BIOORGANIC CHEMISTRY 4, 93–105 (1975)

Structure and Synthesis of Malformin A_1

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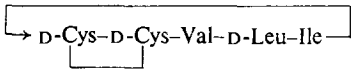
Received July 12, 1974

The structure of malformin A_1, a metabolic product of *Aspergillus niger*, was reexamined and the sequence of its amino acid constituents established as



The cyclopentapeptide-disulfide corresponding to this structure was prepared through stepwise synthesis of the protected pentapeptide derivative, benzyloxycarbonyl-L-isoleucyl-*S*-benzyl-D-cysteinyl-*S*-benzyl-D-cysteinyl-L-valyl-D-leucine methyl ester, which in turn was converted to the hydrazide, partially deprotected, and cyclized via the azide. On removal of the S-benzyl groups and oxidation to the disulfide, a synthetic material was obtained that was indistinguishable from natural malformin A_1 and was as equally potent in causing curvatures on corn roots.

An observation by Curtis (*1*) that metabolic products of *Aspergillus niger* cause curvatures on the roots of some plants led to the isolation (*2*) of the active principle, malformin, from culture filtrates of the microorganism. Structural studies (*3*, *4*) in the same laboratory resulted in the proposal of a cyclopentapeptide-disulfide structure (I, Fig. 1) for the main component, malformin A_1. However, this structure could not be confirmed by synthesis of the corresponding peptide (*5*).

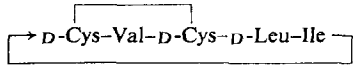


FIG. 1. The structure proposed earlier (*5*) for malformin A_1.

More recently, Schöberl and his associates made a new effort (*6*, *7*) toward the synthesis of malformin A and prepared compound I by a different scheme. Their synthetic material did cause curvatures on corn roots, but for the same effect it had to be applied in a concentration 500 times higher than the natural product. Since malformin is also active as an antibiotic (*8*) and has cytotoxic properties (*9*) as well, further efforts in the same direction seemed to be warranted, and we decided to resynthesize compound I.

Our approach to a protected cyclopentapeptide with the sequence of I was similar to its first synthesis by Isono and Curtis (*5*), except that leucine rather than the hindered isoleucine was chosen as the C-terminal residue of the open-chain intermediates. Also,

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instead of saponification and cyclization through an active ester, the protected pentapeptide derivative benzyloxycarbonyl-L-isoleucyl-*S*-benzyl-D-cysteinyl-L-valyl-*S*-benzyl-D-cysteinyl-D-leucine methyl ester was converted to the hydrazide and, after partial deprotection with hydrobromic acid in acetic acid, cyclized via the azide. Removal of the benzyl groups from the cysteine side chains with sodium in liquid ammonia produced a material that had only marginal effects on corn roots; moderate curvatures could be observed when the crude product was applied at a high (ca. 100 μg/ml) concentration. Oxidation to the disulfide did not improve its potency. These results were consistent with the experience gained earlier in the already mentioned attempts (*5–7*) toward synthetic malformin. Therefore, a re-examination of the structure of malformin A seemed to be necessary.

A sample of natural malformin (mainly A_1) was isolated from filtrates of a culture of *A. niger*, according to the procedure of Curtis (*2*). Amino acid analysis indicated some heterogeneity; the ratios of the constituents were not exactly molar (Table 1). Thus,

TABLE 1

RATIOS OF CONSTITUENTS IN NATURAL AND SYNTHETIC SAMPLES OF MALFORMIN A_1[a]

	Natural	Synthetic
Half-cystine	2.00	2.00
Valine	1.11	0.99
Isoleucine[b]	0.91	0.99
Leucine	1.16	1.01

[a] Hydrolyzed with a 1:1 mixture of concd HCl and AcOH.

[b] Including alloisoleucine.

while only a single spot could be detected on thin-layer chromatograms, the isolated malformin A_1 contained also moderate amounts of additional members of the malformin family, some rich in valine and/or in leucine.[2] This fact, together with the resistance of malformin to degradation with proteolytic enzymes, rendered the structural studies rather difficult. Mass spectra of our preparation demonstrated that fragmentation occurs predominantly at amino acid side chains, and less within the peptide backbone. Therefore, little sequential information could be derived from these spectra.[3] On the other hand, a careful comparison of the fragments formed during partial hydrolysis of the *S,S'*-dibenzyl precursor of compound I and its oxidation

[2] The alloisoleucine found in the hydrolysates stems only in part from the amino acid present in the parent peptides. Racemization of isoleucine with concomitant formation of alloisoleucine was enhanced when a mixture of acetic acid and hydrochloric acid, rather than hydrochloric acid alone, was used for hydrolysis. Yet, addition of acetic acid seemed to be necessary, because of the insolubility of malformins in constant boiling hydrochloric acid, even at 110°C.

[3] The high-resolution mass spectra were recorded and studied by Dr. Rodger L. Foltz of Batelle Memorial Institute, Columbus Laboratories. These spectra showed some peaks that were consistent with the revised sequence, but the evidence was not conclusive. Further attempts are planned in which spectra of derivatives of malformin will be studied.

product with the fragments obtained under identical conditions from *S,S'*-dibenzyl-malformein and performic acid-oxidized malformin (malformeic acid) provided crucial evidence for some error in the structure (I) proposed for malformin and also clues for a revision of this structure.

Hydrolysis of the cyclic *S,S'*-dibenzyl precursor of compound I with a mixture of acetic acid and concentrated hydrochloric acid (11 : 1, v : v) at 110°C for 2.5 hr liberated considerable quantities of the constituent amino acids, but the amount of valine in the hydrolysate was about five times less than that of leucine or isoleucine. This was not too surprising, since the valine residue lies between two *S*-benzylcysteines in the precursors of compound I and the combined hindrance in the partial sequence -Cys(Bzl)-Val-Cys(Bzl)- should result in slow hydrolysis of the amide bonds on both sides of valine. Correspondingly, *S*-benzylcysteine was also less readily liberated than leucine or isoleucine (cf. Table 2 in the preliminary account of this study (*10*)). On the other hand, when *S,S'*-dibenzylmalformein, prepared (*5*) from natural malformin A, was hydrolyzed under the same conditions, valine was released at least as fast as the other amino acid constituents (*10*); therefore, the conclusion had to be drawn that *in malformin A_1 the position of valine is not between two half-cystines*, and also that there is no other amino acid between the two half-cystine residues. This finding was further supported by the rapid release of valine from performic acid-oxidized compound I during hydrolysis with molar hydrochloric acid. The cysteic acid residues on both sides of valine accelerate the hydrolytic cleavage of the adjoining amide bonds in a manner somewhat similar to the effect of aspartyl residues (*11*). In contrast, valine was released more slowly than leucine or isoleucine from malformeic acid.

In the partial acid hydrolysates of our natural malformin preparation (mainly malformin A_1) of *S,S'*-dibenzylmalformein and of malformeic acid, some peptide fragments could be identified by comparison of their elution pattern on the recordings of the amino acid analyzer with the pattern of mixtures of authentic samples prepared for this purpose. The presence of D-leucyl-L-isoleucine and L-valyl-D-leucine was clearly demonstrated, but no dipeptide of valine and isoleucine could be found, even though such dipeptides should have been present in the hydrolysates (*12*) if these two residues had been next neighbors in the sequence. These findings are compatible only with sequence II (Fig. 2), which is therefore the revised primary structure of malformin A_1.

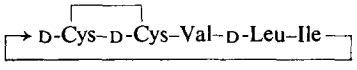

FIG. 2. The revised structure of malformin A_1.

The structure (II) deduced from the above-described degradations was confirmed by synthesis. Starting with D-leucine methyl ester, the protected pentapeptide derivative benzyloxycarbonyl - L - isoleucyl - *S* - benzyl - D - cysteinyl - *S* - benzyl - D - cysteinyl - L - valyl-D-leucine methyl ester was prepared by stepwise chain lengthening (*13*) with *p*-nitrophenyl esters (*14*) as reactive intermediates of the amino acid constituents. The benzyloxycarbonyl group (*15*) was used for the protection of the α-amino functions and hydrobromic acid in acetic acid for its removal (*16*). For the liberation of the

amino component from the hydrobromide, diisopropylethylamine rather than triethylamine (*17*) was applied. Hydrazinolysis of the fully protected pentapeptide methyl ester, partial deprotection with hydrobromic acid in acetic acid, and cyclization through the azide (*18*) yielded the *S,S'*-dibenzyl derivative of the cyclic peptide shown in Fig. 2. Removal of the *S*-benzyl groups with sodium in liquid ammonia (*19*) resulted in a mixture that caused severe curvatures on corn roots, optimally in concentrations

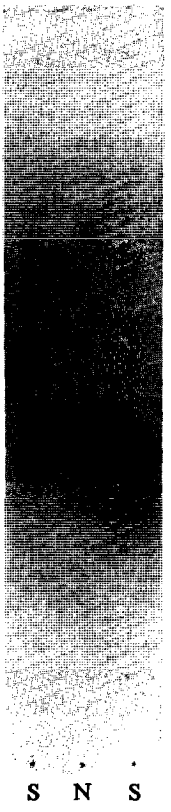

FIG. 3. Thin-layer chromatogram of natural (N) and synthetic (S) malformin A_1, on silica gel with water-saturated ethyl acetate as solvent.

between 0.1 and 1.0 μg/ml. A solution of the crude malformein in dimethylsulfoxide was kept at 100°C for 1 hr to convert it to the more favorable conformation I (*20*). Oxidation to the disulfide, followed by purification, produced a material that was indistinguishable from natural malformin A_1 by thin-layer chromatography (Fig. 3) and cd spectra (Fig. 4). Also, both the (purified) synthetic and natural preparations showed optima in their ability to induce curvatures on corn roots (*1*) at a concentration of 0.1 μg/ml.

The synthesis was not entirely satisfactory in one respect: The final steps, removal of the *S*-benzyl protecting groups and preparation of malformin from the reduced material, proceeded with variable and sometimes rather low yield. Similar yields were encountered, however, when natural malformin was reduced with sodium in liquid

ammonia, benzylated (*5*), and malformin recovered by reduction, etc. The low recoveries in these steps could be explained (*20*) by different stable conformations of malformein and malformin itself. Because of the low yield in the final steps, the synthesis in itself might not be sufficiently unequivocal to prove the revised structure (II). Yet, additional and convincing evidence was found through rigorous comparisons of the products of partial acid hydrolysis of the cyclic *S,S'*-dibenzyl intermediate of the synthesis with the fragments similarly obtained from *S,S'*-dibenzylmalformein.

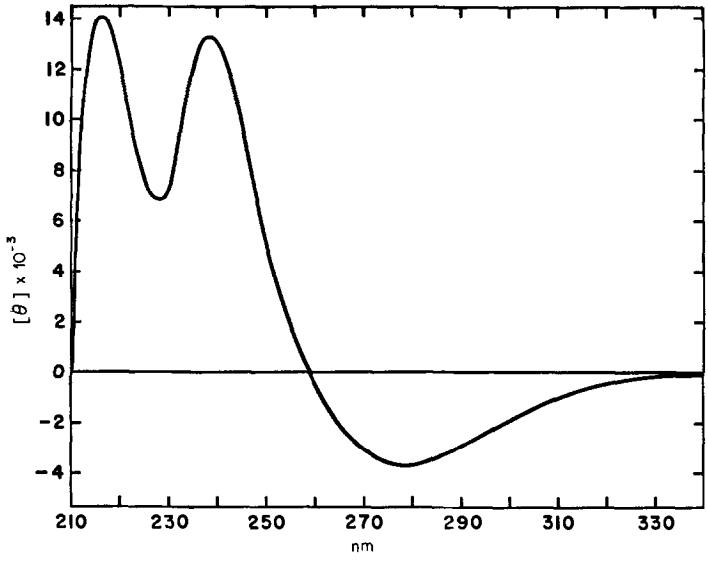

FIG. 4. Circular dichroism spectrum of synthetic malformin A_1 in trifluoroethanol. For natural malformin, cf. Ref. (*21*).

Complete agreement was found between the patterns of the two recordings on the amino acid analyzer; even some so far unidentified peptides appeared at the same elution time when partial hydrolysates of the synthetic and natural material were compared. Also, the release of valine was equally uninhibited in the synthetic and natural *S,S'*-dibenzyl derivatives and was similar in the two performic acid-oxidized samples (cf. Experimental section). Because of these extensive comparisons of malformin with the synthetic product (II) and the good agreements observed between the results, structure II is fully supported by synthesis. It is our hope that our present experiments toward improvements of the synthesis will result in a procedure that can provide malformin A_1 and its analogs in amounts sufficient for studies of their conformation (*21*, *22*), ion-transport properties, and biological activities.

EXPERIMENTAL

Capillary melting points are reported uncorrected. The following systems were used for thin-layer chromatography: A, CH_3OH–$CHCl_3$ (1:19); B, EtOAc saturated with

H_2O; C, $CHCl_3$–CH_3OH (9:1). Spots on tlc were revealed with *t*-butylhypochlorite–KI–starch reagents (*23*). For amino acid analysis, samples were hydrolyzed with constant boiling hydrochloric acid in evacuated sealed ampoules at 110°C for 16 hr, unless otherwise noted, and analyzed by the Spackman–Stein–Moore (*24*) method on a Beckman Spinco 120C amino acid analyzer.[4]

Synthesis of Compound I

*N-Benzyloxycarbonyl-S-benzyl-*D*-cysteinyl-*D*-leucine methyl ester (III).* To a solution of crude D-leucine methyl ester hydrochloride (*25*) (7.25 g, 40 mmole) in water (50 ml), a 50% K_2CO_3 solution (50 ml) was added, followed immediately by extraction with ether (4 × 50 ml each). The ether extracts were washed with water and pooled. The combined extracts were dried with $MgSO_4$, filtered, and added to *N*-benzyloxycarbonyl-*S*-benzyl-D-cysteine *p*-nitrophenyl ester (*26*) (9.13 g, 20 mmole) in ethyl acetate (50 ml). The solution was concentrated under reduced pressure to remove the ether. After 48 hr at room temperature, the solvent was removed *in vacuo* and the resulting oil dissolved in ether (70 ml), washed with eight portions of *N* NH_4OH (50 ml each), followed by washing with water, *N* HCl, and water. The organic layer was dried, filtered, and diluted with hexane (50 ml) until turbid. A few seed crystals from a pilot preparation were added and the product was allowed to crystallize. Gradually, more (250 ml) hexane was added. The crystals were collected, washed with hexane, and dried in air: yield, 7.44 g (79%); mp 77–80°C. A sample was recrystallized from ether–hexane: mp 81–83°C; $[\alpha]_D^{22}$ +40.7° (*c* 2, DMF); tlc R_fA 0.60, R_fB 0.66.

Anal. Calcd for $C_{25}H_{32}O_5N_2S$ (472.6): C, 63.5; H, 6.8; N, 5.9; S, 6.8. Found: C, 63.3; H, 7.0; N, 6.1; S, 6.7.

*Benzyloxycarbonyl-*L*-valyl-S-benzyl-*D*-cysteinyl-*D*-leucine methyl ester (IV).* A portion of compound III (4.15 g, 9.5 mmole) was dissolved in 4.7 *M* HBr in HOAc (18 ml) and was allowed to stand at room temperature for 1.5 hr. Ether (50 ml) was added, followed by hexane (100 ml). The partially deprotected dipeptide, an oil, was washed with ether–hexane, dried *in vacuo* over NaOH, and dissolved in DMF (30 ml). Diisopropylethylamine was added to neutrality (1.3 ml), followed by benzyloxycarbonyl-L-valine *p*-nitrophenyl ester (*26*) (6.72 g, 18 mmole) and by more DIPEA (1.5 ml, 9.5 mmole). After 18 hr at room temperature, the DMF was removed *in vacuo*. The residue, an oil, was dissolved in warm EtOAc (100 ml) and washed with water (100 ml), twice with 0.1 *M* HCl, and four times with water. The organic phase was dried with $MgSO_4$, filtered, and concentrated *in vacuo*. To the resulting oil, ether (100 ml) was added. The crystalline product was collected by filtration, washed well with ether, and dried in air to yield 4.23 g (78%), mp 151–152°C. An analytical sample was recrystallized from ether and EtOAc: mp 151–152°C; $[\alpha]_D^{22}$ +50.3° (*c* 1.87, DMF; tlc R_fA 0.61, R_fB 0.65.

Anal. Calcd for $C_{30}H_{41}O_6N_3S$ (571.7): C, 63.0; H, 7.2; N, 7.4; S, 5.6. Found: C, 62.5; H, 7.0; N, 7.4; S, 5.7.

*N-Benzyloxycarbonyl-S-benzyl-*D*-cysteinyl-*L*-valyl-*D*-cysteinyl-*D*-leucine methyl ester (V).* The protected tripeptide ester IV (4.0 g, 7.0 mmole) was dissolved in 4.5 *M* HBr in HOAc (14 ml). After 70 min, ether (25 ml) and hexane (100 ml) were added, and the

[4] The following abbreviations were used: DMF (dimethylformamide); DIPEA (diisopropylethylamine); TFA (trifluoroacetic acid); DMSO (dimethyl sulfoxide).

partially protected tripeptide hydrobromide was washed with ether–hexane. The precipitate was dried *in vacuo* for 2 hr over NaOH and dissolved in DMF (14 ml); DIPEA was added to neutrality (0.7 ml), followed by *N*-benzyloxycarbonyl-*S*-benzyl-D-cysteine *p*-nitrophenyl ester (*26*) (3.7 g, 8.0 mmole), DMF (9 ml), and more DIPEA (1.2 ml, 7.0 mmole). After 24 hr, the solvent was removed *in vacuo*, the resulting solid dissolved in $CHCl_3$ (100 ml) and washed with 0.1 *M* HCl (2×, 50 ml each), H_2O (4×), each aqueous phase being reextracted with a single portion of $CHCl_3$. The $CHCl_3$ solutions were pooled, dried with $MgSO_4$, and evaporated *in vacuo*. The solid residue was washed with ether, filtered, and dried in air to yield 4.2 g (78%). For analysis, a sample was recrystallized from 75% aqueous ethanol: mp 164–166°C; $[\alpha]_D^{22}$ +63.2° (*c* 1.5, DMF); tlc R_fA 0.62, R_fB 0.65.

Anal. Calcd for $C_{40}H_{52}O_7N_4S_2$ (765.0): C, 62.8; H, 6.9; N, 7.3; S, 8.4. Found: C, 62.7; H, 6.8; N, 7.6; S, 8.3.

Benzyloxycarbonyl-L-*isoleucyl*-*S*-*benzyl*-D-*cysteinyl*-L-*valyl*-*S*-*benzyl*-D-*cysteinyl*-D-*leucine methyl ester* (*VI*). A sample of V (4.0 g, 5.2 mmole) was dissolved in 4.5 *M* HBr in HOAc (10 ml). After 1.5 hr, ether (50 ml) and hexane (100 ml) were added. The hydrobromide was washed with ether–hexane, dried *in vacuo* over NaOH, and dissolved in DMF (10 ml). DIPEA was added to neutrality (0.7 ml), followed by benzyloxycarbonyl-L-isoleucine *p*-nitrophenyl ester (*26*) (3.9 g, 10 mmole) rinsed into the solution with DMF (7 ml). The next day, more DIPEA (0.1 ml) was added. After 2 days, the solvent was removed *in vacuo*, the residue triturated and washed with $CHCl_3$, and dried *in vacuo*. The $CHCl_3$ filtrates were then washed with 0.2 *N* HCl (100 ml) and H_2O (4×, 100 ml), and evaporated to dryness *in vacuo*. The combined chloroform soluble and insoluble material was washed with ether and dried *in vacuo* to yield 4.38 g (96%) of protected pentapeptide ester. A sample was recrystallized from 95% ethanol: mp 209–211°C; $[\alpha]_D^{22}$ +54.2° (*c* 1.5, DMF); tlc R_fA 0.70, R_fB 0.71. Amino acid analysis: Ile, 1.0; Leu, 1.1; Val, 1.2; Bzl-Cys, 2.1.

Anal. Calcd for $C_{46}H_{63}O_8N_5S_2$ (878.2): C, 62.9; H, 7.2; N, 8.0. Found: C, 62.8; H, 7.3; N, 7.8.

Benzyloxycarbonyl-L-*isoleucyl*-*S*-*benzyl*-D-*cysteinyl*-L-*valyl*-*S*-*benzyl*-D-*cysteinyl*-D-*leucine hydrazide* (*VII*). A sample of VI (1.76 g) was dissolved in refluxing methanol (250 ml), and the solution was allowed to cool to room temperature. Hydrazine (15 ml) was added and the solution stirred for 2 days. The precipitate was separated by centrifugation, washed with methanol, and the washings pooled with the decanted solution. The solid was dried *in vacuo*. Yield: 0.60 g. The solution was evaporated under reduced pressure, and traces of hydrazine removed with a stream of N_2. The residue was triturated and washed with methanol, and dried to yield 0.95 g. The two crops were identical by mp (236–238°C) and tlc (R_fA 0.52). The materials were pooled (1.55 g, 86%) and recrystallized from hot methanol. Yield, 1.23 g (80%); mp 239–240°C; $[\alpha]_D^{25}$ +18.2° (*c* 2.4, TFA); tlc R_fA 0.52.

Anal. Calcd for $C_{45}H_{63}N_7O_7S_2$ (878.6): C, 61.5; H, 7.2; N, 11.2. Found: C, 61.7; H, 7.3; N, 11.1.

cyclo-S-Benzyl-D-*cysteinyl*-L-*valyl*-*S*-*benzyl*-D-*cysteinyl*-D-*leucyl*-L-*isoleucyl* (*VIII*). A sample of VII (1.01 g, 1.28 mmole) was suspended in glacial acetic acid (4 ml); 4.5 *M* HBr in acetic acid (6 ml) was added, and the solution was allowed to stand for 2 hr. Ether (150 ml) was added to precipitate the product, which was collected by filtration,

washed with ether (50 ml), and dried *in vacuo*. The material was dissolved in DMF (11 ml), cooled to −20°C, and concd hydrochloric acid (0.10 ml) was added to the solution. Sodium nitrite (1.2 ml, 1 *M* solution) was added, and the solution was stirred for 15 min maintaining a −15 to −20°C temperature. Dimethylformamide (120 ml, precooled) was added, the solution made alkaline with DIPEA (0.6 ml), and allowed to stand for 3 days at 4°C and 2 days at room temperature. Most of the DMF was removed under reduced pressure, and water (100 ml) was added. The product which precipitated was collected by filtration, washed with DMF (60 ml in 3 portions), H_2O (60 ml), and MeOH (60 ml), and dried *in vacuo* (503 mg, 55%). There was slight decomposition at temperatures above 250°C, no melting up to 300°C; $[\alpha]_D^{25}$ +43.9° (*c* 2, TFA).

Anal. Calcd for $C_{37}H_{53}N_5S_2O_5$ (712.0): C, 62.5; H, 7.4; N, 9.9. Found: C, 62.5; H, 7.5; N, 10.1.

A sample (39 mg) of compound VIII was dissolved in liquid ammonia (about 70 ml) and reduced with sodium (*5*). The residue left after the removal of the ammonia was dissolved in DMF (7 ml) and H_2O (63 ml), stirred while exposed to air until the sodium nitroferricyanide reaction was negative, and then tested on corn roots for malformin-like activity (*27*). Moderate curvatures were observed when the peptide was applied at a concentration of 100 μg/ml.

Synthesis of Malformin A_1

*Benzyloxycarbonyl-*L*-valyl-*D*-leucine methyl ester* (*IX*). A sample of D-leucine methyl ester hydrochloride (*25*) (1.2 g) was dissolved in water (10 ml), followed by 50% K_2CO_3 (8 ml), and immediately extracted with ether (3×, 10 ml) and added to benzyloxycarbonyl-L-valine *p*-nitrophenyl ester (*26*) (1.38 g, 3.7 mmole) dissolved in DMF (11 ml). The ether was distilled off under reduced pressure, and the mixture left to stand at room temperature overnight. The solvent was removed *in vacuo*, the residue dissolved in ethyl acetate (50 ml) and washed with 0.1 *M* HCl (3×, 10 ml each), *M* NH_4OH (6×, 10 ml) and water (4×, 10 ml). The organic layer was dried with $MgSO_4$ and the solvent removed under reduced pressure. The crude material was dissolved in hot ether; the product crystallized on cooling. Yield, 1.32 g (95%); mp 135–136°C; $[\alpha]_D^{25}$ +25.4° (*c* 2, DMF); single spot on tlc, R_fA 0.64, R_fB 0.71.

Anal. Calcd for $C_{20}H_{30}N_2O_5$ (378.5): C, 63.5; H, 8.0; N, 7.4. Found: C, 63.6; H, 8.0; N, 7.4.

*N-Benzyloxycarbonyl-S-benzyl-*D*-cysteinyl-*L*-valyl-*D*-leucine methyl ester* (*X*). A sample of compound IX (1.14 g) was dissolved in glacial acetic acid (1 ml) and treated with 4.5 *M* HBr in acetic acid (5 ml). The solution was allowed to stand for 90 min, at which time ether (25 ml) and hexane (75 ml) were added to precipitate the hydrobromide, which was washed with a similar solvent mixture and finally with ether. The material was dried *in vacuo* over NaOH and dissolved in DMF (10 ml). The solution was brought to neutrality with DIPEA (0.1 ml), and *N*-benzyloxycarbonyl-*S*-benzyl-D-cysteine *p*-nitrophenyl ester (*26*) (1.17 g, 2.5 mmole) was added to the solution, followed by DIPEA (0.42 ml, 2.5 mmole). After 18 hr, the solvent was removed under reduced pressure, the residue dissolved in chloroform (50 ml) and washed with 0.1 *M* HCl (3×, 10 ml each), water (2×, 10 ml), *N* NH_4OH (4×, 10 ml) and water (5×, 10 ml). The

organic phase was dried with $MgSO_4$ and the solvent removed under reduced pressure. The resulting solid was dissolved in hot ethyl acetate (20 ml), cooled to room temperature, and diluted with ether (80 ml). The solution was set aside at 4°C and the product crystallized. Yield, 1.17 g (83%); mp 160–161°C; $[\alpha]_D^{25}$ +40.0° (*c* 2, DMF); single spot on tlc, R_fA 0.68, R_fB 0.73. Amino acid analysis: Val, 1.0; Leu, 1.0; *S*-Bzl-Cys, 1.0.

Anal. Calcd for $C_{30}H_{41}N_3O_6S$ (571.7): C, 63.0; H, 7.2; N, 7.4; S, 5.6. Found: C, 62.9; H, 7.3; N, 7.4; S, 5.3.

*N-Benzyloxycarbonyl-S-benzyl-*D*-cysteinyl-S-benzyl-*D*-cysteinyl-*L*-valyl-*D*-leucine methyl ester* (*XI*). Compound X (1.13 g, 2.0 mmole) was suspended in glacial acetic acid (3 ml) and treated with 4.5 *M* HBr in acetic acid. After 90 min, ether (50 ml) and hexane (50 ml) were added to precipitate the partially deprotected tripeptide, the solution decanted, and the precipitate washed twice with ether (50 ml). The material was filtered, dried *in vacuo*, and dissolved in DMF (10 ml). The solution was made neutral with DIPEA (0.03 ml). *N*-Benzyloxycarbonyl-*S*-benzyl-D-cysteine *p*-nitrophenyl ester (*26*) (1.18 g) was added, followed by DIPEA (0.33 ml, 2.0 mmole). Next day the solvent was removed under reduced pressure, the residue dissolved in chloroform (50 ml) and washed with water (2×, 10 ml), *M* NH_4OH (4×, 10 ml), 0.03 *M* HCl (2×, 10 ml), and water (10 ml). The organic layer was evaporated under reduced pressure, and the resulting solid was triturated and washed with ether (100 ml). Yield, 1.25 g (83%); mp 193–194°C; $[\alpha]_D^{25}$ +44.9° (*c* 1.76, DMF); tlc gave a single spot, R_fA 0.58, R_fC 0.63. Amino acid analysis: Val, 1.0; Leu, 1.0; *S*-Bzl-Cys, 2.0.

Anal. Calcd for $C_{40}H_{52}O_7N_4S_2$ (765.0): C, 62.8; H, 6.8; N, 7.3; S, 8.4. Found: C, 62.5; H, 6.8; N, 7.4; S, 8.4.

*Benzyloxycarbonyl-*L*-isoleucyl-S-benzyl-*D*-cysteinyl-S-benzyl-*D*-cysteinyl-*L*-valyl-*D*-leucine methyl ester* (*XII*). A sample of compound XI (1.17 g) was suspended in acetic acid (4.5 ml) and treated with 4.5 *M* HBr in acetic acid (4.0 ml) for 90 min. The partially protected tetrapeptide ester hydrobromide was precipitated with ether (200 ml), filtered, washed with ether (50 ml), and dried *in vacuo* over NaOH. After dissolving it in DMF (6 ml), DIPEA (0.18 ml) was added to neutrality followed by benzyloxycarbonyl-L-isoleucine *p*-nitrophenyl ester (*26*) (1.5 g) and DIPEA (0.27 ml). The reaction was allowed to proceed overnight, after which the solvent was removed under reduced pressure, the solid dissolved in chloroform (100 ml), washed with water (10 ml), 0.1 *N* HCl (2×, 10 ml), and water (5×, 10 ml). The solvent was removed under reduced pressure and the material was triturated, filtered, washed with ether (50 ml each), and dried *in vacuo*. Yield, 1.00 g (76%); mp 219–220°C (unchanged on recrystallization from 95% ethanol); $[\alpha]_D^{25}$ +40.0° (*c* 2.32, DMF); single spot by tlc, R_fA 0.68, R_fC 0.69.

Anal. Calcd for $C_{46}H_{63}N_5S_2O_8$ (878.5): C, 62.9; H, 7.2; N, 8.0; S, 7.3. Found: C, 62.9; H, 7.0; N, 8.0; S, 7.1.

*Benzyloxycarbonyl-*L*-isoleucyl-S-benzyl-*D*-cysteinyl-S-benzyl-*D*-cysteinyl-*L*-valyl-*D*-leucine hydrazide* (*XIII*). A portion of XII (0.86 g) was dissolved in refluxing methanol (170 ml), and the solution was cooled to room temperature. Hydrazine (8 ml, anhyd) was added and the solution stirred overnight. The precipitate was separated by centrifugation, washed with methanol (4×, 30 ml), and dried *in vacuo*. Yield, 0.77 g (90%); mp 239–241°C (unchanged on recrystallization from methanol); $[\alpha]_D^{25}$ +18.5° (*c* 2.16, TFA); single spot on tlc, R_fA 0.34.

Anal. Calcd for $C_{45}H_{63}N_7O_7S_2$ (878.6): C, 61.5; H, 7.2; N, 11.2; S, 7.3. Found: C, 61.4; H, 7.1; N, 11.0; S, 7.5.

*cyclo-S-Benzyl-*D*-cysteinyl-S-benzyl-*D*-cysteinyl-*L*-valyl-*D*-leucyl-*L*-isoleucyl (XIV).* A portion of XIII (0.61 g, 0.69 mmole) was suspended in glacial acetic acid (3 ml), 4.5 *M* HBr in acetic acid (2 ml) was added, and the solution was allowed to stand for 2 hr. Ether (150 ml) was added to precipitate the partially protected pentapeptide hydrazide hydrobromide, which was collected by filtration, washed with ether (50 ml), and dried *in vacuo*. The material was then dissolved in DMF (7 ml), concd hydrochloric acid (0.07 ml) was added, and the solution cooled to −20°C in a dry ice/acetone bath. To the cooled solution, 1 *M* $NaNO_2$ (0.7 ml) was added and stirred for 15 min at -17 ± 3°C. Dimethylformamide (100 ml, precooled) was added, the solution was made alkaline with DIPEA (0.3 ml) and allowed to stand for 3 days at +4°C and 2 days at room temperature. Most of the DMF was removed under reduced pressure, and water (100 ml) was added to precipitate the product. The material was collected by centrifugation, washed with DMF (3×, 20 ml), H_2O (3×, 20 ml), and MeOH (2×, 20 ml), and dried *in vacuo*. Yield, 262 mg (53%); slow decomposition at temperatures higher than 250°C, no mp up to 300°C; $[\alpha]_D^{25}$ +60° (*c* 1.5, TFA). Amino acid analysis: Val, 1.0; Leu, 0.9; Ile, 1.0; *S*-Bzl-Cys, 2.0.

Anal. Calcd for $C_{37}H_{53}N_5S_2O_5$ (712.0): C, 62.4; H, 7.5; N, 9.8; S, 9.0. Found: C, 62.3; H, 7.3; N, 9.8; S, 9.1.

Malformin A_1. A sample of XIV (70 mg) was suspended in liquid ammonia (ca. 120 ml) and treated with sodium at the boiling point of the solution until the blue color persisted for about 5 min. Ammonium chloride (ca. 0.1 g) was added, and the ammonia was allowed to evaporate. The last traces of ammonia were removed *in vacuo* over concd H_2SO_4. The residue was washed with 0.1% HOAc (20 ml) and dried *in vacuo* over P_2O_5. The peptide (47 mg) was dissolved with warming in DMSO (17 ml), and heated at 100°C for 1 hr (*20*). The solution was cooled to room temperature and titrated with a 0.05 *M* iodine solution in DMSO (1.4 ml, calcd 1.8 ml). The small excess of iodine was reacted with 2 drops of 1% aqueous $Na_2S_2O_3$. The solution was then diluted with ethyl acetate (250 ml) and washed with 1% $Na_2S_2O_3$ (75 ml) and water (5×, 50 ml). The precipitate that formed was separated by centrifugation, and each aqueous phase was washed with ethyl acetate. The organic layers were pooled and concentrated under reduced pressure. The last traces of insoluble material were removed by centrifugation. The total insoluble product (probably isomalformin; cf. Ref. *20*) amounted to 20 mg.

The ethyl acetate was removed *in vacuo* and the residue (ca. 25 mg) triturated with dry acetone (*2*). The insoluble material (3.4 mg) and the soluble product (20 mg) were identical on tlc; therefore, they were dissolved in trifluoroethanol, filtered, pooled, and the solvent evaporated with a stream of N_2. The residue was treated with 2 drops of ethyl acetate and then with hexane (5 ml). On trituration, a solid formed that was collected, washed with hexane (5 ml), and dried *in vacuo*. The final product (20 mg, 38%) decomposed without melting above 300°C. It gave a single spot on tlc (R_fB 0.38, R_fC 0.57) when revealed by uv absorption or by charring. Also, a single, opaque spot was observed on spraying with the reagent of Ziminski and Borowsky (*28*). By all these detection methods, the synthetic peptide was indistinguishable from natural malformin A applied to the same plates (Fig. 3). The cd spectrum of synthetic malformin A in trifluoroethanol was superimposable on that of the natural product (Fig. 4; cf. also

Refs. *21* and *22*). Severe curvatures were caused by the synthetic product on corn roots (*27*); the effect was most pronounced when the material was applied at a concentration of 0.1 μg/ml. The potency of the synthetic preparation was confirmed by Curtis (cf. Tables 2 and 3). Amino acid analysis: cf. Table 1.

TABLE 2

EFFECT OF SYNTHETIC MALFORMIN A_1 ON CORN ROOTS

Concn of synthetic Malformin A (ppm)	% Corn roots with 90° (or over 90°) curvature
0 (H_2O)	0.0
1.0	34.8
0.1	95.4
0.01	4.6

[a] The data in this table were determined by the method of Curtis (*27*).

TABLE 3

INHIBITION OF ADVENTITIOUS ROOT FORMATION ON *Phaseolus vulgaris* CV. HARVESTER

Treatment	Root per cutting
H_2O	6.4
Natural malformin, 0.5 ppm	0.3
Synthetic malformin, 0.5 ppm	0.2

[a] The data in this table were determined by the method of Curtis and Fellenburg (30).

Degradations

Partial hydrolysis of the S,S'-dibenzylcyclopentapeptides. *S,S'*-Dibenzylmalformein was prepared according to Isono and Curtis (*5*). Samples of *S,S'*-dibenzylmalformein, VIII and XIV (2 mg each), were dissolved in a few drops of TFA and hydrolyzed in concd HCl and glacial acetic acid (1 : 11 v:v) at 110°C for 2.5 hr. The products of partial hydrolysis were eluted with B buffer (*24*). The retention times of peptides are reported relative to the elution time of phenylalanine added as an internal standard: *S,S'*-Dibenzylmalformein, Leu, 1.0; Ile, 1.0; Val, 1.2; Bzl-Cys, 1.8; Peptides, 55 min, 88 min, 118 min, 128 min; Compound VIII, Leu, 1.0; Ile, 0.9; Val, 0.2; Bzl-Cys, 1.3; Peptide, 89 min; Compound XIV, Leu, 1.0; Ile, 0.9; Val, 1.1; Bzl-Cys, 2.0; Peptides, 55 min, 89 min, 120 min, 130 min. The elution time of authentic samples of L-Val-D-Leu and D-Leu-L-Ile were 55 and 89 min, respectively.

Partial hydrolysis of performic acid-oxidized peptides. Malformin (11.6 mg) was

suspended in formic acid (0.5 ml), and performic acid (*29*) (1.5 ml) was added. The mixture was stirred at room temperature until dissolution occurred, and allowed to stand at 4°C for 4 hr. The solvent was removed by a nitrogen stream and the residue dissolved in water. Samples of compounds I and II were oxidized in an analogous manner. Aliquots of the oxidized samples were hydrolyzed in 1 *M* HCl at 110°C for 2.5 hr and the hydrolysis products eluted from the long column of a Beckman Spinco 120C amino acid analyzer with B buffer (*24*); the amino acid ratios are normalized to isoleucine. Malformin: Ile, 1.0; Leu, 0.8; Val, 0.3; Peptides, 38 min, 56 min, 91 min; Compound I: Ile, 1.0; Leu, 1.0; Val, 1.8; Peptide, 90 min; Compound II: Ile, 1.0; Leu, 0.5; Val, 0.4; Peptides, 37 min, 55 min, 90 min.

ACKNOWLEDGMENTS

This study was supported by a grant from the U.S. Public Health Service (NIH AI-07515). The authors are greatly indebted to Professor R. W. Curtis of Purdue University for his generous assistance in providing a sample of natural malformin A, a strain of *A. niger* for its production, and corn seeds for the testing of the effect of our preparations. He also helped us with advice and encouragement. Professor David Perlman of the University of Wisconsin initiated our studies on malformin, and he prepared for us a charcoal adsorbate from a culture filtrate of *A. niger*. Amino analyses were performed by Mrs. Delores J. Gaut; elemental analyses by the Baron Consulting Co., Orange, CT.

REFERENCES

1. R. W. Curtis, *Science*, **128**, 661 (1958); *Plant Physiol.*, **33**, 17 (1958).
2. N. Takahashi and R. W. Curtis, *Plant Physiol.*, **36**, 30 (1961).
3. S. Marumo and R. W. Curtis, *Phytochemistry*, **1**, 245 (1961).
4. K. Anzai and R. W. Curtis, *Phytochemistry*, **4**, 263 (1965).
5. K. Isono and R. W. Curtis, *Phytochemistry*, **3**, 277 (1964).
6. A. Schöberl, M. Rimpler, and E. Clauss, *Naturwissenschaften*, **56**, 516 (1969); *Chem. Ber.*, **103**, 3159 (1970).
7. A. Schöberl, *Deut. Tieraerztl. Wochenschr.*, **78**, 557 (1971).
8. S. Suda and R. W. Curtis, *Appl. Microbiol.*, **14**, 475 (1966).
9. R. W. Curtis, *Plant Cell Physiol.*, **10**, 203 (1969).
10. M. Bodanszky and G. L. Stahl, *Proc. Nat. Acad. Sci. U.S.A.*, **71**, 2791 (1974).
11. S. M. Partridge and H. F. Davis, *Nature (London)*, **165**, 62 (1950).
12. E. J. Harfenist, *J. Amer. Chem. Soc.*, **75**, 5528 (1953).
13. M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959); M. Bodanszky, *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).
14. M. Bodanszky, *Nature (London)*, **175**, 685 (1955).
15. M. Bergmann and L. Zervas, *Ber. Deut. Chem. Ges.*, **65**, 1192 (1932).
16. D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).
17. M. Bodanszky and A. Bodanszky, *Chem. Commun.*, 541 (1967).
18. T. Curtius, *Ber. Deut. Chem. Ges.*, **35**, 3226 (1902).
19. R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).
20. K. Anzai and R. W. Curtis, *Phytochemistry*, **4**, 713 (1965).
21. J. P. Casey and R. B. Martin, *J. Amer. Chem. Soc.*, **94**, 6141 (1972).
22. M. Ptak, *Biopolymers*, **12**, 1575 (1973).
23. R. H. Mazur, B. W. Ellis, and P. Cammarta, *J. Biol. Chem.*, **237**, 1619 (1962).
24. O. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

25. H. F. SCHOTT, J. B. LARKIN, L. B. ROCKLAND, AND M. S. DUNN, *J. Org. Chem.*, **12**, 490 (1947).
26. M. BODANSZKY AND V. DU VIGNEAUD, *Biochem. Prep.*, **9**, 110 (1962).
27. R. W. CURTIS, *Plant Physiol.*, **36**, 37 (1961).
28. T. ZIMINSKI AND E. BOROWSKI, *J. Chromatogr.*, **23**, 480 (1966).
29. L. C. CRAIG, W. KONIGSBERG, AND T. P. KING, *Biochem. Prep.*, **8**, 70 (1961).
30. R. W. CURTIS AND G. FELLENBURG, *Plant Cell Physiol.*, **13**, 715 (1972).