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Presence of δ-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine in Fermentations of *Penicillium chrysogenum*

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Cultures of *Penicillium chrysogenum*, grown with [^{35}S]sulfate or labeled amino acids, were examined by ion-exchange chromatography for possible peptidic precursors of penicillin. A sulfur-containing compound, present in both the mycelial extracts and the culture filtrates, was eluted at the location of the synthetic LLD-tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. Since this compound was also labeled when the cultures were incubated with DL-[6-^{14}C]α-aminoadipic acid, L-[3,3'-^{3}H]cystine, or DL-[1-^{14}C]valine, its identity with the synthetic LLD-tripeptide can be accepted. No δ-(L-α-aminoadipyl)-L-cysteine or LLL-tripeptide were detected. The implications of these findings for tripeptide and penicillin biosynthesis are discussed.

In 1960, Arnstein et al. (1, 3) described the isolation of δ-(α-aminoadipyl)-cysteinyl-valine from mycelial extracts of *Penicillium chrysogenum*. Bauer (4) observed that a cell-free system of the mold catalyzed the synthesis of the tripeptide from the constituent amino acids. Later, the tripeptide was also found in a cephalosporin C-producing *Cephalosporium* sp. (19, 24, 25). Because of the close structural relationship with the later discovered isopenicillin N (5, 10), this tripeptide was considered as an intermediate in the biosynthesis of penicillin (7).

Although the configuration of the amino acids of the tripeptide was not determined, it was assumed that all amino acids were L-enantiomers, for α-aminoadipic acid and cysteine because this matches the configuration of the corresponding part of the isopenicillin N molecule and for valine because L-valine is a better penicillin precursor than D-valine (2, 6, 21, 22, 25). Since the chiral center of the valine-derived part of penicillin has the D-configuration, it was assumed that inversion occurred at some further step of the biosynthesis. More recently, however, it was shown that the tripeptide of *Cephalosporium* has the LLD-configuration (9, 16). This LLD-peptide is now considered to be a possible precursor of penicillin and cephalosporin (8).

The purpose of this study was to determine whether the tripeptide of *P. chrysogenum* also has the LLD-structure. In addition, we investigated whether the peptide is excreted in the culture medium and if it is present in a non-penicillin-producing strain.

MATERIALS AND METHODS

Radiochemicals. [^{35}S]sulfuric acid, DL-[6-^{14}C]α-aminoadipic acid, and DL-[1-^{14}C]valine were obtained from I.R.E., Mol, Belgium. L-[3,3'-^{3}H]cystine was a product of The Radiochemical Centre, Amersham, England.

Peptides. The LLL- and LLD-isomers of δ-(α-aminoadipyl) cysteinyl-valine were prepared by the following procedure. Bis-δ-(L-α-aminoadipyl)-L-cystinyl-bis-L-valine was obtained by condensing the mixed anhydride of L-1-benzyl-2-carbobenzyloxyaminoadipic acid with L-cystinyl-bis-L-valine. Removal of the protecting groups was carried out with NaOH, followed by HBr in acetic acid and neutralization with aniline. The peptide was pure on paper chromatography and electrophoresis: $[\alpha]_D = -53°$ (C = 2.0, 2 N HCl). Bis-δ-(L-α-aminoadipyl)-L-cystinyl-bis-D-valine was prepared by the same method using L-cystinyl-bis-D-valine: $[\alpha]_D = -9.5°$ (C = 2.0, 2 N HCl). The LLL and LLD-tripeptides were obtained from the respective cystine-peptides by reduction with dithiothreitol in anaerobic conditions. Details of the synthesis will be published elsewhere.

Culture conditions. All fermentations were carried out in 300-ml Erlenmeyer flasks at 27 C and 300 rpm on a rotary shaker. For incubations with ^{35}S, 3.10^7 conidia from 6-day-old cultures of *P. chrysogenum* Wis. 49-2105 or Wis. 49-408 grown on a complete medium (17) were used to inoculate 50 ml of the sulfur-free medium of Halliday and Arnstein (12). Labeled sulfate was added after 24 h; L-valine and L-α-aminoadipic acid (50 μmol of each) were added after 36 h. The mycelia were harvested after 60 h.

To study the incorporation of labeled amino acids, 3.10^7 conidia of *P. chrysogenum* Wis. 49-2105 were used to seed 50 ml of the medium of Jarvis and Johnson (14). After 48 h, the mycelia were

filtered on sterile Whatman no. 1 filter papers and washed three times with sterile water. The mycelia were suspended in 40 ml of Jarvis and Johnson medium, and 10 ml of a sterile solution of the radioactive precursors was added. The cultures were further incubated for 24 h; $^{14}CO_2$ was trapped in two wash bottles containing 40% KOH.

Mycelial extracts. The mycelium of each Erlenmeyer flask was filtered off and washed twice with water; the mixture of culture medium and wash waters is further designated as the culture filtrate. After drying in air, the mycelial mat was ground successively with 5 ml of acetone, 10 ml of 75% acetone, and 15 ml of 80% ethanol, leaving a residue of extracted mycelium. To the combined extracts 2.5 μmol of each of the nonlabeled LLL- and LLD-tripeptides was added as carrier; this solution is further designated as the mycelial extract. For ion-exchange chromatography it was necessary to concentrate the mycelial extract to a volume of 1 to 2 ml. The excess of proteins was removed by addition of 30 to 40 mg of sulfosalicylic acid and centrifugation. The clear supernatant was adjusted to pH 8 with 1 N LiOH and was stored under anaerobic conditions after the addition of 50 mg of dithiothreitol for reduction of cystine-peptides.

Chromatographic conditions. The equipment consisted of a Technicon amino acid analyzer with a 140- by 0.6-cm column and Cromobeads type B in the Li^+ form. The composition of the autograd is shown in Table 1. Buffers of pH 3.01 and 6.50 were prepared as described by Vega and Nunn (23); buffer of pH 2.75 was obtained by acidification with 6 N HCl of the pH 3.01 buffer. Samples (0.3 to 1.0 ml) of the concentrated mycelial extracts or of the culture media were acidified with 10 to 25 μl of 6 N HCl and buffered with 0.20 M lithium citrate, pH 2.20, before loading on the column. Elution was carried out at 37 C with a flow rate of 32 ml/h for 8 h and at 60 C with a flow rate of 40 ml/h for the remainder of the chromatogram (back pressure, 400 lb/in^2). Part of the effluent of the column was used for color development with ninhydrin; the rest of the effluent was directed by a stream splitter to a fraction collector; fractions were taken at 2.5-min intervals. After each run, the column was washed with 0.3 N LiOH for 2 h at 70 C and regenerated with buffer of pH 2.75 for 1 h.

Radioactivity measurements. Counting was performed in a Packard Tri-Carb liquid scintillation spectrometer, model 3390, with absolute activity analyzer, model 544. Standardization was done with [^{3}H]- and [^{14}C]*n*-hexadecane (Packard) and [^{35}S]H_2SO_4 (I.R.E.). All samples labeled with ^{35}S and all fractions of the amino acid analyzer were diluted with water to 5 ml and emulsified with 10 ml of Instagel (Packard). All other samples were combusted with a Packard sample oxidizer, model 306 (Packard Instrument Co., Inc., Downers Grove, Ill.).

TABLE 1. *Gradient for the nine-chambered autograd*

Chamber no.	Buffer 1 pH 2.75 (ml)	Buffer 2 pH 3.01 (ml)	Buffer 3 pH 6.50 (ml)
1	99[a]	—[b]	—
2	50	50	—
3, 4, 5, 6	—	100	—
7, 8, 9	—	—	100

[a] Plus one milliliter of isopropyl alcohol.
[b] —, None.

RESULTS

The isotopes used and their distribution after fermentation are given in Table 2. Incorporation of ^{35}S was tested with two strains of *P. chrysogenum*. Strain Wis. 49-408 does not produce penicillin; strain Wis. 49-2105 produces penicillin in media supplemented with side chain precursors. In cultures of strain Wis. 49-2105 the radioactivity was evenly distributed between culture filtrate, mycelial extract, and extracted mycelium. In strain Wis. 49-408 the isotope balance was in favor of the extracted mycelium; this indicated a higher turnover of sulfur-containing amino acids into mycelial proteins.

Incorporation of radioactive amino acids was studied in two double-labeled experiments; L-[3,3'-^{3}H]cystine was added once with DL-[6-^{14}C]α-aminoadipic acid and once with DL-[1-^{14}C]valine. In this way, compounds that incorporated label of one or more of the constituent amino acids of the tripeptide were easily detected. Recovery of the isotopes (Table 2) ranged from 81 to 92%, except for the label of DL-[1-^{14}C]valine. In that case, more than 50% of the label was lost by decarboxylation, since approximately 50% of the added radioactivity was recovered in the CO_2 trap. The high carbon-14 content of the culture filtrates after feeding of DL-[6-^{14}C]α-aminoadipic acid indicated a poor uptake of the amino acid, presumably of the D-isomer (24). Between 60 and 68% of the tritium of L-[3,3'-^{3}H]cystine was recovered in the culture filtrate. This was probably due to partial degradation of the amino acid to tritiated water since, in unpublished experiments, it was found that the label of L-[U-^{14}C]cystine was well incorporated into the mycelium.

The ninhydrin flow chart up to valine, together with the isotope tracings for sulfate and labeled amino acids, of a mycelial extract from *P. chrysogenum* Wis. 49-2105, is given in Fig. 1. The common physiological amino acids were all present in the extract. Glutamic acid and alanine were the major components, whereas 10 ninhydrin-positive peaks could not be identified.

The ^{35}S distribution in the mycelial extracts was very similar for the producing and the non-

TABLE 2. *Added isotopes and their distribution in P. chrysogenum fermentations*

Isotope	Added labeled precursor		Sp act (mCi/mmol)	Radioactivity (%) in:		
	μmol	μCi		Culture filtrate	Mycelium extract	Extracted mycelium
P. chrysogenum Wis. 49-408						
$(NH_4)_2{}^{35}SO_4$[a]	101.8	1,607.4	15.8	24.5	22.7	43.5
P. chrysogenum Wis. 49-2105						
$(NH_4)_2{}^{35}SO_4$[a]	101.8	1,607.4	15.8	34.5	32.0	26.3
DL-[6-^{14}C]Aad + L-[3,3'-^{3}H]Cys[b]						
^{14}C	19.9	502.8	25.3	45.4	9.2	26.1
^{3}H	14.6	486.1	33.3	68.2	6.7	16.9
DL-[1-^{14}C]Val + L-[3,3'-^{3}H]Cys[c]						
^{14}C	16.6	547.9	33.0	5.6	2.8	35.3
^{3}H	14.4	482.2	33.3	60.7	4.7	18.5

[a] Added nonlabeled precursor: L-valine and L-α-aminoadipic acid, 50 μmol each.
[b] Added nonlabeled precursor: DL-valine, 14.5 μmol.
[c] Added nonlabeled precursor: DL-α-aminoadipic acid, 20.8 μmol.

producing strains. Cysteic acid, taurine, glutathione, methionine, and cystathionine were the major sulfur-containing compounds; no cysteine or cystine could be detected. In addition, a number of unknown ^{35}S peaks were recorded. The extracts of both strains contained a sulfur compound with the same retention time as the synthetic LLD-tripeptide in the sulfhydryl form; this peptide is eluted with glutamic acid. In the analytical system used, none of the tested sulfur compounds is eluted in that part of the chromatogram; the penicilloic acid of isopenicillin N and γ-(L-glutamyl)-L-cysteine are eluted between glutathione and threonine, whereas the synthetic LLL-tripeptide is eluted before the LLD-tripeptide, between serine and asparagine.

To confirm the identity of this sulfur compound with the LLD-tripeptide, feeding experiments were carried out with the constituent radioactive amino acids. Since the peak at the location of the synthetic LLD-tripeptide also incorporated label from DL-[6-^{14}C]α-aminoadipic acid, L-[3,3'-^{3}H]cystine, and DL-[1-^{14}C]-valine, it can be concluded that the LLD-tripeptide is present in mycelial extracts of *P. chrysogenum*.

In contrast, no LLL-tripeptide was detected in the extract. None of the dipeptides, neither δ-(L-α-aminoadipyl)-L-cysteine (running with serine) nor L-cysteinyl-DL-valine (eluted after β-aminoisobutyric acid), could be traced. Several unidentified peaks in the top fractions of the chromatogram were labeled with all the radioactive precursors. It cannot be excluded, however, that these peaks were mixtures, not pure compounds, because the resolution is not optimal in that part of the chromatogram.

The common amino acids were present in lower concentrations in the culture filtrate than in the mycelial extract. After feeding labeled sulfate, the ^{35}S tracing of the culture filtrate was as complex as that of the mycelial extract and contained several unknown compounds. Besides cysteic acid and taurine, only the LLD-tripeptide was identified in the first part of the chromatogram. Since in a nearly sulfur-free basal medium the tripeptide can be assumed to have the same specific activity as the added [^{35}S]sulfate, it could be estimated from the total radioactivity of the peak that the amount of LLD-tripeptide in the culture medium (160 nmol/Erlenmeyer flask) was approximately twice as high as that in the mycelial extract (70 nmol/Erlenmeyer flask). In contrast, only trace amounts of glutathione were excreted in the culture medium, although high concentrations were present in the mycelial extract.

DISCUSSION

The high resolution power of the ion-exchange chromatography system made it possible to analyze total mycelial extracts and culture filtrates without preliminary separations or purifications, which could lead to important losses. The sensitivity and specificity of the analytical system were further increased by feeding labeled precursors. Two strains of *P. chrysogenum* were examined. Both strains produced a sulfur-containing compound which was eluted at the location of synthetic δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. This substance also incorporated the labels from DL-[1-^{14}C]-valine, DL-[6-^{14}C]α-aminoadipic acid, and L-[3,3'-^{3}H]cystine. Hence, all available evidence indicates that these *Penicillium* strains produce the LLD-tripeptide, i.e., with the same

configuration as the tripeptide isolated from *Cephalosporium* (9, 16).

It has been assumed (8) that the LLD-tripeptide can be formed by the addition of valine to δ-(L-α-aminoadipyl)-L-cysteine (further designated as L-Aad-L-CysH). However, this dipeptide was not detected in our cultures of *P. chrysogenum* and has not been found in *Cephalosporium* (16). In the biosynthesis of glutathione, glycine is added to γ-(L-glutamyl)-L-cysteine (20); in that case the dipeptide is present as a phosphate anhydride (18). If L-Aad-L-CysH were activated in the same way, its phosphate anhydride would be eluted very rapidly from the resin used in the present investigations. For that reason, further examination of the unidentified labeled compounds in the top fractions of the chromatogram is indicated.

There are three possible routes for the biosynthesis of the LLD-tripeptide from L-Aad-L-CysH. It cannot be excluded that D-valine is used as such, since D-valine has been isolated from the mycelium of *P. chrysogenum* (13). It should be noted, however, that in those experiments the extraction was preceded by acid hydrolysis of the mycelium, so that the occurrence of free D-valine in mycelium is not yet proved. Furthermore, no LLD-peptide was formed from L-Aad-L-CysH and D-valine in cell-free systems of a *Cephalosporium* sp. (9). One could also assume that the LLD-tripeptide is derived from the LLL-tripeptide, although the

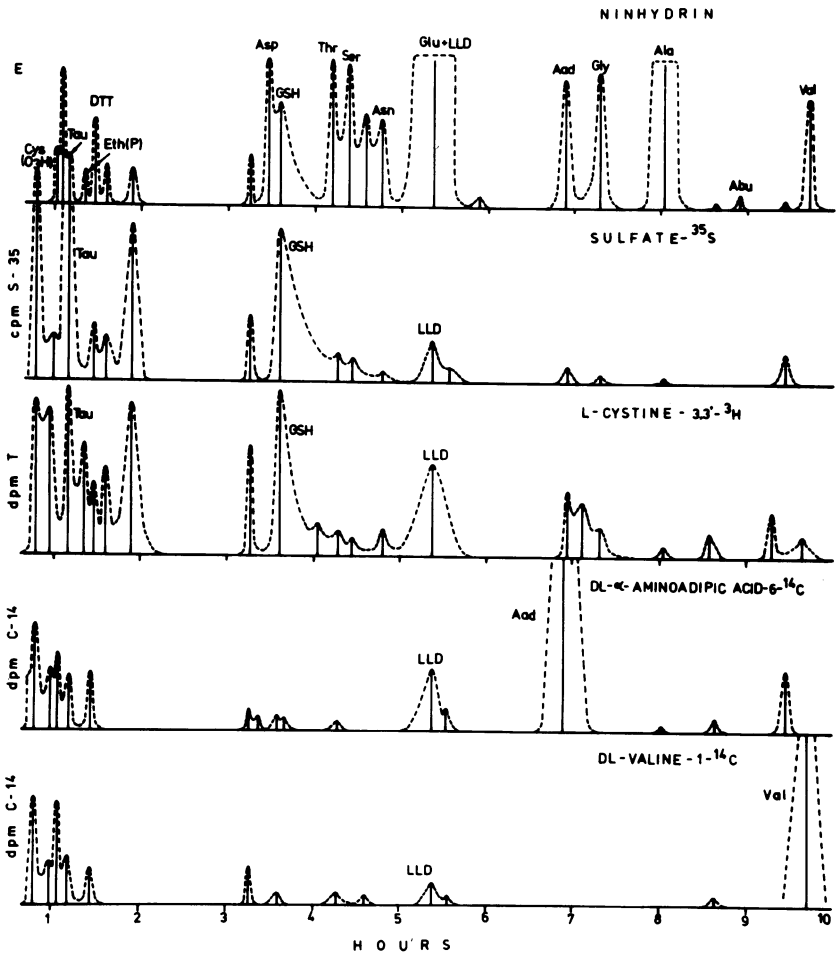

FIG. 1. *Analysis of mycelial extract of P. chrysogenum Wis. 49-2105 by ion-exchange chromatography; first part (up to 10 h) of the ninhydrin flow chart and radioactive tracings after feeding [35S]-sulfate, L-[3,3'-3H]cystine, DL-[6-14C]α-aminoadipic acid, and DL-[1-14C]valine. The following abbreviations were used: Cys (O_3H), cysteic acid; Tau, taurine; DTT, dithiothreitol; Asp, aspartic acid; GSH, glutathione, reduced; Thr, threonine; Ser, serine; Asn, asparagine; Glu, glutamic acid; Aad, α-aminoadipic acid; Gly, glycine; Ala, alanine; Abu, α-aminobutyric acid; Val, valine; LLD, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine.*

latter compound has not yet been detected in mycelial extracts. In contrast, inversion of L-valine and linkage of the D-valine formed to L-Aad-L-CysH might take place on the same enzyme complex, without occurrence of free D-valine or LLL-peptide. This process would be similar to that observed in the conversion of L-phenylalanine to its D-enantiomer in gramicidin S (11, 15). Further studies are needed to clarify these points.

Although the LLD-tripeptide and glutathione have very similar structures, only the former compound was excreted in the culture medium. It is possible that part of the peptide is transformed into penicillin and that the remainder is excreted in the culture filtrate. However, it is also possible that both the LLD-tripeptide and penicillin are excretion products derived from a common precursor, e.g., the LLL-tripeptide, which is rapidly transformed and does not accumulate. In the same way, *P. chrysogenum* Wis. 49-408, which produces LLD-peptide but not penicillin, must be blocked either after the LLD-tripeptide or between the LLL-tripeptide and penicillin. In 1966, it was proposed (7) that the LLL-peptide is transformed into penicillin via an α,β-didehydrovaline intermediate, which would explain the D-configuration of carbon-3 of penicillin. However, if the LLD-peptide were the true penicillin precursor, a hypothetical didehydrovaline intermediate would no longer be necessary to explain the D-configuration of the valine-derived part of penicillin. Further studies are necessary to determine the exact role of the tripeptides in the biosynthesis of penicillin.

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