ISOLATION AND CHARACTERIZATION OF MALFORMIN [1, 2, 3]

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Papers (5, 6) from this laboratory have reported the induction, by culture filtrates of the fungus, *Aspergillus niger*, of curvatures and malformations of bean plants and curvatures of corn roots. The name, malformin, was proposed for the active compound (7). We now present a complete isolation procedure for malformin and describe, in part, the character of the molecule. Isolation of malformin has made it possible to conduct quantitative experiments on its activity (see following report).

MATERIALS & METHODS

Aspergillus niger van Tiegh. strain 56–39 was used in these and the original studies. The culture medium, corn steep-glucose, and shake-flask methods were described earlier (5). Culturing was done at 25 to 26° C.

Cultures of 56–39 were obtained from soil stocks by inoculating agar slants of potato-dextrose medium. After 7 to 10 days, one ml spore suspensions were used to inoculate 100 ml aliquots of corn steep-glucose medium in 500 ml erlenmeyer flasks. After 24 hours on a reciprocating shaker, the cultures were transferred to additional 100 ml aliquots of the same medium in 1 liter erlenmeyer flasks and again incubated for 24 hours on the shaker. Each of these cultures was transferred to New Brunswick Model FS-314 fermentation jars containing 10 liters of corn steep-dextrose medium. The jars were incubated for 4 to 5 days on a New Brunswick fermentor drive assembly (air flow = 6 liters/min./jar; agitation speed = 420 rpm). Antifoam (Dow Polyglycol P-2000) was added as needed. At the end of the fermentation period the mycelium was removed by filtration and discarded.

To follow the isolation of malformin from the filtrates, one of two methods of assay was employed, the bean test (5) or the corn root test (6). The fractions were usually dried and then dissolved in water (containing 4 drops of Tween 80/100 ml for the bean test). In some cases a few drops of acetone were added to the dried residue to assist in dissolving the material.

Additional methods are given later.

RESULTS

I. PURIFICATION OF MALFORMIN: A schematic outline (fig 1) presents the various stages of isolation. After adding activated charcoal to the culture filtrate (step 1) the charcoal-filtrate mixture was stirred for 1 hour. The charcoal settled overnight and was separated from the cleared filtrate by decantating. Additional water was removed by filtering through Whatman no. 1 filter paper. After drying, the charcoal was made into a slurry with technical grade 99 % acetone and poured into a 7 × 90 cm column (step 2). The column was eluted with more acetone. About 1.5 liters of acetone were used for each 100 g of air dried charcoal. When the acetone was removed by distillation (step 3) an aqueous residue remained, its volume depended on the water content of the acetone and the charcoal. This residue was made alkaline (pH 8–9) by adding saturated $NaHCO_3$ solution, or by adding the bicarbonate directly to the aqueous residue. Malformin was removed from the aqueous phase by five extractions with ethyl ether. The ether was then extracted twice with 30 % (v/v) H_2SO_4 to remove basic substances; malformin remained in the neutral ether fraction (step 4). The 30 % H_2SO_4 solution was again extracted with ether twice, and the ether was combined with the neutral ether fraction containing malformin (step 5). Anhydrous Na_2SO_4 was added in excess to the neutral ether fraction for drying (step 6). After filtering and evaporating the ether a yellow syrup remained. By washing this syrup with small quantities of absolute acetone most of the malformin remained as a crude white powder, whereas the yellow substance was dissolved in the acetone. Although malformin is slightly soluble in acetone the use of small quantities minimized the loss. A second crop of crude white powder was usually recovered from the acetone by allowing it to evaporate for 3 to 4 days. Most of the crude white powder, about 600 mg from 30 liters of culture filtrate, could be dissolved by refluxing five times for 30 minutes with 50 ml aliquots of ethyl acetate (reagent grade, step 7). Insoluble material was removed by filtration. The ethyl acetate solution was condensed to approximately 100 ml and added to a 2.2 × 40 cm column of activated alumina (Merck, reagent grade) previously packed with ethyl acetate. The column was eluted with approximately 300 ml of ethyl acetate at a rate of 20 to 30 drops/min. Evaporation of this fraction yielded a small quantity of syrup which had no activity on bean plants. The eluting solvent was then changed to ethyl acetate containing 2 % (v/v) ethyl alcohol. After 150 ml

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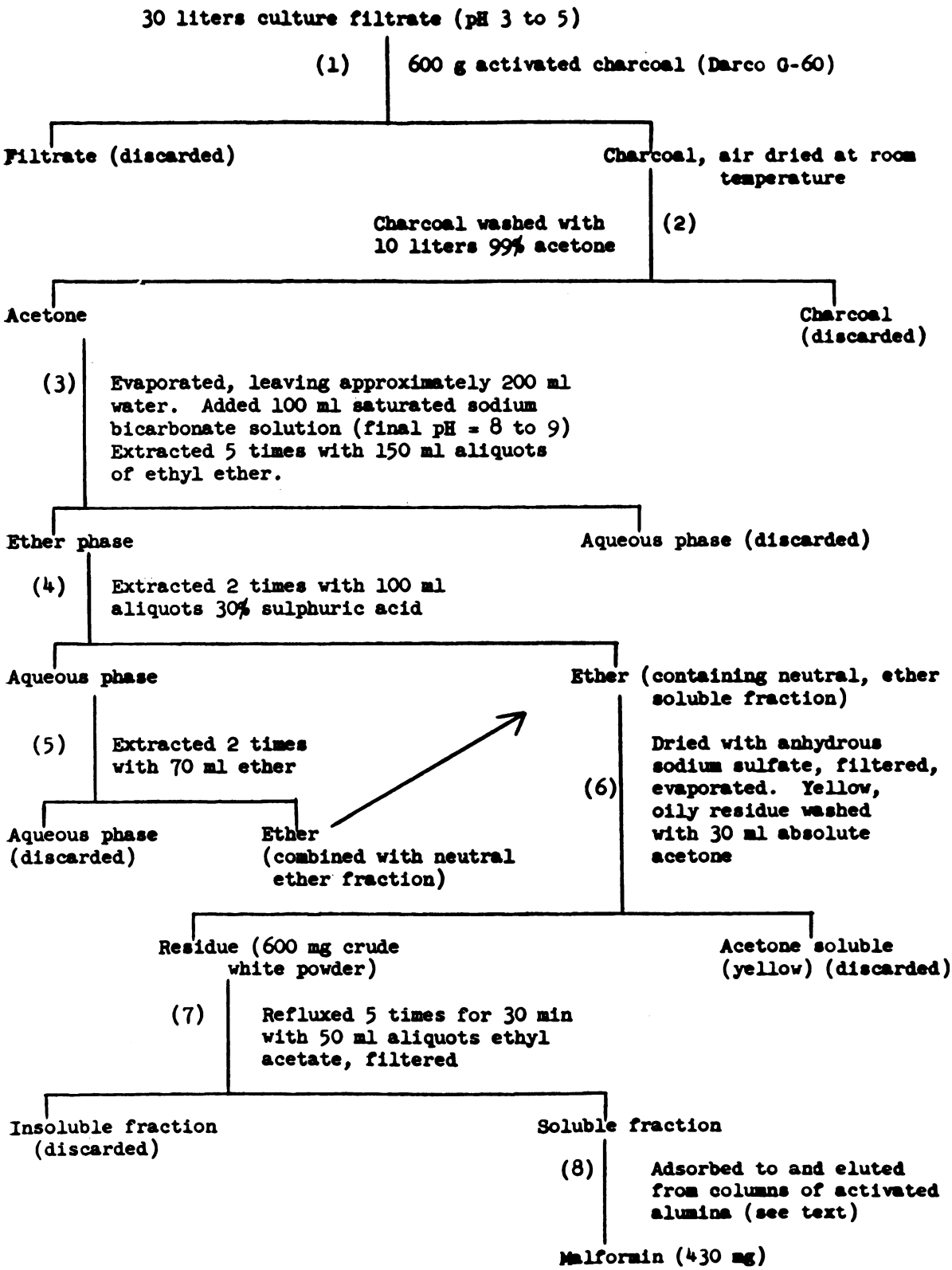


FIG. 1. Isolation procedure for malformin from culture filtrate of *A. niger* 56–39.

had been collected, the solvent was evaporated and 430 mg of a pure white powder remained (from 30 liters of culture filtrate). Recent evidence indicated that using 2 % alcohol in the eluting solvent may not be necessary. The white powder was active on bean seedlings or corn roots when as little as 1×10^{-4} μg/plant were used. Adsorption and elution from alumina columns was repeated for further purification. Malformin did not melt but did decompose at temperatures over 300° C.

The movement of malformin in the solvents indicated that the molecule had neither acidic nor basic groups, and no amphoteric properties; thus, the compound was neutral. These results agree with earlier studies indicating that malformin could not be adsorbed by anion or cation exchange resins (5).

II. Molecular Formula: From the analysis of malformin (table I) for carbon, hydrogen, nitrogen, and sulfur the experimental formulae $(C_{23}H_{39}N_5O_5S_2)_n$ or $(C_{23}H_{41}N_5O_5S_2)_n$ were calculated. They differ only in the number of hydrogen atoms. It was impossible to differentiate the two by the results of elemental analysis.

The molecular weight of malformin was determined by the Barger method (2). A solution of malformin was equilibrated with various concentrations of standard solutions of recrystallized acetanilide in capillary tubes. In two determinations the molecular weight was 530 ± 45 and 495 ± 45. These values agree with the formulae of $C_{23}H_{39}N_5O_5S_2$ or $C_{23}H_{41}N_5O_5S_2$ (table I).

Table I

Elemental Analysis of Malformin

	% Carbon	% Hydrogen	% Nitrogen	% Sulfur	Molecular weight
Found	52.19	7.53	13.35	11.90	512*
$C_{23}H_{39}N_5O_5S_2$	52.11	7.36	13.22	12.10	530
$C_{23}H_{41}N_5O_5S_2$	51.88	7.71	13.16	12.06	532

* Average of two experiments.

III. Physical & Chemical Properties of Malformin:

A. Infrared Spectrum: A Perkin-Elmer Model 21 Spectrophotometer was used for the infrared studies. The infrared spectrum (fig 2) of malformin showed strong adsorption peaks at 3.01, 6.06, and 6.54 μ. These adsorption bands are typical of peptides.

B. Ultraviolet Spectrum: The ultraviolet spectrum was taken on a Beckman Model DU spectrophotometer. Solutions of malformin were prepared with methyl cellosolve and diluted with water. In the region from 220 to 330 mμ no adsorption peaks were observed. End adsorption was observed at the shorter wave lengths (at 220 mμ, $\epsilon = 5400$). The lack of ultraviolet adsorption bands excluded the possibility of any conjugate system in the molecule.

C. Optical Rotation: A 1.1 % solution of malformin in methyl cellosolve was used for optical rotation determinations. Specific rotation was calculated from the readings to be $[\alpha]_D^{25} = -39.0°$.

D. Solubility: Malformin was readily soluble in acetic and formic acid, phenol, methyl cellosolve, and dimethyl formamide; sparingly soluble in ethyl acetate, ethanol, methanol, and acetone; difficultly soluble in water, benzene, petroleum ether (B.R. 60 to 70°), and chloroform.

E. Electrophoretic Mobility: Column electrophoresis was used under acidic and basic conditions. A rectangular, horizontal column (1.6 × 3 × 38 cm), consisting of 19 removable 2 cm sections, was packed with polyvinyl resin (Geon, B. F. Goodrich Chemical Co.) mixed with 0.1 N acetic acid buffer (pH 3.5), or 0.1 M phosphate buffer (pH 8.7) containing 10 % methyl cellosolve. Excess buffer was removed from both ends of the column. Resin from the center section of the column was removed and replaced with resin previously mixed with 0.7 ml malformin solution containing 300 to 500 μg malformin and one mg glucose (control) in 1 ml of buffer. Electrophoresis followed for 17 hours (70 volts/cm, 2.5×10^{-3} amp/cm² under acidic conditions; 70 volts/cm, 5.7×10^{-3} amp/cm² under basic conditions). The resin from each section of the column was removed and washed with 4 ml of water. An aliquot of the eluate from each section was filtered, diluted, and tested for malformin by the corn root test. Malformin was also detected by mixing 1 ml of the eluate with 0.5 ml 15 N H_2SO_4 and hydrolyzing at 121° C for 17 hours. The hydrolysate was adjusted to pH 5.0 with 5 N NaOH, mixed with 2 ml 0.2 N citrate buffer (pH 5.0) and 1.5 ml KCN-ninhydrin solution to detect amino acids (see below). The solutions were heated at 100° C for 15 minutes. The control, glucose, was detected by the anthrone-H_2SO_4 reagent (8).

In acidic conditions malformin and glucose were detected only at the origin and weakly in the first section toward the negative pole. Identical results were obtained in basic solvent except glucose was detected only at the origin. The slight movement of malformin toward the negative pole under basic conditions was not considered significant. It was concluded that malformin was not amphoteric.

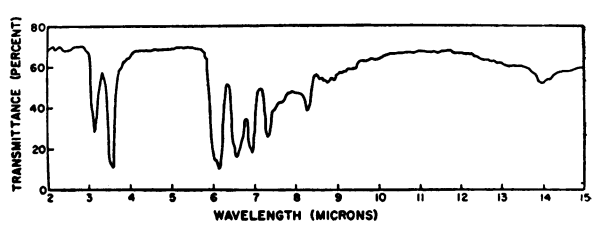


Fig. 2. Infrared spectrum of malformin in Nujol Mull.

IV. Hydrolysis of Malformin: Information derived from the elemental analysis, electrophoresis, and the movement of malformin in various solvents of the purification process led us to expect that malformin was a peptide with no free terminal groups. Malformin was hydrolyzed in 6 N HCl for 20 hours at 121° C. The hydrolyzate gave a strong positive reaction with ninhydrin, thus confirming its peptide nature. Paper and column chromatography were used to identify the amino acids and determine their molar ratio.

A. Paper Chromatography: Malformin (2 mg) was dissolved in 0.4 ml acetic acid and mixed with 2 ml of 6 N HCl. The solution was sealed in a small pyrex test tube and hydrolyzed for 20 hours at 121° C. The hydrolyzate was dried and 0.5 ml of water was used to dissolve the residue. Five μl were applied to strips of Whatman no. 1 paper. The amino acids were separated with the organic phase of a mixture of butanol, acetic acid, and water (50:1:50 v/v) using descending, multiple (two multiplications) chromatography. Four spots were detected with ninhydrin. Their Rf values were 0, 0.37, 0.57, and 0.62. By comparing these spots with those of authentic samples it was concluded that three of the amino acids were valine (Rf 0.37), isoleucine (Rf 0.57), and leucine (Rf 0.62).

The amino acid which remained at the origin was considered to be a sulfur-containing amino acid, and because of its low Rf value, possibly cystine. The intensity of this spot varied with the conditions of hydrolysis. It is known that cystine is not stable to the usual methods of hydrolysis, and peptides or proteins containing cystine are often oxidized by performic acid prior to hydrolysis (19). The oxidation product of cystine, cysteic acid, is stable during hydrolysis. Two mg of malformin were oxidized with performic acid for 4 hours at 4 to 5° C (performic acid was prepared by mixing 22.5 ml of formic acid and 2.5 ml of 30 % H_2O_2 at room temperature for 1 hr). After oxidation the solution was evaporated in vacuo, and the residue was hydrolyzed in 2 ml of 6 N HCl at 121° C for 17 hours. Descending chromatography, with methanol, water, pyridine (80:20:4 v/v) was used to separate the amino acids. Two spots, Rf 0.6 and 0.8, were detected with ninhydrin. The compound with an Rf value of 0.6 was identified as cysteic acid by comparing with authentic material. The other spot corresponded to a mixture of leucine, isoleucine, and valine.

Alkaline hydrolysis of malformin (2 mg) was done in 2 ml of 1 N $Ba(OH)_2$ at 121° C for 20 hours. The hydrolyzed material was brought to pH 5 with H_2SO_4. A precipitate of $BaSO_4$ was removed by centrifugation and washed twice with 3 ml aliquots of water. The hydrolyzate and the $BaSO_4$ washings were combined, evaporated to dryness, and dissolved in 0.5 ml of water. This solution (0.5 μl) was chromatographed using the butanol, acetic acid, water solvent. Four spots were detected with Rf values of 0.15, 0.37 (valine), 0.57 (isoleucine), and 0.62 (leucine). The new spot at Rf 0.15 was derived from cystine during alkaline hydrolysis as shown by similar experiments with authentic cystine.

Carbohydrates were excluded as components of the hydrolyzates by spraying the chromatograms with aniline hydrogen phthalate (16), α-aminophenol-phosphoric acid (10), and ammoniacal $AgNO_3$ (15).

The molar ratio of the component amino acids of malformin was determined by the paper chromatographic method of Kay, Harris, and Entenman (11). In this method the amino acids were detected with ninhydrin, eluted from the paper with alcohol, and measured photometrically. Oxidized and hydrolyzed malformin was chromatographed using butanol, acetic acid, and water as described. Because isoleucine and leucine were not completely separated, they were combined. The molar ratio of cysteic acid, valine, and the leucine-isoleucine mixture was 2:1:1.8, respectively.

B. Column Chromatography: To obtain further evidence for identifying the amino acid components of malformin and their molar ratios the methods of Moore and Stein were used (13) with slight modification. Dowex 50-X8W resin (200–400 mesh) was used to prepare a column (1.0 × 75 cm) with 0.1 N citrate buffer (pH 4.25). The temperature of the column was maintained at 50 ± 1° C by circulating water through the jacket of the column. Malformin (0.3–0.4 mg), hydrolyzed directly or after oxidation with performic acid, was applied to the column in 2.5 ml of 0.1 N citrate buffer (pH 3.42). The sample was washed onto the column with two 0.5 ml aliquots of the same buffer and eluted with 0.1 N citrate buffer (pH 4.25). Because the hydrolyzate contained only four amino acids only this buffer was used for elution. The fractions (1.7 ml) were collected at a constant rate of 5 ml per hour by regulating the position of the buffer reservoir and by air pressure. The amount of amino acid in each fraction was determined by the method of Yemm and Cocking (20) as follows: to each fraction 0.05 ml of 1 N NaOH, 1.5 ml of 0.2 N citrate buffer (pH 5.0), and 1.3 ml of KCN-ninhydrin solution were added. The solutions were heated at 100° C for 15 minutes and cooled in cold water. After adding 5 ml of 60 % ethanol to each fraction the color intensity was measured photometrically at 570 mμ. Known amino acids were used to prepare standard curves. The results of direct hydrolysis of malformin and hydrolysis after oxidation are given in figures 3 and 4. By comparing the position of the peaks with those of standard amino acids, the amino acids were identified as leucine, isoleucine, valine, and cystine (cysteic acid after oxidation). Separation of leucine and isoleucine was complete. The molar ratio (table II) was calculated from the total amino acid content under each curve. The value obtained for ½ cystine, when malformin was hydrolyzed directly, was low because this amino acid is unstable.

If the results of elemental analysis, molecular weight determination, and the paper chromatography are considered the molar ratio of leucine, isoleucine,

TABLE II

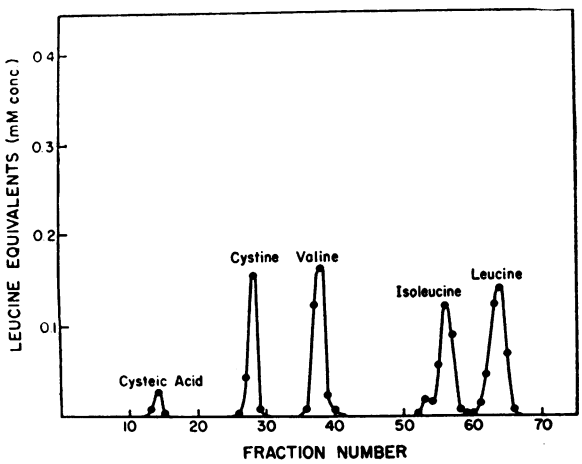
MOLAR RATIO OF AMINO ACID COMPONENTS OF MALFORMIN ISOLATED FROM A. NIGER 56–39

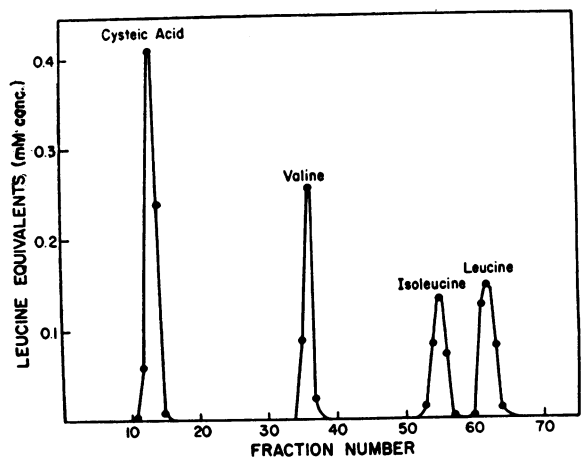
AMINO ACID	MALFORMIN	OXIDIZED MALFORMIN
Leucine	1.00 (1)*	1.00 (1)
Isoleucine	0.79 (1)	0.76 (1)
Valine	0.80 (1)	0.99 (1)
½ Cystine	1.04 (2)	0.00 (0)
Cysteic acid	0.08 (0)	1.97 (2)

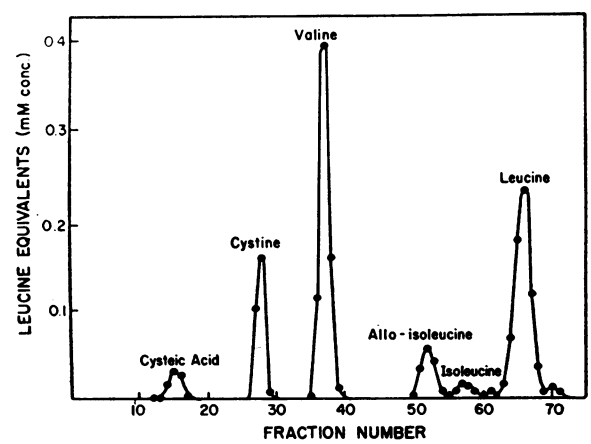
* The values in parentheses were calculated from the experimental formula of $C_{23}H_{41}N_5O_5S_2$.

valine, and ½ cystine must be 1 : 1 : 1 : 2, respectively. A cyclic peptide containing these amino acids in such a ratio has a molecular formula identical with that calculated from the results of elemental analysis. The molar ratio obtained by column chromatography showed slight deviations from the calculated ratio, especially the isoleucine value, but support the 1 : 1 : 1 : 2 ratio.

V. TEST FOR -SH GROUP IN MALFORMIN: The nitroprusside test of Anson (1) and the sodium azide-iodine test of Feigl (9) were used to detect -SH groups. No reaction occurred in either of these tests or after hydrolysis of malformin by a mixture of 90 % formic acid and 6 N HCl (1 : 1 v/v) in evacuated







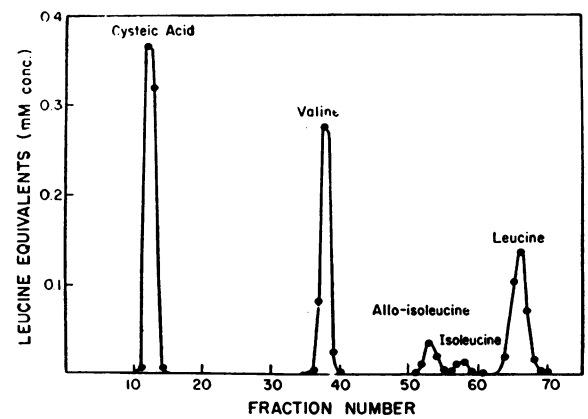


FIG. 3. (*upper left*). Amino acid components of malformin produced by *A. niger* 56–39. Samples hydrolyzed without prior oxidation.

FIG. 4. (*upper right*). Amino acid components of malformin produced by *A. niger* 56–39. Samples hydrolyzed after oxidation with perfomic acid.

FIG. 5. (*lower left*). Amino acid components of malformin produced by *A. niger* 56–30. Samples hydrolyzed without prior oxidation.

FIG. 6. (*lower right*). Amino acid components of malformin produced by *A. niger* 56–30. Samples hydrolyzed after oxidation with performic acid.

and sealed tubes. Under these conditions of hydrolysis, conversion of cysteine to cystine is limited. The lack of -SH groups indicated that, in the isolated malformin, the S-S bridge of cystine was present.

VI. Production of Malformin by Other Strains of A. niger: The yield of purified malformin from culture filtrates of 56–39 gradually decreased from a high of about 15 mg per liter to less than 1 mg per liter. Consequently, a screening program was initiated to obtain higher yielding strains. Strain 56–30 was selected. When grown on a corn steep-glucose medium, it yielded approximately 35 mg per liter of culture filtrate. Because the isolation method, infrared spectrum, and elemental analysis were identical, it was considered that malformin from 56–39 and 56–30 were identical. When hydrolyzates of malformin from 56–30 and 56–39 were compared, differences were found. On paper chromatograms, no differences could be detected in the relative intensity of the leucine, valine, and cystine (or cysteic acid) spots. However, the intensity of the isoleucine spot was much weaker for the amino acids from 56–30 than from 56–39, whether the malformin was hydrolyzed directly or after oxidation with performic acid. For more accuracy, the molar ratio of the component amino acids of malformin isolated from 56–30 was also investigated by the method of Moore and Stein (13). The amino acid curves obtained from malformin hydrolyzed directly (fig 5) and after oxidation (fig 6) indicated that the amino acid which was considered to be isoleucine, on the paper chromatograms, was actually allo-isoleucine. Furthermore, the molar ratio of this amino acid was low (table III). The molar ratio of the other amino acids was essentially the same as that obtained from 56–39. Apparently, the two malformins are different. Strangely, no differences were detected in elemental analysis or infrared spectrum. Clarification must await further experimentation.

Table III

Molar Ratio of Amino Acid Components of Malformin Isolated From A. Niger 56–30

Amino acid	Malformin	Oxidized malformin
Leucine	1.00	1.00
Isoleucine	0.07	0.09
Allo-isoleucine	0.22	0.22
Valine	1.05	1.07
½ Cystine	0.77	0.00
Cysteic acid	0.11	2.13

Malformin, isolated similarly from six other strains, was hydrolyzed and examined for amino acid content by paper chromatography. All strains produced malformin like that of 56–39. Only 56–30 produced the different malformin. The activity of these compounds on bean seedlings and corn roots appeared the same.

Discussion

Evidence obtained earlier (6) indicated that the compounds produced by *A. niger* which caused malformations on beans and curvatures on corn roots were identical. The activity of isolated malformin on both bean stems and corn roots confirmed the results obtained by paper chromatography.

The neutral nature of malformin was shown by: A: no movement by electrophoresis, B: solubility properties, C: no adsorption by anion and cation exchange resins, and D: negative ninhydrin test before hydrolysis. The amino acid components of malformin are all neutral. These facts suggest that malformin is probably a cyclic peptide.

Two formulae are proposed for malformin isolated from strain 56–39. The compounds differ only by two hydrogen atoms, depending on the presence of cystine or two cysteine residues in the molecule. Because no free -SH groups were detected, the first formula should be assigned to malformin. Under the conditions of isolation it seems likely that any free -SH groups present originally would be oxidized to form the S–S bridge. Actually, the activity of this compound may depend on the interconversion of the -SH and S–S forms. The necessity of these groups is emphasized by a complete loss of activity on beans when malformin was oxidized with performic acid to form $-SO_3H$ radicals. Many enzymes, antibiotics, toxins, hormones, and plant growth substances, are believed to be active because they contain or react with sulfhydryl groups (3, 12, 14). Roberts and Lucchese (18) and Pilet (17) have observed that -SH groups are in highest concentration in the apical meristems of plants. These regions respond most strongly to malformin.

Malformin contains an unusually high percentage of cystine (40 % calculated on the basis of ½ cystine). Malformin isolated from strain 56–30 contains an uncommon amino acid, allo-isoleucine, first isolated from a natural compound as a degradation product of actinomycin C (4).

Our results suggest that two malformins exist. The first, from strain 56–39 and other strains of *A. niger*, contains valine, leucine, isoleucine, and ½ cystine in a 1:1:1:2 ratio, respectively. The second malformin, from strain 56–30, appeared identical with respect to method of isolation, elemental analysis, and infrared spectrum. The ratio of valine, leucine, and ½ cystine of the second malformin was similar to that of the first but the ratio of isoleucine was low. Also, the second malformin contained allo-isoleucine. These differences must be resolved before a structure can be proposed. Newly isolated peptides often are later shown to be mixtures. This possibility must also be considered for malformin. We have shown the possibility of two kinds of malformin from strains 56–39 and 56–30. If malformin

from 56–39 is a mixture of the two malformins, the molar ratio for valine, leucine, and ½ cystine would be unchanged. However, because malformin from 56–30 has a low ratio of isoleucine, the isoleucine ratio of the mixture would be decreased. Our data support this possibility, in that the molar ratio of valine, leucine, and ½ cystine closely approximated 1 : 1 : 2, but the molar ratio of isoleucine was low (0.79).

Summary

Malformin was isolated from culture filtrates of the fungus, *Aspergillus niger* 56–39, and found to be a neutral peptide containing four amino acids, valine, leucine, isoleucine, and ½ cystine. The molar ratio obtained for these amino acids supported a 1 : 1 : 1 : 2 ratio, respectively. It was suggested that malformin is a cyclic peptide. The molecular formula, $C_{23}H_{39}N_5O_5S_2$, was proposed. The infrared spectrum showed strong adsorption bands at 3.01, 6.06, and 6.54 μ. A high malformin-producing strain, 56–30, produced malformin which differed from that produced by strain 56–39. This new malformin contained allo-isoleucine.

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