

Monocyclic β -Lactam Tripeptide, 1-(D-Carboxy-2-methylpropyl)-3-L-(δ -L-2-aminoadipamido)-4-L-mercaptoazetidin-2-one†, a Putative Intermediate in Penicillin Biosynthesis

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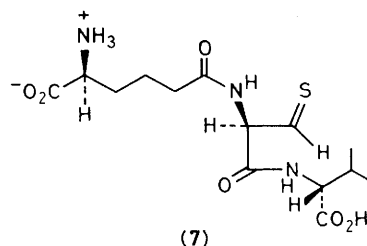
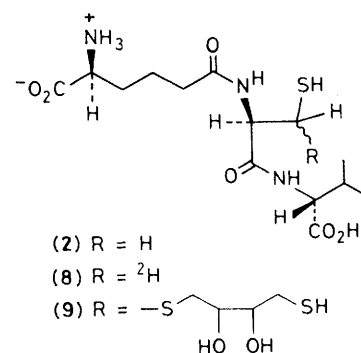
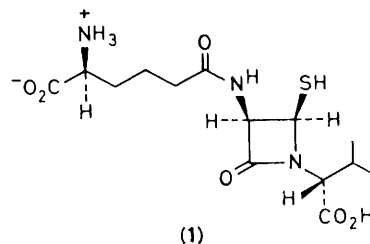
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The disulphide corresponding to the above thiol has been synthesised, but all attempts to reduce this substance to the thiol have been unsuccessful, although an alternative procedure, *via* a thiomercury intermediate, enabled the thiol to be generated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

Recently it was claimed¹ 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*. The evidence supporting this claim was based on the isolation of labelled compounds believed to be (1), from [(3-³H)Cys, (1-¹⁴C)Val]-(2), [(3,4-³H₂, 1-¹⁴C)Val]-(2), and [(2-³H, 1-¹⁴C)Val]-(2). The loss of 49.6% of ³H in the first case, coupled with the retention of all ³H in the last two examples, was adduced to support the structure proposed for (1). Furthermore, the biosynthetic sample of (1) was claimed to be identical with a synthetic sample, as judged by cation-exchange chromatography.† 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 the publication, we had synthesised the disulphide of (1) but had been unable to reduce it to the thiol (1).

The synthetic route to the dimer, (6), from the thiadiazabicycloheptane, (3),³ Scheme 1, involved the final deprotection of the azetidinone (5) followed by sequential ion-exchange and Sephadex-gel filtration chromatography to yield pure (6).§ Reductive cleavage of the disulphide (6) (1 μ mol) with dithiothreitol (10 μ mol) at pH 6.90 and pH



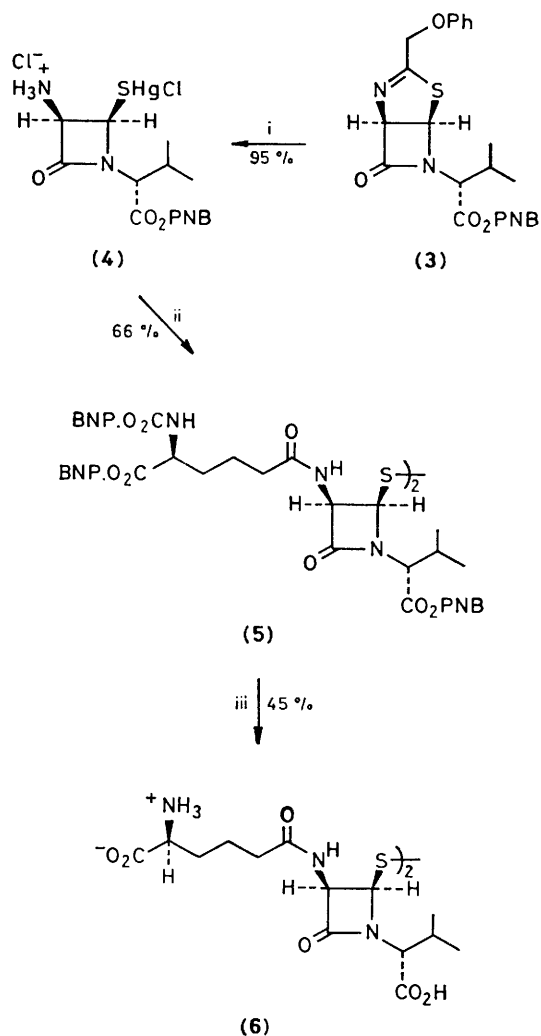
† This system of nomenclature has been retained to be consistent with ref. 1. According to the I.U.P.A.C. system the stereochemistry should be designated as *R* and *S*, *i.e.* 1-[(1*R*)-carboxy-2-methylpropyl]-(3*R*)-[(5*S*)-5-amino-5-carboxypentanamido]-(4*R*)-mercaptoazetidin-2-one.

‡ 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 preparative or spectroscopic data on this synthetic material from the authors.

§ The spectral data for (6) is completely in accord with its formulation: ν_{\max} (nujol) 3200br. (OH, NH), 1750s (β -lactam C=O), 1640s (C=O, -CO₂⁻), 1250m, 1160m, and 1075m cm⁻¹; δ (¹H, D₂O) 0.858 (3H, d, *J* 6.6 Hz, -CHCHMe), 0.915 (3H, d, *J* 6.6 Hz, -CHCHMe), 1.4–1.9 (4H, m, -CH₂CH₂CH₂CO-), 2.1–2.4 (3H, m, -CHCHMe₂ and -CH₂CO-), 3.716 (1H, t, *J* 6.1 Hz, NH⁺CHCO₂⁻), 3.768 (1H, d, -CHCHMe₂), 5.012 (1H, d, *J* 4.0 Hz, β -lactam ring H), and 5.094 (1H, d, *J* 4.0 Hz, β -lactam ring H); δ (¹³C, D₂O) 19.58 (q, -CHMe), 19.83 (q, -CHMe), 21.81 (t, -CH₂CH₂CH₂-), 29.97 (d, -CHMe₂), 30.81 and 35.63 (2 \times t, -CH₂CH₂CH₂-), 55.15 (d, NH⁺CHCO₂⁻), 60.94 and 66.20 (2 \times d, -HNCHCON- and Me₂CHCH-), 77.76 (d, -CHS-), and 169.21, 175.05, and 177.49 p.p.m. (3 \times s, carbonyls, one obscured). Derivatisation (see P. B. Loder and E. P. Abraham, *Biochem. J.*, 1971, **127**, 471) gave the bisethoxycarbonyl dimethyl ester of (6); *m/z*, MH⁺ (weak) 921.362 (C₃₈H₆₁N₆S₂O₁₆ requires 921.359), M⁺/2 - H (base) 459.168 (C₁₉H₃₀N₃S₂O₈ requires 459.166). The disulphide (6) contained less than 0.2 mol% cysteinyl impurity (amino-acid analysis after oxidation to cysteic acid with HCO₃H, 0 °C, 5 h and hydrolysis with 6 M HCl, 24 h, reflux).

7.10 (phosphate buffer) at 21 °C led to the rapid opening of its β -lactam ring. Monitoring these reactions by i.r. and n.m.r. spectroscopy¶ it was possible to show that no β -

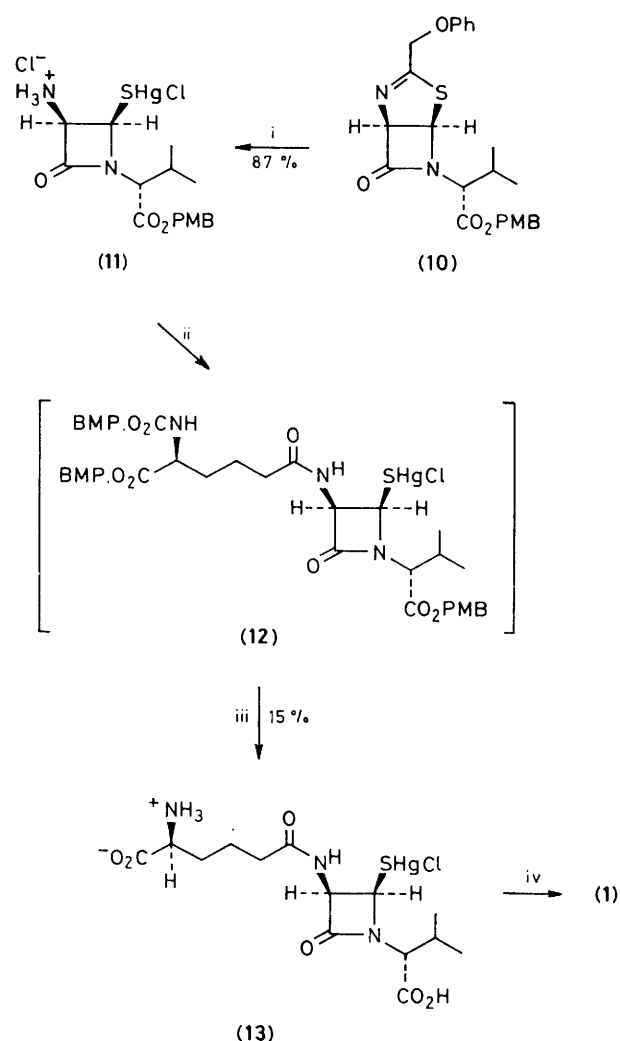
¶ (a) I.r. (CaF₂ cells) ν_{\max} (6), 1740 cm⁻¹ (β -lactam). Within the time for mixing and scanning (*ca.* 30 s) there was no absorption above 1600 cm⁻¹. (b) N.m.r. (D₂O, buffer): β -lactam ring protons of (6) were monitored δ 5.012 and 5.094. Within the time for mixing and scanning (*ca.* 130 s) there were no β -lactam resonances. Compound (8) was obtained as a mixture of isomers at C-3 of the cysteine residue in 80% yield. The β -cysteinyl protons in the n.m.r. spectrum of (8) (D₂O solvent, ²H irradiated) appear as two doublets at δ 3.11 (*J* 5.2 Hz) and 2.79 (*J* 8.7 Hz) of relative area 45:55 respectively. For (9): ν_{\max} (nujol) 3300m and 1630m cm⁻¹; δ (¹H, D₂O) 0.7–1.0 (6H, m, -CHMe₂), 1.5–1.9 (4H, m, -CH₂CH₂CH₂CO-), 2.1–2.3 (1H, m, -CHMe₂), 2.4–2.5 (2H, m, -CH₂CO-), 2.6–2.7 and 2.8–3.0 (2 \times 2H, 2 \times m, 2 \times -CH₂S-), 3.5–3.9 (3H, m, 2 \times -CHO- and NH⁺CHCO₂⁻), and 4.1 (1H, d, *J* 8 Hz, -CHCHMe₂), other resonances not assigned. Compound (9) gave an *N,S,S*-triethoxy-carbonyl dimethyl ester derivative *m/z*, M⁺ = 760.246 (C₂₉H₆₀-N₃S₃O₄ requires 760.245).



Scheme 1. Reagents: i, HgCl_2 , $\text{HOCH}_2\text{C}(\text{Me})_2\text{CH}_2\text{OH}$; ii, NEt_3 , N -(4-nitrobenzoyl)-L- α -amino adipic acid α -(4-nitrobenzyl) ester, ethyl 1,2-dihydro-2-ethoxy-1-quinolinecarboxylate (EEDQ), 24 h; then $\frac{1}{2}\text{I}_2$; iii, Pd-C-H_2 -HOAc (1 M)- $\text{Hg}(\text{OAc})_2$ -tetrahydrofuran. PNB = 4-nitrobenzyl.

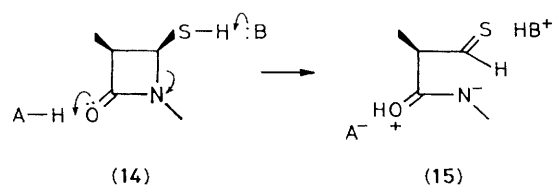
lactam-containing products were formed with lifetimes greater than *ca.* 100 s. The intermediacy of the thioaldehyde peptide, (7), in these reductions was demonstrated by trapping it by reduction with sodium borodeuteride to (8)[¶] and by the formation of (9) on reduction with dithiothreitol. Compound (9) showed spectral data in accord with its structure but could not be isolated in a pure form.[¶] These results are in accord with previous observations on the ring-opening reactions of organic-soluble mercaptoazetidiones.⁴

Since we had been unsuccessful in obtaining the crucial intermediate monocyclic β -lactam, (1), we resorted to an alternative strategy. Thus, we argued that the chloromercury derivative (13), which was readily prepared as in Scheme 2, would represent a precursor which, with hydrogen sulphide, would liberate the thiol, (1), under potentially very mild and controllable conditions. Compound (13) obtained in an unprotected form was purified on Sephadex G-10 and obtained as a foam [$\nu_{\text{max}}(\text{D}_2\text{O}, \text{CaF}_2 \text{ cells})$ 1735s (β -lactam C=O) and 1595s cm^{-1} ($-\text{CO}_2^-$); $\delta(\text{H}, \text{D}_2\text{O})$ 0.718 (3H, d, J 6.4 Hz, $-\text{CHMe}$), 0.813 (3H, d, J 6.4 Hz, $-\text{CHMe}$), 1.5–1.9 (4H, m, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}-$), 2.2–2.4 (3H, m, $-\text{CHCHMe}_2$ and $-\text{CH}_2\text{CO}-$), 3.466 (1H, d, J 10.6 Hz, $-\text{CHCHMe}_2$), 3.526 (1H, t, J 6.2 Hz, $\text{NH}_3^+\text{CHCO}^-$), 4.980 (1H, d, J 4.4 Hz, β -lactam



Scheme 2. Reagents: i HgCl_2 , $\text{HOCH}_2\text{C}(\text{Me})_2\text{CH}_2\text{OH}$; ii, NEt_3 , N -(4-methoxybenzoyl)-L- α -amino adipic acid α -(4-methoxybenzyl) ester,⁶ EEDQ, 24 h; iii, PhH , PhOMe , trifluoroacetic acid; iv, H_2S . PMB = 4-methoxybenzyl.

ring H), and 5.412 (1H, d, J 4.4 Hz, β -lactam ring H)] which was cleanly oxidised ($\text{KI}, \text{I}_2, \text{D}_2\text{O}$) to the previously characterised disulphide, (6). At pH 1.5 ($\text{DCl}, \text{D}_2\text{O}$) (13) was converted into the desired thiol (1) [$\delta(\text{H}, \text{D}_2\text{O}-\text{DCl})$ 0.790 (3H, d, J 6.6 Hz, $-\text{CHMe}$), 0.860 (3H, d, J 6.6 Hz, $-\text{CHMe}$), 1.4–1.9 (4H, m, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}-$), 2.2–2.4 (3H, m, $-\text{CHMe}_2$ and $-\text{CH}_2\text{CO}-$), 3.736 (1H, d, J 9.2 Hz, $-\text{CHCHMe}_2$), 3.850 (1H, t, J 6.2 Hz, $\text{NH}_3^+\text{CHCO}^-$), 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)] was relatively stable ($t_{\frac{1}{2}}$ *ca.* 25 min at 20 °C) and could be reconverted into the stable compound, (13), 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 i.r. spectrum.] as monitored by i.r. and n.m.r. 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 (14) \rightarrow (15) 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 (6) is a perfectly normal tripeptide disulphide.

For the above reasons, we cannot accept that the substance described in the previous report¹ 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, the question of a monocyclic β -lactam intermediate in penicillin biosynthesis is still unresolved.

Incubation of the disulphide (6) (1 mM) with cell-free extracts of *C. acremonium* C-91 or *P. chrysogenum* (SC 6140)⁵ in the presence or absence of dithiothreitol (0–2 mM) gave no isopenicillin N.** In separate experiments there was no

** The incubation mixture was assayed against *Staph. aureus* NCTC 6571 which gave a lower detection limit of 15 $\mu\text{g ml}^{-1}$ of isopenicillin N.

inhibition by (6) of the conversion of LLD-ACV (2) into isopenicillin N by these cell-free systems.

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