Intact Incorporation of δ-(α-L-Aminoadipoyl)-L-[3-^{13}C]cysteinyl-D-[^{15}N]valine† into Isopenicillin N. Observation of One-bond ^{13}C–^{15}N Coupling

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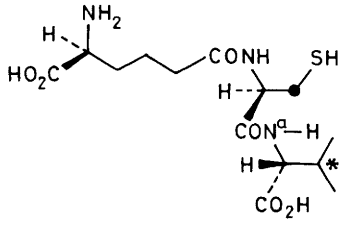
δ-(α-L-Aminoadipoyl)-L-[3-^{13}C]cysteinyl-D-[^{15}N]valine (**1a**) was efficiently transformed into [4-^{15}N, 5-^{13}C]isopenicillin N (**2a**) by a cell-free system prepared from cells of *Cephalosporium acremonium* CW19. ^{13}C N.m.r. spectra of deproteinated incubation mixtures showed ^{13}C–^{15}N coupling of the enriched C-5 resonance of the product, but offered no evidence for the presence of intermediate species.

The evidence currently available from experiments with cell-free systems and protoplasts from penicillin- and cephalosporin-synthesising micro-organisms strongly supports the hypothesis that the Arnstein tripeptide, δ-(α-L-aminodipoyl)-L-cysteinyl-D-valine (**1**), undergoes direct cyclisation to afford isopenicillin N (**2**) which subsequently serves as the precursor for all the natural penicillins and cephalosporins.[1] The isolation of a monocyclic β-lactam, intermediate between structures (**1**) and (**2**), has been reported,[2] but the evidence for the structure originally proposed for this compound is tenuous[3,4] and it seems likely that the material isolated was an artefact. A number of plausible intermediates have been proposed on the basis of chemically feasible mechanisms for the formation of the penam nucleus.[5] However, over the past few years most of these hypothetical intermediates have been precluded on the basis of isotopic labelling studies[6] or, alternatively, by direct assessment of the synthesised 'intermediates' as substrates for cell-free isopenicillin N synthesising systems.[7,8] Attempts to detect free intermediates between compounds (**1**) and (**2**) directly by ^{13}C and ^{1}H n.m.r. spectroscopy of incubation mixtures likewise proved unsuccessful.[9,10]

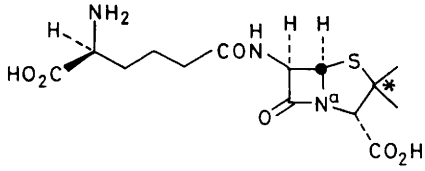
One attractive strategy for the detection and partial characterisation of non-enzyme-bound intermediates involves monitoring both the chemical shift and multiplicity of ^{13}C resonances observed during the transformation of the [*cysteinyl*-3-^{13}C,*valine*-^{15}N]tripeptide (**1a**) to the corresponding [4-^{15}N,5-^{13}C]-isopenicillin N isotopomer. This approach takes advantage of the fact that the presence of an ^{15}N atom adjacent to ^{13}C can be detected through ^{13}C–^{15}N heteronuclear spin-coupling in the ^{13}C n.m.r. spectrum; thus in the final product (**2a**), and in any intermediate structure possessing a β-lactam moiety, the signal derived from the enriched C-3 of the cysteinyl residue of compound (**1a**) should be observed as a doublet. While the magnitude of one-bond ^{13}C–^{15}N coupling constants are variable and are known to depend on the electron density at the coupled nuclei and on the bond length,[11] the earlier observation of a $^{1}J_{CN}$ value of 7 Hz for the coupling between C-5 and the nitrogen of the β-lactam ring in the ^{13}C n.m.r. spectrum of $^{15}N_2$-enriched penicillin G (**3**)[12] lent some confidence to the proposal that appreciable coupling would be observed at least in the case of compound (**2a**).

The synthesis of compound (**1a**) was carried out in a straightforward manner from *N*-benzyloxycarbonyl-L-α-aminoadipic acid,[13] *S*-benzyl-L-[3-^{13}C]cysteine, and D-[^{15}N]valine using the route previously described for the preparation of the analogue (**1**).[14] The product was purified by sequential preparative paper electrophoresis and cation exchange chromatography as the corresponding disulphide (**1b**). The ^{1}H-decoupled ^{13}C n.m.r. spectrum of the disulphide (**1b**) in D_2O–H_2O at pH 4.5 showed the expected singlet at δ 38.7 for the enriched cystine C-3 which was shifted to δ 26.1 in the spectrum of the monomer.[14,15]

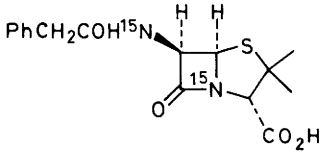
The cell-free enzyme system used in these studies was prepared by ammonium sulphate precipitation of lysates derived from mid-log phase cells of the cephalosoporin C producing *Cephalosporium acremonium* CW19 strain using the



(**1**) N^{α} = ^{14}N; ● = ✱ = ^{12}C
(**1a**) N^{α} = ^{15}N; ● = ^{13}C; ✱ = ^{12}C
(**1b**) disulphide dimer of (**1a**)
(**1c**) N^{α} = ^{14}N; ● = ✱ = ^{13}C



(**2**) N^{α} = ^{14}N; ● = ✱ = ^{12}C
(**2a**) N^{α} = ^{15}N; ● = ^{13}C; ✱ = ^{12}C
(**2b**) N^{α} = ^{14}N; ● = ✱ = ^{13}C



(**3**)

† δ-(α-L-Aminoadipoyl) = 5-(5*S*)-amino-5-carboxypentanoyl

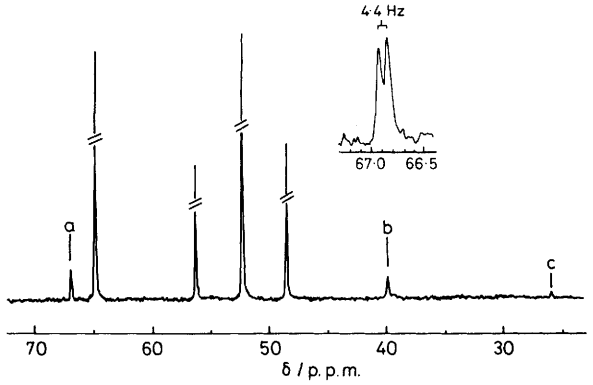


Figure. 50 MHz ^{1}H-Noise decoupled ^{13}C n.m.r. spectrum of the deproteinated incubation mixture obtained after incubation of compound (**1a**) with a cell-free extract from *C. acremonium* for 40 min. The spectrum was the result of 604 pulses with an aquisition time of 2.3 s. No line-broadening factor was applied during the transformation of the free induction decay: a, C-5 of (**2a**); b, cys C-3 of (**1b**); c, cys C-3 of (**1a**)

procedure described by Abraham and his coworkers for *C. acremonium* C91.[1b] Optimum conversions of compound (**1**) into (**2**) with this system (*ca.* 20 μg/mg protein/h) required highly aerobic conditions in the presence of ferrous salts and dithiothreitol.

^{13}C,^{15}N-Enriched tripeptide was administered to aliquots of the cell-free preparation in parallel incubations. The reactions were terminated at predetermined intervals of up to one hour by dilution with acetone and the precipitated protein was subsequently removed by centrifugation. ^{1}H-Decoupled ^{13}C n.m.r. spectra of the supernatant material showed the gradual disappearance of singlets at δ 38.5 and 26.1, corresponding to the enriched C-3 carbons of (**1b**) and (**1a**) respectively, and the synchronous appearance of a doublet ($^1J_{CN}$ 4.4 Hz) at δ 66.9 (Figure). No other signals assignable to ^{13}C,^{15}N-enriched tripeptide-derived species were observed over the range 0—220 p.p.m. The doublet resonance at δ 66.9 can be assigned to the C-5 of enzymatically derived isopenicillin N (**2a**) on the basis of both shift and multiplicity. A chemical shift of 66.7 p.p.m. has been reported for this carbon in [2,5-$^{13}C_2$]isopenicillin N (**2b**) derived from δ-(α-L-aminoadipoyl)-L-[3-^{13}C]cysteinyl-D-[3-^{13}C]valine (**1c**).[9] In a ^{13}C n.m.r. DEPT experiment,[13] the spectrum of a concentrated extract from a one-hour incubation showed negative peaks at δ 38.5 and 26.1 for the cystinyl and cysteinyl methylenes of (**1b**) and (**1a**), while the doublet at δ 66.9 remained positive, consistent with its assignment as a methine resonance.

In an effort to detect other enzymatic products which might have been formed in concentrations below the detection level afforded by direct ^{13}C n.m.r. examination of the incubation mixtures, a series of incubations was carried out with concentrations of compound (**1a**) approximately twice the optimal substrate concentration for maximal conversion into the product (**2a**). After 50 min, the conversion into compound (**2a**) was 50%, as determined *post facto* by bioassay against *Staphylococcus aureus*. At this point the reaction mixtures were pooled, deproteinated, and the concentrated supernatants subjected to rapid column chromatography on charcoal. While the ^{13}C n.m.r. spectrum of the column fractions showed intense peaks corresponding to the ^{13}C-enriched carbons of compounds (**1a**), (**1b**), and (**2a**), no resonances corresponding to other ^{13}C-enriched compounds were observed.

The results fully support the findings of earlier studies on the cyclisation of the tripeptide (**1**) to isopenicillin N (**2**) by cell-free systems[9,10] and show that, at least under the incubation conditions employed in this study, the *in vitro* enzymatic transformation of the labelled analogue (**1a**) to the corresponding product (**2a**) takes place without significant accumulation of any free intermediates. The low turnover of substrate by crude enzyme preparations does not, however, permit distinction between a situation where a low concentration of a transient and unstable intermediate is produced and one where no free intermediates are involved.

Experimental

N.m.r. spectra were recorded on Perkin-Elmer EM360 and Bruker WP80, WP200, WM300, and WH360 spectrometers. Mass spectra were recorded on an AEI MS901 and on a Kratos MS50 instrument fitted with a FAB (fast atom bombardment) source. [^{13}C]Paraformaldehyde (90 atom % ^{13}C) was purchased from Merck, Sharp and Dohm, Cambridge, Massachusetts, U.S.A., and was found to contain *ca.* 30% 1M-HCl unhydrolysed residue. DL-[^{15}N]Valine (95 atom % ^{15}N) was purchased from CEA, Gif-sur-Yvette, France; L-α-aminoadipoic acid, $[\alpha]_D^{20}$ +24° (*c* 2.0, 5M-HCl) and hog kidney acylase (1 846 units/mg) were purchased from Sigma Chemical Co. Inc. Paper electrophoresis was carried out on Whatman 3MM paper using pH 2.1 and 3.5 buffers as described by Ambler.[16] Solvents were purified by standard procedures and organic extracts were dried over anhydrous $MgSO_4$. Ether refers to diethyl ether.

S-*Benzyl*-L-[3-^{13}C]*cysteine*.—This compound was prepared by a modification of the route described by Upson and Hruby[17] for the synthesis of *S*-benzyl-DL-[3-$^2H_2$]cysteine. [^{13}C]Paraformaldehyde (1.0 g, 33.3 mmol), dimethylamine hydrochloride (2.84 g, 35 mmol), and diethyl acetamidomalonate (7.59 g, 35 mmol) were suspended in water (10 cm^3). The suspension was acidified with 1M-HCl (0.1 cm^3) and heated at 100 °C until a clear solution was obtained. The temperature was maintained for a further 0.5 h after which the solution was cooled, saturated with NaCl, and the pH adjusted to 12 with 20% NaOH. The aqueous solution was extracted with ether (30 cm^3 × 3) and the combined extracts dried and evaporated to give diethyl acetamidodimethyl[^{13}C]aminomethylmalonate (2.21 g), m.p. 62—66 °C, δ (60 MHz) 1.30 (6 H, t, *J* 7.0 Hz), 2.08 (3 H, s), 2.30 (6 H, d, $^3J_{HC}$ 5.0 Hz, $CH_3N^{13}CH_2$), 3.30 (2 H, d, $^1J_{HC}$ 131 Hz, $^{13}CH_2$), 4.29 (4 H, q, *J* 7.0 Hz), and 7.03 (1 H, br s, NH).

An ethereal solution of the product (2.21 g, 14 cm^3) was treated with an excess of iodomethane (14 cm^3) and the mixture stirred at 40 °C for 24 h. The solution was cooled to 5 °C, left overnight and the precipitated methiodide (2.77 g) collected by filtration m.p. 171—173 °C (lit.,[17] 174—175).

Phenylmethanethiol (0.8 cm^3, 16.8 mmol) was added to a solution of NaOEt (0.66 g, 9.7 mmol) in dry EtOH (10 cm^3). After 5 min the methiodide (2.71 g) was added and the solution refluxed for 10 h under N_2. The reaction mixture was evaporated under reduced pressure, the residue redissolved in $CHCl_3$ (10 cm^3), and the organic extract washed with water (5 cm^3) and brine (5 cm^3) and dried (K_2CO_3). Evaporation of the $CHCl_3$ solution afforded an oil which was dissolved in 5M-HCl (20 cm^3) and the solution refluxed for 5 h. The cooled solution was filtered through diatomaceous earth and evaporated under reduced pressure. The residue was dissolved in water (10 cm^3) and the pH adjusted to 5.4 with 2M-NH_4OH. Gradual addition of EtOH afforded a precipitate of *S*-benzyl-DL-[3-^{13}C]cysteine which was crystallised from water (0.91 g), m.p. 205 °C (lit.,[17] m.p. 209—211 °C).

The racemic product (0.85 g) was added to a mixture of acetic acid (10 cm^3) and acetic anhydride (1.23 cm^3) and the

suspension stirred at 60 °C until a clear solution was obtained. After a further 15 min the solution was cooled and evaporated under reduced pressure to give an oil which was shaken with water (20 cm^3) and then diluted with EtOH to give a clear solution. Evaporation and crystallisation of the residue from acetone–ether–n-hexane afforded *N*-acetyl-*S*-benzyl-DL-[3-^{13}C]cysteine (0.92 g), m.p. 158—160 °C (lit.,[18] m.p. 157 °C); δ (60 MHz; CD_3OD) 1.67 (3 H, s), 2.48 (2 H, AB or ABMX, δ_A 2.53, δ_B 2.42, *J* 142.0, 4.0, 2.0 Hz, cys 3-H), 3.43 (2 H, SCH_2Ph), 4.27 (1 H, m, cys 2-H), and 6.98 (5 H, s, ArH).

An aqueous solution of the racemic *N*-acetyl amino acid (0.85 g, 100 cm^3) was adjusted to pH 7.5 with 1M-NH_4OH and treated with hog kidney acylase I (0.1 g) with gentle agitation for 24 h at 37 °C. The pH was again adjusted to 7.5, an additional portion of the enzyme (80 mg) added, and the incubation continued for a further 24 h; the solution was then boiled for 5 min, filtered to remove denatured protein and the filtrate subjected to ion exchange on a column (3 × 20 cm) of AG50 × 2 cation exchange resin (H^+ form, 200—400 mesh) which was eluted sequentially with water (600 cm^3) and 1M-NH_4OH (400 cm^3). Evaporation of the basic eluate gave *S*-benzyl-L-[3-^{13}C]cysteine which was crystallised from aqueous EtOH as colourless prisms (0.15 g), $[\alpha]_D$ −16.7° (*c* 1.0, 5M-HCl) (lit.,[19] $[\alpha]_D$ −19.5°); δ (300 MHz; D_2O–NaOD) 2.08 (AB of ABMX, δ_A 2.12, δ_B 2.04, *J* 140.7, 140.3, 13.5, 6.7, 5.3 Hz, 3-H), 2.72 (1 H, ddd, *J* 6.7, 5.3, 4.8 Hz, 2-H), 3.12 (2 H, d, *J* 3.8 Hz, CH_2Ph), and 6.70 (5 H, m, ArH); δ_C (75 MHz; D_2O–NaOD) 36.73 (t, C-3).

D-[^{15}N]*Valine*.—DL-[^{15}N]Valine was acetylated in an identical manner with that used for *S*-benzyl-DL-[3-^{13}C]cysteine and the product treated with hog kidney acylase as before. The unhydrolysed *N*-acetyl D-amino acid was separated from the reaction mixture by ion exchange chromatography as above. Evaporation of the aqueous eluate (700 cm^3) and crystallisation of the residue from acetone–ether gave *N*-acetyl-D-[^{15}N]valine (0.46 g), m.p. 170—171 °C (lit.,[18b] m.p. 168 °C), $[\alpha]_D$ 18.2° (*c* 0.3, H_2O).

The product from above (0.4 g) was refluxed in 5M-HCl (10 cm^3) for 2 h, the solution evaporated under reduced pressure and the product (0.2 g) precipitated from cooled 20% aq. EtOH after adjustment of the pH to 4.5 with aniline; $[\alpha]_D$ −30.3° (*c* 1.0, 6M-HCl) [lit.,[20] $[\alpha]_D$ −28.8° (6M-HCl]; δ (360 MHz; D_2O–DCl) 0.34, 0.38 (6 H, 2d, *J* 7.0 Hz, 4-H), 1.65 (1 H, ddsept., *J* 7.0, 4.4, 3.3 Hz, 3-H), and 3.14 (1 H, d, *J* 4.4 Hz, 2-H); δ_C (50 MHz; D_2O–DCl) 18.8, 18.94 (C-4), 30.35 (C-3), 60.44 (d, $^1J_{CN}$ 6.0 Hz, C-2), and 173.85 (C-1).

δ-(L-α-*Aminoadipoyl*)-L-[3-^{13}C]*cysteinyl*-D-[^{15}N]*valine* (**1b**).—This was prepared from *N*-benzyloxycarbonyl-L-α-aminoadipic acid,[21] and the isotopically enriched amino acids above, as previously described.[13] The disulphide was rigorously purified by paper electrophoresis (pH 3.5; 50 V/cm) and ion exchange on AG50 × 2 resin (200—400 mesh, H^+ form, 18 × 1.5 cm) with a water–pyridine gradient; δ (200 MHz; D_2O) * 0.94 (3 H, d, *J* 7.8 Hz, val 4-H), 0.98 (3 H, d, *J* 7.0 Hz, val 4-H), 1.64—2.01 (4 H, m, α-aminoadipoyl 3-,4-H), 2.16 (1 H, m, val 3-H), 2.80 (2 H, AB of ABMX, δ_A 2.90, δ_B 2.70, *J* 193.9, 12.3, 6.9, 3.6 Hz, cys 3-H), 3.42 (1 H, m, cys 2-H), 3.80 (1 H, t, *J* 6.0 Hz, α-aminoadipoyl 2-H), and 4.16 (1 H, d, *J* 5.6 Hz, val 2-H); δ_C (20 MHz; D_2O)† 39.17 (cys C-3); *m/z* (FAB) 729 $(M+1)^+$.

Growth of the Organism and Preparation of a Cell-free System.—*Cephalosoporium acremonium* CW19 (ATCC 36225) was maintained on agar slants as described by Demain.[22] Seed cultures were prepared by transfer of 7-day old slant cultures to a conidation medium containing soya flour (1.5%), corn meal (2%), methyl oleate (2%), $(NH_4)_2SO_4$ (0.1%), and $CaCO_3$ (0.3%) (pH 6.8 prior to autoclaving) and were grown at 24 °C on a gyrorotatory shaker at 200 rev/min for 72 h. A 10 cm^3 aliquot of the seed culture was used to inoculate 250 cm^3 of fermentation medium which was prepared immediately before use by mixing a sterile solution of glucose (5.4%) and sucrose (7.2%) with a solution of DL-methionine (1%), $(NH_4)_2SO_4$ (1.5%), methyl oleate (0.3%), $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ (3.75 cm^3 of a 2% solution), and salts [67.5 cm^3 of a solution of KH_2PO_4 (11.3%), K_2HPO_4 (11.6%), $Na_2SO_4{\cdot}H_2O$ (1.3%), $MgSO_4{\cdot}7H_2O$ (0.27%), $CaCl_2{\cdot}2H_2O$ (0.6%), $ZnSO_4{\cdot}7H_2O$ (0.02%), $MnSO_4{\cdot}H_2O$ (0.02%), and $CuSO_4{\cdot}5H_2O$ (0.006%)]. Cultures were grown at 24 °C at 200 rev/min on a gyrorotatory shaker for 3 days and the cells harvested by filtration. The cell mat was washed with 5 × 10^{-2}M-sodium morpholinopropanesulphonate buffer (MOPS; pH 7.2; 100 cm^3) at 0 °C, the cells suspended in a further 200 cm^3 of buffer, and the cell suspension homogenised by grinding with glass beads in a Dynomill (blade speed 10 m/s, pump speed 200 cm^3/min) at 0 °C. The homogenate was treated with $(NH_4)_2SO_4$ and the protein precipitated between 50 and 80% saturation, redissolved in 5 × 10^{-2}M-MOPS (25 cm^3) and desalted by passage through a Sephadex G-25 column eluted with the same buffer. The resultant extract was concentrated (×5) by ultrafiltration on an Amicon PM10 Ultrafilter under a N_2 pressure of 4 kg/cm^2.

Substrate Incubations.—These were carried out in MOPS buffer (pH 7.2; 5 × 10^{-2}M; 1.4 cm^3) containing 1.3 × 10^{-3}M-$FeSO_4$ and 2.4 × 10^{-3}M-dithiothreitol, with a final protein concentration of *ca.* 5.5 mg/cm^3. Typically, for concentrations of (**1**) of 0.15—0.6 × 10^{-3}M conversion rates of between 70 and 80% in 1 h were determined by hole-plate bioassay of the isopenicillin N formed with *S. aureus*.[1b]

In a set of parallel incubations, aliquots of (**1a**) (0.125 mg) were incubated as above for periods of 0.25, 0.5, and 1 h. The reactions were stopped by addition of acetone (3 cm^3), centrifuged (3000 *g*, 5 min) to remove precipitated protein, the acetone removed by evaporation under reduced pressure and the solution diluted with D_2O prior to n.m.r. (see text). The concentration of isopenicillin N (**2**) was estimated by bioassay of an aliquot of solution. In a subsequent experiment compound (**1a**) (1.25 mg) was incubated with 8 cm^3 of enzyme solution for 50 min, deproteinated, concentrated, and subjected to rapid column chromatography on charcoal–Celite (1:1, 2 g) eluting with increasing proportions of acetone in water. The fractions were concentrated under reduced pressure, and examined by ^{13}C n.m.r. spectroscopy.

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* ^{1}H Chemical shifts for the unlabelled disulphide given in ref. 13 were referenced to Me_4Si in D_2O. Values given above were referenced to an internal capillary of Me_4Si at 0.00 p.p.m.

† Relative to external Me_4Si at 0.00 p.p.m.; values for ^{13}C chemical shifts of the ^{13}C-enriched carbons of (**1a**), (**1b**), and (**2a**) quoted in the text were referenced to internal dioxane at 67.4 p.p.m.

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