for penicillin N **1**: K_m = 10.34 ± 1.76 μM and k_{cat} = 0.442 ± 0.017 S^{-1} (0.762 ± 0.03 μmol/min/mg). Previously values for recombinant DAOCS from alternative expression systems of 29 μM and 0.432 μmol/min/mg[5,13a] and 33 μM and 0.69 μmol/min/mg,[13b] for K_m and V_{max} respectively have been reported.

Thus, penicillin N **1** is approximately 735 times more efficient as a substrate for DAOCS than adipoyl-6-APA **5**, as judged by k_{cat}/K_m. Deletion of the amine group of the side chain apparently decreases the binding of the penicillin to DAOCS by a factor of about a hundred. In contrast, k_{cat} is only reduced by a factor of about six. Previous substrate analogue studies on IPNS[14] and native DAOCS/DACS[2b] suggest that the side chain binding site for substrates of DAOCS is oriented away from the metal centre at the active site, possibly explaining the relatively modest decrease in k_{cat}. Comparison of the results reported herein with the preliminary results for DAOC/DACS[2b] may suggest that **5** is a much better substrate for DAOC/DACS than for DAOCS. However, in the previous study DAOC/DACS was only partially purified from the native source, *C. acremonium*, and different assays were used in the two studies.[15] Thus, it is unclear if there is a significant difference in the sidechain specificity between DAOCS and DAOC/DACS.

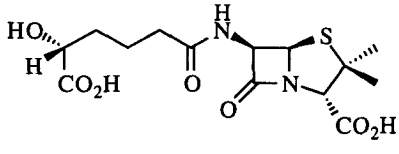
The failures of Yeh *et al.*[3] and Maeda *et al.*[4] to observe *in vitro* conversion of **5** to **6** by DAOCS may result in part from the greatly increased K_m of **5** relative to **1**, since in the former case **5** was incubated with DAOCS at sub-millimolar concentrations[3] (Maeda *et al.*[4] do not explicitly report the concentration of **5** which they used in their incubations). The lower k_{cat} value for the conversion of **5** to **6** relative to that for the conversion of **1** to **2** may also have contributed to the absence of product detection. Additionally in the present study homogenous (>95% by SDS-PAGE analysis) over-expressed enzyme was used compared with the two previous studies[3,4] which used crude cell extracts from *S. clavuligerus*, which may contain materials capable of modifying the activity of DAOCS. DAOCS, like many other ferrous-dependent dioxygenases, apparently undergoes inactivation of activity in the crude extracts. Thus, it is possible and indeed likely that the enzyme concentration was limiting in the previous studies on the relative substrate specificity of different penicillins, using partially purified protein from the native organism.

In addition, the use of a bio-assay to assay cephalosporin products may give false negatives, since the antibacterial activity of cephalosporins is dependent upon the side chain present. Controls produced by incubation of **5** with purified DAOCS showed that 0.63-1.45 nmol of adipoyl-7-ADCA **6** could be readily detectable by the HPLC assay[12], but that these amounts of **6** failed to visibly inhibit the growth of *E. coli* X580 in the standard holed-plate assay.

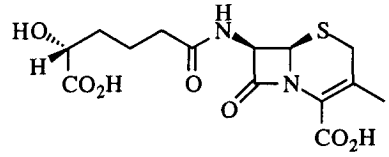
The results of the present study suggest that adipoyl-7-ADCA **6** and adipoyl-7-ACA **8** are produced *in vivo* by ring expansion of adipoyl 6-APA **5** as proposed by Crawford *et al.*[7] In turn **5** is probably biosynthesised *via* exchange of the adipoyl for the *L*-δ-α-aminoadipoyl side chain of isopenicillin N **7**, a process catalysed by an acyl transferase. The possibility that the adipoyl-6-APA **5** is produced directly *via* IPNS catalysed cyclisation of the tripeptide adipoyl-*L*-cysteinyl-*D*-valine is less likely since although this tripeptide is a substrate for IPNS[14], there is no evidence that adipic acid can substitute for *L*-α-aminoadipic acid in the tripeptide synthetase catalysed tripeptide formation reaction. Indeed, adipic acid does not catalyse exchange of labelled phosphate in the ATP-PP_i exchange assay with ACVS from *C. acremonium*[16a] and has been reported not to be a productive substrate for tripeptide formation for ACVS from *C. acremonium* and *S. clavuligerus*.[16]

Recently Alvi *et al.* have reported the isolation of DAOC **2** and 16-hydroxyadipoyl-7-ADCA **9** (Figure

3) from *P. chrysogenum* strains expressing DAOCS activity.[17,18] The isolation of DAOC **2** was taken to suggest that *P. chrysogenum* contains some isopenicillin N epimerase activity, as it has been previously reported by Dotzlaf *et al.* that isopenicillin N **7** is not a substrate *in vitro* for DAOCS from *S. clavuligerus*.[5] However, herein we have demonstrated that *in vitro* work may sometimes give misleading results for some of the penicillins assayed as substrates for DAOCS. Thus it can not be ruled out that isopenicillin N **7** is a (poor) substrate *in vivo* for DAOCS. Furthermore, it is clear that efficient conversion of substrates *in vitro* is not necessarily indicative of the usefulness of bio-transformation *in vivo*, since **5** is apparently a poor substrate *in vitro* for DAOCS, yet this reaction is apparently the key step in the fermentation process developed by Crawford *et al.* for the production of adipoyl-cephalosporins.[7]



16-hydroxyadipoyl-6-APA **10**



16-hydroxyadipoyl-7-ADCA **9**

Figure 3: Structures of **9** and **10**

Finally, the ring expansion of adipoyl-6-APA **5** to give adipoyl-7-ADCA **6** may be used as a convenient assay for routine measurement of DAOCS activity *in vitro*. Although much higher concentrations of adipoyl-6-APA **5** than **1** are required in the assay, the relatively increased stability and ease of preparation of **5** make it an attractive alternative to penicillin N **1**.

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11. Incubations were carried out in a total volume of 2.5 mL at 27° C and 250 r.p.m. Incubation mixtures contained DAOCS (4-5 mg, specific activity = 0.3-0.4 μmol/min), adipoyl-6-APA **5** (10 mM), and cofactors [50 mM $(NH_4)_2SO_4$, 10 mM α-ketoglutarate, 2 mM DTT, 1 mM ascorbate, 50 μM $FeSO_4$, NaOH to pH 7.5 in 50 mM NH_4HCO_3, pH 7.5] in 50 mM NH_4HCO_3. After two hours the incubation was quenched with 7.5 mL acetone, and the protein removed by centrifugation (12,000 *g*, 10 min.). Acetone was removed *in vacuo* and the residue freeze-dried. The crude incubation mixture was purified by HPLC [ODS (250 x 4.6 mm), 25 mM NH_4HCO_3 in 5% (v/v) methanol, 1 mL/min, λ = 254 nm, retention volume 22 mL]: NMR (500 MHz, D_2O and 10% (v/v) [$^2H_6$]-acetone/2,2,3,3-[$^2H_4$]-trimethylsilylpropanoic acid) was consistent with those reported in reference 2b; m/z (electrospray ionisation) = 341 ($M-H^+$, 100%).

12. Production of **6** was monitored by HPLC[13b]: Microsorb Amine (Anachem, 4.6 x 250 mm) retention volume of **6** = *ca.* 8.5 mL. Doping experiments with standards of isolated **6** from preparative scale incubations showed that the new peak observed after incubations of **5** with DAOCS corresponded to **6**. Kinetic experiments were carried out at 30 °C in 50 mM Tris-HCl, pH 7.5 in a total volume of 200 μL. DAOCS and cofactors were pre-incubated for 5 minutes before addition of buffer (140 μL) and substrate (20 μL). At least six different concentrations of substrate were used (at least) in duplicate. The reaction was quenched after 5 minutes by addition of 200 μL of methanol, and the mixture transferred to a 1.5 mL Eppendorf tube. After centrifugation for 2 minutes, 100-150 μL of the reaction mixture was injected onto the anion-exchange HPLC column, which had been equilibrated in 0.24 M sodium acetate titrated to pH 4.0 with acetic acid and analysed at λ = 254 nm and 0.05 AUFS. A calibration curve for the amount of **2** and **6** produced was constructed using DAOC **2**. The concentrations of adipoyl-6-APA **5** used were 0.1-2.0 mM and of penicillin N **1** 0.025-0.3 mM. The reported kinetic data was derived by fitting the data directly to the Michaelis-Menten equation, subsequent to initial Lineweaver and Burk analysis. Protein concentrations were determined by the method of Bradford.[19] k_{cat} was calculated using a mass for DAOCS of 34,500 Da.

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15. Reference 2b reports that *m*-carboxyphenylacetyl-6-APA was expanded by DAOC/DACS to the corresponding cephalosporin at 50% of the rate of penicillin N **1**, compared to 40% of the rate of penicillin N **1** for adipoyl-6-APA **5**. No data on the expansion of *m*-carboxyphenylacetyl-6-APA by recombinant DAOCS has been reported.

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