

## Intact Incorporation of $\delta$ -( $\alpha$ -L-Aminoadipoyl)-L-[3- $^{13}\text{C}$ ]cysteinyl-D-[ $^{15}\text{N}$ ]valinet into Isopenicillin N. Observation of One-bond $^{13}\text{C}$ - $^{15}\text{N}$ Coupling

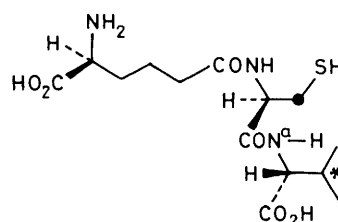
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$\delta$ -( $\alpha$ -L-Aminoadipoyl)-L-[3- $^{13}\text{C}$ ]cysteinyl-D-[ $^{15}\text{N}$ ]valine (**1a**) was efficiently transformed into [4- $^{15}\text{N}$ , 5- $^{13}\text{C}$ ]isopenicillin N (**2a**) by a cell-free system prepared from cells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

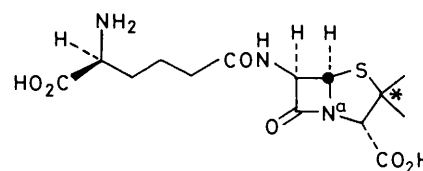
The evidence currently available from experiments with cell-free systems and protoplasts from penicillin- and cephalosporin-synthesising micro-organisms strongly supports the hypothesis that the Arnstein tripeptide,  $\delta$ -( $\alpha$ -L-aminoadipoyl)-L-cysteinyl-D-valine (**1**), undergoes direct cyclisation to afford isopenicillin N (**2**) which subsequently serves as the precursor for all the natural penicillins and cephalosporins.<sup>1</sup> The isolation of a monocyclic  $\beta$ -lactam, intermediate between structures (**1**) and (**2**), has been reported,<sup>2</sup> but the evidence for the structure originally proposed for this compound is tenuous<sup>3,4</sup> 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.<sup>5</sup> However, over the past few years most of these hypothetical intermediates have been precluded on the basis of isotopic labelling studies<sup>6</sup> or, alternatively, by direct assessment of the synthesised 'intermediates' as substrates for cell-free isopenicillin N synthesising systems.<sup>7,8</sup> Attempts to detect free intermediates between compounds (**1**) and (**2**) directly by  $^{13}\text{C}$  and  $^1\text{H}$  n.m.r. spectroscopy of incubation mixtures likewise proved unsuccessful.<sup>9,10</sup>

One attractive strategy for the detection and partial characterisation of non-enzyme-bound intermediates involves monitoring both the chemical shift and multiplicity of  $^{13}\text{C}$  resonances observed during the transformation of the [cysteinyl-3- $^{13}\text{C}$ , valine- $^{15}\text{N}$ ]tripeptide (**1a**) to the corresponding [4- $^{15}\text{N}$ , 5- $^{13}\text{C}$ ]isopenicillin N isotopomer. This approach takes advantage of the fact that the presence of an  $^{15}\text{N}$  atom adjacent to  $^{13}\text{C}$  can be detected through  $^{13}\text{C}$ - $^{15}\text{N}$  heteronuclear spin-coupling in the  $^{13}\text{C}$  n.m.r. spectrum; thus in the final product (**2a**), and in any intermediate structure possessing a  $\beta$ -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  $^{13}\text{C}$ - $^{15}\text{N}$  coupling constants are variable and are known to depend on the electron density at the coupled nuclei and on the bond length,<sup>11</sup> the earlier observation of a  $^1J_{\text{CN}}$  value of 7 Hz for the coupling between C-5 and the nitrogen of the  $\beta$ -lactam ring in the  $^{13}\text{C}$  n.m.r. spectrum of  $^{15}\text{N}_2$ -enriched penicillin G (**3**)<sup>12</sup> lent some confidence to the proposal that appreciable coupling would be observed at least in the case of compound (**2a**).

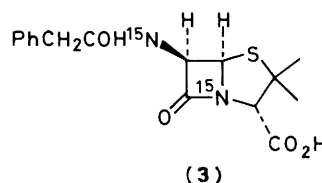
The synthesis of compound (**1a**) was carried out in a straightforward manner from *N*-benzyloxycarbonyl-L- $\alpha$ -aminoadipic acid,<sup>13</sup> *S*-benzyl-L-[3- $^{13}\text{C}$ ]cysteine, and D-[ $^{15}\text{N}$ ]valine using the route previously described for the preparation of the analogue (**1**).<sup>14</sup> The product was purified by sequential



- (**1**)  $\text{N}^{\alpha} = ^{14}\text{N}$ ; ● = \* =  $^{12}\text{C}$   
 (**1a**)  $\text{N}^{\alpha} = ^{15}\text{N}$ ; ● =  $^{13}\text{C}$ ; \* =  $^{12}\text{C}$   
 (**1b**) disulphide dimer of (**1a**)  
 (**1c**)  $\text{N}^{\alpha} = ^{14}\text{N}$ ; ● = \* =  $^{13}\text{C}$



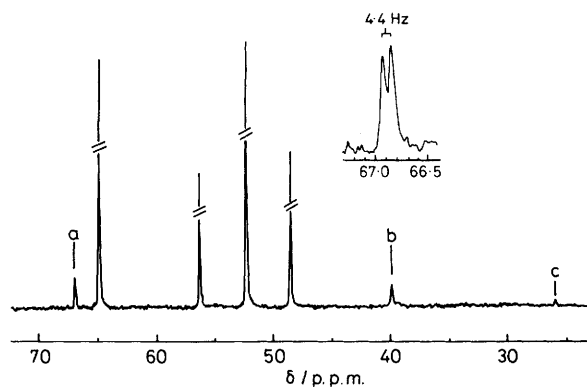
- (**2**)  $\text{N}^{\alpha} = ^{14}\text{N}$ ; ● = \* =  $^{12}\text{C}$   
 (**2a**)  $\text{N}^{\alpha} = ^{15}\text{N}$ ; ● =  $^{13}\text{C}$ ; \* =  $^{12}\text{C}$   
 (**2b**)  $\text{N}^{\alpha} = ^{14}\text{N}$ ; ● = \* =  $^{13}\text{C}$



preparative paper electrophoresis and cation exchange chromatography as the corresponding disulphide (**1b**). The  $^1\text{H}$ -decoupled  $^{13}\text{C}$  n.m.r. spectrum of the disulphide (**1b**) in  $\text{D}_2\text{O}$ - $\text{H}_2\text{O}$  at pH 4.5 showed the expected singlet at  $\delta$  38.7 for the enriched cysteine C-3 which was shifted to  $\delta$  26.1 in the spectrum of the monomer.<sup>14,15</sup>

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 cephalosporin C producing *Cephalosporium acremonium* CW19 strain using the

†  $\delta$ -( $\alpha$ -L-Aminoadipoyl) = 5-(5S)-amino-5-carboxypentanoyl



**Figure 50** 50 MHz  $^1\text{H}$ -Noise decoupled  $^{13}\text{C}$  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 acquisition 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.<sup>1b</sup> Optimum conversions of compound (1) into (2) with this system (ca. 20  $\mu\text{g}/\text{mg}$  protein/h) required highly aerobic conditions in the presence of ferrous salts and dithiothreitol.

$^{13}\text{C}$ ,  $^{15}\text{N}$ -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.  $^1\text{H}$ -Decoupled  $^{13}\text{C}$  n.m.r. spectra of the supernatant material showed the gradual disappearance of singlets at  $\delta$  38.5 and 26.1, corresponding to the enriched C-3 carbons of (1b) and (1a) respectively, and the synchronous appearance of a doublet ( $^1J_{\text{CN}}$  4.4 Hz) at  $\delta$  66.9 (Figure). No other signals assignable to  $^{13}\text{C}$ ,  $^{15}\text{N}$ -enriched tripeptide-derived species were observed over the range 0–220 p.p.m. The doublet resonance at  $\delta$  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- $^{13}\text{C}_2$ ]isopenicillin N (2b) derived from  $\delta$ -( $\alpha$ -L-aminoadipoyl)-L-[3- $^{13}\text{C}$ ]cysteinyl-D-[3- $^{13}\text{C}$ ]valine (1c).<sup>9</sup> In a  $^{13}\text{C}$  n.m.r. DEPT experiment,<sup>13</sup> the spectrum of a concentrated extract from a one-hour incubation showed negative peaks at  $\delta$  38.5 and 26.1 for the cystinyl and cysteinyl methylenes of (1b) and (1a), while the doublet at  $\delta$  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  $^{13}\text{C}$  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  $^{13}\text{C}$  n.m.r. spectrum of the column fractions showed intense peaks corresponding to the  $^{13}\text{C}$ -enriched carbons of compounds (1a), (1b), and (2a), no resonances corresponding to other  $^{13}\text{C}$ -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<sup>9,10</sup> 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. [ $^{13}\text{C}$ ]Paraformaldehyde (90 atom %  $^{13}\text{C}$ ) was purchased from Merck, Sharp and Dohm, Cambridge, Massachusetts, U.S.A., and was found to contain ca. 30% 1M-HCl unhydrolysed residue. DL-[ $^{15}\text{N}$ ]Valine (95 atom %  $^{15}\text{N}$ ) was purchased from CEA, Gif-sur-Yvette, France; L- $\alpha$ -amino-adipic acid,  $[\alpha]_{\text{D}}^{20} + 24^\circ$  (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.<sup>16</sup> Solvents were purified by standard procedures and organic extracts were dried over anhydrous  $\text{MgSO}_4$ . Ether refers to diethyl ether.

*S*-Benzyl-L-[3- $^{13}\text{C}$ ]cysteine.—This compound was prepared by a modification of the route described by Upson and Hruby<sup>17</sup> for the synthesis of *S*-benzyl-DL-[3- $^2\text{H}_2$ ]cysteine. [ $^{13}\text{C}$ ]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  $\text{cm}^3$ ). The suspension was acidified with 1M-HCl (0.1  $\text{cm}^3$ ) and heated at 100  $^\circ\text{C}$  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  $\text{cm}^3 \times 3$ ) and the combined extracts dried and evaporated to give diethyl acetamidodimethyl[ $^{13}\text{C}$ ]aminomethylmalonate (2.21 g, m.p. 62–66  $^\circ\text{C}$ ,  $\delta$  (60 MHz) 1.30 (6 H, t,  $J$  7.0 Hz), 2.08 (3 H, s), 2.30 (6 H, d,  $^3J_{\text{HC}}$  5.0 Hz,  $\text{CH}_3\text{N}^{13}\text{CH}_2$ ), 3.30 (2 H, d,  $^1J_{\text{HC}}$  131 Hz,  $^{13}\text{CH}_2$ ), 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  $\text{cm}^3$ ) was treated with an excess of iodomethane (14  $\text{cm}^3$ ) and the mixture stirred at 40  $^\circ\text{C}$  for 24 h. The solution was cooled to 5  $^\circ\text{C}$ , left overnight and the precipitated methiodide (2.77 g) collected by filtration m.p. 171–173  $^\circ\text{C}$  (lit.,<sup>17</sup> 174–175).

Phenylmethanethiol (0.8  $\text{cm}^3$ , 16.8 mmol) was added to a solution of NaOEt (0.66 g, 9.7 mmol) in dry EtOH (10  $\text{cm}^3$ ). After 5 min the methiodide (2.71 g) was added and the solution refluxed for 10 h under  $\text{N}_2$ . The reaction mixture was evaporated under reduced pressure, the residue redissolved in  $\text{CHCl}_3$  (10  $\text{cm}^3$ ), and the organic extract washed with water (5  $\text{cm}^3$ ) and brine (5  $\text{cm}^3$ ) and dried ( $\text{K}_2\text{CO}_3$ ). Evaporation of the  $\text{CHCl}_3$  solution afforded an oil which was dissolved in 5M-HCl (20  $\text{cm}^3$ ) 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  $\text{cm}^3$ ) and the pH adjusted to 5.4 with 2M- $\text{NH}_4\text{OH}$ . Gradual addition of EtOH afforded a precipitate of *S*-benzyl-DL-[3- $^{13}\text{C}$ ]cysteine which was crystallised from water (0.91 g), m.p. 205  $^\circ\text{C}$  (lit.,<sup>17</sup> m.p. 209–211  $^\circ\text{C}$ ).

The racemic product (0.85 g) was added to a mixture of acetic acid (10  $\text{cm}^3$ ) and acetic anhydride (1.23  $\text{cm}^3$ ) 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<sup>3</sup>) 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-<sup>13</sup>C]cysteine (0.92 g), m.p. 158–160 °C (lit.,<sup>18</sup> m.p. 157 °C);  $\delta$  (60 MHz; CD<sub>3</sub>OD) 1.67 (3 H, s), 2.48 (2 H, AB or ABMX,  $\delta_A$  2.53,  $\delta_B$  2.42, *J* 142.0, 4.0, 2.0 Hz, cys 3-H), 3.43 (2 H, SCH<sub>2</sub>Ph), 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<sup>3</sup>) was adjusted to pH 7.5 with 1M-NH<sub>4</sub>OH 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<sup>+</sup> form, 200–400 mesh) which was eluted sequentially with water (600 cm<sup>3</sup>) and 1M-NH<sub>4</sub>OH (400 cm<sup>3</sup>). Evaporation of the basic eluate gave *S*-benzyl-L-[3-<sup>13</sup>C]cysteine which was crystallised from aqueous EtOH as colourless prisms (0.15 g),  $[\alpha]_D - 16.7^\circ$  (*c* 1.0, 5M-HCl) (lit.,<sup>19</sup>  $[\alpha]_D - 19.5^\circ$ );  $\delta$  (300 MHz; D<sub>2</sub>O-NaOD) 2.08 (AB of ABMX,  $\delta_A$  2.12,  $\delta_B$  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<sub>2</sub>Ph), and 6.70 (5 H, m, ArH);  $\delta_C$  (75 MHz; D<sub>2</sub>O-NaOD) 36.73 (t, C-3).

D-[<sup>15</sup>N]Valine.—DL-[<sup>15</sup>N]Valine was acetylated in an identical manner with that used for *S*-benzyl-DL-[3-<sup>13</sup>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<sup>3</sup>) and crystallisation of the residue from acetone-ether gave *N*-acetyl-D-[<sup>15</sup>N]valine (0.46 g), m.p. 170–171 °C (lit.,<sup>18b</sup> m.p. 168 °C),  $[\alpha]_D 18.2^\circ$  (*c* 0.3, H<sub>2</sub>O).

The product from above (0.4 g) was refluxed in 5M-HCl (10 cm<sup>3</sup>) 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;  $[\alpha]_D - 30.3^\circ$  (*c* 1.0, 6M-HCl) [lit.,<sup>20</sup>  $[\alpha]_D - 28.8^\circ$  (6M-HCl)];  $\delta$  (360 MHz; D<sub>2</sub>O-DCI) 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);  $\delta_C$  (50 MHz; D<sub>2</sub>O-DCI) 18.8, 18.94 (C-4), 30.35 (C-3), 60.44 (d, <sup>1</sup>J<sub>CN</sub> 6.0 Hz, C-2), and 173.85 (C-1).

$\delta$ -(L- $\alpha$ -Amino adipoyl)-L-[3-<sup>13</sup>C]cysteinyl-D-[<sup>15</sup>N]valine (1b).—This was prepared from *N*-benzyloxycarbonyl-L- $\alpha$ -amino adipic acid,<sup>21</sup> and the isotopically enriched amino acids above, as previously described.<sup>13</sup> 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<sup>+</sup> form, 18 × 1.5 cm) with a water-pyridine gradient;  $\delta$  (200 MHz; D<sub>2</sub>O)\* 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,  $\alpha$ -amino adipoyl 3-,4-H), 2.16 (1 H, m, val 3-H), 2.80 (2 H, AB of ABMX,  $\delta_A$  2.90,  $\delta_B$  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,  $\alpha$ -amino adipoyl 2-H), and 4.16 (1 H, d, *J* 5.6 Hz, val 2-H);  $\delta_C$  (20 MHz; D<sub>2</sub>O)† 39.17 (cys C-3); *m/z* (FAB) 729 (*M* + 1)<sup>+</sup>.

\* <sup>1</sup>H Chemical shifts for the unlabelled disulphide given in ref. 13 were referenced to Me<sub>4</sub>Si in D<sub>2</sub>O. Values given above were referenced to an internal capillary of Me<sub>4</sub>Si at 0.00 p.p.m.

† Relative to external Me<sub>4</sub>Si at 0.00 p.p.m.; values for <sup>13</sup>C chemical shifts of the <sup>13</sup>C-enriched carbons of (1a), (1b), and (2a) quoted in the text were referenced to internal dioxane at 67.4 p.p.m.

*Growth of the Organism and Preparation of a Cell-free System.*—*Cephalosporium acremonium* CW19 (ATCC 36225) was maintained on agar slants as described by Demain.<sup>22</sup> Seed cultures were prepared by transfer of 7-day old slant cultures to a conidiation medium containing soya flour (1.5%), corn meal (2%), methyl oleate (2%), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.1%), and CaCO<sub>3</sub> (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<sup>3</sup> aliquot of the seed culture was used to inoculate 250 cm<sup>3</sup> 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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1.5%), methyl oleate (0.3%), Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O (3.75 cm<sup>3</sup> of a 2% solution), and salts [67.5 cm<sup>3</sup> of a solution of KH<sub>2</sub>PO<sub>4</sub> (11.3%), K<sub>2</sub>HPO<sub>4</sub> (11.6%), Na<sub>2</sub>SO<sub>4</sub>·H<sub>2</sub>O (1.3%), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.27%), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.6%), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.02%), MnSO<sub>4</sub>·H<sub>2</sub>O (0.02%), and CuSO<sub>4</sub>·5H<sub>2</sub>O (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<sup>-2</sup>M-sodium morpholinopropanesulphonate buffer (MOPS; pH 7.2; 100 cm<sup>3</sup>) at 0 °C, the cells suspended in a further 200 cm<sup>3</sup> of buffer, and the cell suspension homogenised by grinding with glass beads in a Dynamill (blade speed 10 m/s, pump speed 200 cm<sup>3</sup>/min) at 0 °C. The homogenate was treated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the protein precipitated between 50 and 80% saturation, redissolved in 5 × 10<sup>-2</sup>M-MOPS (25 cm<sup>3</sup>) 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<sub>2</sub> pressure of 4 kg/cm<sup>2</sup>.

*Substrate Incubations.*—These were carried out in MOPS buffer (pH 7.2; 5 × 10<sup>-2</sup>M; 1.4 cm<sup>3</sup>) containing 1.3 × 10<sup>-3</sup>M-FeSO<sub>4</sub> and 2.4 × 10<sup>-3</sup>M-dithiothreitol, with a final protein concentration of ca. 5.5 mg/cm<sup>3</sup>. Typically, for concentrations of (1) of 0.15–0.6 × 10<sup>-3</sup>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*.<sup>1b</sup>

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<sup>3</sup>), centrifuged (3000 g, 5 min) to remove precipitated protein, the acetone removed by evaporation under reduced pressure and the solution diluted with D<sub>2</sub>O 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<sup>3</sup> 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 <sup>13</sup>C n.m.r. spectroscopy.

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