Identification and Characterisation of Shunt Metabolites from Isopenicillin N Synthase

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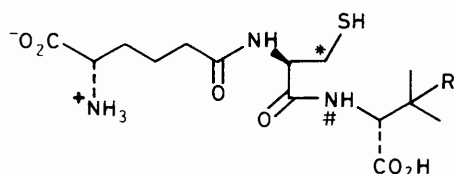
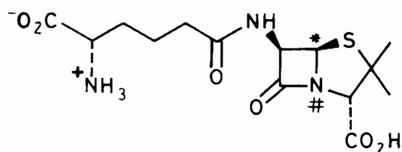
Incubation of [(5*S*)-5-amino-5-carboxypentanoyl]-L-[3-^{13}C]cysteinyl-D-[^{15}N,3-^{2}H]valine with isopenicillin N synthase (IPNS) resulted in the observation of a 'shunt metabolite,' which we believe is formed from the collapse of an enzyme-bound monocyclic β-lactam intermediate, and which from chemical studies has been shown not to be derived from the decomposition of a free thiol monocyclic β-lactam.

During studies on the enzymic conversion of [(5*S*)-5-amino-5-carboxypentanoyl]-L-[3-^{13}C]cysteinyl-D-[^{15}N,3-^{2}H]valine (**1**)† (L,L,D-A[3-^{13}C]C-[^{15}N, 3-^{2}H]V) into isopenicillin N (**2**), under normal incubation conditions,[1] we observed two signals in the ^{13}C n.m.r. spectrum (broad-band-decoupled) at δ_C 89 (dioxane ref. δ_C 67.0) which were absent from spectra of identically treated incubations of the tripeptide L,L,L-A[3-^{13}C]C-[^{15}N, 3-^{2}H]V, from a boiled enzyme control, and present in only trace quantities from incubation of the tripeptide L,L,D-A[3-^{13}C]C-[^{15}N,3-^{1}H]V (**3**).‡ The intensity of this signal derived from (**1**) amounted to approximately 1—5% of that of the corresponding isopenicillin N signal.

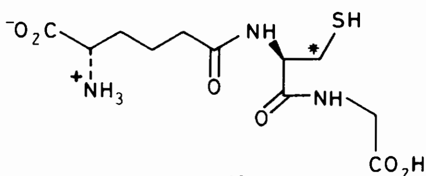
Replacement of the ^{15}N label with ^{14}N in the tripeptide (**1**) afforded L,L,D-A[3-^{13}C]C-[3-^{2}H]V (**4a**) which, upon incubation, also gave rise to the two signals at δ_C 89. These results implied that the apparent 'doublet' at δ_C 89 was not due to ^{13}C–^{15}N coupling within an intact β-lactam ring, but instead suggested a 1:1 ratio of isomers of a possible 'shunt metabolite,' the production of which was enhanced as a consequence of deuteriation in the C-3 position of valine in the tripeptides (**1**) and (**4**). Such a leakage from the enzyme's normal catalytic pathway can be rationalized by considering the role of a primary isotope effect operating as a result of substituting deuterium for hydrogen at the kinetically sensitive C-3 position of valine.[3] This adversely affects the efficiency with which the enzyme isopenicillin N synthase (IPNS) processes the mono-cyclic β-lactam intermediate (**5**) through to the bicyclic penicillin nucleus *via* thiazolidine ring closure. Further evidence for this hypothesis came when the



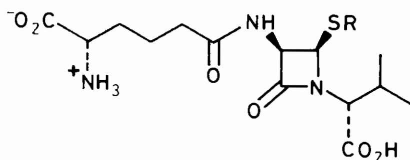
(**1**) * ^{13}C, # ^{15}N, R = ^{2}H
(**3**) * ^{13}C, # ^{15}N, R = ^{1}H
(**4**)**a**; * ^{13}C, # ^{14}N, R = ^{2}H
b; * ^{12}C, # ^{14}N, R = ^{2}H



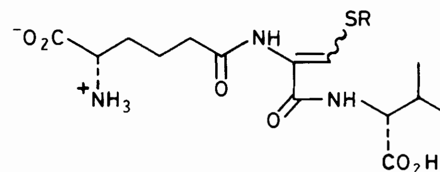
(**2**) * ^{13}C, # ^{15}N



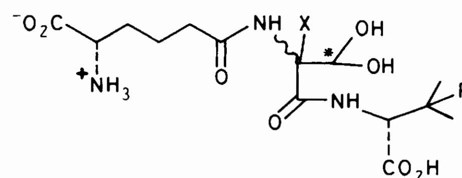
(**6**) * ^{13}C



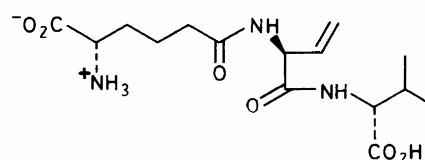
(**7**) R = H
(**8**) R = HgCl



(**9**) R = H
(**10**) R = CH_2CO_2H



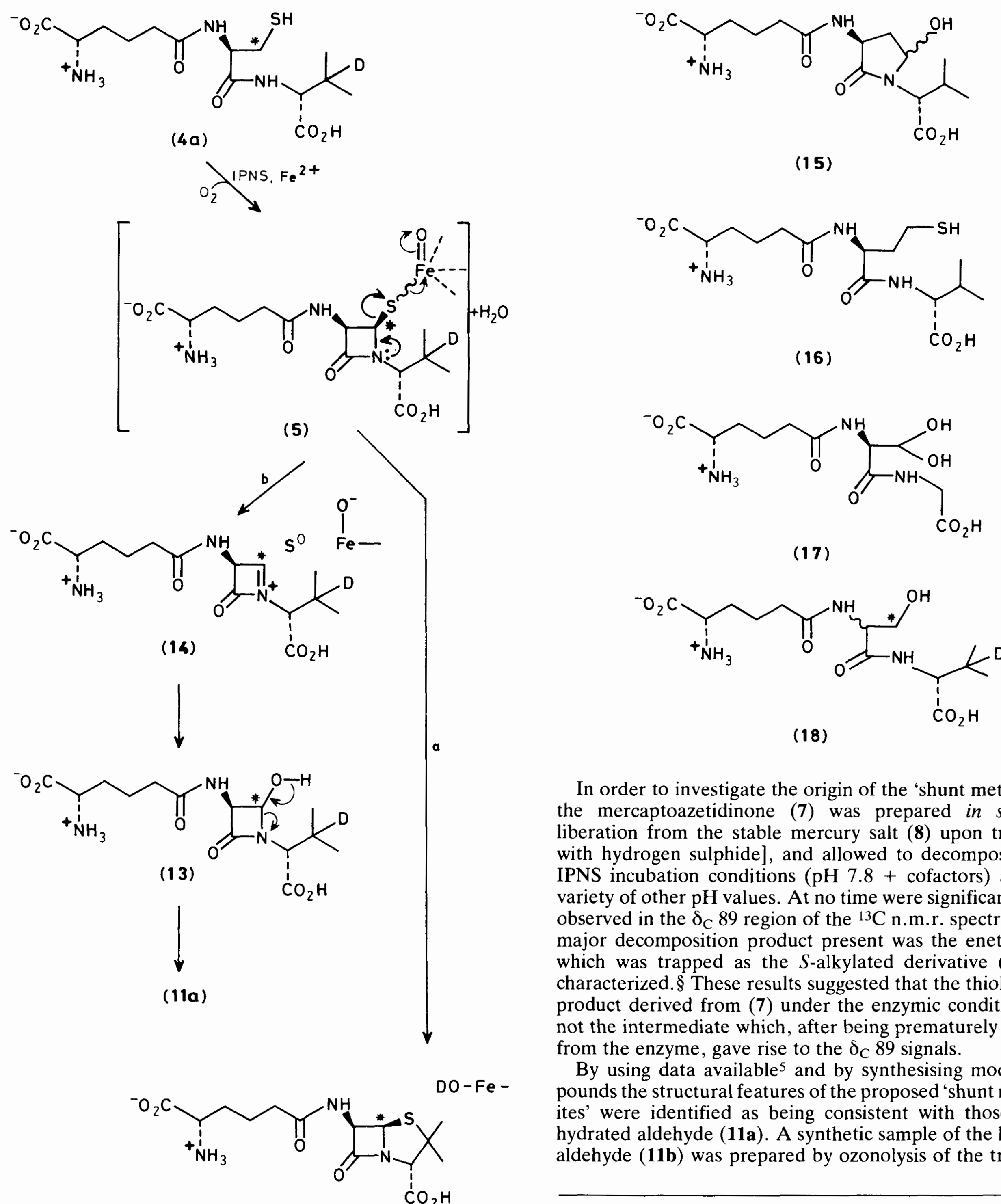
(**11**)**a**; * ^{13}C, R = D, X = H, D
b; * ^{12}C, R = H, X = H, D



(**12**)

† We have previously referred to (5*S*)-5-amino-5-carboxypentanoyl as δ-(L-α-aminoadipoyl).

‡ In a separate study, Scott *et al.*[2] reported that incubation of the tripeptide (**3**) gave isopenicillin N 'without significant accumulation of any free intermediates.' Nor was the presence of any other product reported.



Scheme 1

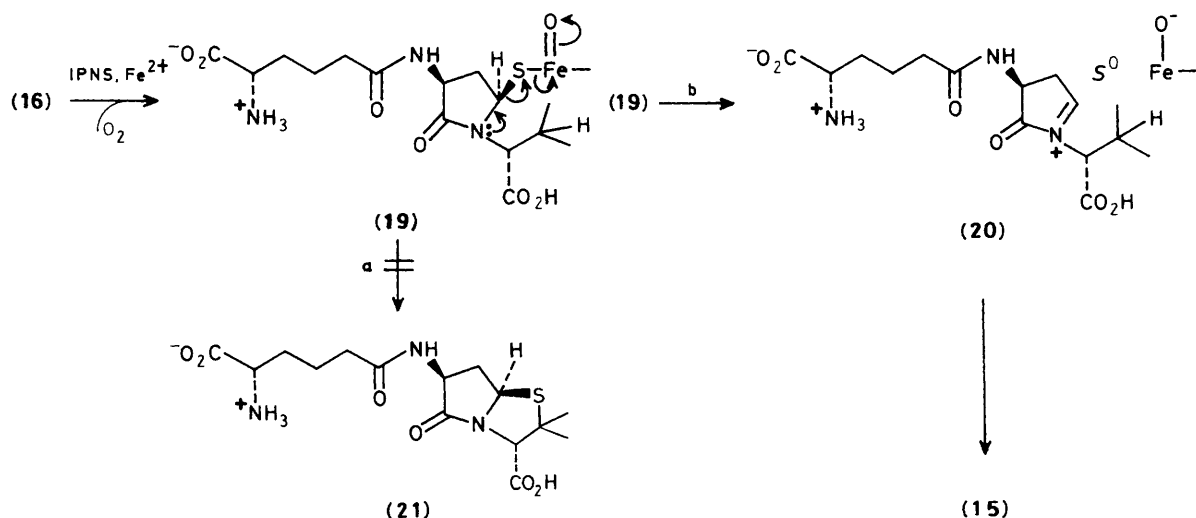
products of an incubation of L,L-A[3-^{13}C]CG (**6**) also gave rise to a signal at δ_C 89, albeit only as one singlet.

Subsequent n.m.r. experiments (broad-band and continuous wave) aimed at elucidating the nature of the moiety giving rise to the signals at δ_C 89 from (**4a**) indicated that one proton was directly bound to this carbon species; a DEPT experiment (in D_2O) also indicated the absence of protons at the carbon atom α to this centre.

In order to investigate the origin of the 'shunt metabolites' the mercaptoazetidinone (**7**) was prepared *in situ*[4] [by liberation from the stable mercury salt (**8**) upon treatment with hydrogen sulphide], and allowed to decompose under IPNS incubation conditions (pH 7.8 + cofactors) and at a variety of other pH values. At no time were significant signals observed in the δ_C 89 region of the ^{13}C n.m.r. spectrum. The major decomposition product present was the enethiol (**9**), which was trapped as the *S*-alkylated derivative (**10**) and characterized.§ These results suggested that the thiol (**7**) or a product derived from (**7**) under the enzymic conditions was not the intermediate which, after being prematurely released from the enzyme, gave rise to the δ_C 89 signals.

By using data available[5] and by synthesising model compounds the structural features of the proposed 'shunt metabolites' were identified as being consistent with those of the hydrated aldehyde (**11a**). A synthetic sample of the hydrated aldehyde (**11b**) was prepared by ozonolysis of the tripeptide

§ *Spectral data* for (**10**) the *S*-alkylated derivative of (**9**): (N.m.r. dioxane ref. δ_C 67.00, sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate ref. δ_H 0.00): δ_H (500 MHz; D_2O) 0.84 and 0.89 (6H, 2 × d, *J* 7 Hz, 2 × Me), 1.72—1.94 (4H, 2 × m, $CH_2CH_2CH_2CO$), 1.97—2.12 (1H, m, $CHMe_2$), 2.46—2.51 (2H, m, CH_2CO), 3.61 (2H, s, SCH_2COO), 3.75 (1H, t, *J* 7 Hz, CH_α of aminocarboxypentanoyl), 4.17 (1H, d, *J* 6 Hz, CH_α of valinyl), and 7.45 (1H, s, $CHSCH_2$); δ_C (126 MHz; D_2O) 17.77 and 19.31 (2 × q, 2 × Me), 21.51 (t, CH_2CH_2CO), 30.52 (t, $CHCH_2$), 31.44 (d, C_β of valinyl), 35.28 (t, CH_2CO), 38.61 (t, SCH_2CO_2H), 55.00 and 61.24 (2 × d, 2 × C_α), 123.65 (s, C=CH), 139.26 (d, C=CHS), and 164.42, 174.95, 175.93, 176.51, and 178.60 (5 × s, 5 × CO); m.s. *m/z* (positive argon fast atom bombardment) 442 (MNa^+), 464 (MNa_2^+), and 486 (MNa_3^+).



Scheme 2

(12) ($MeOH/H_2O$; −50 °C) followed by work-up with dimethyl sulphide. The ^{13}C n.m.r. spectra (broad-band and continuous wave) were consistent with those of the compound derived from enzymic sources, a facile enol(ate)–hydrate equilibrium in deuteriated solvent explaining the apparent loss of proton at the α-position.¶ Purification by h.p.l.c. of the crude products obtained from incubation of the tripeptides (**4a**) and (**4b**) on (a) reverse-phase octadecylsilane, mobile phase 20 mM ammonium hydrogen carbonate; or (b) μ-bond-pack-NH_2, mobile phase 1:4 MeOH–aq. 0.05% HCO_2H, respectively, yielded fractions with retention times under both systems coincident with that of the authentic sample of the hydrate (**11b**). Purification of the crude products obtained from incubation of the tripeptide (**4a**) afforded a fast-running fraction, the ^{13}C n.m.r. spectrum (broad-band decoupled) of which exhibited the δ_C 89 resonances. Reduction of this fraction ($NaBH_4/H_2O$) caused the loss of the δ_C 89 resonances, and gave two new resonances with δ_C 62, consistent with the L,D,D- and L,L,D-A[3-^{13}C]serinyl-[3-^{2}H]V tripeptides (**18**). A proton n.m.r. spectrum of the (partially) purified shunt metabolite [from (**4b**)] exhibited the resonances ass ciated with the hydrate proton [-C*H*$(OH)_2$]. These signals were enhanced by the addition of a synthetic sample of the hydrate (**11b**) to the original solution. ||

A possible route whereby the monocyclic β-lactam intermediate (**5**) gives rise to the shunt metabolite (**11**) and yet completes the stoicheiometric reduction of dioxygen to water in each catalytic cycle[6] is shown in Scheme 1. As already discussed, the deuterium kinetic isotope effect slows carbon–sulphur bond formation to give isopenicillin N (path a), permitting the competing fragmentation (path b) to the iminium ion (**14**), atomic sulphur, and iron(II). Quenching the ion (**14**) would lead to release of the hydroxy β-lactam (**13**) from the enzyme. It has already been shown[7] that such 4-hydroxyazetidinones are unstable with respect to ring opening, forming aldehydes and their derived enols, analogous to those we have observed as the shunt metabolites (**11**). In this scheme the sulphur absorbs the oxidizing equivalent (−2e) normally used for thiazolidine ring formation and hence the overall oxidation stoicheiometry on path b is the same as on path a.

More recently[8] we have shown that the epimeric hydroxy γ-lactams (**15**), which are homologues of (**13**), are the major products obtained after incubation of the L,L,D-A-homocysteinyl-V tripeptide (**16**) with IPNS. This result is readily interpreted by the mechanism in Scheme 1, since the collapse of the corresponding monocyclic γ-lactam (**19**) to the five-membered iminium ion (**20**) would be favoured on stereoelectronic grounds, *i.e.* a double bond endocyclic to a five-membered ring *versus* a four-membered ring as in (**14**) (Scheme 2). Hence in this case this is the sole pathway observed, none of the corresponding bicyclic compound (**21**) being found.

In conclusion, we have observed the formation of a 'shunt'-type metabolite released from IPNS as it converts its natural substrate into isopenicillin N. Deuteriation at the C-3 position of valine in the tripeptide (**4**) enhances the 'shunt pathway' and has allowed isolation and characterization of the 'shunt metabolite' as the hydrated aldehyde (**11a**). This compound is not formed from the decomposition of the monocyclic free thiol β-lactam (**7**) under standard enzymic incubation conditions.††

¶ *Spectral data* for (**11b**): δ_H (500 MHz; D_2O; pH 4) 0.81—0.94 (6H, m, CHMe_2), 1.64—1.88 (4H, 2 × m, CH_2CH_2CH$_2$CO), 2.07—2.12 (1H, m, CH_β of valinyl), 2.37—2.41 (2H, m, CH_2CO), 3.70 and 3.72 (1H, 2 × t, CH_α of aminocarboxypentanoyl), 4.11 and 4.13 (1H, 2 × d, CH_α of valinyl), and 5.25, 5.26 [1H, 2 × s, C*H*$(OH)_2$]; δ_C (126 MHz; D_2O), 17.56 (2 × q, Me), 18.91 and 18.96 (2 × q, Me), 21.24 and 21.33 (2 × t, CH_2CH_2CO), 30.09 and 30.14 (2 × d, C_β, of valinyl), 30.74 and 30.83 (2 × t, $CHCH_2CH_2$), 35.13 (t, CH_2CO), 54.81 and 59.63 (2 × br d, 2 × C_α of aminocarboxypentanoyl and valinyl), 89.06 and 89.14 [2 × d, $CH(OH)_2$], and 169.50—177.00 (4 × s, 4 × CO); *m/z* [positive argon fast atom bombardment *ex.* $(CO_2H)_2/HOCH_2$-$CH(OH)CH_2OH$], 364 (*M*H$^+$, hydrate), and 346 (*M*H$^+$ of enol/aldehyde).

|| Compound (**17**) prepared in an analogous manner to the hydrate (**11b**) gave a ^{13}C n.m.r. spectrum (broad-band decoupled) with one resonance at δ_C 89, as observed in the incubation of (**6**).

†† A structure such as (**11**) may well be equivalent to the 'compound Y' which was derived from L,L,D-A[3-^{3}H]C-[1-^{14}C]V with loss of half the tritium label and also L,L,D-AC[3,4-^{3}H,1-^{14}C]V with retention of all the tritium label upon incubation with IPNS, which was erroneously[4] identified as the free mercaptoazetidinone (**7**).[9]

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