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THE SYNTHESIS AND REACTIONS OF A MONOCYCLIC β-LACTAM TRIPEPTIDE, 1-[(1*R*)-CARBOXY-2-METHYLPROPYL]-(3*R*)-[(5*S*)-5-AMINO-5-CARBOXYPENTANAMIDO]-(4*R*)-MERCAPTOAZETIDIN-2-ONE, A PUTATIVE INTERMEDIATE IN PENICILLIN BIOSYNTHESIS

JACK E. BALDWIN,*[a] SIR EDWARD P. ABRAHAM,[b] ROBERT M. ADLINGTON,[a]
MICHAEL J. CRIMMIN,[a] LESLIE D. FIELD,[a] GAMINI S. JAYATILAKE,[b]
ROBERT L. WHITE[b] and JOHN J. USHER[a]
[a]Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, England
[b]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, England

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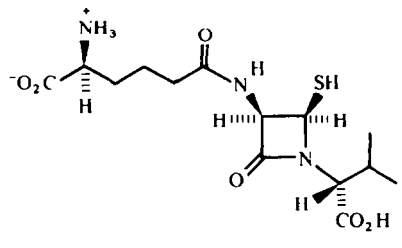
Abstract–The disulphide corresponding to the above thiol has been synthesised, but all attempts to reduce this substance to the thiol were unsuccessful, although an alternative procedure via a thiomercury intermediate, enabled the thiol to be generated *in situ*; the properties of this thiol, however, are not in accord with those previously described for a putative free intermediate in penicillin biosynthesis.[1]

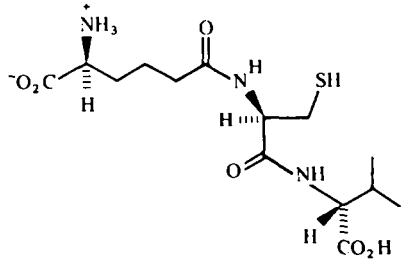
Recently, it was claimed[1] that the monocyclic β-lactam (**1**) was formed from δ - (L - α - aminoadipyl) - L - cysteinyl - D - valine (**2**),[2] by incubation with a protoplast lysate from *Penicillium chrysogenum*. This claim was based on the isolation by cation-exchange chromatography of labelled compounds believed to be (**1**), from [(3-^{3}H)Cys, (1-^{14}C)Val] (**2**), [(3,4-$^3H_2$, 1-^{14}C)Val]-(**2**), and [2-^{3}H, 1-^{14}C Val]-(**2**). The loss of 49.6% of ^{3}H in the first case, coupled with the retention of all ^{3}H in the last two examples, was adduced to support the stucture proposed for (**1**). Furthermore, the biosynthetic sample of (**1**) was claimed to be identical with a synthetic sample as judged by cation-exchange chromatograpy.† The compound (**1**) appeared to be relatively stable since it was stored with an excess of dithiothreitol overnight at room temperature (pH 8.2) and was subsequently subjected to cation-exchange column chromatography. These results were all the more striking to us since prior to the appearance of this publication, we had synthesised the disulphide of (**1**) but had been unable to successfully reduce it to the thiol (**1**). As well as (**1**) there was also isolated from the cell-free extract the penicilloic acid (**3**) of isopenicillin N. Curiously, however, there was observed a relatively poor (28.4% instead of 50.0%) retention of ^{3}H when (**3**) was formed from [(3-^{3}H)Cys] L,L,D-tripeptide (**2**) which was explained by "epimerisation" at C-5. Although the facile epimerisation at C-5 of penicilloic acids is well established[3] it seems more reasonable that thiazolidine ring opening would better explain this epimerisation without loss of hydrogen from C-5, as in Fig. 1.

Recently we have described, in communication form, the preparation of the thiol (**1**) and its dimer (**13**).[4] Herein we report full experimental details of this work. The dimer (**13**) was obtained in seven steps (20% overall yield) from the potassium salt of penicillin V (Scheme 1). Esterification (PNB.bromide,

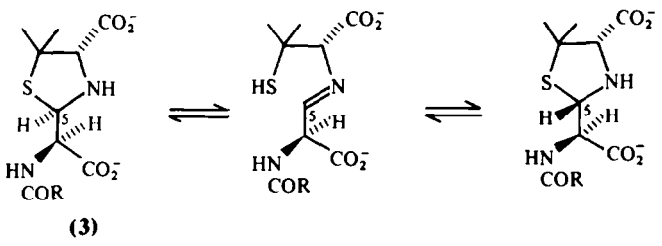
†In Ref. 1, it was stated that the synthetic sample (**1**) was prepared by Roets *et al.* (to be published). Unfortunately we have been unable to obtain a comparison sample, nor any spectroscopic data on this synthetic material from the authors.



(**1**)

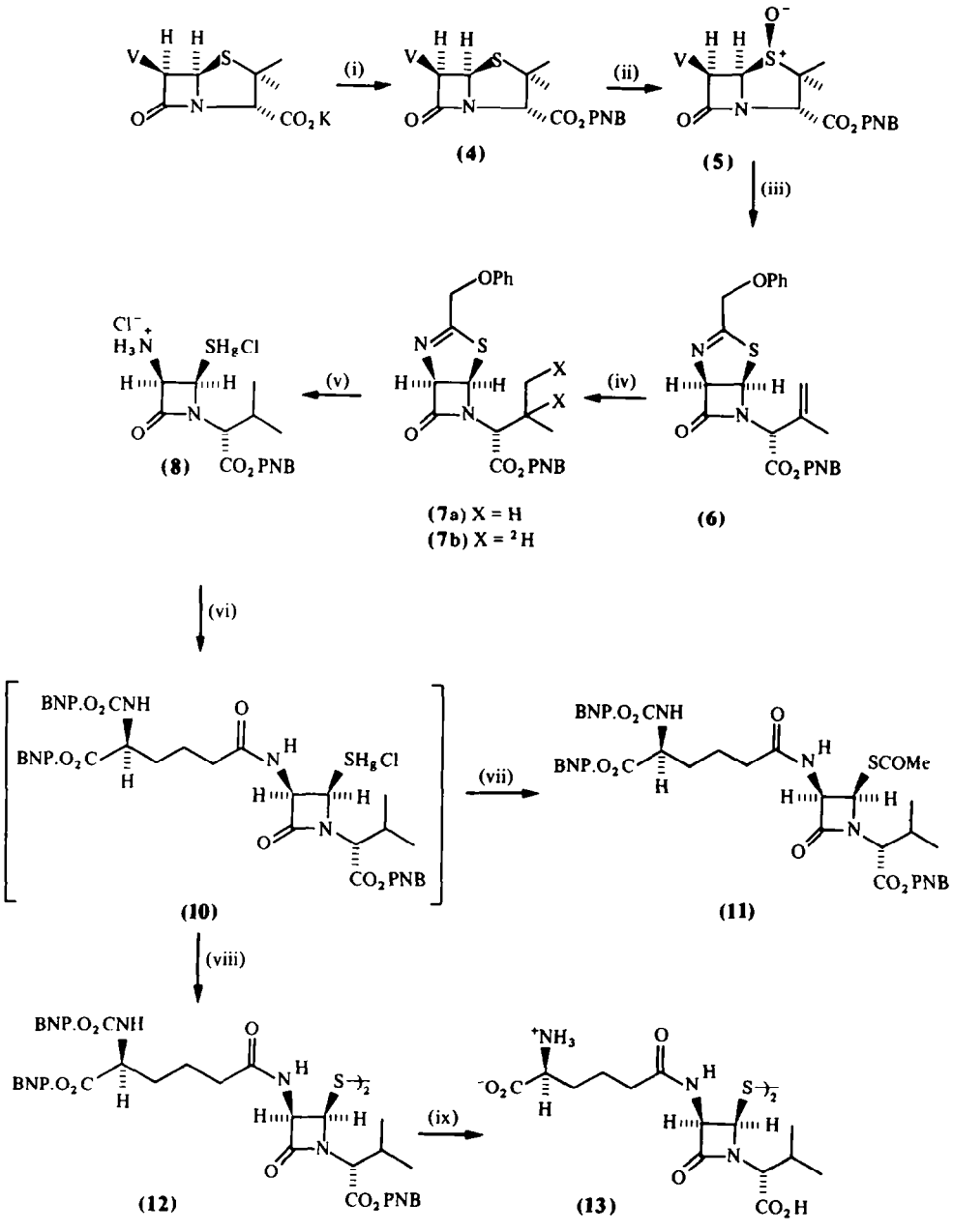


(**2**)



(3)

Fig. 1.



Scheme 1. Reagents (i) PNB.Br, DMF, NaI, 97%; (ii) MCPBA, 92%; (iii) $P(OMe)_3$, PhMe, Δ, 91%; (iv) H_2(87%), or $^2H_2$(84%), $(PPh_3)_3RhCl$, 48 h; (v) $HgCl_2$, $HOCH_2C(Me)_2CH_2OH$, 95%; (vi) NEt_3, (9), EEDQ, 24 h; (vii) CH_3COCl, C_5H_5N, 89%; (viii) NaI, 66%; (ix) Pd/C/H_2/HOAc (1 M)/$Hg(OAc)_2$/THF, 45%. V=$PhOCH_2CONH$–, PNB=4-nitrobenzyl.

DMF, 25°) and oxidation in sequence of the readily available penicillin V potassium salt provided a convenient route to the protected β-sulphoxide (**5**), which was subsequently heated in the presence of trimethyl phosphite to yield the thiazoline azetidin-2-one (**6**).[5] Hydrogenation (H_2/$(PPh_3)_3RhCl$/48h) proceeded smoothly, without rearrangement, to the saturated thiazoline (**7a**) (87%). When deuterium was substituted for hydrogen, two dideuterio-isomers (**7b**) (ratio 70:30, 84%) were produced. Acid hydrolysis of (**7b**) gave a 70:30 ratio of 2*R*,3*R* and 2*R*,3*S*, 3,4-dideuteriovalines respectively, indicating a preference for *re* attack of hydrogen upon the isopropenyl function of (**6**).[6] Reaction of (**7a**) with mercury (II) chloride[7] in the presence of 2,2 - dimethylpropan - 1,3 - diol gave the mercury salt (**8**) (95%). Liberation of the free amine from (**8**) followed by coupling with di - (4 - nitrobenzyl) - protected α-aminoadipic acid (**9**) gave, upon iodine work up, the fully (4-nitrobenzyl)- protected disulphide (**12**) (66%). An alternative work up of the crude coupled product (**10**) with pyridine and acetyl chloride gave the acetylthio derivative (**11**) (89%). Deprotection (Pd–C–H_2) of (12) in the presence of mercury (II) acetate followed by sequential ion-exchange and Sephadex-gel filtration chromatography gave pure (**13**) (45%) (Fig. 2a). The hygroscopic disulphide (**13**) was characterised by standard spectral data (^1H, ^{13}C NMR, IR) and by derivatisation[8] to its bis{N-ethoxycarbonyl dimethyl ester} derivative (**14**), [Found *m/e* MH^{+}

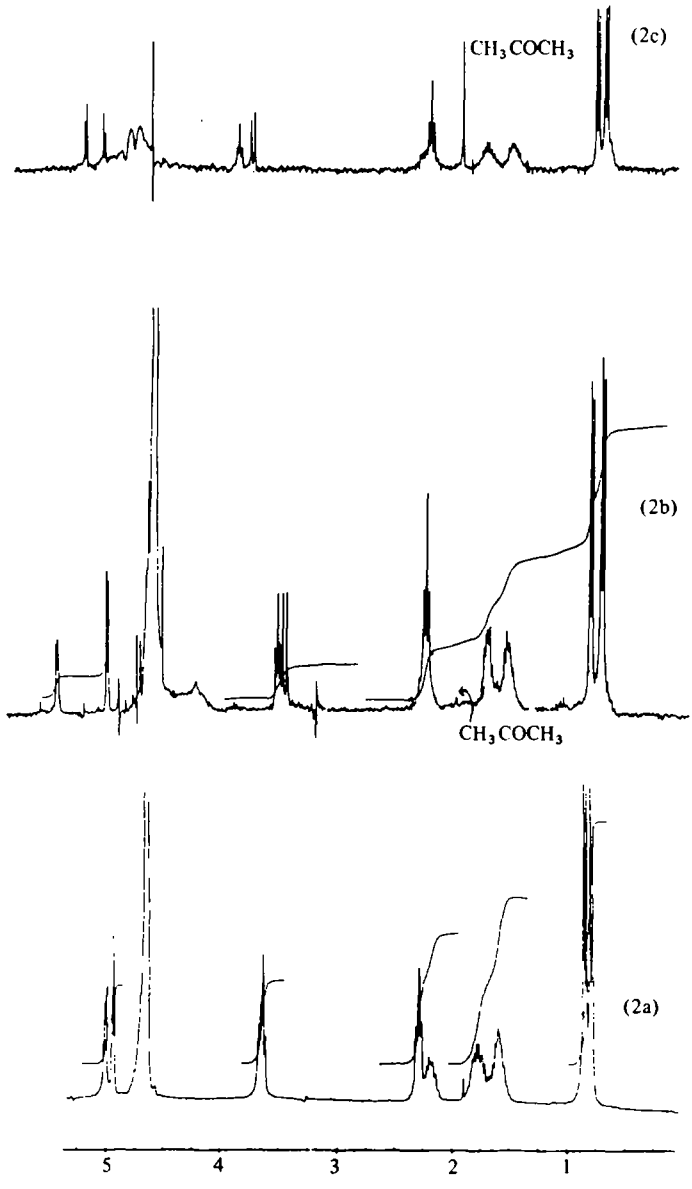
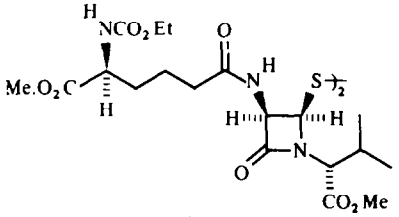


Fig. 2.

(weak) 921.362 $C_{38}H_{61}N_6O_{16}S_2$ requires 921.359); $M^+/2$-H (base, 100%, 459.168 ($C_{19}H_{29}N_3O_8S$ requires 459.166)].



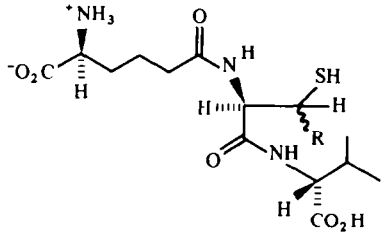
(14)

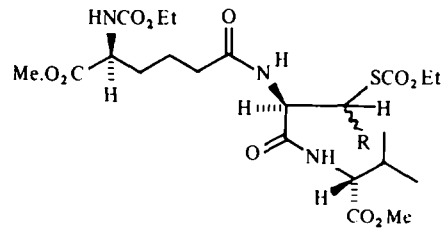
A variety of reagents and conditions (e.g. Zn/HoAc, Sn/HOAc, nBu_3P, Na_2SO_3, electrolytic reduction, UV irradiation, H_2S/Hg^{2+}, and many others) capable of cleaving disulphides to thiols[9] were used for the attempted reduction of (**13**). However in no case could the elusive thiol (**1**) be detected (by electrophoresis, ^1H 300 MHz NMR, and by attempted re-oxidation of products with iodine to the disulphide).

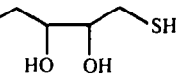
Cleavage of disulphide to thiols by dithiothreitol (DTT) is well documented.[10] Reaction (0–60 min, 0–40°) of the disulphide (**13**) with DTT (1–5 equivs) in acidic, neutral or alkaline aqueous buffered solutions gave partial conversion to a new ninhydrin-positive product (**15**) (by electrophoresis). Excess DTT (10 equivs) led to exclusive formation of (**15**) which was derivitised[8] to its N,S,S-triethoxycarbonyl dimethyl ester derivative (**16**) [Found MH^{+} 760.2461; $C_{29}H_{50}N_3O_{14}S_3$ requires 760.2454]. By monitoring these reactions by IR (pH 6.90) and NMR spectroscopy (pH 6.90), it was possible to show that no β-lactam containing products were formed with lifetimes greater than *ca* 100s.

Cleavage of the disulphide (**13**) with $NaBH_4$ gave the L,L,D - ACV - thiol (**2**), identical to an authentic sample. The derived thiol (**2**) was derivatised[8] to its N,S-diethoxycarbonyl dimethyl ester (**17**) [MH^{+} *m/e* 536], identical to an authentic sample. Thus it is reasonable to assume that no racemisation occured during the conversion of (**13**) to (**2**). Cleavage of the disulphide (**13**) with NaB^2H_4 gave a mono-deuteriated thiol (**18**) which was derivatised[8] to a mono - deuterio - N,S - diethoxycarbonyl dimethyl ester (**19**) [MH^{+} *m/e* 537). Reaction of the disulphide (**13**) with NaB^2H_4 and O_2 in sequence gave the isolated disulphide of thiol (**18**) whose ^1H 300 MHz NMR spectra indicated an almost equal deuterium incorporation into the cysteinyl C-3 pro-R and pro-S [$\delta H(^2H_2O$, ^2H decoupled) 2.99 (d, *J* 5.2 Hz), 2.81 (d, *J* 8.7 Hz) of relative area 45:55]. The products (**15**) and (**18**), and the isomer ratio of (**18**) are consistent with an intermediate thioaldehyde (**20**) (Scheme 2). Such thioaldehydes have precedent.[11]

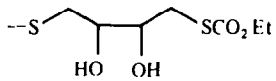
Since we had been unsuccessful in obtaining the crucial intermediate monocyclic β-lactam (**1**), we resorted to an alternative stategy. Thus we argued that the chloromercury derivative (**27**) which was readily prepared by analogous methodology to (**8**), would represent a precursor which with hydrogen sulphide, would liberate the thiol (**1**) under potentially very mild and controllable conditions. Compound (**27**) (Fig. 2b) obtained in an unprotected form was purified by Sephadex G-10 Gel filtration and obtained as a foam [ν_{max} (^2H_2O, CaF_2) 1735 s (β-lactam C=O); $\delta H(^2H_2O)$ 5.034 (1H, d, *J* 4.4 Hz, β-lactam-H, and 5.465 (1H, d, *J* 4.4 Hz, β-lactam-H)], which was cleanly oxidized (KI, I_2, ^2H_2O) to the previously characterised disulphide (**13**). At pH 1.5 (^2HCl, ^2H_2O), (**27**) was converted into the desired thiol (**1**) (Fig. 2c) by treatment with H_2S





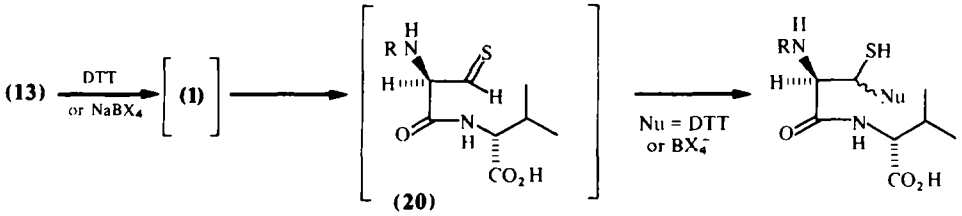
(15) R = 

(18) R = ^2H

(16) R = 

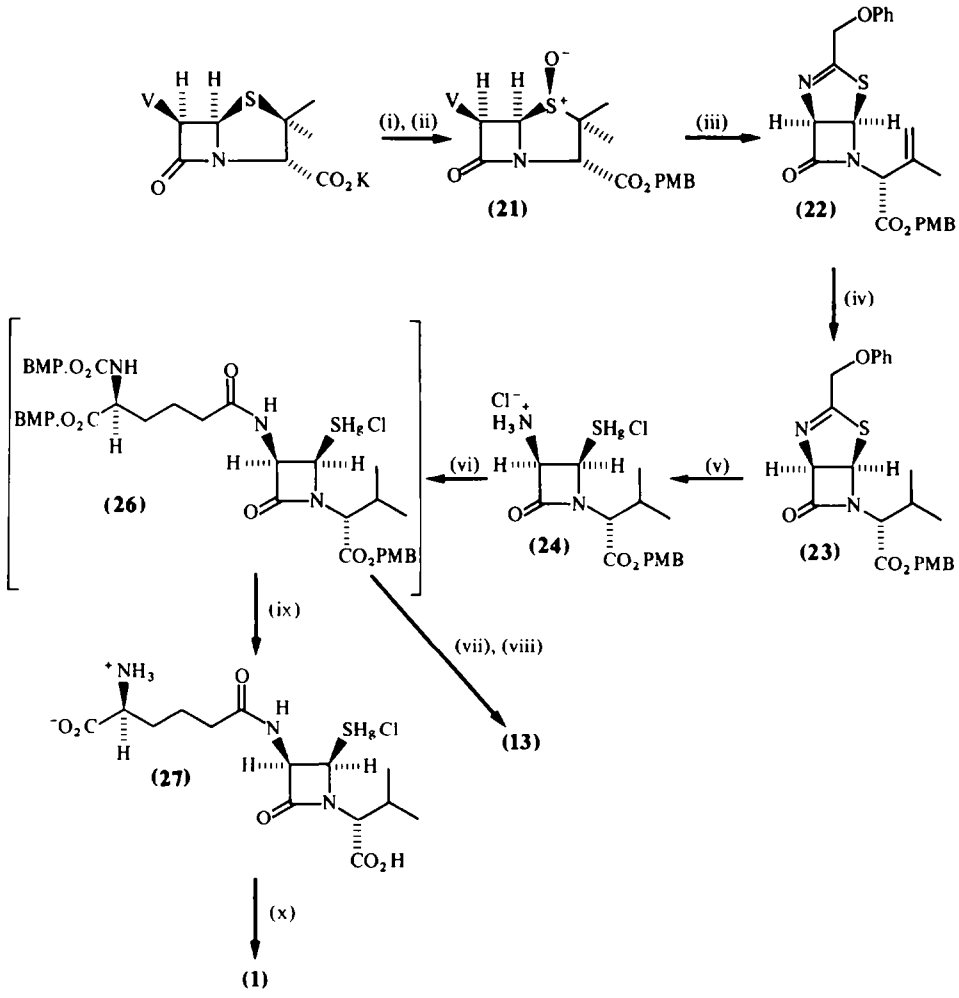
(17) R = H

(19) R = ^2H



(20)

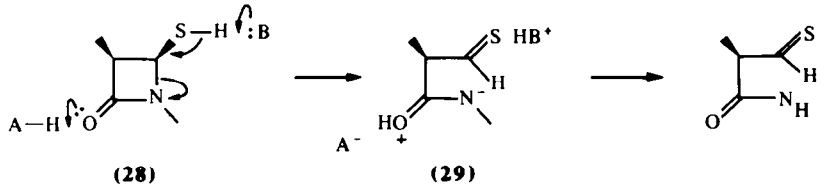
Scheme 2.



Scheme 3. Reagents (i) PMB·Cl, DMF, NaI, (ii) MCPBA, 39%; (iii) $P(OMe)_3$, PhMe, Δ, 96%, (iv) $(PPh_3)_3RhCl$, H_2, 48 h, 56%, (v) $HgCl_2$, $HOCH_2C(Me)_2CH_2OH$, 87%; (vi) NEt_3, EEDQ, (25),[12] 24 h, (vii) NaI_2, (viii) PhH, PhOMe, TFA, 12%; (ix) PhH, PhOMe, TFA, 15%, (x) H_2S. PMB = 4-methoxybenzyl.

followed by removal of the precipitated HgS and excess of H_2S. At this pH, the thiol (1) δH(^2H_2O/^2HCl) 4.987 (1H, d, *J* 4.6 Hz, β-lactam-H), and 5.140 (1H, d, *J* 4.6 Hz, β-lactam-H)] was relatively stable ($t_{1/2}$ *ca* 25 min at 20°) and could be reconverted into the stable compound (27) by treatment with mercury (II) chloride. However as the pH was raised, the lifetime of (1) rapidly decreased [pH 5 *ca* 5 min, pH 6.95 < 3 min. (no β-lactam absorption could be detected after 3 min, the minimum time required to generate the thiol (1) and record the IR spectrum)] as monitored by IR and NMR spectroscopy. We conclude that the thiol (1), which we have generated *in situ* and characterised by spectral and chemical means, undergoes a facile ring opening reaction, probably of the type (28)→(29) at or above neutral pH in aqueous solution. Whereas (1) can be obtained at low pH, the decomposition is so fast at higher pH so as to preclude normal isolation procedures. On the other hand, the dimer of (1) as (13) is a perfectly normal tripeptide disulphide.

For the above reasons, we cannot accept that the



(28) (29)

substance described in the previous report[1] is the thiol (1). Its properties, as far as they were described are completely at variance with our own observations on (1) and until further evidence appears to substantiate the earlier claim, we believe the question of a free monocyclic β-lactam thiol intermediate in penicillin biosynthesis is still unresolved.

Incubation of the disulphide (13) with cell-free extracts of *C.acremonium* C.91 or *P.chrysogenum* (SC 6140)[13] in the presence or absence of dithiothreitol gave no isopenicillin N. In separate experiments there was no inhibition by (13) of the conversion of L,L,D-ACV (2) into isopenicillin N by these cell-free systems. Also the use of the chloromercury derivative (27) in the presence of DTT gave, as in the case of the dimer (13), no evidence of isopenicillin N synthesis, nor its inhibition. However, these negative experiments must be considered in the light of the extremely short lifetime of the monocyclic peptide (1) at these pH levels.

EXPERIMENTAL

Reactions were performed under a dry argon atmosphere at room temp unless otherwise stated. Temperatures were measured in degrees celsius (°). Reaction times are recorded in seconds (s), minutes (min), hours (h), or days (d). Reactions were studies by TLC, IR, or NMR analysis prior to work up. UV inactive compounds on TLC were visualised either with iodine or by concentrated sulphuric acid charring. Reaction mixtures were evaporated at 25° or below on a Büchi rotovapor R110; reaction mixtures containing involatile compounds were further evaporated (< 2 mm Hg). Aqueous solutions were freeze dried. Organic extracts were dried over sodium sulphate. Both TLC, and PLC were carried out on Merck Kieselgel GF_{254}, developing solvents are given in parentheses. Melting points (m.p.) were determined on a Kofler hot stage and are uncorrected. Optical rotations were recorded on a Perkin–Elmer 241 Polarimeter. IR spectra were recorded on a Perkin–Elmer 681 Spectrophotomer, only broad (br), medium (m), or strong (s) bands were reported. UV spectra were recorded on a Perkin–Elmer 555 UV-VIS Spectrophotometer. NMR spectra were recorded upon a Bruker WH 300 MHz NMR spectrometer using tetramethylsilane (organic) or sodium 3-trimethylsilylpropionate - 2,2,3,3 - $^2H_4$ (aqueous) as internal standards unless otherwise stated. Multiplicities were recorded as b (broad peak), s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Mass spectra were recorded upon a V.G. Analytical Ltd ZAB IF Mass spectrometer [for field desorption (FD) or ammonia desorption chemical ionisation (NH_3DCI)] or V.G. Analytical Ltd mm 16F mass spectrometer [for Electron impact (EI) or ammonia chemical ionisation (NH_3CI)]. Microanalyses were recorded by Dr. F. B. Strauss, Microanalytical Laboratory, Dyson Perrins Laboratory, University of Oxford.

All starting material reagents and solvents were purified[14] and dried unless otherwise stated. Paper electrophoresis (both analytical and preparative) was performed upon Whatman no 1 paper using a Locarte power pack. Electrophoresis buffers were prepared as follows: pH 1.8 [water:acetic acid:formic acid (78:20:2)]; pH 3.5 [water: acetic acid:pyridine 135:10:1)]; pH 4.5 [water:acetic acid:pyridine (1000:3:2)].

Preparation of 4 - *nitrobenzyl*(2S,5R,6R) - 3,3 - *dimethyl* - 7 - *oxo* - 6 - *phenoxyacetamido* - 1 - *aza* - 4 - *thiabicyclo*[3.2.0]*heptane* - 2 - *carboxylate* (4)

The potassium salt of penicillin V (15.56 g, 40.10 mmol), 4-nitrobenzyl bromide (8.64 g, 40.00 mmol), and sodium iodide (0.60 g) were suspended in dry DMF (90 ml) and the mixture was stirred for 15 h. The soln was poured into water (150 ml) and dichloromethane (150 ml), the organic layer separated, re-extracted with water (4 × 150 ml), dried, filtered, and evaporated. Recrystallisation of the residue from dichloromethane and light petroleum gave the title compound (4) (18.76 g, 97%); TLC [diethyl ether: dichloromethane (1:9)] R_f 0.50; m.p. 100–1°; ν_{max} ($CHCl_3$) 1792 s (β-lactam C=O), 1755 m (ester C=O), 1690 s (amide C=O), 1528 s (Ar–NO_2), 1352 s (Ar–NO_2), and 1294 m cm^{-1}; δH(C^2HCl_3) 1.440 (3H, s, 3-Me), 1.596 (3H, s, 3-Me), 4.534 (1H, s, 2-H), 4.530 and 4.557 (2H, ABq, *J* 15.0 Hz, $PhOCH_2$), 5.267 and 5.323 (2H, AB q, *J* 13.0 Hz, CO_2CH_2), 5.586 (1H, d, *J* 4.2 Hz, 5-H), 5.750 (1H, dd, *J* 4.2, 9.2 Hz, 6-H), 6.90–6.95 (2H, m, phenyl 2-H), 7.00–7.07 (1H, m, phenyl 4-H), 7.30–7.35 (3H, m, phenyl 3-H, plus NH), 7.54–7.57 (2H, m, aryl 2-H), and 8.23–8.27 (2H, m, aryl 3-H).

Preparation of 4 - *nitrobenzyl*(2S,4S,5R,6R) - 3,3 - *dimethyl* - 7 - *oxo* - 6 - *phenoxyacetamido* - 1 - *aza* - 4 - *thiabicyclo*[3.2.0]*heptane* - 2 - *carboxylate* 4-*oxide* (5)

The ester (4) (4.89 g, 10.07 mmol) was dissolved in dry dichloromethane (50 ml) and MCPBA (1.91 g, 11.07 mmol) was added over 5 min. The soln was stirred for 30 min, and washed with saturated aq sodium hydrogen carbonate solution (100 ml). The aqueous phase was separated, re-extracted with dichloromethane (100 ml), and the organic layers were combined, dried, filtered, and evaporated. Purification by chromatography on flash silica "H" [(i) 50 g, (ii) 40 g, eluant diethyl ether:dichloromethane (0:1–1:4)] gave the title β-sulphoxide (5) (4.62 g, 92%); TLC [dichloromethane:diethyl ether (9:1)] R_f 0.30; m.p. 176–8°: $[\alpha]_D^{20}$ +150° (c 1.07, $CHCl_3$); ν_{max} ($CHCl_3$) 1800 s (β-lactam C=O), 1755 m (ester C=O), 1685 s (amide C=O), 1598 m (aryl C=C), 1520 s (Ar–NO_2), 1490 m, 1347 s (Ar–NO_2), 1062 m, and 1037 m cm^{-1}; λ_{max} (CH_3CN) 268.5 nm (ε 12,000); δH (C^2HCl_3) 1.156 (3H, s, 3-Me), 1.713 (3H, s, 3-Me), 4.542 (2H, s, $PhOCH_2$), 4.744 (1H, s, 2-H), 5.057 (1H, d, *J* 4.6 Hz, 5-H), 5.313 and 5.353 (2H, AB q, *J* 12.9 Hz, CO_2CH_2), 6.120 (1H, dd, *J* 10.4, 4.6 Hz, 6-H), 6.91–7.04 (3H, m, phenyl 2,4-H), 7.27–7.33 (2H, m, phenyl 3-H), 7.55–7.58 (2H, m, aryl 2-H), 8.25 (1H, obs, NH), and 8.25–8.28 (2H, m, aryl 3-H). Found: C, 55.21; H, 4.50; N, 8.25; S, 6.44. $C_{23}H_{23}N_3O_8S$ requires C, 55.08; H, 4.62; N, 8.38; S, 6.39%.

Preparation of 4-*nitrobenzyl* (2R) - 3 - *methyl* - 2 - [(1R,5R)-7 - *oxo* - 3 - *phenoxymethyl* - 4 - *thia* - 2,6 - *diazabicyclo*[3.2.0]*hept* - 2 - *en* - 6 - *yl*]*but* - 3 - *enoate* (6)

The sulphoxide (5) (6.82 g, 13.6 mmol) and freshly distilled trimethylphosphite (1.70 ml, 14.4 mmol) in dry toluene were slowly heated to relux in a Dean and Stark apparatus over 250 min. Evaporation and recrystallisation of the residue from dichloromethane and light petroleum gave crude (6). Purification by chromatography on flash silica "H" [(50 g), eluant dichloromethane:diethyl ether (1:0–93:7)] and recrystallisation from dichloromethane and light petroleum gave the title compound (6) (5.80 g, 91%); TLC [dichloromethane: diethyl ether (92.5: 7.5)] R_f 0.50; m.p. 131.5–2°; $[\alpha]_D^{20}$ −114° (c 0.43, $CHCl_3$); ν_{max} ($CHCl_3$) 1774 s (β-lactam C=O), 1750 m (ester C=O), 1612 m, 1593 m, 1526 s (Ar–NO_2), 1497 m, 1449 m, 1380 m, 1365 m, 1351 s (Ar–NO_2), 1333 m, 1304 m, 1290 m, 1174 m, 1155 m, 1052 m, 1018 m, 967 m, 956 m, 916 m, and 852 m cm^{-1}; λ_{max} (CH_3CN) 267 nm (ε 11,400); δH(C^2HCl_3) 1.777 (3H, s, vinyl-Me), 4.896 and 4.969 (2H, dABq, *J* 1.1, 14.2 Hz, $PhOCH_2$), 4.893 (2H, s, CH_2=), 5.110 (1H, d, *J* 1.5 Hz, $CHCO_2$), 5.249, 5.296 (2H, ABq, *J* 13.1 Hz, CO_2CH_2), 5.902 (1H, d, *J* .4.2 Hz, 1-H), 6.031 (1H, bd, *J* 4.2 Hz, 5-H), 6.90–7.03 (3H, m, phenyl 2,4-H), 7.27–7.33 (2H, m, phenyl 3-H), 7.49–7.52 (2H, m, aryl 2-H), and 8.21–8.25 (2H, m, aryl 3-H); δC(C^2HCl_3) 21.38 (q, Me), 58.58, 66.94, 91.98 (3 × d, 6-C, 1C, 5C), 65.74 (t, CH_2Ar), 67.39 (t, CH_2OPh), 114.50 (d, phenyl 2C), 117.59 (t, CH_2=), 121.75 (d, phenyl 4C), 123.67 (d, aryl 3C), 128.49 (d, phenyl 3C), 129.45 (d,

aryl 2C), 137.21 (s, C=CH$_2$), 141.76 (s, aryl 1C), 147.69 (s, aryl 4C), 157.33 (s, phenyl 1C), 164.79, 168.27 (2 × s, 7C, 3C), and 173.06 (s, CO$_2$); *m/e* (F.D.) 467 (M$^+$). Found: C, 59.28; H, 4.61; N, 8.73; S, 7.05. $C_{23}H_{21}N_3O_6S$ requires C, 59.09; H, 4.53; N, 8.99; S, 6.86%.

Preparation of 4-*nitrobenzyl* (2R) - 3 - *methyl* - 2 - [(1R,5R) - 7 - *oxo* - 3 - *phenoxymethyl* - 4 - *thia* - 2,6 - *diazabicyclo*[3.2.0]*hept* - 2 - *en* - 6 - *yl*]*butanoate* (**7a**)

Thiazoline (**6**) (5.75 g, 12.30 mmol) and Wilkinson's catalyst (0.40 g) were dissolved in dry benzene (120 ml) and the solution hydrogenated at 1 atm for 24 h. Further catalyst (0.30 g) was added, and the sample re-hydrogenated for an additional 24 h. The solution was evaporated and the residue purified by chromatography on flash silica "H" [(60 g × 2), eluant dichloromethane:diethyl ether (1:0–9:1)] and triple recrystallisation from dichloromethane and light petroleum to give the title compound (**7a**) (5.03 g, 87%); TLC [dichloromethane:diethyl ether (9:1)] R_f 0.70; m.p. 123°; $[\alpha]_D^{20}$ −6.5° (c 0.49, $CHCl_3$); ν_{max} ($CHCl_3$) 1775 s (β-lactam C=O), 1745 s (ester C=O), 1620 m 1610 m, 1602 m, 1593 m, 1528 s (Ar-NO$_2$), 1497 m, 1370 m, 1352 s, (Ar-NO$_2$), 1335 m, 1292 m, 1153 s, and 1045 m cm^{-1}; λ_{max} (CH_3CN) 268 nm (ε 11,300); δH(C^2HCl_3) 0.897 (3H, d, *J* 6.8 Hz, CHMe$_2$), 0.948 (3H, d, *J* 6.8 Hz, CHMe$_2$), 2.29–2.36 (1H, m, CH Me$_2$), 4.185 (1H, d, *J* 8.5 Hz, 6-CH), 4.577 and 4.963 (2H, dAB q *J* 1.1, 14.4 Hz, PhOCH$_2$), 5.246 (2H, *ca* d, *J* 1.7 Hz, CO$_2$CH$_2$), 5.854 (1H, d, *J* 4.0 Hz, 1-H), 6.003 (1H, d, *J* 4.0 Hz, 5-H), 6.91–7.03 (3H, m, phenyl 2,4-H), 7.26–7.32 (2H, m, phenyl 3H), 7.50–7.53 (2H, m, aryl 2-H), and 8.22–8.25 (2H, m, aryl 3-H); δC(C^2HCl_3) 18.98, 19.42 (2 × q, Me), 29.90 (d, CHMe$_2$), 61.04, 67.79, 92.53 (3 × d, 6-C, 1C, 5C), 65.47 (t, CH$_2$Ar), 67.37 (CH$_2$OPh), 114.49 (d, phenyl 2C), 121.78 (d, phenyl 4C), 123.68 (d, aryl 3C), 128.56 (d, phenyl 3C), 129.45 (d, aryl 2C), 141.83 (s, aryl 1C), 147.70 (s, aryl 4C), 157.35 (s, phenyl 1C), 165.20, 169.02 (2 × s, 7C, 3C), and 171.86 (s, CO$_2$); *m/e* (FD) 469 (M$^+$). Found: C, 58.70; H, 5.05; N, 8.79; S, 6.59. $C_{23}H_{23}N_3O_6S$ requires C, 58.84; H, 4.94; N, 8.95; S, 6.83%.

Preparation of 4-*nitrobenzyl* (2R,3RS) - 3,4 - *dideuterio* - 3-*methyl* - 2 - [(1R,5R) - 7 - *oxo* - 3 - *phenoxymethyl* - 4 - *thia* - 2,6 - *diazabicyclo*[3.2.0]*hept* - 2 - *en* - 6 - *yl*]*butanoate* (**7b**)

Thiazoline (**6**) (523 mg, 1.12 mmol) and Wilkinson's catalyst (80 mg) were dissolved in dry benzene (40 ml) and the solution was reacted with deuterium gas (1 atm) for 24 h. Further catalyst (90 mg) was added and the sample re-deuteriated for 24 h. An identical work up to (**7a**) gave a 70:30 mixture of 2*R**, 3*R** (**7b**): 2*R**, 3*S** (**7b**) (442 mg, 84%), TLC as for (**7a**), δH(C^2HCl_3) {for 2*R**, 3*R**-isomer 0.89 (3H, bs, Me), 0.92–0.94 (2H, bm, CH$_2$^{2}H); for 2*R**, 3*S**-isomer 0.87–0.89 (2H, bm, CH$_2$^{2}H), 0.94 (3H, br s CH$_3$)}, other resonances as for (**7a**) except 4.184 (1H, s, CHCO$_2$), *m/e* (FD, M$^+$) found 471.1434; $C_{23}H_{21}{}^2H_2N_3O_6S$ requires 471.1433.

A sample (105 mg) was dissolved in 6N hydrochloric acid (40 ml) and the solution refluxed for 24 h. Evaporation, purification on Dowex 50W-X8 [H$^+$ form, load in water, elute with 1M pyridine in water (500 ml)] and by preparative paper electrophoresis [pH 1.8 buffer, 4.0 kV, 1 h, elution of ninhydrin active band from the paper with water (4 × 25 ml)] gave crude 3,4-dideuteriovaline. Repurification by ion-exchange on Dowex 50W-X8 (H$^+$ form, as before) gave a 70:30 mixture of 2*R*,3*R*: 2*R*,3*S* 3,4-dideuteriovaline (13 mg, 49%), δH(^2H_2O) {for 2*R*,3*R*-isomer 0.82–0.85 (2H, m, CH$_2$^{2}H), 0.924 (3H, s, Me); for 2*R*,3*S*-isomer 0.845 (3H, s, Me), 0.89–0.92 (2H, m, CH$_2$^{2}H)}; for both 3.024 (1H, s, CHCO$_2$).

Preparation of (2R,3R) - 2 - *chloromercuriothio*) - 1 - [(1R)-2 - *methyl* - 1 - (4 - *nitrobenzyloxycarbonyl*)*propyl*] - 4 - *oxoazetidin* - 3 - *yl ammonium chloride* (**8**)

Thiazoline (**7a**) (2.24 g, 4.78 mmol) was dissolved in dry dichloromethane (60 ml). 2,2 - Dimethylpropan - 1,3 - diol (3.00 g, 28.8 mmol) and dry mercury (II) chloride (1.30 g, 4.79 mmol) were added and the mixture stirred for 23 h. A white precipitate was filtered off, and washed with dry dichloromethane to give the title compound (**8**) (2.84 g, 95%); m.p. 158–61°; ν_{max} (Nujol) 3150 m (N–H), 1768 s (β-lactam C=O), 1730 s (ester C=O), 1604 m, 1550 m, 1517 m (Ar-NO$_2$), 1392 m, 1350 m, 1308 m, 1260 m, 1243 m, 1213 s, 1204 s, 1164 m, 1103 m, 987 m, 953 m, 947 m, 892 m, 859 m, 844 m, 838 m, 815 m, 769 m, 736 s, and 720 m cm^{-1}; δH($C^2H_3SOC^2H_3$) 0.898 (3H, d, *J* 6.6 Hz, CHMe$_2$), 1.030 (3H, d, *J* 6.6 Hz, CHMe$_2$), 4.036 (1H, d, *J* 8.9 Hz, 1-CH), 4.690 (1H, d, *J* 4.7 Hz, 2-H), 5.336 (2H, s, CO$_2$CH$_2$), 5.690 (1H, d, *J* 4.7 Hz, 3-H), 7.70–7.73, and 8.24–8.27 (4H, 2 × m, aryl-H), δC($C^2H_3SOC^2H_3$), 19.00 (q, Me), 19.60 (q, Me), 28.84 (d, CHMe$_2$), 58.34, 61.41, 61.76 (3 × d, 1-C, 3C,2C), 65.26 (t, CH$_2$), 123.55 (d, aryl 3C), 128.81 (d, aryl 2C), 143.23 (s, aryl 1C), 147.14 (s, aryl 4C), 161.90 (s, 4C), and 168.31 (s, CO$_2$). Found: C, 28.74; Cl, 11.02; H, 3.24; N, 6.60; S, 5.12. $C_{15}Cl_2H_{19}HgN_3O_6S$ requires C, 28.83; Cl, 11.35; H, 3.06; N, 6.72; S, 5.13%.

Preparation of 1 - (4 - *nitrobenzyl*) *hydrogen* (2S) - 2 - (4 - *nitrobenzyloxycarbonylamino*)*hexanedioate* (**9**)

L-α-Amino adipic acid (2.72 g, 16.89 mmol) was dissolved in sodium hydroxide (2M, 17.0 ml) and water (3 ml) at 0°. 4-Nitrobenzyloxycarbonyl chloride (5.50 g, 25.5 mmol) in dioxan (40 ml) and sodium hydroxide (2M, 17.0 ml) were individually added dropwise over 18 min (at the same rate), the suspension was warmed to 25° and stirred for 45 min. Water (150 ml) was added, the soln filtered, the filtrate extracted with ethyl acetate (3 × 100 ml), acidified to pH 2 (2M hydrochloric acid) and re-extracted with ethyl acetate (3 × 150 ml). The final organic layers were combined, dried, filtered, evaporated, and recrystallised from ethyl acetate and light petroleum to give crude (2*S*) - 2 - (4 - nitrobenzyloxycarbonylamino)hexanedioate (4.60 g): m.p. 137–8°; $[\alpha]_D^{20}$ −4.7 [C 1.04, CH_3SOCH_3]: δH(C^2H_3·SO·C^2H_3) 1.56–1.78 (4H, m, 3,4-H), 2.214 (2H, t, *J* 6.9 Hz, 5-H), 3.91–4.01, (1H, m, 2-H), 5.190 (2H, s, CH$_2$Ar), 7.60–7.63 (2H, m, aryl 2-H), 7.768 (1H, d, *J* 8.0 Hz, NH), and 8.20–8.25 (2H, m, aryl 3-H).

A sample (4.47 g) was dissolved in dry DMF (10 ml) and the soln warmed to 75°. Dry dicyclohexylamine (2.60 ml, 13.05 mmol) were added and the solution stirred for 5 min. 4-Nitrobenzylbromide (3.41 g, 15.8 mmol) in dry DMF (11.3 ml) was added, the suspension stirred for 120 s, then cooled to 0° immediately. Ethyl acetate (100 ml) was added, the suspension filtered, the filtrate washed with water (3 × 150 ml), dried, filtered, and evaporated. Purification by chromatograpy on flash silica "H" [(60 g), eluant dichloromethane:ethyl acetate (1:0—0:1)] and recrystallisation from dichloromethane and light petroleum gave the title compound (**9**) (3.245 g, 42% from L - α - aminoadipic acid); TLC (ethyl acetate) R_f 0.40; m.p. 109–9.5°; $[\alpha]_D^{20}$ −1.8° (c 1.04, $CHCl_3$); ν_{max} (Nujol) 3300 m (N–H), 3080 m, 1745 s (ester C=O), 1695 s (CO$_2$H), 1612 m, 1550 s, 1520 s (Ar-NO$_2$), 1410 m, 1347 m (Ar-NO$_2$), 1330 m, 1248 m, 1220 m, 1070 s, 973 m, 858 m, 850 s, 747 m, and 697 m cm^{-1}; λ_{max} (CH_3CN) 268 nm (ε 19,500); δH(C^2H_3CN) 1.61–2.00 (4H, m, 3,4-H), 2.317 (2H, t, *J* 7.2 Hz, 5-H), 4.20–4.31 (1H, m, 2-H), 5.16–5.30 (4H, m, CH$_2$Ar), 6.239 (1H, d, *J* 7.9 Hz, NH), 7.53–7.58 (4H, m, aryl 2-H), and 8.16–8.20 (4H, m, aryl 3-H); δC(C^2HCl_3) 20.27 (t, 4C), 31.35, 32.94 (2 × t, 3, 5C), 53.67 (d, 2C), 65.41, 65.62 (2 × t, CH$_2$Ar), 123.58, 123.70 (2 × d, aryl 3C), 127.92, 128.37 (2 × d, aryl 2C), 142.24, 143.53 (2 × s, aryl 1C), 147.48; 147.69 (2 × s, aryl 4C), 155.54 (s, NHCO$_2$), 171.71 (s, CO$_2$CH$_2$), and 178.36 (s, CO$_2$H; *m/e* (FD) 476 (MH^{+}); Found: C, 53.14; H, 4.53; N, 8.71. $C_{21}H_{21}N_3O_{10}$ requires C, 53.05; H, 4.45; N, 8.84%.

Preparation of 4-*nitrobenzyl* (2S) - 5 - {(2R, 3R) - 2 - *acetylthio* - 1 - [(1R) - 2 - *methyl* - 1 - (4 - *nitrobenzyl-oxycarbonyl*)*propyl*] - 4 - *oxoazetidin* - 3 - *ylcarbamoyl*} - 2 - (4 - *nitrobenzyloxycarbonylamino*)*pentanoate* (**11**)

To a soln of the mercury salt (**8**) (2.68 g, 4.29 mmol) and triethylamine (0.60 ml, 4.32 mmol) in dichloromethane (100 ml) was added EEDQ (1.06 g, 4.28 mmol) and the diprotected aminoacid (**9**) (2.04 g, 4.29 mmol). The solution was stirred for 24 h, treated with pyridine (1.20 ml) and acetyl chloride (1.0 ml), and the soln was stirred for an additional 6 h, extracted into ethyl acetate (300 ml), washed with 2M hydrochloric acid (2 × 70 ml), saturated sodium hydrogencarbonate solution (100 ml), brine (2 × 100 ml), dried, filtered, and evaporated. Purification by column chromatography on flash silica "H" [(50 g), eluant (i) dichloromethane:diethyl ether (1:0–0:1), (ii) diethyl ether:ethyl acetate (1:0–0:1)] gave the title compound (**11**) (3.25 g, 89%); TLC (ethyl acetate) R_f 0.80; white foam m.p. 54°; $[\alpha]_D^{20}$ +5.7° (c 0.44, $CHCl_3$); ν_{max} (film) 3370 m (N–H), 3315 m (N–H), 3075 m, 2960 m, 2930 m, 1780–1700 brs (C=O), 1612 s, 1525 s (Ar–NO_2), 1450 m, 1345 s (Ar–NO_2), 1320 m, 1266 s, 1128 m, 1107 m, 1060 m, 1012 m, 952 m, 850 m, 734 s, 698 m, and 660 m cm^{-1}; λ_{max} (CH_3CN) 267.5 nm (ϵ 33,440); δH(C^2HCl_3), 0.936 (3H, d, J 6.7 Hz, CHMe$_2$), 1.058 (3H, d, J 6.7 Hz, CHMe$_2$), 1.74–2.00 (4H, m, 3′,4′-H), 2.15–2.50 (3H, m, 2′-H, CHMe$_2$) 2.306 (3H, s, CH_3CO), 3.954 (1H, d, J 9.0 Hz, 1-CH), 4.40–4.50 (1H, m, 5′-H), 5.20–5.35 (6H, m, CO_2CH_2), 5.344 (1H, dd, J 4.8, 8.0 Hz, 3-H), 5.83 (1H, obs, NH), 5.837 (1H, d, J 4.8 Hz, 2-H), 6.720 (1H, d, J 8.0 Hz, NH), 7.40–7.60 (6H, m, aryl 2-H), and 8.15–8.30 (6H, m, aryl 3-H); δC(C^2HCl_3) 19.38, 19.73 (2 × q, CHMe$_2$), 20.93 (t, 3′C), 29.27 (d, CHMe$_2$), 31.02 (q, CH_3CO), 31.44, 34.68 (2 × t, 2′,4′C), 53.55 (d, 5′C), 59.99, 62.70, 64.75 (3 × d, 3, 2C, 1-CH); 65.52, 65.64, 65.68 (3 × t, CO_2CH_2), 123.67, 123.81 (3 × d, one obs., aryl 3C), 128.00, 128.40, 128.60 (3 × d, aryl 2C), 142.18, 142.30, 143.58 (3 × s, aryl 1C), 147.77, 147.81 (3 × s, one obs., aryl 4C), 155.69 (s, NHCO$_2$), 165.83, 168.68, 171.67, 172.59 (4 × s, NHCO, CO$_2$), and 193.43 (s, CH_2CO); *m/e* (FD) 853 (MH^{+}), 852 (M^{+}), 808 (MH^{+}–CH_2CHO). Found: C, 53.32; H, 4.74; N, 9.66; S, 3.91. $C_{38}H_{40}N_6O_{16}S$ requires C, 53.52; H, 4.73; N, 9.85; S, 3.76%.

Preparation of di - {4 - nitrobenzyl (2S) - 5 - {(2R,3R) - 2-mercapto - 1 - [(1R) - 2 - methyl - 1 - (4 - nitrobenzyloxycarbonyl)propyl] - 4 - oxoazetidin - 3 - ylcarbamoyl} - 2 - (4 - nitrobenzyloxycarbonylamino)pentanoate}disulphide (**12**)

To a soln of the mercury salt (**8**) (1.26 g, 2.02 mmol) and triethylamine (282 μl, 2.03 mmol) in dichloromethane (50 ml) was added EEDQ (0.50 g, 2.02 mmol) and the diprotected aminoacid (**9**) (962 mg, 2.02 mmol). The solution was stirred for 24 h. A soln of iodine (256 mg, 1.01 mmol) in dry THF (3.60 ml) was added dropwise over 5 min. Evaporation and direct purification by chromatography on flash silica "H" [2 × 50 g, eluant (i) dichloromethane:ethyl acetate (1:1), (ii) ethyl acetate] gave the title compound (**12**) (1.09 g, 66%); TLC (ethyl acetate) R_f 0.70; m.p. 70°; $[\alpha]_D^{20}$ −56.5° (c 0.81, $CHCl_3$); ν_{max} ($CHCl_3$) 1775 m (β-lactam C=O), 1738 m (ester C=O), 1527 s (Ar–NO_2), 1476 m, 1424 m, 1351 s (Ar–NO_2), 931 m, and 850 m cm^{-1}; λ_{max} (CH_3CN) 267 nm (ϵ 65,700); δH/2(C^2H_3CN) 0.897 (3H, d, J 6.6 Hz, CHMe$_2$), 1.015 (3H, d, J 6.6 Hz, CHMe$_2$), 1.65–2.00 (4H, m, 3′,4′H), 2.295 (2H, t, J 6.8 Hz, 2′-H), 2.35–2.50 (1H, m, CHMe$_2$), 4.039 (1H, d, J 9.9 Hz, 1-CH), 4.20–4.35 (1H, m, 5′-H), 5.00–5.35 (8H, m, CH_2Ar, 3,2-H), 6.376 (1H, d, J 7.7 Hz, NH), 7.10 (1H, m, NH), 7.50–7.59 (6H, m, aryl 2-H), and 8.13–8.19 (6H, m, aryl 3-H); δC/2(C^2H_3CN) 19.51, 19.90 (2 × q, CHMe$_2$), 22.30 (t, 3′-C), 30.49 (d, CHMe$_2$), 31.55, 35.48 (2 × t, 2′,4′C), 55.19 (d, 5′C), 62.14, 62.96 (2 × d, 1-CH, 3C), 65.91, 66.22, 66.48 (3 × t, CH_2Ar), 78.03 (d, 2C), 124.50, 124.53, 124.60 (3 × d, aryl 3C), 128.80, 129.22, 129.66 (3 × d, aryl 2C), 143.94, 144.43, 145.64 (3 × s, aryl 1C), 148.43, 148.58, 148.71 (3 × s, aryl 4C), 156.98 (s, NHCO$_2$), 166.58, 169.91, 172.98, and 174.22 (4 × s, NHCO, CO$_2$); *m/e* (FD) 809/808 ($M^{+}/2+H$, $M^{+}/2$). Found: C, 53.52; H, 4.64; N, 10.22; S, 3.84. $C_{72}H_{74}N_{12}O_{28}S_2$ requires C, 53.40; H, 4.60; N, 10.38; S, 3.96%.

Preparation of di - {1 - [(1R) - carboxy - 2 - methylpropyl]-(3R) -] - (5S) - 5 - amino - 5 - carboxypentanamido] - (4R)-mercapto azetidin - 2 - one}disulphide (**13**)

The protected β-lactam disulphide (**12**) (201 mg, 0.28 mmol) was dissolved in THF (20 ml) and 1M acetic acid (20 ml). Mercury (II) acetate (100 mg) and 10% palladium on carbon (400 mg) were added. The mixture was hydrogenated for 100 min filtered through Celite 543, THF evaporated off and the residue extracted with ethylacetate (2 × 50 ml). The aqueous layer was freeze dried and purified by ion-exchange on Dowex 1 × 8 400 acetate form resin [15 × 1.5 cm column, load in water, wash 0.75 M acetic acid, elute with 1.0 M acetic acid (500 ml)] and by gel filtration on sephadex G-10 (20 × 1.5 cm column, load and elute with 0.1 M acetic acid] to give the title compound (**13**) (40 mg, 45%); ninhydrin active material which moves 15.0 cm towards the anode upon paper electrophoresis (pH 4.5 buffer, 4.0 kV, 40 min); ν_{max} (Nujol) 3200 br (O–H, N–H), 1750 s (β-lactam C=O), 1640 s, (C=O, amino acid), 1250 m, 1160 m, and 1075 m cm^{-1}; ν_{max} (^2H_2O, CaF_2 cells) 1740 s (β-lactam C=O) cm^{-1}; δH/2(^2H_2O) 0.948 (3H, d, J 6.7 Hz, CHMe$_2$), 1.005 (3H, d, J 6.7 Hz, CHMe$_2$), 1.4–1.9 (4H, m, CH_2 CH_2CH_2CO), 2.1–2.4 (3H, m, CHMe$_2$ and CH_2CO), 3.806 (1H, t, J 6.1 Hz, $\overset{+}{N}H_3CHCO_2$), 3.858 (1H, d, J 10.1 Hz, CHCHMe$_2$), 5.102 (1H, d, J 4.5 Hz, β-lactam ring-H), and 5.163 (1H, d, J 4.5 Hz, β-lactam ring H), δC/2(^2H_2O, dioxan = 67.04 ppm) 19.58, 19.83 (2 × q, CHMe), 21.81 (t, $CH_2CH_2CH_2$), 29.97 (d, CHMe$_2$), 30.81, 35.63 (2 × t, $CH_2CH_2CH_2$), 55.15 (d, $\overset{+}{N}H_3CHCO_2^-$), 60.94, 66.20 (2 × d, 3C, 1-CH), 77.76 (d, 4C), and 169.21, 175.05, 177.49 (4 × s, one obscured, C=O).

A sample of (**13**) (1.4 mg) was oxidized with 3% performic acid in formic acid (0.25 ml) at 0° for 5 h. Water (1 ml) was added and the mixture freeze dried. 6 M hydrochloric acid (10 ml) was added, the soln was refluxed for 24 h, and evaporated. Aminoacid analysis indicated a total of 7.2 nmol of cysteic acid, equivalent to less than 0.2 mol% initial cysteinyl impurity.

A sample of (**13**) (2 mgs) was derivatised[8] to the bis - N - ethoxycarbonyl dimethyl ester (**14**), *m/e* (NH_3Cl) 460 ($M^{+}/2$), 459 ($M^{+}/2$-H), 230 [EtO$_2$CNH·C(CO$_2$Me)(CH$_2$)$_3$CO$^+$, base]; (FD) 920 (M^{+}), 889 (M^{+}–OMe), 459 ($M^{+}/2$-H), [found MH^{+} (weak) 921.362, ($C_{38}H_{61}N_6O_{16}S_2$ requires 921.359), and $M^{+}/2$-H (base) 459.168 ($C_{19}H_{29}N_3O_8S$ requires 459.166)].

Preparation of dithiothreitol adduct (**15**)

The β-lactam disulphide (**13**) (5.0 mg, 7×10^{-3} mmol) was treated with a solution of dithiothreitol (10.7 mg, 7×10^{-2} mmol) in 50 mM pH 7.0 phosphate buffer (0.50 ml) and the mixture stood for 5 min. Purification by ion-exchange on Dowex 1 × 8 400 acetate form resin (10 × 2.0 cm column, eluant 0.50 M acetic acid) gave upon freeze drying, the dithiothreitol containing peptide (**15**) (5.0 mg, 70%), single ninhydrin active spot which moves 8.5 cm towards the anode upon paper electrophoresis (pH 4.5 buffer, 4.0 kV, 45 min); δH(^2H_2O) 0.99–1.14 (6H, m, CHMe$_2$), 1.80–2.10 (4H, m, $CH_2CH_2CH_2$CO), 2.20–2.30 (1H, m, CHMe$_2$), 2.54–2.64 (2H, m, CH_2CO), 2.80–2.95 and 3.00–3.15 (2 × 2H, 2 × m, 2 × CH_2S), 3.80–3.90 and 4.00–4.10 (2H, 2 × m, CHOH), 3.86 (1H, t, J 7 Hz, $\overset{+}{N}H_3CHCO_2^-$), 4.23 (1H, d, J 8 Hz, CHCHMe$_2$), and 4.69 (1H, d, J 7 Hz, CHCHS, ν_{max} (Nujol) 3300 m br (O–H), and 1640 m br (CO_2^-).

The β-lactam disulphide (**13**) (1.5 mg) and DTT (1.5 mg) were dissolved in 50 mm pH 7.5 phosphate buffer (2 ml) and the solution stirred for 35 min. Derivatisation gave the N,S,S-triethoxycarbonyl dimethyl ester derivative (**16**) *m/e* (FD) MH^{+} found 760.2461; $C_{29}H_{50}N_3O_{14}S_3$ requires 760.2454.

Spectroscopic study of the reaction of the β-lactam disulphide (**13**) *with dithiothreitol*

(a) *By infra-rad spectroscopy.* The β-lactam disulphide (**13**) (0.80 mg, 1.1 μmol) was dissolved in deuteriated 1 M, pH 6.90 phosphate buffer, ν_{max} (CaF_2 cells) 1740 s (β-lactam

C=O), 1600 s (carboxylate C=O). A soln of DTT (1.6 mg, 10.4 μmol) in ^2H_2O was added, the solution quickly mixed for 30 s and the IR spectrum over 1800–1500 cm^{-1} immediately recorded (time for scan = 27 s). No β-lactam carbonyl absorption could be detected, ν_{max} 1600 s (carboxylate C=O) cm^{-1}.

(b) *By* ^1H *NMR spectroscopy* (300 MHz). The β-lactam disulphide (**13**) (2.0 mg) was dissolved in deuteriated 1 M phosphate buffer (pH 7.10 or 6.90). A soln of DTT (3.0 mg) in ^2H_2O was added and the NMR spectrum immediately recorded. Within the time for mixing and scanning (*ca* 130 s) there were no β-lactam resonances.

Preparation of the N,S-*diethoxycarbonyl dimethyl ester of* L,L,D - *ACV-thiol* (**17**)[8]

The L,L,D-ACV-thiol (**2**) (10.50 mg, 2.9×10^{-2} mmol) and DTT (4.5 mg) were dissolved in water (1 ml) and saturated sodium hydrogencarbonate (100 μl). The mixture was stirred for 20 min, treated with diethylpyrocarbonate (100 μl) and stirred for 50 min. The mixture was extracted into water (10 ml) and dichloromethane (50 ml), the aqueous layer was separated and re-extracted with dichloromethane (2 × 50 ml), acidified to pH 2 (2M hydrochloric acid) and then extracted with ethyl acetate (3 × 50 ml). The ethyl acetate layers were combined, dried, filtered, reacted with excesss diazomethane in ether at 0° for 15 min, then evaporated. Double recrystallisation from dichloromethane and petroleum gave the title compound (**17**) (8.0 mg, 51%); foam; TLC [ethylacetate:dichloromethane (1:2)] R_f 0.25; ν_{max} ($CHCl_3$) 1720 s (C=O), 1690 s (NHCO), 1500 m, and 1150 s (C–O) cm^{-1}; δH(C^2HCl_3) 0.907 (3H, d, *J* 6.9 Hz, CHMe$_2$), 0.956 (3H, d, *J* 6.9 Hz, CHMe$_2$), 1.242 (3H, t, *J* 7.2 Hz, CH_2Me), 1.324 (3H, t, *J* 7.2 Hz, CH_2Me), 1.60–2.00 (4H, m, $CH_2CH_2CH_2CO$), 2.15–2.30 (3H, m, CHMe$_2$ and CH_2CO), 3.20–3.29 (2H, m, CH_2S), 3.743 (3H, s, CO_2Me), 3.752 (3H, s, CO_2Me), 4.112 (2H, q, *J* 7.2 Hz, CH_2Me), 4.30–4.40 (1H, obs, CH·$(CH_2)_3$), 4.313 (2H, q, *J* 7.2 Hz, CH_2Me), 4.463 (1H, dd, *J* 4.9, 8.6 Hz, ·CHCHMe$_2$), 4.65–4.80 (1H, m, CHCH$_2$S), 5.526 (1H, d, *J* 7.2 Hz, NH), 6.629 (1H, d, *J* 6.9 Hz, NH), and 7.043 (1H, d, *J* 8.7 Hz, NH); *m/e*[15] (E.I.) 536 ($MH^{+\cdot}$, 5%), 430 (5%), 405 (10%), 377 (45%), 331 (15%), 273 (30%), 230 (100%), 148 (55%), 98 (50%), and 72 (40%); (NH_3CI) 536 ($MH^{+\cdot}$, 65%), 480 (5%), 430 (10%), 405 (30%), 377 (55%), 331 (15%), 273 (30%), 230 (100%), 148 (45%), 98 (55%), 72 (40%), and 55 (30%); (NH_3DCI) 536 ($MH^{+\cdot}$, 100%): [(EI). Found: ($MH^{+\cdot}$) 536.2276, $C_{22}H_{38}N_3O_{10}S$ requires 536.2276, (M^+) 535.2202, $C_{22}H_{37}N_3O_{10}S$ requires 535.2200).

Reaction of the β-lactam disulphide (**13**) *with sodium borohydride*

The β-lactam disulphide (**13**) (5.0 mg, 6.9 μmol), was dissolved in water (1 ml) containing a trace of selenocysteine. Sodium borohydride (20 mg) was added, the mixture stirred for 20 min, treated with DTT (5.0 mg, 32.5 μmol) and stirred for 20 min. Purification by preparative paper electrophoresis (pH 4.5 buffer, 4.0 kV, 60 min), gave upon extraction of the ninhydrin active material at 17.5–25.0 cm towards the anode with water (5 × 25 ml), the crude L,L,D–ACV–thiol (**2**). Concentration, followed by oxidation at pH 8 (aqueous NH_4OH) with oxygen for 1 h, gave upon repeated preparative eletrophoresis, the L,L,D–ACV–disulphide dimer of the thiol (**2**) identical by ^1H NMR and electrophoresis to an authentic sample of the L,L,D–tripeptide disulphide.

The reaction was repeated with the β-lactam disulphide (**13**) (4.3 mg) and sodium borohydride (10.0 mg) to yield the crude thiol (**2**). This was directly derivatised[8] to the N,S-diethoxycarbonyl dimethyl ester derivative (**17**), identical by ^1H NMR, *m/e*, and TLC to an authentic sample.

Reaction of the β-lactam disulphide (**13**) *with sodium borodeuteride*

The β-lactam disulphide (**13**) (9.0 mg, 12.5 μmol) and sodium borodeutide (12.0 mg) in deuterium oxide (3.0 ml) were stirred at 20° for 6 d. Oxidation at pH 8 ($N^2H_4O^2H$ solution) followed by purification by ion-exchange on Dowex 1X8-400 acetate form resin [10 × 1.5 cm column, load and wash in water, elute with 1 M acetic acid (500 ml)] and gel-filtration on Sephadex G-15 (0.1 M acetic acid as eluant) gave the disulphide of thiol (**18**) (7.2 mg, 80%), identical to the disulphide of thiol (**2**) by electrophoresis at pH 4.5, and identical by ^1H 300 MHz spectroscopy except that the cysteinyl-3 protons appeared as two pairs of doublets of relative ratio 45:55, δH(^2H_2O, ^2H irradiated, $CH_3CO_2{}^2H$ = 1.91 ppm) 2.99 (1H, d, *J* 5.2 Hz, cysteinyl-3-H), 2.81 (1H, d, *J* 8.7 Hz, cysteinyl-3-H). Irradiation at δ4.57 (cysteinyl-2-H) caused collapse of the doublets to two singlets. The peptide was shown to be completely converted into an isopenicillin N derivative with an active strain from *C. Acremonium* by both bioassay and direct ^1H NMR examination.[16]

The reaction was repeated using the β-lactam disulphide (**13**) (4.3 mg) and sodium borodeuteride (10.0 mg) to yield the crude deuteriated thiol (**18**). This was directly derivatised[8] to its N,S-diethoxycarbonyl dimethyl ester derivative (**19**), identical by TLC and ^1H NMR to an authentic sample (**17**) except that the cysteinyl 3-protons, δH 3.26, appeared as two overlapping doublets and the cysteinyl C-2 protons, δH 4.70 appeared as a double-doublet. Irradiation at δH 4.70 caused collapse of the cysteinyl 3-proton to two singlets, δH(C^2HCl_3) 3.25 (1H, s) and 3.28 (1H, s), relative ratio 45:55, *m/e* (NH_3CI) 537 ($MH^{+\cdot}$, 60%), 491, 431, 406, 378, 308, 230 (base), 170, 98, and 72: relative intensities for (**19**) [$MH^{+\cdot}$ *m/e* 536: 537: 538: 539: 540 = 0: 100: 28: 9: 2], for (**17**) [$MH^{+\cdot}$ *m/e* 535: 536: 537: 538: 539 = 0: 100: 29: 9: 2].

Preparation of 4-*methoxybenzyl* (2S,4S,5R,6R) - 3,3 - *dimethyl* - 7 - *oxo* - 6 - *phenoxyacetamido* - 1 - *aza* - 4 - *thiabicyclo*[3.2.0]*heptane* - 2 - *carboxylate* 4-*oxide* (**21**)

The potassium salt of penicillin V (2.72 g, 7.01 mmol), 4-methoxybenzyl chloride (1.10 g, 7.02 mmol), and sodium iodide (20 mg) were dissolved in dry DMF (20 ml) and the solution stirred for 24 h. The soln was extracted into dichloromethane (250 ml), washed with water (4 × 200 ml), dried, filtered, and evaporated. TLC [ethylacetate: dichloromethane (1:9)] indicated the desired ester, R_f 0.70 (*ca* 95%). The product was dissolved in dry dichloromethane (50 ml), MCPBA (1.80 g, 85%, 8.90 mmol) added, and the soln was stirred for 50 min. The soln was extracted into dichloromethane (200 ml), washed with saturated aq sodium hydrogencarbonate solution (200 ml), dried, filtered, and evaporated. Purification by chromatography on flash silica "H" [(50 g), eluant dichloromethane:ethylacetate (1:0–4:1)] gave the title sulphoxide[17] (**21**) (1.325 g, 39%); TLC [ethyl acetate:dichloromethane (1:9)] R_f 0.30; m.p. 124–5°; $[\alpha]_D^{20}$ +163° (c 0.45, $CHCl_3$); ν_{max} ($CHCl_3$) 3020 m (aryl-H), 1805 s (β-lactam C=O), 1755 m (ester C=O), 1695 s (amide C=O), 1515 s, 1495 m, 1445 m, 1305 m, 1290 m, 1175 s, 1160 m, 1065 m, and 1035 m cm^{-1}; λ_{max} (CH_3CN) 221.5 nm (ε 22,000); 268 nm (ε 2,450) 273.5 nm (ε 2,400); δH(C^2HCl_3) 1.060 (3H, s, 3-Me), 1.667 (3H, s, 3-Me), 3.829 (3H, s, OMe), 4.542 (2H, s, $PhOCH_2$), 4.679 (1H, s, 2-H), 5.020 (1H, d, *J* 4.6 Hz, 5-H), 5.113 and 5.267 (2H, AB q, *J* 11.7 Hz, CO_2CH_2), 6.104 (1H, dd, *J* 4.6, 10.6 Hz, 6-H), 6.89–7.04 (5H, m, aryl-H), 7.27–7.35 (4H, m, aryl-H) and 8.26 (1H, d, *J* 10.6 Hz, NH); δC(C^2HCl_3) 18.14, 19.19 (2 × q, 3-Me), 55.04, 66.07 (2 × d, 2,6C), 55.23 (q, MeO), 67.57 (t, CH_2Ar), 75.11 (s, 3C), 76.28 (d, 5C), 113.87, 114.62 (2 × d, aryl 3C, phenyl 2C), 121.91 (d, phenyl 4C), 126.54 (s, aryl 1C), 129.45, 130.53 (2 × d, phenyl 3C, aryl 2C), 156.77 (s, phenyl 1C), 159.82 (s, aryl 4C), 167.49, 167.97, and 173.03 (3 × s, C=O); *m/e* (F.D.) 487 ($MH^{+\cdot}$), 486 (M^+), 470 ($M–O^{+\cdot}$), 468 ($M–H_2O^{+\cdot}$). Found: C, 59.38; H, 5.38; N, 5.72. $C_{24}H_{26}N_2O_7S$ requires C, 59.25; H, 5.39; N, 5.76%.

Preparation of 4-*methoxybenzyl* (2R) - 3 - *methyl* - 2 - [(1R,5R) - 7 - *oxo* - 3 - *phenoxymethyl* - 4 - *thia* - 2,6 - *diazabicyclo*[3.2.0]*hept* - 2 - *en* - 6 - *yl*]*but* - 3 - *enoate* (**22**)

The sulphoxide (**21**) (1.22 g, 2.51 mmol) and freshly distilled trimethylphosphite (320 μl, 2.71 mmol) were dissolved in dry toluene (100 ml) and the solution slowly refluxed into a Dean and Stark apparatus over 270 min. The solvent was evaporated and purification by chromatography on flash silica "H" [(55 g), eluant dichloromethane:ethyl acetate (1:0–9:1)] and recrystallisation from dichloromethane and light petroleum gave the title compound (**22**) (1.09 g, 96%); TLC [ethylacetate:dichloromethane (1:9)] R_f 0.75; m.p. 95–95.5°; $[\alpha]_D^{20}$ −118° (c 0.98, $CHCl_3$); ν_{max} ($CHCl_3$) 1770 s (β-lactam C=O), 1740 s, (ester C=O), 1175 m, and 1155 m cm^{-1}; λ_{max} (CH_3CN) 223 nm (ε 18,300), 268.5 nm (ε 2,700), 274 nm (ε 2,550); δH(C^2HCl_3) 1.728 (3H, s, vinyl-Me), 3.817 (3H, s, MeO), 4.847 (2H, bs CH_2C=), 4.880 and 4.967 (2H, d AB q, *J* 1.3, 14.2 Hz, $PhOCH_2$), 5.045 (1H, d, *J* 1.3 Hz, 6-CH), 5.097 and 5.150 (2H, AB q, *J* 11.9 Hz, CO_2CH_2), 5.916 (1H, d, *J* 4.0 Hz, 1-H), 6.011 (1H, bd *J* 4.0 Hz, 5-H), 6.87–7.03 (5H, m, aryl-H), and 7.26–7.33 (4H, m, aryl-H); δC(C^2HCl_3) 21.56 (q, vinyl-Me), 55.25 (q, MeO), 58.82, 67.09, 92.10 (3 × d, 6-C, 1C, 5C), 67.45, 67.63 (2 × t, CH_2O), 114.02, 114.68 (2 × d, aryl 3C, phenyl 2C), 117.47 (t, CH_2=), 121.90 (d, phenyl 4C), 126.87 (s, aryl 1C), 129.60, 130.35 (2 × d, phenyl 3C, aryl 2C), 137.51 (s, MeC=), 157.57 (s, phenyl 1C), 159.91 (s, aryl 4C), 165.03, 168.72, and 173.27 (3 × s, 3C, 7C, CO_2); *m/e* (NH_3CI) 453 (MH^{+}), 121 (base), and 98. Found: C, 63.53; H, 5.27; N, 6.33; S, 7.20. $C_{24}H_{24}N_2O_5S$ requires C, 63.70; H, 5.35; N, 6.19; S, 7.08%.

Preparation of 4-*methoxybenzyl* (2R), 3 - *methyl* - 2 - [(1R,5R) - 7 - *oxo* - 3 - *phenoxymethyl* - 4 - *thia* - 2,6 - *diazabicyclo*[3.2.0]*hept* - 2 - *en* - 6 - *yl*]*butanoate* (**23**)

Isopropenyl ester (**22**) (1.04 g, 2.29 mmol) and Wilkinson's catalyst (0.15 g) were dissolved in dry benzene (100 ml) and the soln hydrogenated for 1 d. Further catalyst (0.15 g) was added and the solution rehydrogenated for 1 d. Evaporation and purification by chromatography on flash silica "H" [(80 g), eluant dichloromethane:ethyl acetate (1:0–9:1)] gave (**22**) contaminated by *ca* 10% of starting material (**21**). The solid was redissolved in benzene (100 ml), treated with Wilkinson's catalyst (150 mg) and hydrogenated for 1 d. Further catalyst (100 mg) was added and the solution re-hydrogenated for 2 d. Evaporation, purification by chromatography on flash silica "H" (as above) and recrystallisation for dichloromethane and light petroleum gave the title compound (**23**) (581 mg, 56%); TLC [ethyl acetate:dichloromethane (1:9)] R_f 0.70; m.p. 94–5°; $[\alpha]_D^{20}$ −2.04 (c 0.25, $CHCl_3$); ν_{max} ($CHCl_3$) 1770 s (β-lactam C=O), 1735 m (ester C=O), 1515 m, 1495 m, 1335 m, and 1155 m cm^{-1}; λ_{max} (CH_3CN) 224 nm (ε 16,000), 268 nm (ε 2,400), 274 nm (ε 2,300); δH(C^2HCl_3) 0.864 (3H, d, *J* 6.9 Hz, $CHMe_2$), 0.908 (3H, d, *J* 6.9 Hz, $CHMe_2$), 2.20–2.30 (1H, m, $CHMe_2$), 3.816 (3H, s, MeO), 4.141 (1H, d, *J* 8.8 Hz, 6-CH), 4.863 and 4.970 (2H, d AB q, *J* 1.3, 15.6 Hz, $PhOCH_2$), 5.057 and 5.123 (2H, AB q, *J* 11.7 Hz, CO_2CH_2), 5.818 (1H, d, *J* 4.0 Hz, 1-H), 5.957 (1H, d, *J* 4.0 Hz, 5-H), 6.88–7.03 (5H, m, aryl-H), and 7.27–7.33 (4H, m, aryl-H), δC(C^2HCl_3) 19.07, 19.55 (2 × q, CMe_2), 30.09 (d, $CHMe_2$), 55.25 (q, MeO), 61.25, 67.83, 92.61 (3 × d, 1C, 5C, 6-CH), 67.06, 67.63 (2 × t, CH_2Ar), 114.05, 114.71 (2 × d, aryl 3C, phenyl 2C), 121.93 (d, phenyl 4C), 127.05 (s, aryl 1C), 129.60, 130.35 (2 × d, phenyl 3C, aryl 2C), 157.60 (s, phenyl 1C), 159.91 (s, aryl 4C), 165.48, 169.34, and 172.10 (3 × s, 3C, 7C, CO_2); *m/e* (NH_3CI) 455 (MH^{+}), 263, 192, 191, 121 (base), and 98. Found: C, 63.25; H, 5.84; N, 6.19; S, 7.06. $C_{24}H_{26}N_2O_5S$ requires C, 63.42; H, 5.76; N, 6.16; S, 7.05%.

Preparation of (2R,3R) - 2 - *chloromercuriothio*) - 1 - [(1R)-2 - *methyl* - 1 - (4 - *methoxybenzyloxycarbonyl*)*propyl*] - 4-*oxoazetidin* - 3 - *yl ammonium chloride* (**24**)

Thiazoline (**23**) (585 mg, 1.29 mmol), 2,2 - dimethyl - 1,3-propandiol (0.81 g, 7.79 mmol) and mercury (II) chloride (350 mg, 1.29 mmol) were dissolved in dry dichloromethane (50 ml) and the soln stirred for 18 h, evaporated, and the residue was recrystallised from dichloromethane and diethyl ether to give the title compound (**24**) (685 mg, 87%); m.p. 115° (dec); ν_{max} (Nujol) 3100 m, 1760 s, (β-lactam C=O), 1615 m, 1515 m, 1250 m, and 1175 m cm^{-1}; δH($C^2H_3SOC^2H_3$) 0.838 (3H, d, *J* 6.6 Hz, CHMe), 0.995 (3H, d, *J* 6.6 Hz, CHMe), 2.45–2.55 (1H, m, $CHMe_2$), 3.750 (3H, s, MeO), 3.930 (1H, d, *J* 8.8 Hz, 1-CH), 4.618 (1H, d, *J* 4.7 Hz, 2-H), 5.080 and 5.129 (2H, AB q, *J* 12.0 Hz CO_2CH_2), 5.659 (1H, d, *J* 4.7 Hz, 3-H), 6.92–6.95 (2H, m, aryl 3-H), and 7.34–7.37 (2H, m, aryl 2-H). Found: C, 31.15; H, 3.74; N, 4.74; S, 5.34%. $C_{16}Cl_2H_{22}HgN_2O_4S$ requires C, 31.51; H, 3.63; N, 4.59; S, 5.26%), decomposes in solution (by ^1H NMR in C^2HCl_3 or $C^2H_2SOC^2H_3$).

Preparation of 1 - (4 - *methoxybenzyl*) *hydrogen* (2S) - 2 - (4-*methoxybenzyloxycarbonylamino*)*hexandioate* (**25**)

The dicyclohexylamine salt of (**25**)[12] (577 mg), m.p. 108–12° (lit[12] 108–13°) was dissolved in ethyl acetate (50 ml) and pH 3.0 phosphate buffer (30 ml). The solns were mixed for 2 min, the aqueous phase separated and re-extracted with ethyl acetate (2 × 50 ml). The organic layers were combined, dried, filtered, and evaporated. Purification by chromatography on flash silica "H" [(40 g), eluant ethyl acetate] gave the title compound (**25**) (307 mg); TLC (ethyl acetate) R_f 0.80; δH($C^2H_3COC^2H_3$) 1.60–2.00 (4H, m, 3,4-H) 2.309 (2H, t, *J* 7.1 Hz, 5-H), 3.793 (3H, s, MeO), 3.799 (3H, s, MeO), 4.15–4.25 (1H, m, 2-H), 4.95–5.15 (4H, m, CH_2Ar), 6.642 (1H, d, *J* 9.3 Hz, NH), 6.89–6.93 (4H, m, aryl 3-H), and 7.28–7.36 (4H, m, aryl 2-H).

Preparation of the β-*lactam disulphide* (**13**) *via hydrochloride salt* (**24**)

Diprotected L-α-aminoadipic acid (**25**) (46 mg, 0.10 mmol) was dissolved in dry dichloromethane (10 ml) and the hydrochloride salt (**24**) (61 mg, 0.10 mmol) was added. Triethylamine (14 μl, 0.10 mmol) and EEDQ (25 mg, 0.10 mmol) were added and the soln stirred for 24 h. A solution of iodine (12.6 mg, 0.05 mmol) in THF (2.1 ml) was added over 5 min, the solution evaporated and purified by PLC (1 × 20 × 20 × 0.1 cm plate, dichloromethane:ethyl acetate (1:1) × 2) to yield a fully 4-methoxybenzyl protected β-lactam disulphide (11 mg, 14%), TLC [ethyl-acetate:dichloromethane (1:1)] R_f 0.65. The sample was dissolved in benzene:anisole:trifluoroacetic acid (20:1:3) (5 ml), the soln stirred for 30 min, then evaporated. The residue was redissolved in toluene (2 × 5 ml) and re-evaporated. The residue was partitioned between ethyl acetate (20 ml) and water (20 ml), the aqueous phase re-extracted with ethyl acetate (20 ml), and freeze dried. Purification by preparative paper electrophoresis (pH 3.5 buffer, 4.0 kV, 1 h) gave upon extraction of the ninhydrin active band with water (3 × 25 ml) and evaporation, the β-lactam disulphide (**13**) (4.3 mg, 83%), identical to an authentic sample by ^1H NMR and electrophoresis.

Preparation of 1 - [(1R) - *carboxy* - 2 - *methylpropyl*] - (3R)-[(5S) - 5 - *amino* - 5 - *carboxypentanamido*] - (4R) - *chloromercuriothioazetidin* - 2 - *one* (**27**)

The amine hydrochloride salt (**24**) (162 mg, 0.27 mmol) was dissolved in dry dichloromethane (5 ml) at 25°. Triethylamine (37 μl, 0.27 mmol), EEDQ (66 mg, 0.27 mmol) and protected amino-acid (**25**) (120 mg, 0.27 mmol) were added and the soln stirred for 30 h, then evaporated. Benzene:anisole:trifluoroacetic acid (20:1:3) (5 ml) was added and the solution stirred for 30 min, then evaporated. The residue was redissolved in toluene (3 × 5 ml) and re-evaporated. The solid was washed with dichloromethane (20 ml), extracted into water (30 ml) and dichloromethane (30 ml), the aqueous layer separated and freeze dried to yield crude (**27**) (109 mg). Purification of a sample (32 mg) by gel filtration on sephadex G-10 (16 × 2 cm column, load and elute with water) gave the title compound (**27**) (7.1 mg, 15%); as a foam which upon electrophoresis (pH 4.5 buffer, 4.0 kV, 40 min), moves 14.5 cm towards the anode; ν_{max}

(CaF_2 cells, ^2H_2O) 1735 s (β-lactam C=O), 1615 s and 1595 s (amide and carboxylate C=O); δH(^2H_2O, CH_3COCH_3 = 2.058 ppm) 0.770 (3H, d, *J* 6.4 Hz, CHMe), 0.866 (3H, d, *J* 6.4 Hz, CHMe), 1.5–1.9 (4H, m, $(CH_2)_2CH_2$), 2.2–2.4 (1H, m, $CHMe_2$), 2.300 (2H, t, *J* 7.1 Hz, CH_2CO), 3.519 (1H, d, *J* 10.6 Hz, $CHCHMe_2$), 3.579 (1H, t, *J* 6.2 Hz, $NH_3CHCO_2^-$), 5.034 (1H, d, *J* 4.4 Hz, β-lactam ring H), and 5.465 (1H, d, *J* 4.4 Hz, β-lactam ring H).

Oxidation of the thiomercury (II) species (**27**) *to the β-lactam disulphide* (**13**)

The thiomercury (II) species (**27**) (2.0 mg), δH (^2H_2O) 0.770 (3H, d, *J* 6.4 Hz, CHMe), 0.866 (3H, d, *J* 6.4 Hz, CHMe), 5.034 (1H, d, *J* 4.4 Hz, β-lactam ring-H), and 5.465 (1H, d, *J* 4.4 Hz, β-lactam ring-H) was dissolved in ^2H_2O and then oxidized with a dilute soln of iodine in aq potassum iodide. The soln was immediately neutralised with aq sodium thiosulphate soln, then filtered. NMR analysis indicated a smooth conversion (*ca* 80%) to the β-lactam disulphide (**13**), δH(^2H_2O) 0.948 (3H, d, *J* 6.7 Hz, $CHMe_2$), 1.005 (3H, d, *J* 6.7 Hz, $CHMe_2$), 5.102 (1H, d, *J* 4.5 Hz, β-lactam ring-H), and 5.163 (1H, d, *J* 4.5 Hz, β-lactam ring H) with some decomposed material, δH 0.80–0.95 (m, $CHMe_2$). Electrophoresis (pH 4.5 buffer, 4.0 kV, 45 min) indicated conversion of the species (**27**) (17.5 cm towards anode) to the β-lactam disulphide (**13**) (18.5 cm toward anode) (80%), identical to an authentic sample. An authentic sample (2.0 mg) of the β-lactam disulphide (**13**) was added to the crude product. NMR analysis indicated enhancement of the β-lactam disulphide (**13**) resonances.

In situ *preparation of* 1 - [(1R) - *carboxy* - 2 - *methylpropyl*- (3R) - [(5S) - 5 - *amino* - 5 - *carboxypentanamido*] - (4R) - *mercaptoazetidin* - 2 - *one* (**1**)

The thiol mercury compound (**27**) (2.0 mg) was dissolved in deuteriohydrochloric acid/deuterium oxide (pH 1.5) at 20° and the soln treated with hydrogen sulphide (g) for 30 s. The black precipitate was filtered off, and the NMR spectrum of (**1**) recorded 8–10 min after hydrogen sulphide treatment, δH(^2H_2O/^{2}HCl CH_3COCH_3 = 2.013 ppm) 0.790 (3H, d, *J* 6.6 Hz, CHMe), 0.860 (3H, d, *J* 6.6 Hz, CHMe), 1.4–1.9 (4H, m, $(CH_2)_2CH_2CO$), 2.2–2.4 (1H, m, $CHCHMe_2$), 2.307 (2H, t, *J* 7.2 Hz, CH_2CO), 3.736 (1H, d, *J* 9.2 Hz, $CHCHMe_2$), 3.850 (1H, t, *J* 6.2 Hz, $NH_3CHCO_2^-$), 4.987 (1H, d, *J* 4.6 Hz, β-lactam ring-H), and 5.140 (1H, d, *J* 4.6 Hz, β-lactam ring H). Under these conditions, (pH 1.5, 20°) the thiol slowly decomposed ($t_{1/2}$ *ca* 25 min). After 2 h, no β-lactam resonances could be detected by NMR. The $CHMe_2$ region, 0.75–0.95 ppm, contained many resonances, indicating several decomposition products.

Conversion of the thiol (**1**) *to the chloromercuriothio species* (**27**)

Treatment of the chloromercuriothio compound (**27**) (2.0 mg) with hydrogen sulphide at pH 1.5 (as before) gave a clean conversion to the β-lactam thiol (**1**), δH_{max}(^2H_2O/^{2}HCl/pH 1.5) 0.790 (3H, d, *J* 6.6 Hz, CHMe), 0.860 (3H, d, *J* 6.6 Hz, CHMe), 4.987 (1H, d, *J* 4.6 Hz, β-lactam ring-H), and 5.140 (1H, d, *J* 4.6 Hz, β-lactam ring-H) ppm. The soln was briefly degassed (under reduced pressure for 1 min), then treated with a soln of mercury (II) chloride in deuterium oxide. The soln was filtered to yield **27**), δH (^2H_2O, ^{2}HCl, $CH_3CO_2{}^2H$ = 2.010 ppm) 0.792 (3H, d, *J* 6.7 Hz, $CHMe_2$), 0.860 (3H, d, *J* 6.7 Hz, $CHMe_2$), 5.010 (1H, d, *J* 4.6 Hz, β-lactam ring H), and 5.488 (1H, d, *J* 4.6 Hz, β-lactam ring H), as the only β-lactam species. An authentic sample of (**27**) (2.0 mg) was added. NMR analysis indicated enhancement of the chloromercuriothio species (**27**) resonances.

Generation of the β-lactam-thiol (**1**) *at pH* 5-7

(a) The thiomercury species (**27**) (2.0 mg) was dissolved in deuterium oxide and treated with hydrogen sulphide for 30 s. The soln (pH 5) was filtered and direct NMR examination [4–5 min after thiol (**1**) generation] indicated thiol (**1**) (*ca* 20%), δH (^2H_2O, pH 5) 0.835 (3H, d, *J* 6.6 Hz $CHMe_2$), 5.003 (1H, d, *J* 4.6 Hz, β-lactam ring H), and 5.122 (1H, d, *J* 4.6 Hz, β-lactam ring H), and another species, (*ca* 80%), δH_{max}(^2H_2O, pH 5) 0.715 (3H, d, *J* 6.9 Hz, $CHMe_2$), and 0.753 (3H, d, *J* 6.9 Hz, $CHMe_2$). Re-examination by NMR [7–8 min after thiol (**1**) generation] indicated *no* β-lactam containing species.

(b) The thio-mercury species (**27**) (2.0 mg) was dissolved in deuteriated 1 M phosphate buffer (pH 7.15) and the soln treated with H_2S for 30 s, then filtered (pH 7.0). NMR examination [4–9 min after generation of (**1**), (the minimum time required to generate the thiol (**1**) and record its proton NMR spectrum at 300 MHz)] indicated *no* β-lactam containing species.

The thio-mercury species (**27**) (2.0 mg), ν_{max} (CaF_2, ^2H_2O) 1735 s (β-lactam C=O), 1615 and 1595 s (amide and carboxylate C=O) cm^{-1}, was dissolved in deuteriated pH 6.95 phosphate buffer, the solution treated with hydrogen sulphide (g), for 30 s., then filtered (pH *ca* 6.9). IR examination (CaF_2, deuteriated phosphate buffer, pH 6.95) [200–227 s after generation of the thiol (**1**)] indicated no β-lactam containing species, ν_{max} 1650 (CO_2^-) cm^{-1}.

Incubation of 1 - [(1R) - *carboxy* - 2 - *methylpropyl*] - (3R)- [(5S) - 5 - *amino* - 5 - *carboxypentanamido*] - (4R) - *mercaptoazetidin* - 2 - *one* (**1**)

Partially purified preparations of isopenicillin N synthetase from *Cephalosporium acremonium* C-91 and *Penicillium chrysogenum* SC6140 respectively were used in these experiments. The enzyme in *C. acremonium* was extracted by grinding the mycelium, suspended in 3(N-morpholino) propane sulphonic acid (MOPS) buffer, 50 mM, pH 7.2, with glass beads in a Dyno-Mill (Glen Creston, Stanmore, Middlesex, UK). Nucleic acids were precipitated with protamine sulphate and the synthetase then precipitated with $(NH_4)_2SO_4$ (80% saturation). Further purification was carried out by chromatography on DEAE Sephadex A-50.[13] The synthetase was precipitated from the active fractions with $(NH_4)_2SO_4$ and then dissolved in (MOPS) buffer (50 mM, pH 7.2). The enzyme from *P. chrysogenum* was extracted and purified similarly, but after chromatography on DEAE Sephadex A-50 was purified further by gel filtration through Sephadex G-100. Details of the production and purification of these synthetases are to be published.

The enzyme, in MOPS buffer pH 7.2 (366 μl, about 0.5 mg/protein/ml, was added to a solution containing L-ascorbic acid (8 μl, 50 mM), $FeSO_4$ (8 μl, 5 mM), DTT (8 μl, 100 mM) and the monocyclic β-lactam tripeptide dimer (**13**) (10 μl, 20 mM; corresponding to a final monomer concn of 1 mM) or the chloromercury derivative of the monomer (**27**) (10 μl, 20 mM). The solution (400 μl) in a test tube (10 cm × 1.4 cm internal diam) was aerated in a reciprocal shaker at 100 cycles/min in a water-bath at 27° for 60 min. The enzyme was then inactivated by the addition of acetone (930 μl). After centrifugation the supernatant was concentrated to 400 μl under a stream of air and its isopenicillin N content determined by microbiological assay with *Staphylococcus aureus* NCTC 6571 as the test organism.[18] In other experiments with the β-lactam tripeptide (**13**), DTT was omitted from the incubation mixture. In all cases the formation of isopenicillin N from δ - (L - aminoadipyl) - L - cysteinyl - D - valine (LLD-ACV) (**2**) (final concn 1 mM on reduction of the dimer *in situ* by DTT) was determined under similar conditions as a control.

To determine whether the reduction product of the monocyclic tripeptide dimer (**13**) or the chloromercury derivative of the monomer (**27**) acted as a strong inhibitor or inactivator of the synthetase from *C. acremonium*, each of these compounds (final concn. 1 mM) was incubated for 15 min at 27° with shaking in the reaction mixture (600 μl, containing DTT, as described above) and LLD-ACV (**2**)

(final concentration 0.4 mM) then added. Samples (200 μl) were removed after 5, 15 and 30 min for determination of the isopenicillin N formed. In parallel control experiments no tripeptides (13) or (27) were added.

In control experiments up to 60% of LLD-ACV (2) was converted to isopenicillin N in the presence of partly purified synthetases from *Cephalosporium acremonium* and *Penicillium chrysogenum*. A minimal conversion of about 3% was detectable by the bioassay used for the determination of isopenicillin N. In parallel experiments with the monocyclic tripeptide dimer (13) (in the presence and absence of DTT) and the chloromercury derivative of the monomer (27) in the presence of DTT no product with activity against the test organism (*Staphylococcus aureus*) was detected. Hence, there was no indication that these compounds and the products obtained from them on treatment with DTT could function as free intermediates in isopenicillin N biosynthesis.

Within the limit of accuracy of the assay (ca 10%) neither compound in the presence of DTT behaved as an inactivator or inhibitor of the synthetase from *C. Acremonium*.

Note added in proof. Some 8 months after the appearance of our first communication on this subject there appeared a note describing a similar synthesis of thiol (1).[19] These authors claimed that the reduction of the disulphide (13) to thiol (1) can be "accomplished either by treatment with dithiothreitol (DTT) in a pH 7 buffer or by treatment with Zn in DCl/D_2O". In our hands, at pH 7, the disulphide (13) was reduced with DTT to the DTT-adduct (15), without any detectable thiol (1) (monitored by IR, NMR). Also, we re-examined the reduction with zinc in DCl/D_2O of disulphide (13) and observed formation of a product (B), which appears to be the same as the reported[1] thiol (1) δH 5.6338 (d, J 3.8 Hz, 1H) at 5.9740 (d, J. 3.8 Hz, 1H). However this is certainly not the first formed product of this reaction as we noted when the disulphide (13) is treated with Zn (excess) in DCl/D_2O for 15 min. (20°) until approximately 50% reaction of (13) (δH D_2O/DCl 4.95 (1H, d, J 4 Hz)), 5.10 (1H, d, H 4 Hz) then an initial product (A) is seen (δH D_2O/DCl, 5.05 (1H, d, J 4 Hz)), 5.20 (1H, d, J 4 Hz) which on standing at 20° for 1 hr. is converted to (B). The formulation of these species in DCl/D_2O containing excess of Zn^{2+} is, in our view, uncertain. It follows that the claim[19] that "incubations of thiol (1) (equivalent to (B)), with a cell-free preparation of *C. acremonium* did not yield a detectable quantity of isopenicillin N" must be taken with caution.

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REFERENCES

[1]B. Meesschaert, P. Adriaens and H. Eyssen, *J. Antibiotics* 722 (1980).

[2]This peptide (2) has been previously shown to be the precursor of isopenicillin N in *C. acremonium*, *cf* J. O'Sullivan, R. C. Bleaney, J. A. Huddleston and E. P. Abraham, *Biochem. J.* **184**, 421 (1979); T. Konomi, S. Herchen, J. E. Baldwin, M. Yoshida, N. A. Hunt and A. L. Demain, *Ibid.* 427.

[3]R. Busson, P. J. Claes and H. Vanderhaeghe, *J. Org. Chem.* **41**, 2556 (1976); L. D. Sabath, M. Jago and E. P. Abraham, *Biochem. J.* **96**, 739 (1965).

[4]E. P. Abraham, R. M. Adlington, J. E. Baldwin, M. J. Crimmin, L. D. Field, G. S. Jayatilake, R. L. White and J. J. Usher, *J. Chem. Soc.*, Chem. Commun. 1130 (1982).

[5]R. D. G. Cooper and F. L. José, *J. Am. Chem. Soc.* **92**, 2575 (1970).

[6]D. H. G. Crout, M. Lutstorf, P. J. Morgan, R. M. Adlington, J. E. Baldwin and M. J. Crimmin, *J. Chem. Soc.*, Chem. Commun. 1175 (1981).

[7]We thank Dr. L. Hatfield of the Lilly Research Laboratories for details of this procedure.

[8]P. B. Loder and E. P. Abraham, *Biochem. J.* **123**, 471 (1971).

[9]S. Patai [Ed.], *The Chemistry of the Thiol Group*. Wiley, London (1974).

[10]W. Konigsberg, *Methods in Enzymology* (Edited by C. H. W. Hirs and S. N. Timasheff), Vol. 25, p. 185. Academic Press, New York (1972).

[11]J. E. Baldwin and M. Jung, *J. Chem. Soc.*, Chem. Commun. 609 (1978).

[12]J. J. Usher, B. Loder and E. P. Abraham, *Biochem. J.* **151**, 729 (1975).

[13]E. P. Abraham, J. A. Huddleston, G. S. Jayatilake, J. O'Sullivan and R. L. White, In *Recent Advances in the Chemistry of β-lactam Antibiotics* (*2nd Int. Symp.*) (Edited by G. I. Gregory). Royal Society of Chemistry, Special Publication, No. 38, 125 (1981).

[14]D. D. Perrin, W. L. F. Armarego and D. R. Perrin, *Purification of Laboratory Chemicals*, 1st Edn. Pergamon Press, London (1966).

[15]Further details of the mass spectra of these types of compounds will be reported elsewhere.

[16]G. Bahadur, J. E. Baldwin, L. D. Field, E.-M. M. Lehtonen, J. J. Usher, C. A. Vallejo, E. P. Abraham and R. L. White, *J. Chem. Soc.*, Chem. Commun. 917 (1981).

[17]R. R. Chauvette, P. A. Pennington, C. W. Ryan, R. D. G. Cooper, F. L. José, I. G. Wright, E. M. V.-Heyningen and G. W. Huffman, *J. Org. Chem.* **36**, 1259 (1971).

[18]G. S. Jayatilake, J. A. Huddleston and E. P. Abraham, *Biochem. J.* **194**, 645 (1981).

[19]S. K. Chung, R. Shankaranarayan and A. I. Scott, *Tetrahedron Letters* 2941 (1983).