Phytochemistry, 1967, Vol. 6, pp. 287 to 292. Pergamon Press Ltd. Printed in England

CHEMICAL STUDIES ON MALFORMIN—V.*

MALFORMIN B_1 AND B_2

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(*Received* 28 *June* 1966)

Abstract—Malformin B separated into two cyclic pentapeptides, B_1 and B_2, by thin-layer and column chromatography. The biological activity of both compounds was identical. Malformin B_1, $C_{23}H_{39}O_5N_5S_2$, contains cysteine, valine, leucine and allo-isoleucine in the ratio 2:1:1:1. Malformin B_2, $C_{22}H_{37}O_5N_5S_2$, contains cysteine, valine, and leucine in the ratio 2:2:1. Malformin A also separated into two compounds. The major fraction, A_1, approximately 87 per cent of the mixed sample, is identical with malformin A of earlier reports. The minor component, A_2 ($C_{22}H_{37}O_5N_5S_2$), contains cysteine, valine, and leucine or isoleucine in the ratio 2:2:1. Biological activity of malformin A_2 was questionable. Mass spectra of A_1 and B_1 were identical.

INTRODUCTION

THE structure, cyclo-L-isoleucyl-D-cysteinyl-L-valyl-D-cysteinyl-D-leucyl, was assigned to malformin A,[1,2] a plant growth regulator produced by the fungus *Aspergillus niger*.[3] Although malformin A was first isolated from *A. niger* 56–39, we are presently using a higher yielding strain, 58–883, for production of this compound. During a search for higher yielding strains of the fungus one isolate, 56–30, produced malformin which differed from malformin A.[4] We referred to this "new" compound as malformin B.[1] Although malformin B has similar physical properties and biological activity, it liberates low yields of isoleucine (0·08 mole) and allo-isoleucine (0·22 mole) after acid hydrolysis. The molar ratio of leucine, valine, and cysteine was similar to that obtained from malformin A. This report concerns chemical and mass spectrometry studies on malformin B, with emphasis on its separation into two active compounds, B_1 and B_2.

RESULTS AND DISCUSSION

Separation of Malformin B_1 and B_2 by Thin-Layer Chromatography

Malformin B was isolated from culture filtrate of *A. niger* 56–30 as described earlier.[4] From 3 g of crude malformin B applied to alumina chromatography we obtained 1·7 g of purified material. To check the purity of this sample 2 mg were dissolved in 2 drops of acetic acid and chromatographed on thin-layer plates using silica gel H as adsorbent and

* Journal Paper No. 2857 of the Purdue Agricultural Experiment Station. This work was supported by the American Cancer Society, grant E-146 D, and The National Science Foundation, grant G-20989. The mass spectrometry was supported by the National Aeronautic and Space Administration and by the National Institutes of Health.

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[1] S. MARUMO and R. W. CURTIS, *Phytochem.* 1, 245 (1961).
[2] K. ANZAI and R. W. CURTIS, *Phytochem.* 4, 263 (1965).
[3] R. W. CURTIS, *Plant Physiol.* 33, 17 (1958).
[4] N. TAKAHASHI and R. W. CURTIS, *Plant Physiol.* 36, 30 (1961).

ethyl acetate (water saturated) as solvent. After drying, the plates were sprayed with 30% sulfuric acid and heated. Two spots, R_f=0·84 and R_f=0·80, were detected. The faster-moving compound was designated as malformin B_1 and the slower, B_2. When the plates were sprayed with water, rather than sulfuric acid, the compounds appeared as white spots on a translucent background. Both compounds were active in the corn root curvature test.[5] Mass spectrometry analysis indicated approximately 50% each of B_1 and B_2 in the mixed sample.

Separation of Malformin B_1 and B_2 by Column Chromatography

Separation of malformin B_1 and B_2 by silica gel column chromatography, using water-saturated ethyl acetate as solvent, was attempted. Thin-layer chromatography was used to detect elution of the compounds and their purity. Although a degree of purification was achieved, separation was not complete. The fractions were divided into two groups, one rich in malformin B_1 and the other rich in B_2, and each chromatographed again on silica gel columns. The process was repeated, if necessary, until fractions were obtained containing only malformin B_1 or B_2 as judged by thin-layer chromatography. By mass spectrometry the pure sample of B_1 contained less than 1% B_2, but B_2 was contaminated with about 20% of B_1. Although adequate quantities of pure malformin B_1 were obtained, we were able to obtain only small quantities of B_2.

Biological Activity of Malformin B_1 and B_2

The optimum concentration of both compounds for induction of curvatures in corn roots was 0·1 ppm. Higher or lower concentrations diminished root curvatures. The activity of the separated compounds was identical with that of the mixture and with that of malformin A.

Chemical Properties of Malformin B_1 and B_2

The molecular formula of malformin B_1 was determined as $C_{23}H_{39}O_5N_5S_2$ by mass spectrometry (Table 1) and elemental analysis. In this respect it is identical with malformin

TABLE 1. HIGH RESOLUTION MASS MEASUREMENTS OF THE MOLECULAR ION OF MALFORMINS

Malformin	Elemental composition	Calc. mass*	Measured mass	Error (mmu)
A_1	$C_{23}H_{39}O_5N_5S_2$	529·2392	529·2418	2·6
A_2	$C_{22}H_{37}O_5N_5S_2$	515·2236	515·2224	1·2
B_1	$C_{23}H_{39}O_5N_5S_2$	529·2392	529·2386	0·6
B_2	$C_{22}H_{37}O_5N_5S_2$	515·2236	515·2230	0·6

* Calculated on basis C=12·0000.

A, as this formula corresponds to amino acid components of two cysteine, two leucine (any isomer) and one valine. The amino acids present in acid hydrolysates of malformin B_1 were determined qualitatively by paper chromatography. The results were the same as that reported earlier,[4] i.e., the relative intensity of the leucine, valine, and cysteine spots was

[5] R. W. CURTIS, *Science* **128**, 661 (1958).

similar, whereas the intensity of the allo-isoleucine (or isoleucine) spot was weaker than that for malformin A.

The molecular formula of malformin B_2 by mass spectrometry, $C_{22}H_{37}O_5N_5S_2$ (Table 1), corresponded to a compound containing one less CH_2 group than malformin B_1, e.g., replacement of a leucine by a valine. In addition, the ratio of amino acids in malformin B_2 appeared to differ from that of malformin B_1. Compared to malformin B_1, the valine spot obtained on paper chromatograms of acid hydrolysates of B_2 was considerably darker, and the leucine–isoleucine spot considerably lighter. These observations suggested that malformin B_2 contained cysteine, valine, and a member of the leucine family in the ratio of 2:2:1.

The i.r. spectra of malformin B_1 and B_2 were identical with that of malformin A. None of these compounds adsorb in u.v. light. Malformin B_1, B_2, and A decompose rapidly at temperatures over 300°. Optical rotation of malformin B_1 and B_2 differed slightly (B_1; $[\alpha]_D^{25} = -40{\cdot}3°$, $c = 0{\cdot}72$, methyl cellosolve: B_2; $[\alpha]_D^{25} = -49{\cdot}5°$, $c = 0{\cdot}73$, methyl cellosolve).

Mass Spectrometry of Malformin B_1 and B_2

Chromatographically pure samples of malformin B_1 and B_2 were employed to obtain the mass spectra shown in Figs. 1 and 2. Mass spectra were recorded with a double focusing

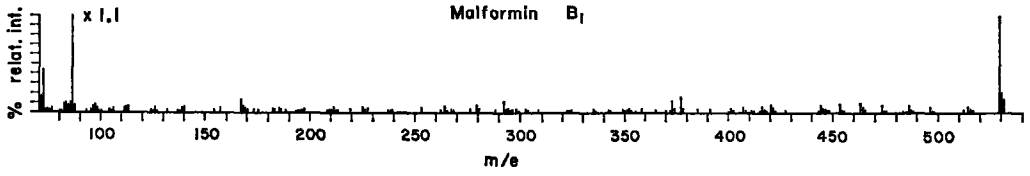


FIG. 1. MASS SPECTRUM OF MALFORMIN B_1.

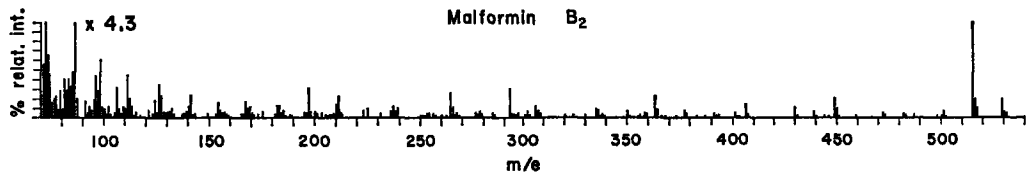


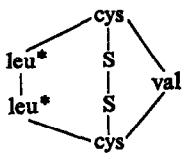
FIG. 2. MASS SPECTRUM OF MALFORMIN B_2.

instrument (Consolidated Electrodynamics Corporation 21-110B) using photoplate recording for high resolution data reduction. The spectra shown in Figs. 1 and 2 were obtained by utilizing an electron multiplier. The samples were introduced into the ion source through a direct introduction system and were recorded at ionizing voltage 70 eV, ionizing current 50 μA.

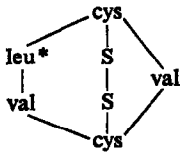
The stability of the bicyclic ring systems caused the fragment ions to be of relatively low abundance, but exact mass measurements on these confirm the presence of the amino acids two cysteine, one valine and two leucine (or isomers) in malformin B_1 (I) and two cysteine, two valine and one leucine (or isomer) in B_2 (II). For example, the presence of abundant ions at m/e 72 ($C_4H_{10}N^+$) and 86 ($C_5H_{12}N^+$) are indicative of the presence of valine and leucine respectively.[6] The amino acid sequence analyses were carried out with desthio derivatives of the malformins, prepared by desulphurization with Raney nickel. Details of these

[6] B. J. MILLARD, *Tetrahedron Letters* 3041 (1965).

determinations are reported separately.[7] The major fragment ions of these spectra are consistent with the structures illustrated in formulae (I) and (II), but there are minor ions that could be due either to isomeric impurities or to rearrangements in the mass spectra.



(I)



(II)

* leucine or isomer

From earlier studies, one mole of leucine, 0·22 mole of allo-isoleucine, and 0·08 mole of isoleucine were liberated after acid hydrolysis of malformin B[4]. Therefore, one of the two members of the leucine-group in B_1 must be leucine itself, and the molecule contains cysteine, valine, leucine and allo-isoleucine or isoleucine in the ratio of 2:1:1:1.

These studies explain in part the low yield of allo-isoleucine (0·22 mole) from acid hydrolysates of malformin B, which contains almost equal quantities of B_1 and B_2. An equimolar mixture of two peptides, cyclo- allo-isoleucyl-cysteinyl-valyl-cysteinyl-leucyl (B_1) and cyclo-valyl-cysteinyl-valyl-cysteinyl-leucyl (B_2) can liberate only 0·50 mole of allo-isoleucine. From such a mixture, 1·50 moles of valine should have been obtained, whereas 1·06 moles were found (71 % yield). However, under similar conditions of hydrolysis the yield of valine from malformin A was only 80 per cent. Because of the greater yield of allo-isoleucine, as compared to isoleucine, we propose that the structure of malformin B_1 is the same as indicated above.

Separation of Malformin A_1 and A_2

In view of the separation of malformin B into two compounds, malformin A was chromatographed in the same manner. Two spots were detected and were designated as malformin A_1 for the major, faster-moving compound (R_f=0·82), and as A_2 for the slower compound (R_f=0·77). When chromatographed on the same plates with malformin B_1 and B_2 the slight differences in R_f value of the four compounds was consistently obtained. Despite repeated attempts we were unable to obtain chromatographically pure samples of A_2 by column chromatography. Using chromatographically pure samples of A_1 the optimum concentration for induction of corn root curvatures was unchanged from that of the mixed sample, i.e., 0·1 ppm. Because of the small quantities of A_2 in the original mixed sample (see below) it was not surprising that the activity was unchanged. We estimated that the activity of samples relatively rich in malformin A_2 was approximately $\frac{1}{10}$ of A_1. Since the A_2 preparation was contaminated with A_1, the biological activity may have been due to the latter.

Our earlier work on malformin A employed samples containing approximately 88 % malformin A_1 and 12 % of A_2 by mass spectrometry. We feel that the small quantities of A_2 present in these samples did not influence the results.

Mass Spectrometry of Malformin A_1

The amount of A_2 in malformin A is considerably less than the amount of B_2 in malformin B. Chromatographically pure samples of malformin A_1 contained about 93 per cent A_1 and 7 per cent A_2.

[7] M. SENN and F. W. MCLAFFERTY, to be submitted to *Tetrahedron Letters*.

The mass spectrum of malformin A_1 is identical to that of B_1 within experimental error. This is also true of the corresponding desthio derivatives, and indicates that these compounds differ only in their isomeric composition. We were unable to obtain suitable quantities of malformin A_2. However, the elemental composition was determined from the spectrum of the mixed sample (Table 1) indicating a structure similar to that of malformin B_2. Several properties of the various malformins are summarized in Table 2.

TABLE 2. PROPERTIES OF MALFORMIN A_1, A_2, B_1, AND B_2

	Molecular formula	Biologically active	$[\alpha]_D^{25}$	R_f	Amino acids
A_1	$C_{23}H_{39}O_5N_5S_2$	+	−39·0°	0·82	cys:val:leu:ileu (2:1:1:1)
A_2	$C_{22}H_{37}O_5N_5S_2$	?	?	0·77	cys:val:leu or ileu (2:2:1)
B_1	$C_{23}H_{39}O_5N_5S_2$	+	−40·3°	0·84	cys:val:leu:allo-ileu (2:1:1:1)
B_2	$C_{22}H_{37}O_5N_5S_2$	+	−49·5°	0·80	cys:val:leu (2:2:1)

The malformins constitute a family of closely related cyclic pentapeptides at least three of which are biologically active. We have demonstrated the formation of biologically active and inactive conformational isomers of malformin A_1 and thiolmalformin A_1.[2,8] Although naturally-occurring conformational isomers have not been demonstrated, neither have they been excluded. Conformational isomers or isomers which differ only in optical configuration of one or more amino acids would not readily separate and could not be differentiated in mixed samples by mass spectrometry. The malformins described here all contain 2 moles of cysteine, 1 or 2 moles of valine, and 1 or 2 moles of the leucine family of amino acids. Whether or not these represent basic requirements for biological activity can only be determined by isolation and characterization of additional compounds with malformin-like activity.

EXPERIMENTAL

Separation of Malformin B_1 and B_2 by Column Chromatography

Malformin B, 300 mg, was dissolved in warm, glacial acetic acid, 100 ml, concentrated *in vacuo* to 10 ml, and treated with chromatographic grade silica gel, 10 g. The silica gel slurry was dried *in vacuo*, suspended in ethyl acetate (water saturated), and added to the top of a 4·0 × 115 cm column of silica gel previously packed with water-saturated ethyl acetate. The column was eluted, 20–30 drops per min, with water-saturated ethyl acetate. Two hundred 12 ml fractions were collected. The presence of malformin B_1 and B_2 in the various fractions was determined by thin-layer chromatography. Malformin B_1 was found in fractions 132–138 and B_2 in fractions 153–180. When the fractions were pooled separately, concentrated, and again chromatographed on thin-layer plates they were found to be contaminated, each with the other. However, it appeared that a degree of purification had been achieved. The B_1-rich samples were chromatographed on 2·2 × 40 cm silica gel columns as described, except 10 ml fractions were collected. Malformin B_1 was found in fractions 34–40, B_2 in fractions 55–63.

Fractions containing malformin B_1 and B_2 were separately combined, washed with aqueous $NaHCO_3$, 30% H_2SO_4, and water and dried *in vacuo*. The final products were pure as judged by thin-layer chromatography.

[8] K. ANZAI and R. W. CURTIS, *Phytochem.* 4, 713 (1965).

Molecular Formula of Malformin B_1 and B_2

Chromatographically pure samples of malformin B_1 and B_2 were used for elemental analysis. Malformin B_1: Required for $C_{23}H_{39}O_5N_2S_2$ (M.W. 529·7): C, 52·11; H, 7·36; N, 13·22; S, 12·10. Found: C, 52·03; H, 7·38; N, 13·18; S, 11·92%. Malformin B_2: Required for $C_{22}H_{37}O_5N_5S_2$ (M.W. 515·68): C, 51·24; H, 7·23; N, 13·58; S, 12·43. Found: C, 51·48; H, 7·32; N, 13·28; S, 11·88%.