THE CHEMISTRY OF MOLD TISSUE

XIII. ISOLATION OF SOME MONOAMINOMONOCARBOXY AND SOME MONOAMINODICARBOXY ACIDS FROM ASPERGILLUS SYDOWI*

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For some time we have been investigating the nitrogenous constituents of the mycelium of Aspergillus sydowi by the use of a systematic fractionation scheme. The isolation of the three diamino acids, arginine, histidine, and lysine, during the course of this fractionation has been previously reported (1). In this paper will be described the isolation and identification of two monoaminodicarboxy acids, aspartic and glutamic, and of eight monoaminomonocarboxy acids, leucine, isoleucine, tryrosine, proline, valine, serine, threonine, and tryptophane.

The literature concerning the amino acids of mold mycelium is rather meager. Abderhalden and Rona (2) isolated glycine, alanine, leucine, glutamic acid, and aspartic acid from Aspergillus niger and identified them by analysis. Vorbrodt (3) recognized alanine, leucine, and tyrosine in this same organism. The presence of tyrosine in a limited number of the mold fungi has been postulated on the evidence of color tests (4–6). Similarly the existence of tryptophane in the same organisms has been inferred from colorimetric procedures. The presence of leucine and isoleucine in Aspergillus sydowi has been shown by isolation and analysis of the free acids and by preparation and examination of derivatives (7).

The fractionation scheme used in this investigation was as follows: The amino acids were rendered soluble by autolysis under

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controlled conditions as previously described (1), the solution was treated with mercuric acetate and sodium carbonate, and the nitrogen compounds were freed from the resulting precipitate with $\text{H}_2\text{S}$. The dicarboxyamino acids were then precipitated in the usual manner with barium hydroxide and alcohol; tryptophane, histidine, purines, and pyrimidines were precipitated with $\text{HgSO}_4$, and lysine with phosphotungstic acid. From the resulting filtrate tyrosine was crystallized. Next the unhydroxylated monoamino acids were extracted with butyl alcohol, and serine and threonine were obtained from the residual aqueous solution. The separation of the latter two acids was possible mainly because the threonine was present as a complex which formed a copper salt readily soluble in ethyl alcohol.

The isolation of tryptophane was difficult, for there appeared to be a wide variety of compounds present in the mycelium which were precipitated in sulfuric acid solution by $\text{HgSO}_4$ and were also extracted from neutral aqueous solution by butyl alcohol. Fractionation of the butyl alcohol extract with copper and silver salts failed to yield pure tryptophane, but the actual isolation was eventually effected by butyl alcohol extraction, first from acid, then from alkaline, and finally from neutral solution.

The amounts of the various amino acids isolated must not be regarded as the amounts present in the mycelium, but rather as the minimum quantities there. For example, frequent use was made of mercuric acetate and sodium carbonate to precipitate the amino acids, but these reagents do not quantitatively precipitate such compounds, as was shown by the fact that it was possible to isolate amino acids from some of the resulting filtrates. Further, considerable nitrogen was lost by adsorption on the sulfides resulting from the decomposition of such precipitates. When it was possible to isolate an amino acid by a more or less direct method, as in the case of proline, the amount obtained was much greater than that found in the course of the rather complicated fractionation scheme necessary to isolate the other amino acids. In Table I are listed the quantities of the amino acids isolated in this investigation together with the percentage of the total nitrogen of the mycelium and of the sodium carbonate-mercuric acetate precipitate which they contain. For completeness the amounts of the diamino acids previously reported (1)
are also listed. It can be seen that 17.1 per cent of the total nitrogen of the mycelium or 47.7 per cent of that of the sodium carbonate-mercuric acetate precipitate has been obtained as definite compounds. It is interesting to note the relatively large amounts of the hydroxyamino acids, serine and threonine, which were found.

The presence of phenylalanine, glycine, and hydroxyproline could not be detected even by color reactions. For example, an aliquot of the nitrogenous compounds precipitable by mercuric acetate and sodium carbonate in an autolysate corresponding to 1.1 gm. of dry mycelium gave no Kapeller-Adler test (8) for phenylalanine, while if 1 mg. of phenylalanine was added to a similar aliquot and the test repeated, a deep purple color was obtained. Subsequent tests on the monoamino fraction likewise failed to reveal any phenylalanine. The monoamino fraction gave no test for glycine with o-phthalic dialdehyde (9), and failed to give any hydroxyproline test (10). No evidence for the presence of alanine was found during the copper salt fractionation. It is not, of course, justifiable to conclude that these amino acids were not present.
acids are not present in the mycelium, for they may have been destroyed during autolysis.

**EXPERIMENTAL**

The mold was grown and autolyzed as described in a previous paper (1). For this investigation 2 kilos of dry mycelium, containing 103 gm. of nitrogen, were used. After autolysis 64.5 gm. of nitrogen were soluble, of which 10.3 per cent was ammonia nitrogen. The latter was removed by making the solution alkaline and concentrating under reduced pressure. The concentrate was then precipitated with mercuric acetate and sodium carbonate as previously described (1) and it was found that 9.0 gm. of nitrogen were not precipitated. When the precipitate was decomposed with H₂S, and the HgS was filtered and washed, there were 37.4 gm. of nitrogen in the filtrate. Tyrosine was determined on an aliquot of this filtrate by the method of Folin and Ciocalteu (11). A total of 12.0 gm. was indicated, and in subsequent operations 8.4 gm. were actually isolated.

The solution was concentrated under reduced pressure to 5 liters, and an excess of hot, saturated barium hydroxide was added. On the following day the precipitate was collected, washed with dilute barium hydroxide, and discarded, since it was practically nitrogen-free. The filtrate and washings were concentrated under reduced pressure to 1.5 liters, and treated with barium hydroxide and alcohol in the usual manner for the separation of the dicarboxyamino acids (12). The precipitate was allowed to form for a week, and then filtered, washed with alcohol, dissolved in water, and reprecipitated with alcohol. After a week the purified precipitate was filtered and washed with alcohol, and the filtrate and washings were combined with those from the first barium hydroxide-alcohol precipitate.

**Isolation of Aspartic Acid**—The alcohol-insoluble barium salts were suspended in 1.5 liters of water, H₂SO₄ was added until the solution was strongly acid to thymol blue, and the mixture was heated on the steam bath for 10 hours. This acid treatment was necessary in order to hydrolyze a water-soluble, non-reducing carbohydrate complex which was present. Barium sulfate was filtered off and washed, and the filtrate was concentrated under reduced pressure to about 1 liter, made to 5 per cent acid with
H₂SO₄, and treated with an excess of HgSO₄ in 5 per cent H₂SO₄. The precipitate which formed was filtered and washed with a dilute solution of the precipitating reagent. Since the solution resulting when the precipitate was decomposed with H₂S did not give a ninhydrin reaction, further investigation of this fraction will not be described here. The filtrate from the HgSO₄ precipitate was freed of mercury with H₂S and the resulting filtrate was treated with mercuric acetate and sodium carbonate in order to separate the amino acids from the sugar present. After the precipitate was decomposed with H₂S there were 3.0 gm. of nitrogen in the solution; 330 mg. of nitrogen were left in the filtrate. The solution of amino acids was concentrated under reduced pressure to about 750 cc., boiled with an excess of copper hydroxide, centrifuged, and cooled. Copper aspartate slowly crystallized out. After 2 months the crystals were filtered and washed; 9.0 gm. were obtained. When the filtrate was concentrated to dryness and treated with water, no residue remained undissolved.

C₈H₁₉O₄N₂Cu·3H₂O. Calculated, N 5.62; found, N 5.63

This amount of copper aspartate is equivalent to 4.12 gm. of aspartic acid.

The copper salt was decomposed with H₂S, and the free acid crystallized from dilute alcohol.

C₆H₁₇O₄N. Calculated. N 10.5, neutral equivalent 133

Found. " 10.7, " 133

The neutral equivalent was determined by titration in aqueous solution.

Isolation of Glutamic Acid—The filtrate from copper aspartate was freed of copper with H₂S, concentrated under reduced pressure to about 100 cc., and saturated with HCl at 0°. No crystals formed until the solution had stood in the refrigerator for about 2 weeks. After a month had elapsed, the crystals were filtered, washed with concentrated HCl, and dried. The filtrate was concentrated under reduced pressure to about 40 cc., again saturated with HCl, and allowed to stand in the refrigerator for a month. The crystals were separated as before, and it was found that a
total of 2.57 gm. of the hydrochloride, corresponding to 2.0 gm. of glutamic acid, was obtained.

C₅H₅O₄N·HCl. Calculated, N 7.65, Cl 19.3; found, N 7.60, Cl 19.4

The crystals melted at 210° when heated rapidly. Glutamic acid hydrochloride melts at 210° under the same conditions (13).

A portion of the hydrochloride was decomposed with Ag₂O, excess silver was removed from the filtrate with H₂S, and the resulting filtrate was concentrated under reduced pressure. When alcohol was added, crystals of the acid separated.

C₅H₅O₄N. Calculated, N 9.51; found, N 9.50

**Removal of Basic Substances**—The combined filtrates and washings from the alcohol-insoluble barium salts were concentrated to about 3 liters under reduced pressure to remove alcohol, and enough H₂SO₄ was added to give a concentration of 5 per cent. After the BaSO₄ was removed, HgSO₄ in 5 per cent H₂SO₄ was added until precipitation ceased. The precipitate was allowed to form for a week, and was then filtered and washed with dilute H₂SO₄. Considerable fractionation of the material precipitated by HgSO₄ was carried out with the aim of isolating tryptophane, but since no pure tryptophane was isolated from this run, these procedures will not be described. Suffice it to say that besides giving various color reactions for tryptophane, one of the fractions yielded a small amount of 3,4,5,6-tetrahydro-4-carboline-5-carboxylic acid (14). Also from some of these fractions a total of 240 mg. of recrystallized tyrosine (7.85 per cent N, calculated 7.74) was obtained. The isolation of tryptophane from another run is described below.

The filtrate from the HgSO₄ precipitate was freed of mercury with H₂S, but during this step 19 per cent of the solution was accidentally lost. The filtrate from HgS was then treated with phosphotungstic acid and the resulting filtrate was freed of reagents in the usual manner. Since considerable ash was found in the solution at this point, the amino acids were precipitated with mercuric acetate and sodium carbonate; 1.38 gm. of nitrogen were not precipitated. In order to recover some of this nitrogen, the filtrate was acidified with HCl, treated with H₂S, filtered, concentrated under reduced pressure to dryness, and extracted.
with alcohol. The alcohol was evaporated under reduced pressure and the residue dissolved in water. The solution was adjusted to pH 6.5 with NaOH and extracted continuously under reduced pressure with butyl alcohol. The extracted amino acids were separated and identified by analysis, as described below for the main fraction. On correction for the 19 per cent loss mentioned above, 1.48 gm. of leucine, 254 mg. of proline, and 1.46 gm. of isoleucine plus valine would have been obtained.

Isolation of Tyrosine—After the mercuric acetate-sodium carbonate precipitate was decomposed with H₂S, 10.10 gm. of nitrogen, 9.04 gm. of which were α-NH₂ nitrogen (Van Slyke), were found in solution. This solution was concentrated under reduced pressure to about 400 cc., allowed to stand overnight, and filtered. The crystals were recrystallized from water and the filtrate was added to the main filtrate. 6.6 gm. of typical tyrosine crystals were obtained.

C₉H₇O₂N. Calculated, N 7.74; found, N 7.70

The ethyl ester hydrochloride was prepared from 200 mg. of the crystals and was found to melt at 161–162°. Authentic tyrosine ethyl ester hydrochloride in the same bath melted at 162°. On correction for the 19 per cent loss and addition of the amount isolated from the HgSO₄ precipitate, 8.4 gm. of tyrosine were obtained. As mentioned above, 12.0 gm. were indicated by a colorimetric analysis.

Isolation of Leucine—The tyrosine filtrates were concentrated under reduced pressure to 350 cc. and extracted continuously under reduced pressure with butyl alcohol until no more material was observed to precipitate in the boiling flask. The residual aqueous solution was reserved for the isolation of serine and threonine. The butyl alcohol was removed from the extract under reduced pressure, and the residue dissolved in hot water. The amino acids were converted to their copper salts by heating with copper hydroxide and the excess of the latter was centrifuged down and washed. The solution was concentrated to dryness and the residue was washed with water until the washings were only pale blue. 13.2 gm. of leucine copper salt (8.75 per cent N, calculated 8.66) remained, and 1.1 gm. more were obtained from the methyl alcohol-insoluble copper salts, as de-
scribed below. Further, the excess copper hydroxide contained considerable leucine copper salt, for after it had been decomposed with H₂S, 9.7 gm. of leucine were obtained by concentrating the filtrate from the CuS and adding alcohol. When a correction is made for the 19 per cent loss mentioned above, it can be seen that a total of 26.2 gm. of leucine would have been obtained, and on addition of that isolated from the second mercuric acetate-sodium carbonate filtrate, it follows that the total yield was 27.7 gm. The identity of this amino acid was further established in the same manner as previously described (7).

Water-Soluble, Methyl Alcohol-Insoluble Salts—The filtrate from the leucine copper salt was evaporated and thoroughly dried in vacuo, and the dry salts were powdered and repeatedly extracted with cold methyl alcohol until the extract was only pale blue. The combined extracts were reserved for the isolation of isoleucine, valine, and proline. The insoluble portion was then extracted with water, but a residue of 1.1 gm. of what proved to be leucine copper salt remained undissolved. The water-soluble portion was again dried and fractionally crystallized from water and from methyl alcohol; 0.4 gm. of a mixture of the copper salts of the isomeric leucines (8.62 per cent N, calculated 8.66) and 5.0 gm. of a mixture containing considerable ash were obtained. The nitrogen in the latter mixture amounted to only 110 mg. No pure compounds were obtained from the water-soluble, alcohol-insoluble salts. It is reasonable to assume that this fraction contained small amounts of tyrosine, serine, threonine, and the leucines.

Isolation of Proline—The methyl alcohol-soluble copper salts when dry proved to be completely soluble in methyl alcohol, so they were dissolved in water and treated with H₂S. The resulting filtrate, which contained 1.27 gm. of nitrogen, of which 1.04 gm. would react in the Van Slyke determination, was concentrated under reduced pressure, treated with alcohol, filtered, concentrated to dryness, and allowed to stand with absolute alcohol. The insoluble material was reserved for the isolation of isoleucine and valine. The alcohol was removed from the filtrate under reduced pressure and the residue was dissolved in 0.25 N H₂SO₄, treated with 9.5 gm. of ammonium rhodanilate (15), and placed in the refrigerator. After an hour the crystals were filtered and
washed; 8.0 gm., corresponding to 1.54 gm. of proline, were obtained.

\[ C_7H_{10}O_2N_2S_4Cr \cdot H_2O \]
Calculated, N 16.2; found, N 16.6

The high result was probably due to the presence of some ammonium rhodanilate. The crystals were converted to free proline by treating with pyridine (15), and the acid recrystallized from butanol.

\[ C_7H_4O_2N \]
Calculated, N 12.2; found, N 12.1

Some of the amino acid was racemized by autoclaving with barium hydroxide and the copper salt of the racemic acid was prepared, crystallized from alcohol, and thoroughly dried in vacuo.

\[ C_{10}H_{15}O_4N_2Cu \]
Calculated, N 9.64; found, N 9.45

The picrate, which was prepared from some of the free acid and recrystallized from water, melted at 154°. Proline picrate melts at 154° (16).

That the losses of proline during the fractionation were considerable can be seen from the following experiment. 550 gm. of dried mycelium were allowed to autolyze and the resulting solution was concentrated to 4 liters and treated with 60 cc. of concentrated \( H_2SO_4 \) and then with an excess of \( HgSO_4 \) in 5 per cent \( H_2SO_4 \). After mercury was removed from the filtrate with \( H_2S \), the solution was neutralized (pH 6.5) with \( NaOH \) and concentrated to dryness under reduced pressure, and the residue was thoroughly extracted twice with alcohol. The alcohol was removed from the combined extracts under reduced pressure, the residue was dissolved in dilute \( H_2SO_4 \), and ammonium rhodanilate equivalent to the non-amino nitrogen was added to precipitate the proline; 15.0 gm. of crystals, equivalent to 2.88 gm. of proline, were obtained.

\[ C_{11}H_{10}O_4N_2S_4Cr \cdot H_2O \]
Calculated, N 16.2; found, N 16.1

The free amino acid was prepared and from it the picrate, which melted at 155°.

Isolation of Isoleucine and Valine—The alcohol-insoluble ma-
material obtained from the methyl alcohol-soluble copper salts contained 11.2 per cent nitrogen. After two recrystallizations from 95 per cent alcohol 4.5 gm. of beautiful crystals were obtained, but the nitrogen content was unchanged. This nitrogen content indicated a mixture of 60 per cent isoleucine and 40 per cent valine. 380 mg. were treated according to the method of Levene and Van Slyke (17) to separate these two acids. The insoluble lead salt weighed 404 mg., while theory is 410 mg. if the mixture was actually 60 per cent isoleucine and 40 per cent valine.

\[ C_{12}H_{24}O_4N_2Pb \] Calculated, N 6.00; found, N 5.94

The further identification of isoleucine was performed as in a previous publication (7).

From the filtrate, 145 mg. of crystalline amino acid were obtained in the usual way (17).

\[ C_6H_10O_2N \] Calculated, N 12.0; found, N 11.9

50 mg. were oxidized with 100 mg. of chloramine-T and the p-nitrophenylhydrazone of the resulting volatile aldehyde was prepared. After several recrystallizations the melting point and mixed melting point with authentic isobutyraldehyde p-nitrophenylhydrazone were 130–131°. Pure isobutyraldehyde p-nitrophenylhydrazone melted at 131–132°. Since several recrystallizations were necessary in order to obtain the pure hydrazone, it is probable that the valine obtained still contained a trace of isoleucine.

**Isolation of Serine**—The residual aqueous solution from the butanol extraction, which contained 5.77 gm. of nitrogen, was heated with excess copper hydroxide, and the excess of the latter was centrifuged out and washed. The deep blue solution was concentrated and treated with alcohol, and finally concentrated to dryness and extracted with alcohol. The alcohol solution was reserved for the isolation of threonine. Since the alcohol-insoluble copper salt was not quite pure serine copper salt, as judged by its nitrogen content (in a previous run analytically pure serine copper salt was obtained at this point), an aliquot was decomposed with H_2S, and the free amino acid crystallized with the aid of alcohol. An amount equivalent to 29.7 gm. of
serine in the total quantity of copper salt was obtained, and on correction for the 19 per cent loss mentioned above, 36.7 gm. would have been found.

C₅H₁₂O₅N. Calculated, N 13.3; found, N 13.5

The amino acid did not yield a volatile aldehyde when oxidized with chloramine-T, but from the reaction mixture a deep red crystalline \( p \)-nitrophenylosazone melting at 299–300° with decomposition was obtained. The \( p \)-nitrophenylosazone of glyoxal melts at 298–300° with decomposition (18).

Some of the amino acid was racemized by autoclaving with barium hydroxide, and from 200 mg. of the racemic acid the phenylhydantoin was prepared. After recrystallization from water it melted at 161°. The phenylhydantoin prepared from synthetic serine melted at 160°, and the two when mixed melted at the same temperature. This melting point is slightly lower than that recorded in the literature (19).

C₁₉H₂₂O₄N₁. Calculated, N 13.6; found, N 13.6

The \( \beta \)-naphthalenesulfonyl derivative of the racemic acid melted at 210–212°. \( \text{N}-\beta \)-Naphthalenesulfonyl serine melts at 210° (20).

Isolation of Threonine—The ethyl alcohol-soluble copper salt, which contained 1.3 gm. of nitrogen, was dissolved in water and decomposed with \( \text{H}_₂\text{S} \). Since only a part of the nitrogen in the filtrate was amino nitrogen (Van Slyke), the solution was concentrated under reduced pressure to about 25 cc., treated with 3 cc. of concentrated \( \text{H}_₂\text{SO}_₄ \), and heated for 6 hours on the steam bath. After this treatment all the nitrogen was in the amino form. The solution, however, when oxidized with chloramine-T gave no volatile aldehyde. Since some ash was present, sodium carbonate and mercuric acetate were added, and the resulting precipitate was filtered off and decomposed with \( \text{H}_₂\text{S} \). The filtrate from the \( \text{HgS} \) was concentrated under reduced pressure to a small volume and treated with absolute alcohol; 5.94 gm. of crystals formed.

C₁₀H₁₁O₄N₁. Calculated, N 11.8; found, N 11.9

When recrystallized from dilute alcohol, hexagonal plates were obtained. After correction for the 19 per cent loss mentioned above, 7.34 gm. would have been found.
The copper salt was prepared and crystallized from ethyl alcohol.

\[ \text{CsH}_{16} \text{O}_6 \text{N}_2 \text{Cu} \]. Calculated, N 9.35, Cu 21.2; found, N 9.30, Cu 21.0

When 400 mg. of the acid were oxidized with chloramine-T according to the directions of Meyer and Rose (21), and the reaction mixture was warmed with a solution of 2,4-dinitrophenylhydrazine, a precipitate formed which when recrystallized from pyridine melted at 298–300° with decomposition. The 2,4-dinitrophenylosazone of methylglyoxal melts at 299–301° with decomposition (22). In a similar manner the p-nitrophenylosazone, melting at 299–301°, and the phenylosazone, melting at 142–144°, were prepared. The corresponding osazones of methylglyoxal melt at 302–304° and 143–144° (23).

A small amount of the acid was reduced with red phosphorus and concentrated HI (21), and the resulting acid was oxidized with chloramine-T. The p-nitrophenylhydrazone of the volatile aldehyde which was formed melted at 119–120°. The p-nitrophenylhydrazone derived from synthetic aminobutyric acid melted at 121°.

**Isolation of Tryptophane**—The autolysate from 1100 gm. of dried mycelium was concentrated under reduced pressure to 2 liters and filtered to removed tyrosine which had crystallized. Enough concentrated \( \text{H}_2\text{SO}_4 \) was added to the filtrate to give a concentration of 5 per cent, 10 per cent \( \text{HgSO}_4 \) in 5 per cent \( \text{H}_2\text{SO}_4 \) was added until no more precipitate formed, and the mixture was allowed to stand overnight in the refrigerator. The precipitate was then filtered, thoroughly washed with 5 per cent \( \text{H}_2\text{SO}_4 \), suspended in water, and decomposed with \( \text{H}_2\text{S} \). The filtrate from the \( \text{HgS} \) was concentrated under reduced pressure to 300 cc., and while still quite acid was extracted continuously for 12 hours with butyl alcohol under reduced pressure. Dilute barium hydroxide was added to the extract and the butanol was removed under reduced pressure. The solution, which was alkaline with barium hydroxide, was then extracted continuously for 70 hours with butyl alcohol under reduced pressure. The residual aqueous solution then gave only a faint Hopkins-Cole test. Water was added to the extract and the butanol was removed under reduced pressure. The solution was adjusted to approxi-
I>. W. Woolley and W. H. Peterson 519

mately pH 6, and extracted three times with 6 volumes of butyl alcohol each time; 10 mg. of tryptophane as determined by the Folin-Ciocalteu method (11) remained in the aqueous phase. After the butanol was removed from the extracts, the solution was acidified with H₂SO₄ and extracted continuously with ether for 24 hours in an unsuccessful attempt to remove an acidic compound which was present. The residual aqueous solution was then treated with silver oxide and H₂SO₄, and the precipitate produced by these reagents was centrifuged off and washed with dilute H₂SO₄. After the filtrate was freed of silver with H₂S, it was adjusted to approximately pH 6 with barium hydroxide, filtered from BaSO₄, and concentrated under reduced pressure. Glistening plates soon formed which were filtered and washed with alcohol; 443 mg. were obtained. They gave good Hopkins-Cole and bromine water tests for tryptophane.

C₉H₁₄O₂N₂. Calculated, NH₂-N 6.86; found, NH₂-N 6.88

By the Folin-Ciocalteu method (11) it was found that the crystals contained 99 per cent tryptophane.

25 mg. were dissolved in 1 cc. of dilute HCl and 2 cc. of concentrated HCl were added. The pure white needles which separated melted at 250° with decomposition. Tryptophane hydrochloride melts at 251° with decomposition (24).

The picrate, prepared from 50 mg. of the crystals and 50 mg. of picric acid, crystallized from water in clusters of red needles and melted at 198-200° with decomposition. Tryptophane picrate in the same bath melted at 198-200° with decomposition. This melting point is slightly higher than that recorded by Mayeda (25).

SUMMARY

Aspartic and glutamic acids, tyrosine, leucine, proline, isoleucine, valine, serine, threonine, and tryptophane have been isolated from an autolysate of the mycelium of Aspergillus sydowi and identified by analysis of the free acids and by examination and analysis of suitable derivatives. A considerable percentage of the nitrogen of the mycelium was contained in these pure compounds.
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