Isopenicillin N Synthase: A New Mode of Reactivity

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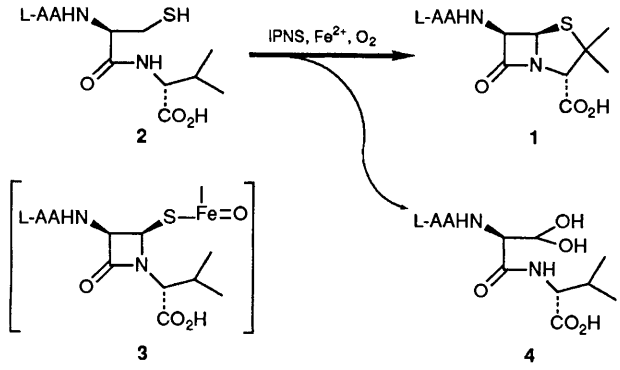
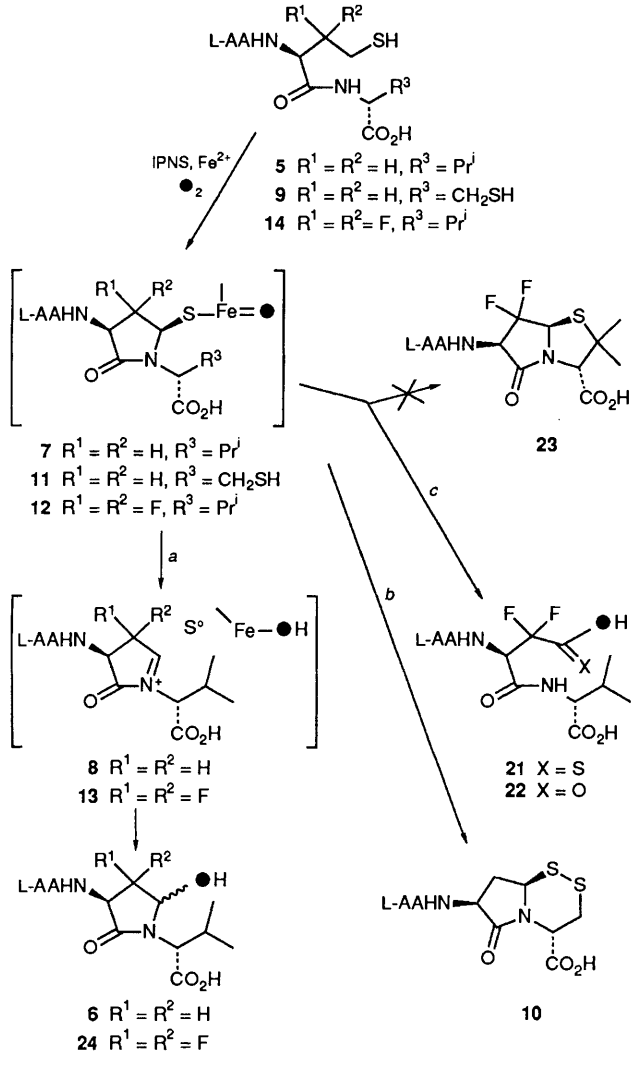
Incubation of δ-(L-α-aminoadipoyl)-L-(3,3-difluorohomocysteinyl)-D-valine with isopenicillin N synthase gave a thiocarboxylic acid, consistent with the formation of a monocyclic lactam intermediate.

The biosynthesis of isopenicillin N **1** has been shown to proceed *via* enzyme catalysed desaturative bicyclisation of the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (L,L,D-ACV, **2**).† We have investigated the mechanism by the incubation of a wide range of substrate analogues with isopenicillin N synthase (IPNS), and have proposed that the formation of the bicyclic β-lactams so obtained can be rationalised by a mechanism involving an enzyme bound monocyclic intermediate, in which the sulphur is directly bonded to an iron oxo species **3**.[1] Recent spectroscopic studies are also in accord with the formation of an iron–sulphur linkage in the initial binding of ACV **2** to IPNS.[2] In addition kinetic data[3] and the isolation of a shunt metabolite **4**[4] have also supported the prior formation of the β-lactam before the thiazolidine ring.

† δ-(L-aminoadipoyl) = 5-(5*S*)-amino-5-carboxypentanoyl.

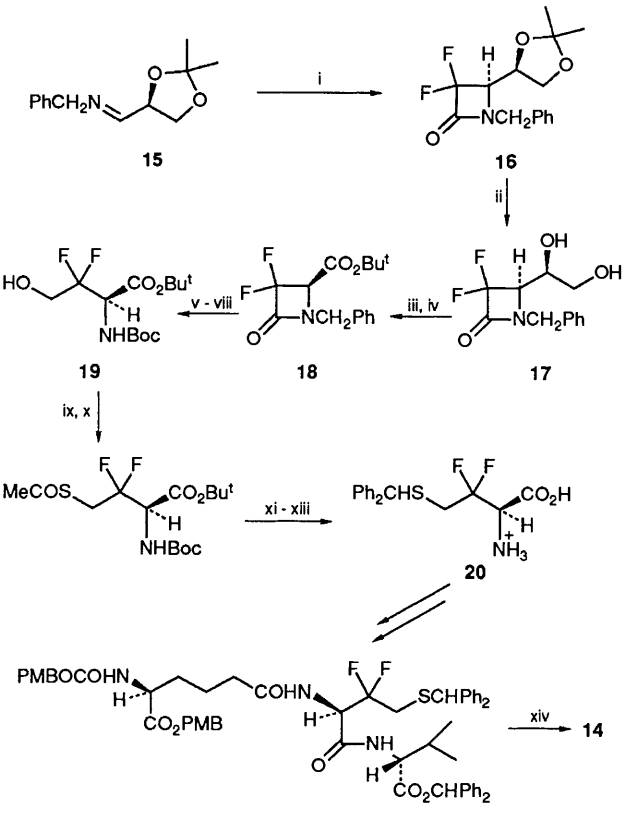


Scheme 1 L-AA = δ-(L-α-aminoadipoyl)†



Scheme 2

Recently we reported that incubation of δ-(L-α-aminoadipoyl)-L-homocysteinyl-D-valine, (L,L,D-AHCV, **5**) with IPNS did not result in the formation of a bicyclic lactam, but gave instead the monocyclic 5-hydroxy-γ-lactams **6** (Scheme 2, path *a*).[5] We reasoned that the lack of bicyclic products from the homocysteinyl substrates resulted from the relatively rapid collapse of the γ-lactam intermediate **7**, compared to that of the analogous β-lactam species **3**, to give an acyl iminium ion **8**. Subsequently we showed that incubation of δ-(L-α-aminoadipoyl)-L-homocysteinyl-D-cysteine **9** with IPNS resulted in the formation of the unusual bicyclic lactam **10**[6]



Scheme 3 *Reagents*: i, $BrCF_2CO_2Et$, Zn, tetrahydrofuran (THF), reflux; ii, *p*-$MeC_6H_4SO_3H$, MeOH, 55 °C; iii, $CrCO_3$, H_5IO_6, acetone–H_2O, 0 °C; iv, $Cl_3CC(NH)OBu^t$, $BF_3{\cdot}Et_2O$, CH_2Cl_2–cyclohexane, 0 °C; v, Bu^i_2AlH, ether, −78 °C; vi, $NaBH_4$, EtOH, 0 °C; vii, H_2, 10% Pd–C, EtOH; viii, $(Boc)_2O$, $KHCO_3$, dioxane–H_2O; ix, $(CF_3SO_2)_2O$, pyridine, CH_2Cl_2, 0 °C; x, MeCOSK, dimethylformamide, 0 °C; xi 0.2 mol dm^{-3} NaOH, MeOH; xii, 6 mol dm^{-3} HCl, dioxane; xiii, Ph_2CHOH, CF_3CO_2H; xiv, CF_3CO_2H, anisole. PMB = *p*-methoxybenzyl.

(Scheme 2, path *b*) containing an intramolecular disulphide bridge, consistent with cleavage of the Fe–S bond of the enzyme substrate complex **11** by the thiolate of the terminal cysteine.

Thus, in contrast to the natural pathway, incubation of the homocysteinyl tripeptide **5** with IPNS resulted in the formation of **6** in which only one half of the oxidising potential of dioxygen was realised in the product. Thus, we proposed that in the case of **5**, collapse of the intermediate iminum ion **8** resulted in the production of atomic sulphur (Scheme 2, path *a*). We reasoned that the introduction of strongly electron withdrawing fluorine atoms at the 3-position of the homocysteinyl residue would stabilise the intermediate **12** relative to iminium ion **13** formation, and may bias the enzymic reaction to afford a product with the same oxidation state as isopencillin N **1**.

The requisite tripeptide **14** was synthesised *via* an extension of the methodology of Kobayashi:[7] Reformatsky reaction of ethyl bromodifluoroacetate with the benzylimine **15**, derived from (*S*)-glyceraldehyde acetonide, provided the difluoroazetidinone **16** as the major product (Scheme 3). Removal of the acetonide protecting group and cleavage of the resultant diol **17** with concomitant oxidation was followed by esterification to the *tert*-butyl ester **18**.[8] Elaboration to the protected difluorohomoserine **19** involved three sequential reductions followed by protection of the amine as the *tert*-butoxycarbonyl derivative. Introduction of sulphur was achieved *via* triflate formation and displacement with thioacetate. Base hydrolysis to the thiol, removal of the amino acid protecting groups and protection of the thiol as its benzhydryl derivative gave the

desired difluorohomocysteine **20**. Conversion to the tripeptide **14** was achieved by standard methods.[9]

Incubation of **14** with IPNS gave after protein precipitation and HPLC purification (ODS reverse phase, 25 mmol dm^{-3} NH_4HCO_3) two products which have been assigned on the basis of ^1H and ^{19}F NMR data and mass spectral analyses as the thiocarboxylic **21**‡ and the carboxylic **22** acids.§ Analysis of the crude incubation by ^1H NMR spectroscopy (500 MHz) indicated that **21** was the major product (**21**:**22**, >5:1). Furthermore, purified thiocarboxylic acid **21** was shown to decompose to the carboxylic acid **22** at pH 7.5, consistent with **21** being the sole isolated enzymic product. It was anticipated that the bicyclic γ-lactam **23**, and/or the hydroxy lactams **24**, may also have been products of the incubation; however, we have thus far been unable to find any NMR spectroscopic evidence for their formation.

The incubation of **14** under an atmosphere of $^{18}O_2$ gas was carried out and for the so-derived **21** we observed *m/z* (electrospray) 452 (MNa^+, 21%), 430 (MH^+, 100), consistent with >90% incorporation of one ^{18}O atom into the product **21**. This level of incorporation is consistent with that observed in the formation of hydroxylated bicyclic β-lactams using IPNS but is higher than that observed into the hydroxylated γ-lactams **6**. It is likely that the lower incorporation levels observed in this latter case are a result of relatively facile non-enzymatic exchange of the hydroxy group.

We reexamined the incubation of the protiated homocysteinyl peptide **5** with IPNS, and were unable to isolate any thiocarboxylic acid. Thus, it would appear that the introduction of two fluorine atoms into the homocysteinyl residue biases the reaction pathway from the production of monocyclic γ-lactams such as **6**, towards production of the thiocarboxylic acid **21**. These results are entirely in accord with our previously postulated mechanism for the formation of the shunt metabolite **4**[4] and the monocyclic γ-lactams **6**[5] since the inductive effect of the two fluorine atoms should stabilise the γ-lactam intermediate **12** against heterolytic collapse to an iminium cation **13**. The intermediate **12** now apparently undergoes oxidation of the remaining 4-H bond by the attached iron–oxo species and ring opening to the thiocarboxylate **21** (Scheme 2, path *c*).

In summary, the incubation of **14** with IPNS has resulted in a new mode of reactivity for IPNS, in which both equivalents of the dioxygen molecule are utilised in the oxidation of a single carbon of the homocysteinyl residue to form the thiocarboxylic acid **21**, thereby extending still further the range of product types produced by IPNS.

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‡ *Spectral data* for **21**: δ_H (500 MHz, D_2O, ref. sodium 3-trimethylsilyl[2,2,3,3,-$^2H_4$]propionate) 0.89 and 0.93 (2 × 3H, d, *J* 7.0 Hz, CH_3), 1.65–1.78 and 1.82–1.94 (2 × 2H, m, $CH_2CH_2CH_2CO$), 2.12–2.18 (1H, m, $CHMe_2$), 2.43 (2H, t, *J* 7.0 Hz, CH_2CO), 3.74 (1H, t, *J* 6.0 Hz, Hα-AA), 4.12 (1H, d, *J* 5.0 Hz, Hα-Val), 5.62 (1H, dd, *J* 14.5, 11.5 Hz, $CHCF_2$); δ_F (235 MHz, D_2O, ^1H decoupled) −106.4, −107.8, (ABq, *J* 244 Hz); *m/z* (electrospray) 450 (MNa^+, 27%), 428 (MH^+, 100). The product from the $^{18}O_2$ experiment was derivatised as its *N*-ethoxycarbonyl trimethyl ester: *m/z* (desorption chemical ionisation, NH_3) 544 (MH^+).

§ *Spectral data* for **22**: δ_H (500 MHz, D_2O, ref. sodium 3-trimethylsilyl[2,2,3,3,-$^2H_4$]propionate) 0.88 and 0.91 (2 × 3H, d, *J* 7.0 Hz, CH_3), 1.65–1.76 and 1.80–1.94 (2 × 2H, m, $CH_2CH_2CH_2CO$), 2.10–2.16 (1H, m, $CHMe_2$), 2.43 (2H, t, *J* 7.5 Hz, CH_2CO), 3.72 (1H, t, *J* 6.0 Hz, Hα-AA), 4.11 (1H, d, *J* 6.0 Hz, Hα-Val), 5.33 (1H, dd, *J* 14.0, 12.5 Hz, $CHCF_2$); δ_F (235 MHz, D_2O, ^1H decoupled) −111.6 −113.0 (ABq, *J* 250 Hz); *m/z* (electrospray) 434 (MNa^+, 29%), 412 (MH^+, 100). Derivatised as its *N*-ethoxycarbonyl trimethyl ester: *m/z* (DCI, NH_3) 526 (MH^+)

References

1 J. E. Baldwin and E. P. Abraham, *Nat. Prod. Rep.*, 1988, **5**, 129.
2 V. J. Chen, A. M. Orville, M. R. Harpel, C. A. Frolik, K. K. Surerus, E. Munck and J. D. Lipscomb, *J. Biol. Chem.*, 1989, **264**, 21677.
3 J. E. Baldwin, R. M. Adlington, S. E. Moroney, L. D. Field and H.-H. Ting, *J. Chem. Soc., Chem. Commun.*, 1984, 984; J. E. Baldwin, E. P. Abraham, C. G. Lovel and H.-H. Ting, *J. Chem. Soc., Chem. Commun.*, 1984, 902.
4 J. E. Baldwin, R. M. Adlington, M. Bradley, W. J. Norris, N. J. Turner and A. Yoshida, *J. Chem. Soc., Chem. Commun.*, 1988, 1125.
5 J. E. Baldwin, W. J. Norris, R. T. Freeman, M. Bradley, R. M. Adlington, S. Long-Fox and C. J. Schofield, *J. Chem. Soc., Chem. Commun.*, 1988, 1128.
6 J. E. Baldwin, J. M. Blackburn, M. Sako and C. J. Schofield, *J. Chem. Soc., Chem. Commun.*, 1989, 970.
7 T. Taguchi, O. Kitagawa, Y. Suda, S. Ohkawa, A. Hashimoto, Y. Iitaka and Y. Kobayashi, *Tetrahedron Lett.*, 1988, **41**, 5291.
8 A. Armstrong, I. Brackenbridge, R. F. W. Jackson and J. M. Kirk, *Tetrahedron Lett.*, 1988, **29**, 2483.
9 J. E. Baldwin, S. R. Herchen, B. L. Johnson, M. Jung, J. J. Usher and T. Wan, *J. Chem. Soc., Perkin Trans. 1*, 1981, 2253.