STUDIES ON THE BIOSYNTHESIS OF ISOPENICILLIN N WITH A CELL-FREE PREPARATION OF *PENICILLIUM CHRYSOGENUM*

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When δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine was added to a cell-free system prepared by lysis of *Penicillium chrysogenum* protoplasts, "compounds X and Y" were detected after analysis on a cation-exchange column. The chromatographic position as well as results of experiments with double labelled tripeptides showed "compound X" to be the penicilloic acid of isopenicillin N. LLD-Tripeptide labelled with tritium at carbon-2 of the valine part was incorporated into isopenicillin N with retention of label. "Compound Y" retained all hydrogens on the valine part of the peptide, but lost half of the tritium on carbon-3 of the cysteine part. The results are consistent with the hypothesis that the LLD-tripeptide is converted into isopenicillin N *via* a monocyclic β-lactam and without a dehydrovalinyl intermediate. Extensive transacylase activity was observed between isopenicillin N and 6-aminopenicillanic acid.

The biosynthetic pathway leading to the natural cephalosporins has been successfully investigated using cell-free preparations of *Cephalosporium acremonium*. A cell-free system prepared by ultrasonic treatment of mycelium of *C. acremonium* was used to study the biosynthesis of the intermediate δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (=LLD-tripeptide)[1]. With a preparation of lysed *C. acremonium* protoplasts the LLD-tripeptide was transformed into penicillin N *via* isopenicillin N[2,3,4]. Similar preparations converted penicillin N into deacetoxycephalosporin C[5,6,7].

Although it was apparent that the LLD-tripeptide could be the biosynthetic precursor of isopenicillin N in *Penicillium chrysogenum*, direct evidence has never been obtained. The nature of the intermediate between the LLD-tripeptide and isopenicillin N in *C. acremonium* and *P. chrysogenum* also remained unknown.

The present study deals with the conversion of the LLD-tripeptide into isopenicillin N by a cell-free preparation of *P. chrysogenum* obtained by lysis of protoplasts. Evidence is also given for an intermediate between the LLD-tripeptide and isopenicillin N.

Materials and Methods

Materials

Labelled bis-δ-(L-α-aminoadipyl)-L-cystinyl-bis-D-valine peptides were prepared by modifying the method of VANDERHAEGHE and ADRIAENS[8]. Two successive couplings using dicyclohexylcarbodiimide/1-hydroxybenzotriazole were performed. The first reaction was between di-N-benzyloxycarbonyl-L-cystine and D-valine ethyl ester. After cleaving the benzyloxycarbonyl groups of the protected dipeptide with HBr in acetic acid, the dipeptide ethyl ester was coupled to 1-benzyl-N-benzyloxycarbonyl-L-α-aminoadipic acid. Peptides were obtained after removal of benzyloxycarbonyl and benzyl ester groups with HBr in acetic acid and hydrolysis of the ethyl esters with alkali. Four labelled peptides were synthesized. The first product contained D-[1-^{14}C] valine (([1-^{14}C] Val) LLD-tripeptide; specific activity 1,186 μCi/mmol). A second tripeptide was prepared with L-[3,3′-^{3}H] cystine (([3,3′-^{3}H] Cys) LLD-tripeptide; specific activity 1,790 μCi/mmol). Another [^{3}H] labelled peptide was synthesized with D-

[2-^{3}H] valine (([2-^{3}H] Val) LLD-tripeptide; specific activity 2,625 μCi/mmol). A peptide was also prepared with D-[3,4-^{3}H] valine (([3,4-^{3}H] Val) LLD-tripeptide; specific activity 4,599 μCi/mmol); L- and D-[3,4-^{3}H] valine were obtained from N-acetyl-DL-[3,4-^{3}H] valine. A [^{3}H] NMR spectrum which was recorded by Prof. ELVIDGE (University of Surrey, U. K.) on L-[3,4-^{3}H] valine showed that no tritium was present on carbon-2 of valine; 13.3 and 86.7% of the isotope was on carbon-3 and -4 respectively.

ATP (disodium salt), NAD (free acid) and sodium pyruvate were from E. Merck, Darmstadt, BRD. Phosphoenolpyruvate (trisodium salt, hydrate), pyruvate kinase (from rabbit muscle) and lactic dehydrogenase (from pig heart) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Neutrapen was a product of Kettelhack Riker Pharma GmbH, Borken, BRD.

6-Aminopenicillanic acid was a gift of Gist-Brocades, Delft, The Netherlands. Isopenicillin N was prepared by VANDERHAEGHE *et al.*[9] Phenylacetic acid was a product of U.C.B. Brussels, Belgium. Dithiothreitol (DTT) was a product of E. Merck. Bacto-peptone was from Difco-Laboratories, Detroit, Michigan, U.S.A. Chitin was purchased from Koch-Light Laboratories Ltd., Colnbrook, U.S.A. A laminarin-rich extract of *Laminaria hyperborea* fronds was obtained from the University of Trondheim, Norway.

(1-^{14}C) Phenylacetic acid was purchased from The Radiochemical Centre, Amersham, U. K. 6-Aminopenicillanic acid labelled with ^{3}H in the 2-β-methyl group was synthesized as previously described[10].

Culture conditions

P. chrysogenum E-15 (ATCC 26818) was maintained on the minimal sporulation medium described by LEMKE *et al.*[11] Spores were obtained on the same medium; $3\sim5\times10^7$ spores were used to seed 50 ml of JARVIS and JOHNSON medium[12], without phenylacetic acid. Fermentations were carried out at 27°C on a G10 Gyrotory shaker (325 rev./min.; New Brunswick Scientific Co. Inc., Brunswick, N. Y., U.S.A.). The mycelium of 20 Erlenmeyer flasks was collected on a sterile sintered glass filter after 60 hours of fermentation, which is the beginning of the rapid antibiotic production phase. The mycelium was washed by 3 successive resuspensions in 200 ml of H_2O and one resuspension in 200 ml of 0.7 M NaCl.

Preparations of lytic enzymes

Protoplasts were prepared as described by ANNÉ[13]. *Trichoderma harzianum* CBS 354.22 and *Streptomyces graminofaciens* ATCC 12705 were used for the preparation of cell wall lytic enzymes; the strains were maintained on the complete medium of MCDONALD *et al.*[14] and on the complete sporulation medium of LEMKE & NASH[15], respectively. Both strains were grown in the following medium: glucose, 1.0 g; Bacto-peptone, 0.1 g; urea, 0.3 g; $(NH_4)_2SO_4$, 1.4 g; $CaCl_2\cdot6H_2O$, 0.3 g; *Laminaria*-extract, 10.0 g; chitin, 5.0 g; salt mixture of JARVIS and JOHNSON medium[12], 4.0 g; mycelium of *P. chrysogenum* E-15 from one liter of fermentation obtained as described above and desintegrated; H_2O, 1 liter. For *S. graminofaciens* a 24-hour preculture in the same medium was necessary. Spores of *Trichoderma* ($3\sim5\times10^7$) and 2 ml of *Streptomyces*-preculture were used to inoculate 50 ml of the medium in 300 ml Erlenmeyer flasks. After fermentation of $3\sim4$ days for *T. harzianum* and $6\sim7$ days for *S. graminofaciens*, the cultures were centrifuged. The unpurified extracellular fluid was freeze-dried. From 1 liter of culture medium $7\sim10$ g of crude *Trichoderma* lytic enzyme (=T.L.E.) or Strepzyme were obtained. The enzyme preparations were stored at −20°C.

Induction of protoplasts

Strepzyme and T.L.E. were suspended in 0.7 M NaCl at a concentration of 10 mg/ml and 20 mg/ml, respectively. The preparation was stirred at room temperature for 15 minutes and the pH was adjusted to 6.3 where necessary. The mixture was centrifuged for 15 minutes at 5°C and 15,000$\times g$ and the supernatant was sterilized by filtration.

Mycelium obtained as above was suspended in the lytic enzyme solution (maximum 1 g/10 ml) and incubated at 27°C and stirred at $150\sim200$ rev./min. Digestion of the mycelium was complete within 3 hours. Mycelial debris was removed by filtration through a sintered glass filter (porosity 1). Protoplasts were centrifuged (8 min., 700$\times g$) and were washed twice with 0.7 M NaCl. The resulting suspension of protoplasts was cooled in ice. With this method, $3\sim3.5$ ml of protoplasts (10^8/ml) were obtained from 10 g of air dry mycelium of *P. chrysogenum* E-15.

Precursor solutions

The [^{14}C] labelled tripeptide and DTT were dissolved in 0.1 M Tris/HCl buffer of pH 7.2 to final concentrations of 10 μmol/ml and 80 μmol/ml respectively. The solutions were brought to pH 8 with 2 M NaOH and were stored overnight under anaerobic conditions. The pH was then lowered to 7.2 with 1 M HCl. Of this solution 0.25 ml, containing 5 μmol of the cysteinyl peptide (=2.95 μCi) and 20 μmol of DTT, was transferred to a sterile test tube (2 cm × 10 cm). ATP (5 μmol) was added with or without an energy generating system composed of phosphoenolpyruvate (10 μmol) and pyruvate kinase (20 I.U.). The effect of NAD (5 μmol) supplemented with a NAD regenerating system of pyruvate (1 μmol) and lactic dehydrogenase (20 I.U.) was also investigated. Phenylacetic acid was supplemented in 5 μmol amounts. In some experiments [1-^{14}C] phenylacetic acid (specific activity 100 μCi/mmol) was added. Additional DTT was added as 100 μl of a 0.2 M solution in the Tris buffer. 6-Aminopenicillanic acid and isopenicillin N were added in an amount of 1 μmol in 100 μl of buffer solution.

In the double labelled experiments a [^{3}H] labelled LLD-tripeptide and the [^{14}C] labelled tripeptide were mixed in [^{3}H]: [^{14}C]-ratios of 3: 1 to 7: 1. The total concentration of [^{3}H-^{14}C] labelled tripeptide was 20 μmol per ml (after reduction to the thiol form).

Preparation of the cell-free system

The cooled protoplasts were diluted with an equal volume of cooled 0.1 M Tris/HCl buffer containing the salt mixture of the JARVIS and JOHNSON medium (without KH_2PO_4).[12] The suspension was gently mixed and 1.5 ml was immediately transferred to tubes containing 0.25~0.50 ml of precursor solution (=5 μmol LLD-tripeptide). Microscopic examination showed some protoplasts in the system before it was added to the precursor solution and after that, no protoplasts were found. In control experiments the system was first boiled for one minute before being transferred to the precursor solutions. The tubes were stoppered and incubated for 2~3 hours at 28°C and 175 rev./min. (under an angle of 45°). The penicillin content of the cell-free preparation was assayed by the agar diffusion method with *Sarcina lutea* ATCC 9341 as test organism.

Extraction of the cell-free preparation

After incubation, acetone was added up to a concentration of 75% (v/v). After centrifugation the supernatant was removed and the precipitate was suspended in 1 ml H_2O. After addition of 3 ml of acetone the precipitate was again centrifuged and the supernatants were combined (=acetone extract). A second extract was obtained after mixing with 1.5 ml of 0.1 M LiOH and centrifugation; the precipitate was washed once more with 1 ml H_2O and the two supernatants were combined (=the LiOH extract). Both extracts were evaporated and each residue was taken up in 2 ml of 0.1 M Li_2HPO_4 pH 8.2 and 15 mg of DTT was added. The acetone and LiOH extracts were usually combined (pH 8~8.5) and evaporated; the residue was taken up in 2 ml of buffer and 30 mg of DTT was added. The pH was adjusted to pH 8.2 with 0.3 M LiOH and insoluble matter was removed by centrifugation. The clear supernatants were stored overnight under anaerobic conditions. Each extract was then acidified with 5 M HCl (to pH 3~3.5) and sulfosalicylic acid (to pH 2.2), cooled in crushed ice for 15~30 minutes and centrifuged. Approximately 1.5~2 ml of nearly colorless extract was obtained.

Analytic procedure

The extracts were analyzed on a cation-exchange column (Chromobeads type B, Li^+-form; 140 cm × 6 mm; Technicon Co., Tarrytown, N.Y., U.S.A.). The lithium buffer gradient and elution conditions were described earlier; the system was calibrated for a wide range of ninhydrin-positive compounds and for the degradation products of different penicillins[16~19]. The penicilloic acid of isopenicillin N eluted between aspartic acid and threonine; δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine coeluted with glutamic acid. Part of the effluent was collected in fractions of 4 minutes (=1.6 ml). After addition of 3.6 ml of Lumagel (Lumac N.V., Meise, Belgium), the samples were counted with a Packard model 3390 Liquid Scintillation Counter, with a 544 Absolute Activity Analyzer; calibration was performed using [^{3}H]- and [^{14}C]-hexadecane (The Radiochemical Centre, Amersham, U. K.). When this procedure was applied to the labelled LLD-tripeptides (reduced with an eightfold excess of DTT) a radiochemical purity of 99±1% was calculated. Part of the extracts (0.50~0.75 ml) was mixed with 0.25 ml of 0.3 M lithium citrate of pH 2.2 and loaded on the amino acid analyzer.

Results and Discussion

Cell-free Conversion of δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine into Isopenicillin N

In a typical experiment 1.5 ml of a cell-free preparation was mixed with 0.5 ml of a precursor solution containing 5 μmol ([1-^{14}C]Val)LLD-tripeptide (=2.95 μCi), ATP and NAD, and an ATP and NAD generating system. The control system was deactivated by heating before being added to the precursor solution. The pH was 6.5 after incubation. The acetone extracts contained 52~62% of the added radioactivity; 15~20% of the label was present in the alkaline extracts. The extracts were analyzed on the amino acid analyzer; recovery of radioactivity was about 80%. Fig. 1a shows the radioactivity in the effluent of the column during chromatography of a sample of the acetone extract of the intact system. Labelled valine could not be detected in either extract. Besides ([1-^{14}C] Val) LLD-tripeptide, the extracts contained another labelled compound ("compound C"); 0.8% and 5.2% of the radioactivity in the acetone and the alkaline extracts, respectively, was present in this product. The presence of "compound C" in the deactivated system indicated that a chemical reaction was involved in its formation. "Compound C" was no longer present in the extract if the NAD and ATP regenerat-

Fig. 1. Tracing of the radioactivity in the effluent of the cation-exchange column after loading an extract of a cell-free preparation of *P. chrysogenum* which was incubated with 5 μmol ([1-^{14}C]Val) LLD-tripeptide and 20 μmol of DTT.
a: to 1.5 ml of cell-free preparation was added 5 μmol ATP, 5 μmol NAD and the regenerating systems of both.
b: the experiment was carried out with 2.5 ml of cell-free preparation and no cofactors were added.

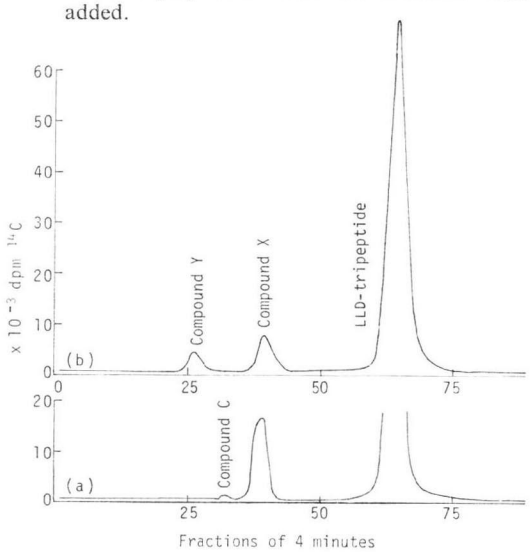


Table 1. [^{3}H]: [^{14}C]-ratio in the LLD-tripeptide, "compound X" and "compound Y" after extraction from a cell-free preparation of *P. chrysogenum* E-15 and analysis on a cation-exchange column.
1.5 ml (A) or 2.5 ml (B) of cell-free preparation was used for incubation with double labelled LLD-tripeptide.

Exp.	Tripeptide	[^{3}H]: [^{14}C]-ratio in the LLD-tripeptide	"Product X"		"Product Y"	
			[^{3}H]: [^{14}C]-Ratio	% Rel. to the ratio in the LLD-tripeptide	[^{3}H]: [^{14}C]-Ratio	% Rel. to the ratio in the LLD-tripeptide
A	([3-^{3}H] CysH-([1-^{14}C] Val) LLD-tripeptide	7.045	2.004	28.4%	—	—
	([3,4-^{3}H-1-^{14}C] Val) LLD-tripeptide	5.945	5.228	87.9%	—	—
	([2-^{3}H-1-^{14}C] Val) LLD-tripeptide	4.980	5.022	100.8%	—	—
B	([3-^{3}H] CysH-[1-^{14}C] Val) LLD-tripeptide	5.038	1.738	34.5%	2.500	49.6%
	([3,4-^{3}H-1-^{14}C] Val) LLD-tripeptide	5.301	4.564	86.1%	5.358	101.1%
	([2-^{3}H-1-^{14}C] Val) LLD-tripeptide	4.662	4.669	100.2%	4.607	98.8%

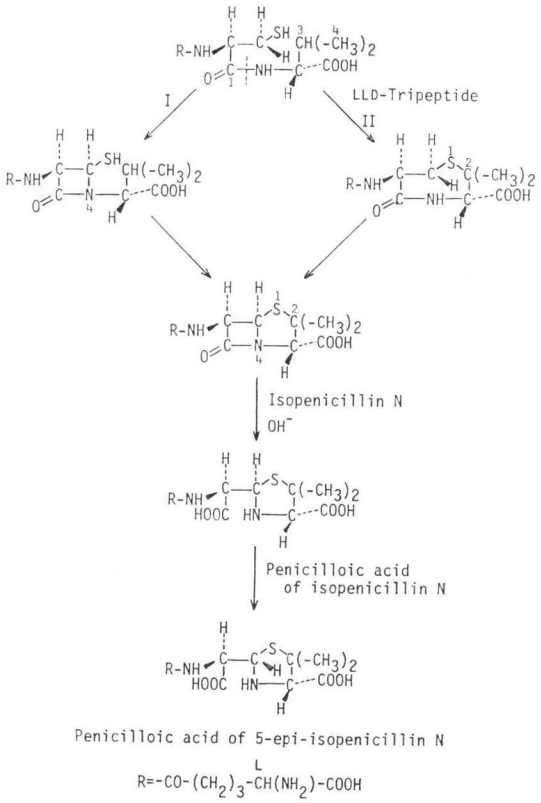
ing systems were omitted from the incubation mixture. Clearly "compound C" was the result of a chemical reaction between the LLD-tripeptide and one of the constituents of the regenerating systems.

However, 8.1% and 11.7% of the recovered activity in the acetone and alkaline extract from the intact system respectively, was present in a product ("compound X") which could not be detected in the extracts of the denatured system. The experiment was repeated several times and the acetone and alkaline extracts were combined. The conversion of LLD-tripeptide to "compound X" ranged from 2 to 8%. When a precursor solution containing 5 μmol of ([1-^{14}C]Val) LLD-tripeptide and all cofactors was incubated with 1 ml of the lytic enzyme solution which was used for protoplast formation, no "compound X" could be detected. This excluded the possibility that conversion of the LLD-tripeptide to "compound X" was due to enzymes of the lytic solution.

In different chromatographic conditions (with and without a Varigrad system) and on two cation-exchange columns[16], "compound X" invariably coeluted with the penicilloic acid of isopenicillin N. In addition, when isopenicillin N was put on the column it was eluted as its penicilloic acid. This suggested that "compound X" was the penicilloic acid of isopenicillin N and was formed from isopenicillin N during extraction or chromatography.

To confirm the structure of "compound X", suspected to be the penicilloic acid of isopenicillin N, 1.5 ml of cell-free preparation was incubated with mixtures of a [^{3}H] labelled LLD-tripeptide and ([1-^{14}C] Val) LLD-tripeptide. After extraction and analysis, the [^{3}H]:[^{14}C]-ratio was determined in the LLD-tripeptide and in "compound X". Eventual loss of tritium in "compound X", due to the appearance of intramolecular chemical bonds during its formation from the LLD-tripeptide, is easily expressed by its [^{3}H]:[^{14}C]-ratios; *e.g.* as per cent relative to the ratio in the LLD-tripeptide. In this way, the results are independent, within experimental error, of the yield of the enzymatic conversion.

Fig. 2. Two mechanisms for the closure of the β-lactam-thiazolidine ring system.
I: *via* a monocyclic β-lactam intermediate.
II: *via* a cyclic cysteinylvaline derivative.
Also, the epimerisation at carbon-5 of the penicilloic acid of isopenicillin N is shown.



Three double-labelled experiments were carried out; ATP and NAD were added. The results are summarized in Table 1A. Total conversion, based upon the [^{14}C] isotope was 6%; the isotope ratios calculated for the LLD-tripeptide agreed with those of the corresponding precursor solutions. The loss of 12.1% of [^{3}H] activity, when "compound X" was formed from ([3,4-^{3}H] Val) LLD-tripeptide, corresponded well with the label which was initially present on position-3 of valine (13.3%) and proved the formation of the S_1-C_2-bond (Fig. 2). The loss of label when "compound X" was formed from ([3-^{3}H] CysH) LLD-

tripeptide (71.6%) is explained by the formation of the β-lactam ring: retention of label should however be 50%[20,21]. These experiments were repeated several times and results were consistent with those reported.

It is clear from the loss of [^{3}H] activity when "compound X" was formed from ([3,4-^{3}H] Val) LLD-tripeptide and ([3-^{3}H] CysH]) LLD-tripeptide, as well as from the position on the chromatogram, that "compound X" is the penicilloic acid of isopenicillin N. Hence, we may conclude that the LLD-tripeptides were converted into isopenicillin N; hydrolysis of isopenicillin N presumably occurred during extraction. The poor retention of label when "compound X" was formed from ([3-^{3}H] CysH) LLD-tripeptide (28.4%) is explained by epimerization at carbon-5 of the penicilloic acid of isopenicillin N (Fig. 2); BUSSON *et al.*[22] reported the spontaneous formation of the 5-epibenzylpenicilloate from the natural isomer.

The isotope ratio in the penicilloic acid of isopenicillin N, when it was formed from ([2-^{3}H-1-^{14}C] Val) LLD-tripeptide, showed that no dehydrovalinyl intermediate was involved in the biosynthesis of isopenicillin N. FAWCETT *et al.*[2] also reported that the isotope of ([2-^{3}H] Val) LLD-tripeptide was retained when the compound was transformed into (iso)penicillin N by a cell-free preparation of *C. acremonium*. These results suggest the mechanism of penicillin formation in *P. chrysogenum* and in *C. acremonium* to be similar.

Biosynthesis of Isopenicillin N

When large samples of cell-free preparation (2.5~3 ml) and a constant amount of substrate (5 μmol) and DTT (20 μmol) were used, a new product ("compound Y") was detected; Fig. 1b shows the position of this compound. We assumed that the occurence of "compound Y" was related to the relative concentration of DTT (ratio of enzyme to DTT). Indeed, after the addition of an extra amount of DTT, to a duplicate of an incubation which produced "compounds X and Y", only "compound X" was detected. Also after denaturation by heat of a duplicate of a system which produced both compounds, only the LLD-tripeptide could be detected. After the addition of ATP and/or NAD more of the penicilloic acid of isopenicillin N was detected; this indicated that isopenicillin formation from the LLD-tripeptide was stimulated. In contrary the amount of "product Y" diminished after the addition of NAD and/or ATP (Table 2). From these results we suggest that "compound Y" is an intermediate in the formation of isopenicillin N from the LLD-tripeptide and that conversion of "compound Y" to isopenicillin N is DTT dependent.

Two different mechanisms were suggested for the closure of the bicyclic β-lactam-thiazolidine ring system (Fig. 2). In the first possibility, the formation of the (N_4-C_5) bond would lead to a monocyclic β-lactam ring intermediate; this mechanism was suggested by DEMAIN[23]. In a second pathway, the closure of the (S_1-C_2) bond would be the first reaction involved; in this way a cyclic cysteinylvaline structure would be the intermediate between the LLD-tripeptide and isopenicillin N[24,25]. These two mechanisms can be easily distinguished using double-labelled peptides.

Table 2. Effect of different cofactors on the enzymatic conversion of ([1-^{14}C] Val) LLD-tripeptide to "compounds X and Y" by a cell-free preparation of *P. chrysogenum* E-15.

For details see preparation of precursor solutions. Results are given as % of total recovered activity.

	% X	% Y	% X / % Y	% Enzymatic conversion
Without cofactors	4.20	2.51	1.67	6.71
+NAD	5.01	2.28	2.20	7.29
+ATP	4.80	1.47	3.27	6.27
+NAD+ATP	6.54	1.61	4.06	8.15

The monocyclic β-lactam intermediate would retain the isotope on carbon-3 of valine but would lose half of the isotope on carbon-3 of cysteine. On the contrary the cyclic cysteinylvaline intermediate would retain all isotope on carbon-3 of cysteine; but would lose the isotope on carbon-3 of valine.

Similar experiments as those described for "compound X" were set up with 2.5 ml samples of cell-free preparation being used; no NAD or ATP was added. "Compound Y" was present (4% of the recovered activity based on the [^{14}C] isotope), together with the penicilloic acid of isopenicillin N (only 2% of the recovered [^{14}C] activity). The experimental data are summarized in Table 1B. The isotope ratios in the penicilloic acid of isopenicillin N agreed with its structure. The ratios in "compound Y" showed that half of the tritium on carbon-3 of cysteine was lost and that the isotope on carbon-3 of valine was retained. These data were in agreement with a monocyclic β-lactam structure and excluded a cyclic cysteinylvaline structure for "compound Y". This result could be confirmed in similar experiments.

Furthermore, the full retention of label from ([3,4-^{3}H] Val) LLD tripeptide excluded the possibility that "compound Y" was a peptide containing β-hydroxyvaline or a degradation product of isopenicillin N (or any other penicillin). Such a peptide, with β-hydroxyvaline and an additional glycine, was isolated from *C. acremonium*[1] and from *Paecilomyces persicinus*[26].

However besides the formation of a monocyclic β-lactam intermediate, some other reactions may also explain the isotope ratio in "compound Y" when it is formed from ([3-^{3}H] CysH-[1-^{14}C] Val) LLD-tripeptide. Hydroxylation at position-3 of the cysteine part as well as formation of a double bond between carbon-2 and -3 of the cysteine part would result in loss of half of the [^{3}H] activity. Both of these reactions would lead to compounds which are not intermediate in the biosynthesis of isopenicillin N. In the first case, elimination of H_2S would lead to an aldehyde. On the other hand, ARNSTEIN and CRAWHALL[20] reported that an α-β-dehydrocysteinyl intermediate is not involved in the biosynthesis of penicillin. "Compound Y" is formed at a low concentration of DTT. We cannot exclude that this reaction condition favours one of the above reactions, but suggest that "compound Y" is an intermediate in the formation of isopenicillin and that the conversion of "compound Y" to isopenicillin is DTT dependent. BALDWIN and WAN[27] proposed a carbon radical at the isopropyl part of a monocyclic β-lactam intermediate which is trapped by a disulphide linkage; such a disulphide may arise from the product itself and a thiol-containing enzyme. The extreme sensitivity to DTT of the isopenicillin N formation may be an indication for such a mechanism.

From the isotope ratios it is also obvious that "compound Y" does not correspond to a dehydrovalinyl structure. The retention of isotope at the asymmetric centre of valine in both "compound Y" and isopenicillin N, is in agreement with a precursor product relationship between "compound Y" and isopenicillin N.

The obtained isotope ratios in "compound Y" indicate that the product may correspond to a monocyclic β-lactam intermediate. Monocyclic β-lactams, in general, are rather stable molecules and decomposition is not expected to arise during extraction and column chromatography. The position on the chromatogram, relative to the penicilloic acid of isopenicillin N and to the LLD-tripeptide, is probably explained by the strong acidic properties of a thiol function on a β-lactam ring.

The monocyclic β-lactam ring intermediate (=1-(1-D-carboxy-2-methylpropyl)-3-L-(5-L-aminoadipamido)-4-L-mercaptoazetidin-2-one) was prepared by ROETS *et al.* (to be published). On analysis of our cation-exchange column this product behaved as "compound Y". This proved that "compound Y" indeed was the monocyclic β-lactam ring intermediate and that β-lactam ring formation is the first step in the cyclization of the LLD-tripeptide to isopenicillin N.

Other Reactions in the Cell-free Preparation

Although the mycelium was thoroughly washed before being transformed into protoplasts and although the protoplasts were resuspended twice in 0.7 M NaCl, the cell-free preparations expressed some antibiotic acitivity (equivalent to 1~4 μg penicillin G/ml). This activity was due to penicillins, since it rapidly disappeared upon the addition of a β-lactamase (Neutrapen), and corresponded to the penicillins which were present intracellularly. Since mycelium of *P. chrysogenum* E-15 was developed in a medium without added side chain precursor, the penicillins present were a mixture of the penicillins F, FH_2 and K[28]. Penicillin slowly disappeared from the preparation (pH 6.5~7). This loss of antibiotic activity was stimulated at a higher pH-value (pH 8); but activity was more stable at pH 5.5. These reaction features correspond to those of a penicillin acylase (=amidase) for which the aliphatic penicillins are good substrates[29]. It was thus not surprising that addition of phenylacetic acid and [^{3}H] labelled 6-aminopenicillanic acid (=6-APA) to the cell-free preparation did not result in the formation of penicillin G, since the reaction conditions induced deacylation. Also [^{14}C] phenylacetic acid was not used for the formation of penicillin G from isopenicillin N which was added or was biosynthesized from ([2-^{3}H] Val) LLD-tripeptide.

Simultaneous addition of ([1-^{14}C] Val) LLD-tripeptide and [^{3}H] labelled 6-APA resulted in double labelled penicilloic acid of isopenicillin N. Also when unlabelled 6-APA was added together with ([2-^{3}H] Val) LLD-tripeptide, 6-APA and its degradation products became [^{3}H] labelled. It can thus be concluded that an equilibrium exists between 6-APA and isopenicillin N. Additional proof for this equilibrium and for the nature of "compound X" was obtained after incubation of 1.5ml of cell-free preparation with 20 μmol DTT, and 1 μmol of both isopenicillin N and [^{3}H] labelled 6-APA: after extraction and column chromatography 10% of the [^{3}H] activity was present in "compound X", the penicilloic acid of isopenicillin N.

ABRAHAM[30] has reported an enzyme which converted isopenicillin N into 6-APA and α-aminoadipic acid. The equilibrium between 6-APA and isopenicillin N suggests an enzyme with multiple activities and explains the result of FAWCETT *et al.*[31], who reported that isopenicillin N and 6-APA were equivalent precursors of penicillin G. Further studies are however necessary to clarify the exact role of isopenicillin N and 6-APA in the biosynthesis of penicillins.

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