Tetrahedron Vol. 39, No. 7, pp. 1061 to 1068, 1983
Printed in Great Britain.

0040-4020/83/071061-08$03.00/0

CONVERSION OF $^{17}O/^{18}O$ LABELLED δ-(L-α-AMINOADIPYL)-L-CYSTEINYL-D-VALINE TO $^{17}O/^{18}O$ LABELLED ISOPENICILLIN N IN A CELL-FREE EXTRACT OF CEPHALOSPORIUM ACREMONIUM. A STUDY BY ^{17}O-NMR SPECTROSCOPY AND MASS SPECTROMETRY.

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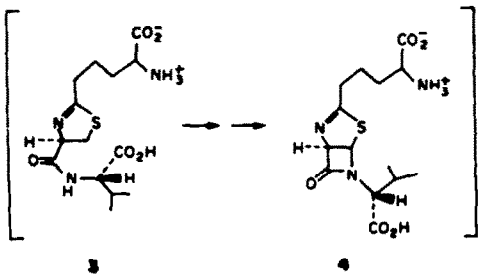
(*Received in USA 9 December* 1982)

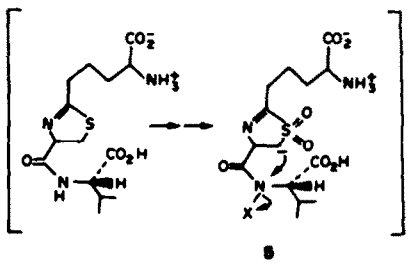
Abstract: δ-(L-α-Amino[1,1,6-$^{17}O/^{18}O$]-adipyl)-L-cysteinyl-D-valine was converted into isopenicillin N in cell-free extracts of Cephalosporium acremonium with no loss of $^{17}O/^{18}O$ label as shown by ^{17}O NMR spectroscopy and mass spectrometry. Incubation of unlabelled tripeptide in a cell-free system containing $^{17}O/^{18}O$ enriched water produced isopenicillin N with no incorporation of $^{17}O/^{18}O$.

The biosynthesis of isopenicillin N 1 from L-valine, L-cysteine and L-α-aminoadipic acid proceeds via the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV, 2)[1]. Among the mechanisms which have been proposed for the transformation of 2 into 1, several have included steps involving hydration/dehydration of intermediate products. Cooper[2] has proposed a scheme involving thiazolines and thiazolineazetidinones eg 3,4, as possible intermediates in the biosynthesis of cephalosporins and penicillins. The feasibility of this approach has been demonstrated by Kishi et al[3] in "biogenetic-type" syntheses of penam and cepham ring systems.



3 4

The possible use of thiazolinesulfones eg 5 as potential intermediates in biomimetic syntheses of β-lactam antibiotics has been investigated by Scott and coworkers[4]. In this scheme, nucleophilic attack by an anion, generated at the β-carbon of a cysteine-thiazolinesulphone, upon the nitrogen of a hydroxamic acid derivative yields a β-lactam ring.



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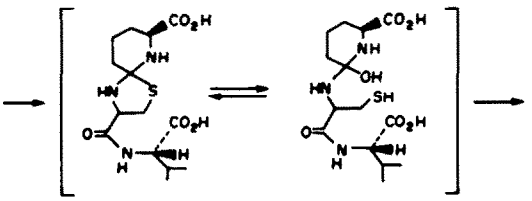
In an alternative scheme, Morin et al[5] have proposed a cyclic orthothioamide, 6, as a biogenetic precursor of isopenicillin N, following their isolation of 6-oxo-piperidine-2-carboxylic acid from the fermentation broth of Penicillium chrysogenum.



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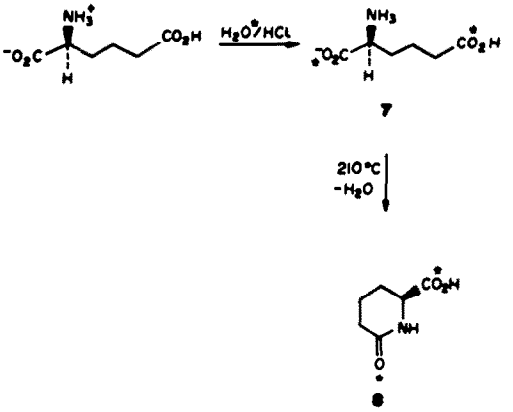
The possibility of dehydration/hydration steps in the conversion of LLD-ACV to isopenicillin N can be tested by conducting the biosynthesis in a $^{17}O/^{18}O$ isotopically enriched medium or by employing $^{17}O/^{18}O$ isotopically labelled precursors. In a cell-free extract of *C. acremonium*[6], the progress of the conversion of LLD-ACV to isopenicillin N can be followed directly by ^{1}H NMR spectroscopy[7] and the fate of ^{17}O labelled atoms (either from the medium or from labelled substrates) can be studied by ^{17}O NMR spectroscopy.

^{17}O NMR spectroscopy has been employed only rarely as a tool in organic chemistry because of the low natural abundance of ^{17}O and also because ^{17}O resonances are generally broadened by quadrupolar relaxation ($I = {}^{3}/_{2}$). The linewidth of ^{17}O resonances increases dramatically with molecular size but most oxygen-containing functional groups can be distinguished[8] by ^{17}O NMR even in molecules up to molecular weight ca 1000. The rapid relaxation of ^{17}O nuclei allows reliable integration of spectra and using an internal standard, eg natural abundance ^{17}O resonances in a solvent, the levels of ^{17}O enrichment, in various functional groups can be estimated.

Synthesis and ^{17}O NMR Spectra of $^{17}O/^{18}O$ Enriched LLD-ACV.

L-α-aminoadipic acid was isotopically enriched with $^{17}O/^{18}O$ in both the α- and δ-carboxyl groups by dissolving it in $^{17}O/^{18}O$ enriched aqueous hydrochloric acid (3M HCl in H_2O; $H_2{}^{16}O$: $H_2{}^{17}O$: $H_2{}^{18}O$ = 10:50:40) and allowing the solution to stand for 48 hours at room temperature. The ^{17}O NMR spectrum of the enriched compound, 7, was a single resonance (255 ppm from $H_2{}^{17}O$, $W_{1/2}$ = 760 Hz, pH = 1) which indicated a total enrichment of ca. 1.3 moles of ^{17}O per mole. On heating, 7 was dehydrated to the lactam, 8, where the lactam amide and carboxyl groups could be distinguished by ^{17}O NMR (377 and 253 ppm from $H_2{}^{17}O$ respectively at pH3). Integration of the resonances in the ^{17}O NMR spectrum of 8, showed that 7 had been enriched in both the α- and δ- carboxyl groups ca. 40:60 respectively.



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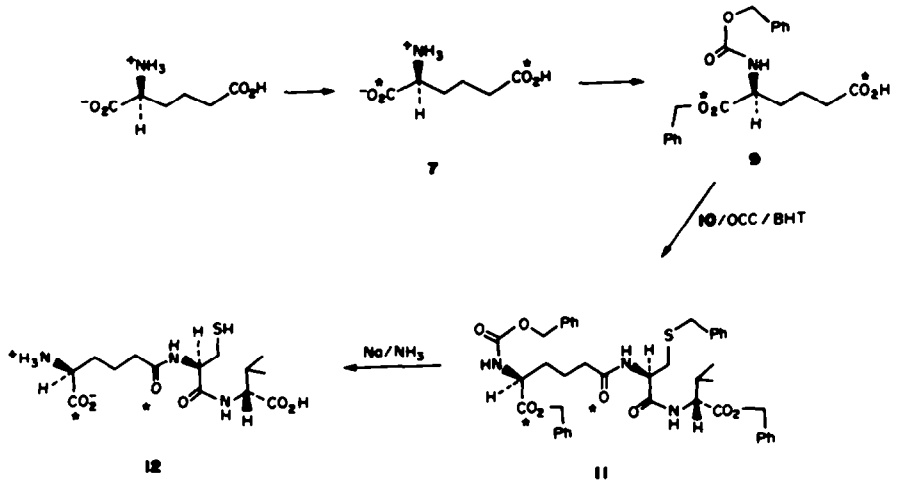
The acid 7, was protected as its N-benzyloxycarbonyl-α-benzyl ester derivative, 9, and coupled with S-benzyl-L-cysteinyl-D-valine benzyl ester, 10 (Scheme 1)[10]. The tripeptide 11, was deprotected to yield isotopically enriched LLD-ACV, 12.

Figure 1 shows the ^{17}O NMR spectra of compounds 7, 9, 11 and 12 and, as expected, the linewidths of the resonances increase as the size of the molecules increases. In the ^{17}O NMR spectrum of 9, enrichment in both the ester and carboxyl groups can be distinguished clearly. Even in 11, where the width of the resonances at half height approaches 4kHz, carbonyl and alkoxy sites are resolved, although further distinction between the amide and ester carbonyl functions is not possible. In 12, the amide carbonyl and the terminal carboxyl group are not separated and integration of the spectrum against an internal H_2O reference indicated a total enrichment of ca. 1 mole ^{17}O per mole of 12.



Scheme 1

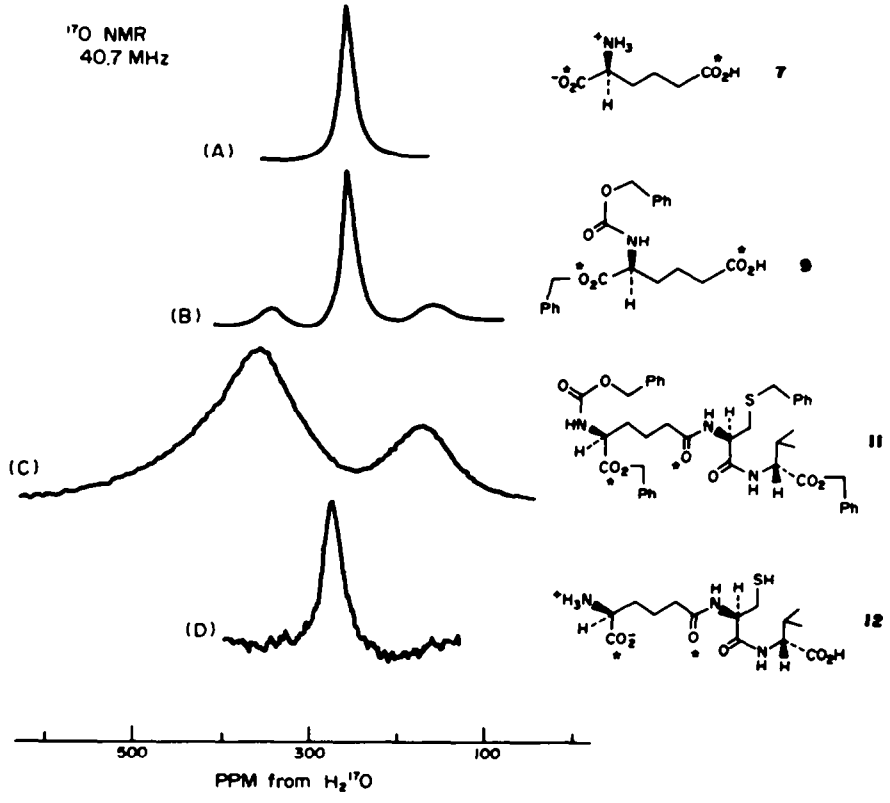
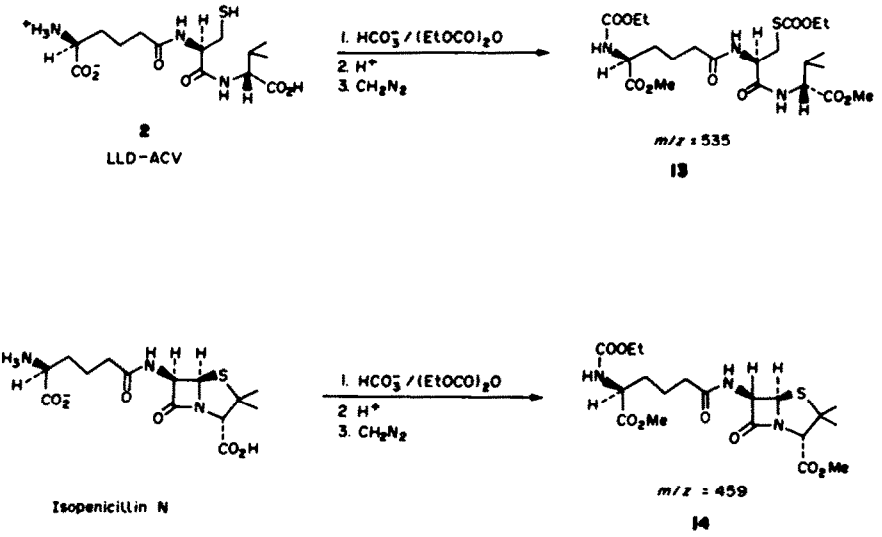


Figure 1 ^{17}O NMR spectra (40.7 MHz) of intermediates in the synthesis of $^{17}O/^{18}O$ enriched LLD-ACV. (A) and (D) D_2O solvent, (B) and (C) $CDCl_3$ solvent.



Scheme 2

Mass Spectrometry

LLD-ACV peptides and isopenicillin N were derivatised[11] for mass spectrometry as their -N-ethoxycarbonyl, -S-ethoxycarbonyl, -carboxylmethyl derivatives (Scheme 2).

The protonated molecular ions in the ammonia desorption chemical ionisation (DCI) mass spectrum of unlabelled derivatives 13 and 14 exhibit near theoretical intensities:

13, $C_{22}H_{38}N_3O_{10}S^+$, found 536: 537: 538: = 100: 25: 9
calc'd 536: 537: 538: = 100: 27.5: 10.1

14, $C_{19}H_{30}N_3O_8S^+$, found 460: 461: 462 = 100: 21: 13
calc'd 460: 461: 462 = 100: 23.9: 8.8

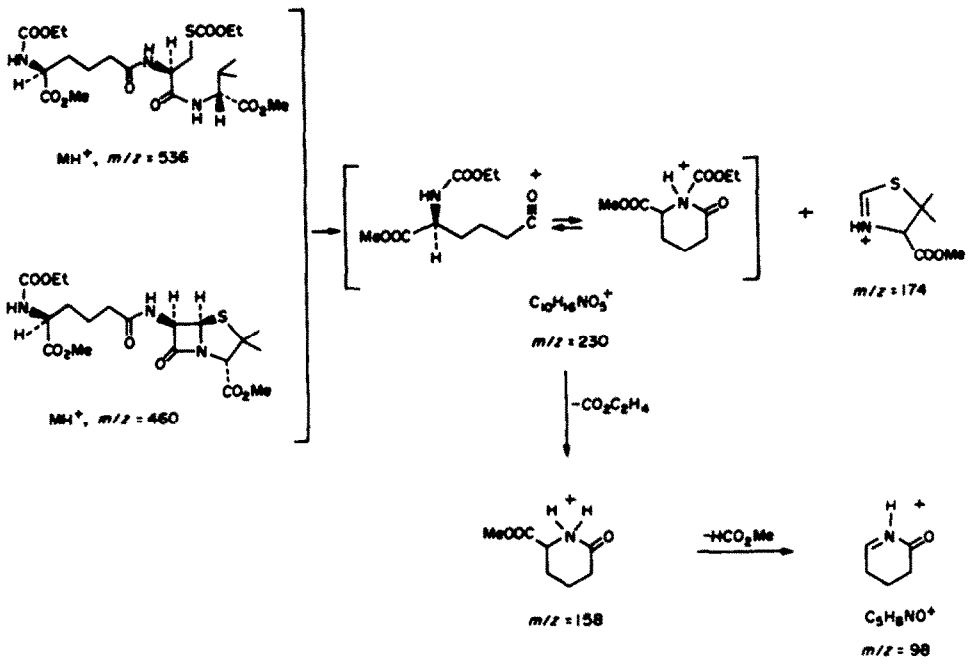
Under electron impact (EI) conditions, both 13 and 14 fragment to ions m/z 230, 158 and 98 (relative ion intensity: 13 - 100%, 31%, 49%; 14 - 7%, 7%, 18% m/z 174 = 100%) arising from cleavage of the adipyl sidechain from the rest of the molecule (Scheme 3). The sequential unimolecular fragmentation scheme was confirmed by B/E linked scan measurements and the intermediate ions were identified by accurate mass measurement: m/z 230 (found 230.1028, $C_{10}H_{16}NO_5$ requires 230.1028); m/z 158 (found 158.0816, $C_7H_{12}NO_3$ requires 158.0817); m/z 98 (found 98.0605, C_5H_8NO requires 98.0606).

When the labelled tripeptide, 12, was derivatised, the distribution of protonated molecular ions in its DCI mass spectrum indicated $^{17}O/^{18}O$ enrichment at three sites in the molecule (MH^+, 536: 537: 538: 539: 540: 541: 542 = 26: 65: 100: 95: 69: 34: 13). In the in beam EI spectrum of derivatised 12, the intensities of the fragment ions $C_{10}H_{16}NO_5^+$ (m/z, 230: 231: 232: 233: 234: 235: 236 = 39: 73: 100: 88: 56: 23: 7) and $C_5H_8NO^+$ (m/z, 98: 99: 100 = 89: 100: 74) were consistent with the intensity of the molecular ions and verified that all of the labels were in the aminoadipyl residue in the labelled tripeptide 12.

A least squares analysis of the intensities of the molecular ions and the fragment ions indicated enrichment in the adipyl amide bond (^{16}O: ^{17}O: ^{18}O = 36: 37: 27) and in each of the adipyl α-carboxyl sites (^{16}O: ^{17}O: ^{18}O = 51: 28: 21).

Conversion of LLD-ACV to Isopenicillin N

Incubation of unlabelled LLD-ACV with a cell-free extract of C. acremonium in $^{17}O/^{18}O$ enriched water (15 mole % ^{17}O) produced isopenicillin N quantitatively (by bioassay[6] and ^1H - NMR[7]).



Scheme 3

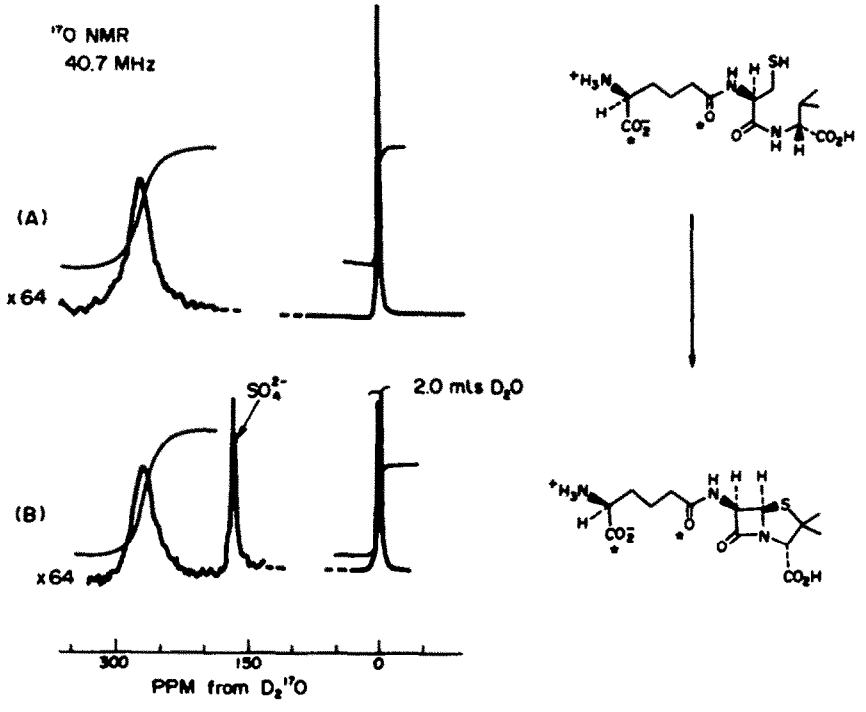


Figure 2 ^{17}O NMR spectra (40.7 MHz) (A) $^{17}O/^{18}O$ enriched LLD-ACV; (B) the product isopenicillin N after incubation with a cell-free extract of C. acremonium. (B) contains SO_4^{2-} (with natural abundance ^{17}O) from the cell-free extract. 2.0 mls D_2O (solvent) provided internal calibration for the levels of ^{17}O enrichment.

The ^{17}O NMR spectrum of the triply lyophilised product mixture showed no detectable incorporation of ^{17}O. The DCI mass spectrum of derivatised isopenicillin N, isolated from the reaction mixture showed a molecular ion distribution (MH^+, 460: 461: 462 = 100: 21: 13) identical to that obtained from derivatised isopenicillin N arising from incubation of LLD-ACV in normal (i.e. unenriched) water.

Incubation of labelled LLD-ACV, 12, with a cell-free extract of C. acremonium produced isopenicillin N quantitatively. By ^{17}O NMR spectroscopy no nett loss of ^{17}O could be detected (Figure 2). The isopenicillin N product was derivatised in situ and isolated from the incubation mixture. The protonated molecular ions in the DCI mass spectrum of the derivatised isopenicillin N showed an identical isotope distribution (MH^+, 460: 461: 462: 463: 464: 465: 466 = 26: 65: 100: 94: 67: 33: 13) to that of the derivatised labelled tripeptide precursor. Moreover, the $^{17}O/^{18}O$ isotope distributions in the fragment ions ($C_{10}H_{16}NO_5^+$, $C_5H_8NO^+$) were consistent with those in derivatised labelled LLD-ACV although the absolute intensities of these fragments was too low to be measured accurately in the presence of other products derived from the incubation mixtures.

Conclusions

During the enzymatic biosynthesis of isopenicillin N from LLD-ACV, the fact that no oxygen is incorporated from an isotopically enriched medium and no oxygen atoms are lost from isotopically labelled LLD-ACV precludes any mechanism involving a dehydration/hydration step. The formation of intramolecular thiazolines, orthothioamides etc as intermediates in the biosynthesis is clearly inconsistent with our experimental results. The possible formation[11] of any intermolecular, covalently-linked intermediate (eg enzyme-bound thioesters, esters, amidines etc) involving any of the oxygen sites of LLD-ACV is also excluded[12].

Thomas et al[13] have reported the loss of one oxygen atom, from a labelled L-valine precursor, in the biosynthesis of penicillin V by intact mycelia of Penicillium chrysogenum. Assuming that the biosynthesis in this organism follows the same path as in a cell-free extract of C. acremonium, our results suggest that this oxygen atom is lost during the biosynthesis of LLD-ACV from its constituent amino acids (or possibly during the exchange of the phenoxymethyl for the aminoadipyl sidechain) and not during the oxidative cyclisation of LLD-ACV.

Penicillin biosynthesis can involve the oxygen sites of LLD-ACV only in non-covalent interactions eg hydrogen bonding with the synthetase enzyme. We have demonstrated that δ-(L-α-aminoadipyl)-L-serinyl-D-valine is neither a substrate nor a competitive inhibitor for the synthetase enzyme[14], thus we believe that the SH group of LLD-ACV may play a decisive role in binding the substrate to the active site during penicillin biosynthesis.

Acknowledgements

We thank Professor R. Thomas of the University of Surrey for helpful discussions. We also thank SERC and BTG for financial support and the SERC for a grant for the WH300 Bruker spectrometer and the VG Micromass mass spectrometer. One of us (R.L.W.) is indebted to the NSERC of Canada for a Postdoctoral Fellowship.

Experimental

NMR spectra were recorded on a Bruker WH300 NMR spectrometer at 20°C. ^{17}O NMR spectra were recorded at 40.7 MHz, typically 25,000 scans were accumulated with a repetition rate of 0.02 sec. Mass spectra were obtained on a VG Micromass ZAB 1F mass spectrometer. In beam EI spectra were obtained using the standard DCI probe under EI conditions. Ammonia DCI spectra were recorded at an indicated source pressure of 6×10^{-5} torr. High resolution measurements were run at a resolution of 10,000 using peak matching against perfluorotributylamine as internal standard.

$^{17}O/^{18}O$ Enriched N-benzyloxycarbonyl-L-α-aminoadipic acid δ-benzyl ester 9. Dry hydrogen chloride was dissolved in water (1.00 ml) isotopically enriched in ^{17}O and ^{18}O (KOR isotopes[9], $H_2{}^{16}O$: $H_2{}^{17}O$: $H_2{}^{18}O$ = 10: 50: 40) until the concentration of the acid was approximately 3M. L-α-aminoadipic acid (540 mg, 3.3 mmole) was dissolved in the acid and allowed to stand for 48 hours at 20°C. The solution was neutralised with sodium bicarbonate, the solvent was removed under vacuum, and the residue was dissolved in aqueous sodium hydroxide (20 mls, 0.35 M) at 5°C. A solution of benzyl chloroformate (860 mg, 5 mmole) in dioxan (20 mls) was added dropwise, concurrently with an aqueous solution of sodium hydroxide (20 mls, 0.25M). The solution was stirred for 1 hour at 20°C then washed with ethyl acetate (3 x 50 mls), acidified (pH ~1) and extracted with ethyl acetate

(3 x 50 mls). The combined extracts were dried ($MgSO_4$) and the solvent removed to yield crude $^{17}O/^{18}O$ isotopically enriched N-benzyloxycarbonyl-L-α-aminoadipic acid (930 mg) as a white crystalline solid which was used without further purification.

Crude N-CBZ-L-α-aminoadipic acid (930 mg) was dissolved in dry DMF (5 mls) under argon, cyclohexylamine (350 mg, 3.3 mmole) was added and the solution was heated to 80°C. Benzyl bromide (510 mg, 3 mmole) in dry DMF (2 mls) was added, the solution was stirred for 10 minutes at 80°C then poured into water (150 mls) and extracted with ethyl acetate (3 x 50 mls). The extracts were washed successively with water (3 x 50 mls) and aqueous sodium bicarbonate (3 x 50 mls, 0.01N) then dried ($MgSO_4$) and the solvent removed to yield a crystalline solid (720 mgs). The combined aqueous phases were acidified (pH ~1) and extracted with ethyl acetate (3 x 50 mls). The extracts were dried ($MgSO_4$) and removal of the solvent afforded unreacted N-CBZ-L-α-aminoadipic acid (400 mg) which was recycled through the esterification procedure (twice). The combined crude product (1.1 g) was purified by flash chromatography on silica (30 cm x 2 cm) using chloroform/acetone/acetic acid (80: 20: 1) as eluent. After a forerun of diesterified material (120 mg) the required monoester, 9, (750 mg) was obtained. Recrystallisation (benzene/light petroleum) afforded pure $^{17}O/^{18}O$ enriched N-benzyloxycarbonyl-L-α-aminoadipic acid α-benzyl ester (500 mg, 1.3 mmol, 39%) m.p. 90-92°C; $[\alpha]_D^{20}$ -14.8° (c = 0.95, acetone) (lit.[15] m.p. 92-94°C; $[\alpha]_D^{25}$ -14°, c = 2, acetone).

9 was converted to $^{17}O/^{18}O$ enriched LLD-ACV following literature procedures[16].

Derivatisation for Mass Spectrometry

Peptides and penicillins were routinely derivatised for mass spectrometry using a procedure analogous to that of Abraham et al[11]. Typically, the compound (ca 1 mg) was dissolved in water (2 mls) and saturated sodium bicarbonate solution (50 μl). Diethylpyrocarbonate (100 μl) was added and the suspension stirred for 1 hour, adjusted to pH2 (2N HCl) and extracted with ethyl acetate (3 x 3 mls). The extracts were dried (Na_2SO_4), filtered and treated directly with freshly prepared diazomethane in diethyl ether at 0°C for 15 minutes. Evaporation of the solvents gave the crude derivatives which could be purified by preparative layer chromatography on silica plates.

LLD-ACV (10.5 mg) was derivatised as above to yield a crude product which was purified by preparative layer chromatography (ethyl acetate: dichloromethane, 1:2, x 2). N,S-diethyoxycarbonyl-LLD-ACV dimethyl ester, 13, was obtained as a foam (8.0 mg, 52%), R_f 0.25 (silica, ethyl acetate: dichloromethane, 1:2); ν_{max} ($CHCl_3$) 3420 (NH), 2950 (CH), 1720 (C=O), 1690 (NHCO), 1500 and 1150 (C-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.903 (3H, d, J = 6.9Hz, -CHMe), 0.952 (3H, d, J = 6.9Hz, -CHMe), 1.238 (3H, t, J = 7.2Hz, $-CH_2Me$), 1.320 (3H, t, J = 7.2Hz, $-CH_2Me$), 1.5-2.5 (7H, broad mult., $-CHMe_2$, $-(CH_2)_3-$), 3.20-3.29 (2H, m, $-CH_2S-$), 3.737 (3H, s, -COOMe), 3.748 (3H, s, -COOMe), 4.12 (2H, m, $-CH_2Me$), 4.25-4.35 (3H, m, $-CH_2CH_3$, $-CH-CH_2$), 4.46 (1H, dd, J = 4.9, 8.7Hz, $-CH-CHMe_2$), 4.76 (1H, m, $-CH-CH_2-S-$), 5.51, 6.62, 7.03 (3x broad doublets, 3x-NH) ppm; m/z 535.2202 ($C_{22}H_{37}N_3O_{10}S$ requires 535.2200).

Synthetic L,L,D- isopenicillin N[16] (12.0 mg) was derivatised as above to yield a crude product which was purified by preparative layer chromatography (ethyl acetate: dichloromethane, 1:2, x 2). N-ethoxycarbonyl-L,L,D-isopenicillin N, dimethyl ester, 14, as a foam (4.5 mg); R_f 0.50 (silica, ethyl acetate: dichloromethane, 1:2, x 2); ν_{max} ($CHCl_3$) 3440 (NH), 1790, 1750, 1715 (C=O), 1510 cm^{-1}; δ H($CDCl_3$) 1.25 (3H, t, $-CH_2Me$), 1.501 (3H, s, -S-CMe), 1.669 (3H, s, -S-CMe), 1.5-2.5 (6H, bm, $-(CH_2)_3-$), 3.750 (3H, s, -COOMe), 3.782 (3H, s, -COOMe), 4.12 (2H, m, $-CH_2-CH_3$), 4.20 (1H, bm, $-CH-CH_2-$), 4.427 (1H, s, $-CH-CMe_2-$), 5.54 (1H, d, J = 4.4Hz, -CH-S-), 5.70 (1H, dd, J = 4.4, 9.3Hz, -CH-CH-S-), 5.3, 6.3 (2x broad doublets, 2 x N-H) ppm; m/z 459.1683 $C_{19}H_{29}N_3SO_8$ requires 459.1675.

Conversion of LLD-ACV to Isopenicillin N. LLD-ACV (0.03 mmole) in a total of 5 ml of cell-free extract plus cofactors was reciprocally shaken at 100 cycles/min at 27°C for 60 minutes. Protein was precipitated with acetone and the mixture was filtered and lyophilised.

The biosynthesised isopenicillin N and unreacted LLD-ACV were derivatised directly in the crude lyophilised reaction mixture using the procedure above. Purification by preparative layer chromatography provided derivatives for mass spectrometry.

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