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CLEAVAGE OF THE 5-AMINO-5-CARBOXY-2-OXAPENTANOYL SIDE CHAIN FROM ENZYMATICALLY SYNTHESISED PENICILLINS AND CEPHALOSPORINS

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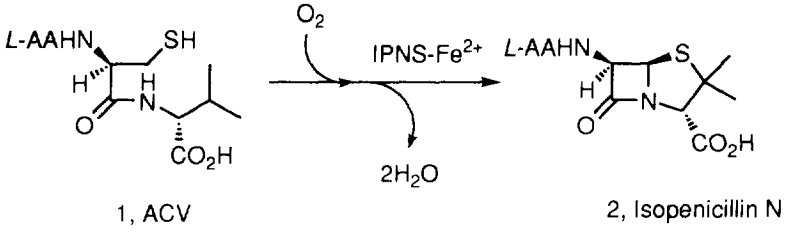
Abstract: Both 5*R*- and 5*S*-5-amino-5-carboxy-2-oxapentanoyl side chains can substitute for the *L*-δ-α-aminoadipoyl side chain in the isopenicillin N synthase catalysed formation of penicillins from tripeptides. The 5*R*- side chain analogue can be cleaved from penicillins and cephalosporins by treatment with *D*-amino acid oxidase followed by oxidative decarboxylation and decarboxylative elimination.

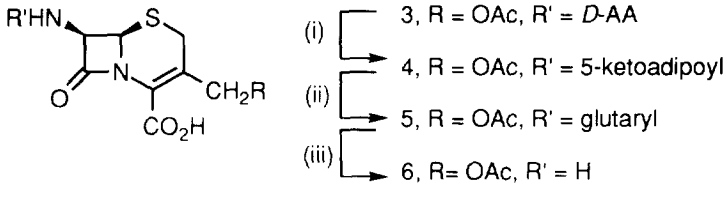
The biosynthesis of pencillins is mediated by isopenicillin N synthase (IPNS) which catalyses the desaturative bicyclisation of a simple tripeptide, *L*-δ-α-aminoadipoyl-*L*-cysteinyl-*D*-valine (*L,L,D*-ACV, **1**) to give isopenicillin N **2** in a single step (Scheme 1).



1, ACV 2, Isopenicillin N

Scheme 1, L-AA = *L*-δ-(α-aminoadipoyl).

IPNS has been shown to have a remarkably broad substrate specificity and to accept unnatural tripeptides containing substitute amino acids for each of the three residues in ACV.[1,2] Modifications to the valinyl residue are of particular interest since in many cases they have been shown to lead to novel bicyclic β-lactam structures. Many of these structures would be relatively inaccessible by synthesis.



Scheme 2, (i) *D*-Amino acid oxidase, (ii) H_2O_2, (iii) Cephalosporin acylase.
D-AA = *D*-δ-(α-aminoadipoyl).

A drawback to the *in vitro* use of IPNS for the generation of new β-lactams is that the small scale exchange of the *L*-α-aminoadipoyl side chain for other side chains is problematic. In contrast cleavage of the *D*-α-aminoadipoyl side chain from cephalosporin C **3** has been extensively studied and can be effected by its

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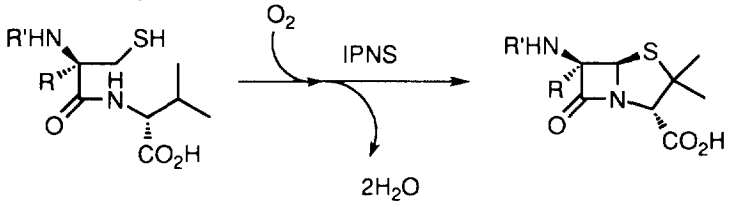
reaction first with a *D*-amino acid oxidase to give a keto acid **4** which undergoes hydrogen peroxide mediated oxidative decarboxylation to give **5**, followed by removal of the glutaryl side chain with a 'cephalosporin acylase' to give 7-aminocephalosporanic acid **6** (Scheme 2).[3,4] However, application of this process for the removal of side chains from the variety of β-lactams produced by IPNS requires that the specificity constraints of two separate enzymatic steps are overcome.



(a) 7, R = OMe, R' = *L*-2-oxa-AA ⟶ 8, R = OMe, R' = *L*-2-oxa-AA

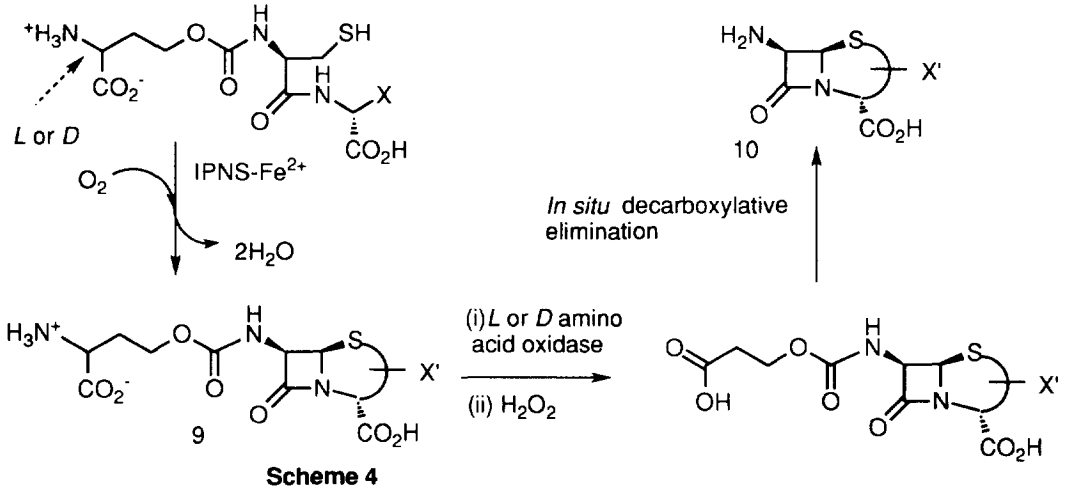
(b) 11, R = H, R' = *L*-2-oxa-AA ⟶ 12, R = H, R' = *L*-2-oxa-AA

(c) 15, R = H, R' = *D*-2-oxa-AA ⟶ 16, R = H, R' = *D*-2-oxa-AA

13, R = R' = H
14, R = H, R' = $PhCH_2CO$

Scheme 3, *L*-2-oxa-AA = (5*S*)-5-amino-5-carboxy-2-oxapentanoyl, *D*-2-oxa-AA = (5*R*)-5-amino-5-carboxy-2-oxapentanoyl.

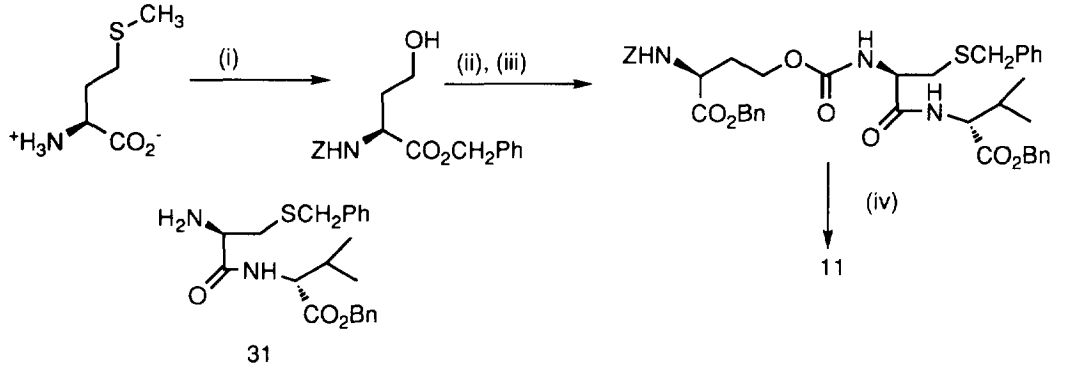
IPNS has been shown to accept the (5*S*)-5-amino-5-carboxy-2-oxapentanoyl side chain in the case of the conversion of the α-methoxycysteinyl analogue **7** to a penicillin **8** (Scheme 3, (a)).[5] We considered that cleavage of an (5*S*)- or (5*R*)-5-amino-5-carboxy-2-oxapentanoyl side chain from bicyclic β-lactams **9** synthesised using IPNS could be effected in 'one pot' by treatment with an *L*- or *D*-amino acid oxidase respectively, with *in situ* oxidative decarboxylation (mediated by the H_2O_2 produced in the first reaction) followed by decarboxylative elimination (Scheme 4), to give a bicyclic β-lactam with a free amine **10**, ready for coupling to a side chain of choice. Herein we demonstrate the feasibility of this approach.



Scheme 4

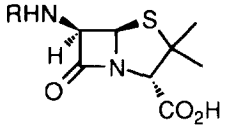
The *L,L,D*-ACV analogue **11** was synthesised from methionine, via homoserine[6] (Scheme 5). Incubation with IPNS from *Aspergillus nidulans* (5.5 -10 I.U.) under standard conditions [5] led to efficient

[>80% yield by ^1H n.m.r. (500 MHz) analysis] conversion to the desired penicillin **12** (Scheme 3, (b)), which showed comparable bioactivity to isopenicillin N **2** versus *Staphylococcus aureus*. Incubation of the h.p.l.c. purified material with *L*-amino acid oxidase from *Crotalus adamanteus* did not, however, lead to the production of the desired 6-aminopenicillanic acid (6-APA, **13**) by h.p.l.c. or ^1H n.m.r. analysis. Treatment of the crude reaction mixture with phenylacetylchloride followed by bioassay for penicillin G **14** (versus *S. aureus*) also implied side chain cleavage had not occurred.

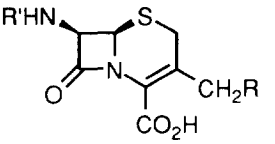


Scheme 5, (i) Methyl iodide, MeOH, H_2O (1:10), evaporation then $PhCH_2OCOCl$, Na_2CO_3, 1,4-dioxan, evaporation then $PhCH_2Br$, KI (cat.), DMF (50%); (ii) 1,1'-carbonyldiimidazole, CH_2Cl_2, (90%); (iii) (31), 4-dimethylaminopyridine (cat.), MeCN (53%); (iv) $Na/NH_3(l)$ (74% after oxidation to disulphide). (15) was prepared in a similar manner from *D*-methionine, yields, (i) 58%, (ii) 95%, (iii) 61%, (iv) 66%.

D-Amino acid oxidases reportedly have a wider substrate specificity than *L*-amino acid oxidases and have been used for the oxidation of the side chain of cephalosporins (*vide supra*). Hence the *D,L,D*-ACV analogue **15** was synthesised from *D*-methionine (Scheme 5) and incubated with IPNS (Scheme 3, (c)). A reasonable (*ca.* 40% unoptimised) conversion to the penicillin **16** was observed and the structure of the product confirmed by synthesis from the benzyl ester of 6-APA **13**.[7] Attention was then directed towards finding a *D*-amino acid oxidase which would effect the desired oxidation. Initial model studies were carried out on penicillin N **17**. **17** was not a substrate for pig kidney *D*-amino acid oxidase, but incubation with partially purified enzyme from *Trigonopsis variabilis* [8,9] in the presence of catalase led to the production of α-keto acid **18** in low yield (*ca.* 10%).[11] We believe that this is the first reported instance of the oxidative deamination of a penicillin side chain and indicated that modified penicillin **16** may also be substrate. Indeed incubation of the *D*-side chain analogue **16** with crude *T. variabilis* *D*-amino acid oxidase in the presence of NaN_3 (which inhibits catalase activity) gave, in low yield,6-APA **13** which was characterised by ^1H n.m.r., mass spectrometry, by doping with authentic material, and by conversion to penicillin G **14**.

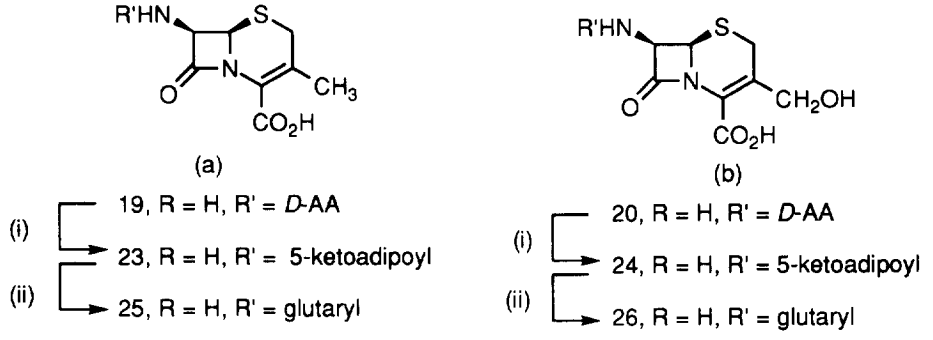


16, R = *D*-2-oxa-AA
17, R = *D*-AA
18, R = 5-ketoadipoyl



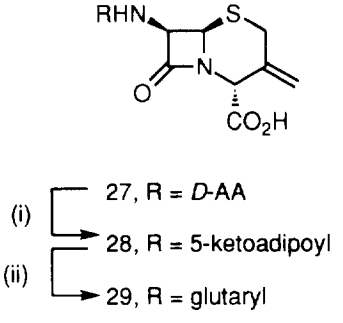
21, R = H, R' = *D*-2-oxa-AA
22, R = OH, R' = *D*-2-oxa-AA
30, R = OH, R' = H

The utility of this methodology for the cleavage of side chains from cephems synthesised *in vitro* using deacetoxy **19**/deacetyl **20** cephalosporin C (DAOC/DAC) synthase from *Cephalosporium acremonium* was also explored. Thus, penicillin **16** with the 5*R*- side chain was converted by treatment with DAOC/DAC synthase (0.2 - 0.4 I.U.) under standard conditions [14] to a mixture of cephems **21** and **22** in a *ca.* 1:9 ratio respectively. The former was shown to be converted to the latter by further treatment with the enzyme.



Scheme 6, (i) *D*-Amino acid oxidase, (ii) H_2O_2.

Model reactions were then carried out on the substrate specificity of the crude *D*-amino acid oxidase preparation from *T. variabilis* with repect to cephem substrates.[11] Cephalosporin C **3** was found to be a good substrate (as previously described[15,16]) to give either **4** or **5** in the presenceor absence of catalase respectively (Scheme 2). DAOC **19** and DAC **20** were also quantitatively processed to either the glutaryl cephems, **25** or **26**, in the absence of catalase or the α-keto acids, **23** or **24**[17] in the presence of catalase activity (Scheme 6, (a) and (b)). The exomethylene cepham **27** was converted in *ca.* 40-50% yield to either **28** or **29** under the same conditions (Scheme 7).[18] The modified cephalosporin **22** was then incubated with the *D*-amino acid oxidase under decarboxylating conditions, leading to a *ca.* 40% conversion to the desired 7-aminocephem **30**, which was characterised by ^1H n.m.r. and mass spectrometry.



Scheme 7, (i) *D*-Amino acid oxidase, (ii) H_2O_2.

This study demonstrates the feasibility of substituting the 5-amino-5-carboxy-2-oxapentanoyl group for the α-aminoadipoyl side chain in order to facilitate the cleavage of side chains from enzymatically

synthesised penicillins and cephams. At present yields for the production of the 6-amino penams from tripeptides are low, but it is possible with appropriate optimisation involving the use of alternative or engineered amino acid oxidases this process may be of considerable utility. This is also the first report of the oxidative deamination of a penicillin side chain and the first time the isolation and characterisation by n.m.r. and mass spectrometry of a product of a DAOC/DAC synthase catalysed reaction from a substrate with a hetereoatom in the side chain has been described.

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7. N-Benzyloxycarbonyl-O-carbonylimidazole-2*R*-homoserine benzyl ester was reacted with the benzyl ester of 6-APA **13** (DMF, 50 °C) to give a protected penicillin which was hydrogenated over Pd/C (40 p.s.i.) to give **16**.

8. Berg, C.P. and Rodden, F.A. *Analytical Biochem.*, **1976**, *71*, 214.

9. *T. variabilis* was grown[8] and purified *ca.* 20 fold according to modified literature procedures[10] The pellet obtained after ammonium sulphate fractionation (35-60%) was resuspended in 10 mM sodium pyrophosphate buffer (pH 8.1) containing FAD at a concentration of *ca.* 3mg/ml protein and desalted by gel filtration (Pharmacia NAP-5 column) or by dialysis. Other amino acid oxidases were obtained commercially.

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11. *D*-Amino acid oxidase activity was assayed by measuring hydrogen peroxide production.[15] Preparative scale incubations in the presence of catalase were carried out in 10 mM sodium pyrophosphate buffer (pH 8.1) containing crude amino acid oxidase (0.9 mg/ml), 0.5 μM FAD, and catalase (25 I.U. ml^{-1}). The mixture was agitated at 250 r.p.m. and 30 °C for 16 hours. Acetone (7ml) was added and the suspension centrifuged at 11 500 g for 10 minutes. (In the absence of catalase the same protocol was used except catalase was omitted and sodium azide at a conc. of 1.5 μM added.) The supernatant was decanted and the acetone removed *in vacuo* and the residue freeze dried to give a crude product, which was analysed by ^1H n.m.r. and purified by reverse phase h.p.l.c. The following kinetic results were obtained under initial rate conditions: for cephalosporin C **3**, apparent K_m 0.25 mM, specific activity 4.9 nmol $H_2O_2 min^{-1} mg$ $protein^{-1}$ (Note a higher K_m value of 13mM has been previously reported for a purified *D*-amino oxidase isozyme from *T. variabilis*.[10]); for DAOC **19**, K_m 1.9 mM, specific acitvity 35 nmol H_2O_2 $min^{-1} mg^{1}$; DAC **20** gave differing kinetic values at different

protein concentrations: at 0.06 mg protein ml^{-1}, K_m = 0.6 mM specific activity = 9.2 nmol H_2O_2 $min^{-1}mg^{-1}$; at 0.12 mg ml^{-1}, K_m = 0.9 mM, specific activity = 11 nmol H_2O_2 $min^{-1}mg^{-1}$. Similar phenomena have been previously noted and attributed to a decrease in substrate affinity as dimerisation of the enzyme occurs.[13]

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17. DAOC **21** has been previously reported not to be a substrate for *purified D*-amino acid oxidase from *T. variabilis*.[10]

18. Since IPNS has been shown to produce a C-3 exomethylene cepham from *L*-(δ-(α-aminoadipoyl)-L-cysteinyl-*D*-dehydrovaline it is possible that the methodology described in this Letter may be used in a new route to the valuable 7-amino exomethylene cepham nucleus. See Baldwin, J.E., Adlington, R.M., King, L.G., Parisi, M.F., Sobey, W.J., Sutherland, J.D., and Ting, H.-H. *J. Chem. Soc., Chem. Commun.*, **1988**, 1635.

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