*Tetrahedron* Vol 41 No 16, pp 3339 to 3343 1985
Printed in Great Britain

0040-4020/85 $3 00+ 00


# PENICILLIN BIOSYNTHESIS THE IMMEDIATE ORIGIN OF THE SULPHUR ATOM

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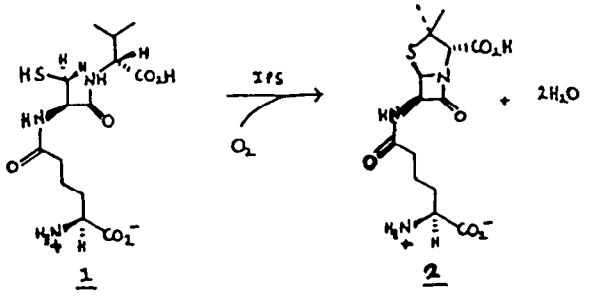
(*Received in UK 23 April* 1985)

**Abstract** A mixture of tripeptide isotopomers δ-(L-α-aminoadipyl)-L-cysteinyl-D [2-$^2$H]-valine and δ-(L-α-aminoadipyl)-L-($^{33}$S-cysteinyl)-D-valine were converted by the enzyme isopenicillin-N-synthetase into isopenicillin N The distribution of the $^2$H and $^{33}$S in this product, determined by mass spectrometry, showed there was no transfer of the sulphur between the precursor molecules during conversion to penicillin

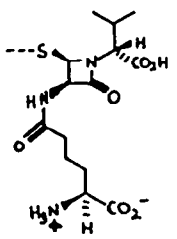
## INTRODUCTION

It is now well established that the biosynthesis of penicillin involves conversion of the tripeptide δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) (1) into isopenicillin N (2)[1] by the enzyme isopenicillin N synthetase (IPS),[2] an iron and oxygen dependent desaturase of an unusual type, fig 1




1 2

Fig 1



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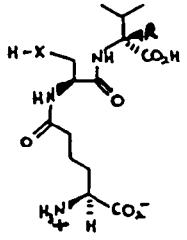
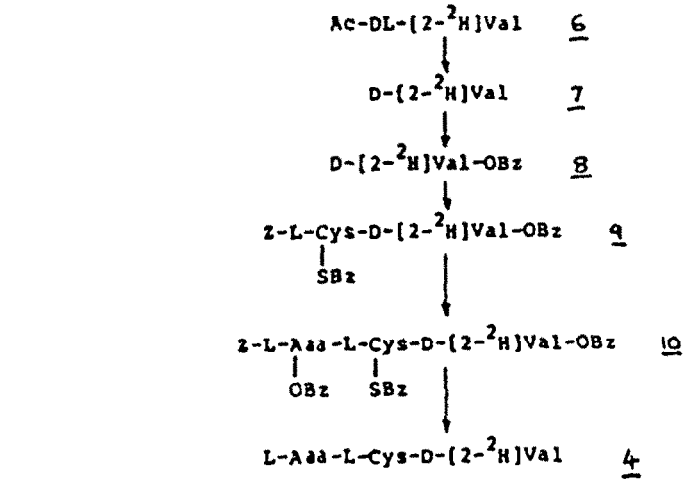


4 X = $^{33}$S, R = $^2$H

5 X = $^{34}$S, R = $^1$H

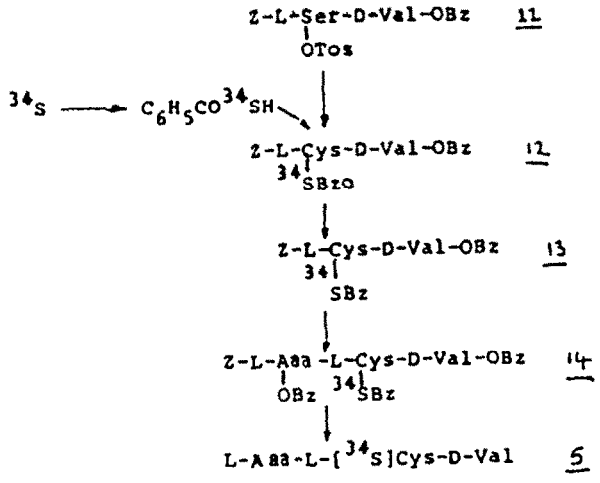
Although the precise details of this conversion are still uncertain it has recently been proved by applications of kinetic isotope effects, that the 3-cysteinyl hydrogen is removed before the 3-valinyl hydrogen and that an enzyme bound intermediate, as (3), is probably involved [3] Since the IPS enzyme contains at least one cysteinyl thiol, whose blockade inhibits the enzyme's activity,[4] it is by no means clear that the penicillin bound sulphur is in fact the same atom as exists in the precursor tripeptide Previous work on the incorporation of $^{35}$S-cystine by intact cell preparations into benzyl penicillin did not allow an answer to this question [5] However the

possibility that the sulphur atom of penicillin is derived by a transfer mechanism, via the IPS enzyme, may be tested by an appropriate mixed labelling experiment Thus, if a mixture of $^{34}S$ labelled tripeptide and $^{2}H$ labelled tripeptide were enzymatically converted to penicillin then the distribution of $^{34}S$ between unlabelled and $^{2}H$ labelled products would reveal any such sulphur transfer process




Aaa = α-aminoadipoyl

Scheme 1




Scheme 2

In the event, a mixture of isotopically labelled tripeptides was obtained from two samples bearing deuterium at the valine C-2 and $^{34}S$ at the cysteinyl C-3 position respectively, which were prepared as in Scheme 1 and Scheme 2 The isotopomer composition of this sample was determined by desorption chemical ionisation mass spectrometry (DCI-MS) on the N,S-diethyoxycarbonyl-dimethyl esters, Table 1 [6]

Table 1 Isotopomer Composition of N,S-diethoxycarbonyl-dimethyl esters of (1), (4) and (5)

| (1)[6] | | Mixture (4) and (5) | |
|---|---|---|---|
| m/e | Intensity % | m/e | Intensity % |
| 536 | 100 0 | 536 | 11 2 |
| 537 | 25 0 | 537 | 72 6 |
| 538 | 9 0 | 538 | 100 0 |
| | | 539 | 27 9 |
| | | 540 | 6 9 |

Composition of mixture (4) and (5)

| | |
|---|---|
| LLD-ACV | 6 9% |
| LLD-AC($2^2$H)V | 42 9% |
| LLD-A($^{34}$S)CV | 50 2% |

This sample of the isotopic mixture was transferred to isopenicillin N by incubation with a purified sample of IPS enzyme in the presence of ferrous ion, ascorbic acid and oxygen [2] The so-formed isopenicillin N was separated for the protein and converted to its N-ethoxycarbonyl-dimethyl ester for DCI-MS analysis The results are shown in Table 2, along with the isotopomer composition derived from this data

Table 2 Isotopomer Composition of N-ethoxycarbonyl-dimethylester of isopenicillin N, (2)

| (2)[6] | | Product from (4) and (5) | |
|---|---|---|---|
| m/e | Intensity % | m/e | Intensity % |
| 460 | 100 0 | 460 | 10 5 |
| 461 | 21 0 | 461 | 73 0 |
| 462 | 13 0 | 462 | 100 0 |
| | | 463 | 25 5 |
| | | 464 | 6 5 |

Composition of product from (4) and (5)

| | |
|---|---|
| Isopenicillin N | 6 4% |
| 3-$^2$H Isopenicillin N | 42 9% |
| $^{34}$S Isopenicillin N | 50 7% |

As can be seen there is no change in the distribution of $^{34}$S between the isotopomers of the products isopenicillin N, within experimental error If a sulphur transfer mechanism were in operation during penicillin synthesis then the heavy sulphur $^{34}$S would become randomly distributed among the deuterium or proton containing penicillin molecules In this case the expected distribution of ions of the derivatised product would be -

| | Cal[d](%) | Found (%) |
|---|---|---|
| M | 23 0 | 5 0 |
| M+1 | 22 0 | 34 9 |
| M+2 | 30 1 | 47 8 |
| M+3 | 24 9 | 12 2 |

We therefore conclude that during the enzymatic conversion of the tripeptide (1) into isopenicillin N (2) the cysteinyl 3C-S bond maintains its integrity

Acknowledgment We wish to thank Dr R T Aplin (Dyson Perrins Laboratory) for the mass spectral data The work at ETH was supported financially by Sandoz AG, Basel

## GENERAL EXPERIMENTAL

$^1$H NMR spectra were recorded on a Bruker WM-300 (300 MHz) Mass spectra were recorded on a Hitachi-Perkin-Elmer spectrometer RMU-6A except for ammonia DCI mass spectra which were recorded on a VG Micromass ZAB IF mass spectrometer at an indicated source pressure of $6 \times 10^{-5}$ torr

The synthesis of the labelled tripeptides was performed in Zürich The incubations with isopenicillin N synthetase and subsequent product (and mixed starting material) determinations by mass spectral analysis were performed at Oxford

The derivative mixture from (4) and (5) was prepared by the method described [6]

The derivatised isopenicillin N products from (4) and (5) was prepared as follows -

A sample (3mg) containing (4) and (5) was incubated with isopenicillin N synthetase under standard conditions [2] The crude product was protein precipitated and the mother liquor freeze dried Water (3ml) was added (pH ~ 6-7), then saturated $NaHCO_3$ solution was added to pH 7-8 (ca 50 µl ) $(EtOCO)_2O$ (50µl ) was added and the mixture was stirred vigorously at 20° for 1 h The solution was extracted with ether (3 x 2 ml), acidified to pH2 (2N HCl), and extracted into ethylacetate (4 x 2 ml) The ethylacetate layers were combined, dried ($Na_2SO_4$), filtered and treated with excess $CH_2N_2$ in ether at 0° for 15 minutes Evaporation gave the derivatised labelled penicillin product whose structure was confirmed by 500 MHz n m r (as described) [6]

Preparation of Acetyl-DL-[2-$^2$H]Valine (6)

A method similar to that employed by D E Brundish et al[7] was employed Thus L-valine (6 44g, 55 mmol), $D_2O$ (99 8%, 49 5 mls) and acetic anhydride (250 ml) were mixed, and the solution stirred at 100°C for 10 min until homogeneous Further acetic anhydride (27 ml) was added and the solution stirred at 100°C for 5 min The reaction mixture was carefully quenched with $D_2O$ (20 ml) and the solvent removed *in vacuo* The residue was dissolved in water (3 x 50 ml) and the solvent evaporated *in vacuo* (3 times) The residue was dissolved in ethyl acetate (700 ml), the solution dried ($Na_2SO_4$), concentrated to 450 ml volume, and stored at 0° overnight to give the derivative 6 (6 69, 76%), as white crystals m p 145-6° An 80% level of α-deuteriation was estimated by $^1$H n m r methods

The compound 6 thus obtained (6 69 g, 40 9 mmols) was dissolved in $D_2O$ (74 ml) and acetic anhydride (300 ml) and the reaction stirred at 100°C for 10 min Further acetic anhydride (97 ml) was added, the reaction stirred for another 6 min at 100° then carefully quenched with $D_2O$ (20 ml) Work-up (as before) gave the derivative (6) (5 26 g, 80%) as white crystals m p 145-6°, $\nu_{max}$ (KBr) 3380 s, 1730 s, 1715 s, 1600 s, and 1550 s $cm^{-1}$, δH($CD_3OD$) 0 96(3H, d, J 6 9Hz, $CH_3$), 0 98(3H, d, J 6 9Hz, $CH_3$), 2 00(1 5H, s, $CH_3$)[+], 2 14(1H, m, J 6 9Hz, β-CH), 4 31(0 08H, d, J 5 6Hz, α-CH)

Preparation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-$^2$H-valine (4)

The conversion of the N-acetyl derivative (6) to the deuteriated tripeptide (4) (Scheme 1) follows from literature methods Thus the racemic (6) was resolved enzymatically[8,9] using Acylase I (FLUKA, salt free) to give L-[2-$^2$H]valine $[\alpha]^2\beta$ = +26 5° (C=1, 5N HCl) and N-Acetyl-D-[2-$^2$H]-valine $[\alpha]^2\beta$ = -10 8° (C=1, acetic acid) The latter was acid hydrolysed[9] to D-[2-$^2$H]valine (7) $[\alpha]^2\beta$ = -26 7° (C=1, 5N HCl) in 75% yield (7) was transformed according to the literature procedures[10] to the tripeptide (4) The degree of deuteriation was analysed by mass spectroscopy on the protected dipeptide (9) which gave a fragment m/e 444 ($C_{23}H_{26}DN_2O_5S^+$) showing a level of 91 5% ± 0 5% deuteriation

Preparation of tosylate (11)

The benzyl ester of N-Benzyloxycarbonyl-L-seryl-D-valine was prepared by the method of König and Geiger[11] from the p-toluenesulponate salt of D-valine benzyl ester[12] and N-Benzyloxycarbonyl-L-serine (FLUKA, puriss), yield 81% Crystals from ethyl acetate/pentane, m p 116-7°, $[\alpha]^2\beta$ = +9 1° (C = 1, acetone) The dipeptide was tosylated at -10° in pyridine[13] and gave the tosylate (11) in 91% yield After trituration with pentane the product gave crystals from ethyl acetate/pentane, m p 82-3°C, $[\alpha]^2\beta$ = +13 9° (C = 1, acetone)

Preparation of [$^{35}$S]-Thiobenzoic acid

In a high melting glass tube 204 mg (6 0 mmol) of [$^{35}$S] sulphur (Amersham, $^{35}$S > 93%), 335 mg (6 0 mmol) iron filings and 20 mg of norite were heated over a Bunsen burner until glowing red and then immediately put into a test tube (15 ml) containing ice (1 g) Dropwise addition of concentrated sulphuric acid (2 ml) gave an easily controllable flow of hydrogen sulphide

For the preparation of [$^{35}$S]-dibenzoylsulphide a modified version of the procedure of Adkins et al[14] was employed Benzoyl chloride (12 mmol), methylene chloride (12 ml) and pyridine (1 4 ml) in a 40 ml reaction vessel were cooled to -15° [$^{35}$S]-Hydrogen sulphide was introduced (via a teflon tube, diameter 0 9 mm) into the vigorously stirred reaction mixture using a slow stream of nitrogen as a carrier gas After 10 minutes a white precipitate formed, the ice cooling bath was removed and the reaction was stirred for a further 40 minutes while a slow stream of nitrogen was bubbled through it

To the so-formed [$^{35}$S]-dibenzoylsulphide solution, sodium methoxide (1M, 18 mls) was added, and the mixture stirred for 15 minutes at room temperature To the yellow reaction mixture sodium bicarbonate solution (5%, 120 mls) was added, the aqueous layer extracted with ether (3 x 50 mls), poured into ice and brought to pH 1 with concentrated hydrochloric acid The solution was extracted with ether (2 x 80 ml), dried ($Na_2SO_4$) and evaporated to yield crude thiobenzoic acid

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[+] The N-acetyl group was found to be partially deuteriated (ca 50% mixed by $^1$H n m r integration)

(722 mgs) Iodotitration (0 01 N in ethanol) of a small aliquot dissolved in DMF and acidified with acetic acid indicated that 73% (4 4 mmol) of the sulphur had been transformed into [$^{35}$S]-thiobenzoic acid

Preparation of N-Benzyloxycarbonyl-S-benzoyl-L-[$^{35}$S]cysteinyl-D-valine benzyl ester (12)[15]

The tosylate (11) (4 13 mmol, 2 40 g) and [$^{35}$S]-thiobenzoic acid (4 35 mmol) were dissolved in DMF (19 ml) and a methanolic solution of sodium methoxide (1M, 4 35 ml) added The reaction mixture was stirred under a hydrogen atmosphere for 24 h at room temperature when iodotitration of an aliquote showed that 95% of the thiobenzoic acid had been consumed Ice water (50 ml) was added and crude (12) (2 10 g) crystallised out, m p 148-9°C The crude product was purified by flash chromatography [using methylene chloride/ethyl acetate (6 1) as eluant] to yield (12) (1 98 g, 88%) of white crystals m p 151-2°, $[\alpha]^{2}_{\beta}$ -25 5° (C = 1, acetone) m/e 550($M^+$, <1 %), 316(6), and 105(100), $\nu_{max}$ (KBr) 3300 s, 1735 s, 1700 s, 1660 s, and 1650 s $cm^{-1}$, δH ($C^2HCl_3$) 0 82(3H, d, J 6 9Hz, $CH_3$), 0 89(3H, d, J 6 9Hz, $CH_3$), 2 16(1H, m, β-CH, D-Val) 3 43, 3 54(AB part of ABX system with δA 3 43, δB 3 54, $J_{AB}$ 14 0Hz, $J_{AX}$ 7 8Hz, $J_{BX}$ 4 6Hz, β-$CH_2$ L-Cys), 4 50(1H, m, α-CH, L-Cys), 4 57(1H, dd, $J_1$ 4 8Hz, $J_2$ 8 7Hz, which collapsed to a doublet $J_1$ 4 8Hz upon exchange with $^2H_2O/NaO^2H$, α-CH D-Val), 5 09(2H, d, J 1 3Hz, $OCH_2Ar$), 5 10, 5 18(2H, 2 X d, J 12 2HZ, $OCH_2Ar$), 5 70(1H, bd, exchangeable with $^2H_2O/NaO^2H$, NH), 6 84(1H, bd, exchangeable with $^2H_2O/NaO^2H$, NH), and 7 25-7 96 (15H, m, ArH)

Preparation of N-Benzyloxycarbonyl-S-benzyl-L-[$^{35}$S]cysteinyl-D-valine benzyl ester (13)[15]

(12) (1 98 g, 3 61 mmol) was dissolved in DMF methanol (1 1, 50 ml) and sodium methoxide (3 50 mls, 1 0 M) was added under a hydrogen atmosphere After 10 minutes, benzyl chloride (1 90 ml) was added and the reaction mixture concentrated at room temperature to 25 ml volume Ethyl acetate (200 ml) was added, and the organic layer washed with saturated sodium bicarbonate solution (2 x 50 ml), saturated brine (2 x 50 ml), dried ($Na_2So_4$), filtered and evaporated The oily residue was purified by column chromatography [using hexane ethyl acetate (4 1) as eluant] to yield (13) (749 mgs, 39%) as white crystals The $^1$H n m r , i r , m p and optical rotation were in agreement with the literature values[10] Mass spectroscopic analysis of the fragment m/e 445 ($C_{23}H_{27}N_2O_5S^+$) gave a [$^{35}$S] content of 93 5% (± 0 5%)

Preparation of δ-(L-α-Aminoadipyl)-L-[$^{35}$S]cysteinyl-D-valine (5)

The dipeptide (13) was converted into the tripeptide (5) by the literature procedures [10]

## REFERENCES


1 (a) J O'Sullivan, R C Bleaney, J A Huddleston, and E P Abraham, Biochem J , 1979, 184, 421, (b) T Konomi, S Herchen, J E Baldwin, M Yoshida, N A Hunt, and A L Demain, Biochem J , 1979, 184, 427

2 C-P Pang, E P Abraham, R M Adlington, J E Baldwin, B Chakravarti, G S Jayatilake, H-H Ting, and R L White, Biochem J , 1984, 222, 789

3 (a) J E Baldwin, R M Adlington, S E Moroney, L D Field, and H-H Ting, J Chem Soc Chem Commun , 1984, 984, (b) J E Baldwin, E P Abraham, C G Lovel, and H-H Ting, J Chem Soc Chem Commun , 1984, 902

4 J E Baldwin, H-H Ting, E P Abraham, and D Perry, unpublished work

5 H R V Arnstein and P T Grant, Biochem J , 1954, 57, 360

6 R M Adlington, R T Aplin, J E Baldwin, B Chakravarti, L D Field, E-M M John, E P Abraham, and R L White, Tetrahedron, 1983, 39, 1061

7 D E Brundish, D F Elliott, and R Wade, J Lab Compd , 1971, VII(4), 473

8 J P Greenstein and M Winitz, "Chemistry of the Amino Acids", Wiley, New York, 1961, p 2375

9 C B Baker and H A Sober, J Amer Chem Soc , 1953, 75, 4058

10 J E Baldwin, S R Herchen, B L Johnson, M Jung, J J Usher, and T Wan, J Chem Soc , Perkin Trans I, 1981, 2253

11 W König and R Geiger, Chem Ber , 1970, 103, 788

12 L Zervas, M Winitz, and J P Greenstein, J Org Chem , 1957, 22, 1515

13 C Zioudrou, M Wilchek, and A Patchomik, Biochem , 1965, 4, 1811

14 H Adkins and Q E Thompson, J Amer Chem Soc , 1949, 71, 2242

15 Procedures developed from C Zioudrou et al, Biochem 1965, 4, 1811