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CHEMICAL STUDIES ON MALFORMIN—III.*

STRUCTURE OF MALFORMIN *A*

KENTARO ANZAI† and ROY W. CURTIS

Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana

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Abstract—The amino acid sequence of malformin *A*, cyclo-L-isoleucyl-D-cysteinyl-L-valyl-D-cysteinyl-D-leucyl, was confirmed. The presence of an S—S bond in the molecule was proved by iodine oxidation of thiolmalformin *A* to malformin *A*. A stereoisomer, isomalformin *A*, was also produced during oxidation. Both S—S compounds were interconvertible via the SH compound. Possible steric configurations of malformin *A* were discussed.

INTRODUCTION

MALFORMIN *A*, a metabolic product of the fungus *Aspergillus niger* which induces malformations of bean plants[1,2] and severe curvatures of corn roots,[3] was assigned the structure, cycol-L-isoleucyl-D-cysteinyl-L-valyl-D-cysteinyl-D-leucyl.[4,5] The structure surrounding the sulfur was not clarified and some results were inconsistent with the presence of an S—S bond. By reduction of malformin *A* with zinc in acetic acid[5] or with sodium in liquid ammonia,[6] thiolmalformin *A* was prepared. Uncertainty regarding the structure surrounding the sulfur was excluded in this compound.

Synthesis of thiolmalformin *A* was attempted,[6] but the synthetic compound induced no curvature of corn roots[3] at a concentration of 100 ppm, while natural thiolmalformin *A* had optimum activity at 1 ppm. A significant difference in optical rotations of the benzylated derivatives of natural thiolmalformin *A* and the synthetic compound was also observed. Whereas the specific rotation of S,S′-dibenzylmalformin *A* was $+62{\cdot}7°$ ($c = 2\%$, trifluoroacetic acid), the synthetic compound gave a value of $+40{\cdot}2°$ under similar conditions. If the proposed structure of malformin *A* is correct, these differences could be ascribed to dissimilarities in the third-dimensional structure. We know of no reports suggesting the presence of stable stereoisomers in cyclic peptides as small as malformin *A*. We considered malformin *A* from this and other viewpoints, especially the amino acid configuration, sequence, and structure surrounding the sulfur.

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† Institute of Physical and Chemical Research, Bunkyo-ku, Tokyo, Japan.

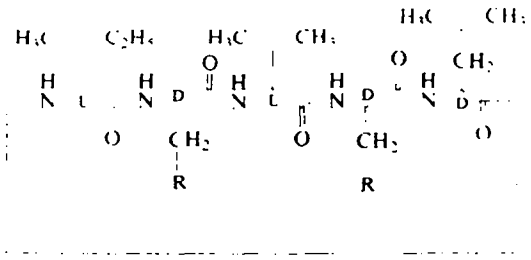
[1] R. W. CURTIS, *Plant Physiol.* **33**, 17 (1958).
[2] R. W. CURTIS, *Plant Physiol.* **36**, 37 (1961).
[3] R. W. CURTIS, *Science*, **128**, 661 (1958).
[4] N. TAKAHASHI and R. W. CURTIS, *Plant Physiol.* **36**, 30 (1961).
[5] S. MARUMO and R. W. CURTIS, *Phytochem.* **1**, 245 (1961).
[6] K. ISONO and R. W. CURTIS, *Phytochem.* **3**, 277 (1964).

RESULTS AND DISCUSSION

Amino Acid Configuration, Sequence and Molar Ratio

The molar ratio of amino acids in malformin *A* (I), oxidized malformin *A* (II) and desthiomalformin *A* (III) supported a 1:1:1:2 ratio of L-valine, L-isoleucine, D-leucine, and D-half cystine, respectively.[4,5] Oxidized malformin *A* (II) and desthiomalformin *A* (III), obtained from malformin *A* by performic acid oxidation and reduction with Raney nickel, respectively, were hydrolyzed and the component amino acids were separated by column chromatography. Results on the optical configuration of half-cystine were conflicting. Although desthiomalformin *A* yielded 2 moles of D-alanine, 2 moles of DL-cysteic acid were obtained from oxidized malformin *A*.[5] It was concluded that both moles of half-cystine in the original molecule were D-form and that they were racemized during performic acid oxidation and hydrolysis.

To clarify these results malformin *A* itself was hydrolyzed, and cystine was isolated by cellulose column chromatography. Although some degradation and racemization of cystine



FORMULAE I–IV

Formulae: I. R = S
II R = SO_3H
III R = H
IV R = SH

was expected, the $[\alpha]_D^{23}$ was +40°. We concluded that both half-cystine residues in the molecule were D-form and calculated 81 per cent racemization during hydrolysis. Authentic L-cystine treated similarly racemized 92·5 per cent. Because DL-cysteic acid was obtained from acid hydrolysates of malformin *A* previously oxidized with performic acid, and because authentic cystine and cysteine were not racemized by performic acid oxidation, the possibility of other than an S—S bond in malformin *A* was suggested earlier.[5] Racemization of authentic cysteic acid during hydrolysis was investigated. After heating at 110° for 4 hr in a mixture of concentrated HCl and acetic acid (1:1) authentic cysteic acid was 88 per cent racemized. Under the same conditions in 20% HCl and without acetic acid, racemization decreased to 58 per cent. Acetic acid appeared to promote racemization.[7] Thus, racemization of D-cysteic acid in oxidized malformin *A* during hydrolysis explains this anomaly. From the $[\alpha]_D$ values reported earlier[5] we calculated 88·5 per cent racemization of D-cysteic acid during hydrolysis of oxidized malformin *A*.

We confirmed the optical configuration of the other amino acid components of malformin *A* as reported earlier.[5] In addition, the molar ratio of these amino acids was confirmed by gas

[7] M. BERGMANN and L. ZERVAS, *Biochem. Z.* **203**, 280 (1924).

chromatography of the DNP-methylesters.[8] Valine, leucine, and isoleucine are present in equimolar ratios in malformin *A* (Fig. 1). Cystine was excluded from the hydrolysate before dinitrophenylation.

Thiolmalformin *A* (IV) was partially converted to thiazoline-containing malformin *A* (V) in concentrated HCl by attachment of the sulfur to the carbonyl groups of the amino acid residues connected to each cysteine residue.[5] The appearance of allo-isoleucine from bacitracin after acid hydrolysis was explained as a result of the double bond in such a thiazoline ring shifting to the α-carbon of isoleucine during hydrolysis, resulting in the racemization of L-isoleucine to D-allo-isoleucine.[9] During hydrolysis of thiazoline-containing malformin *A* the two amino acid residues adjacent to each half-cystine residue should racemize. The appearance of allo-isoleucine in acid hydrolysates of thiazoline-containing malformin *A* supported the presence of an isoleucyl-cysteinyl linkage in the molecule.[5] If the proposed amino acid sequence is correct, valine should also be racemized during hydrolysis of thiazoline-containing

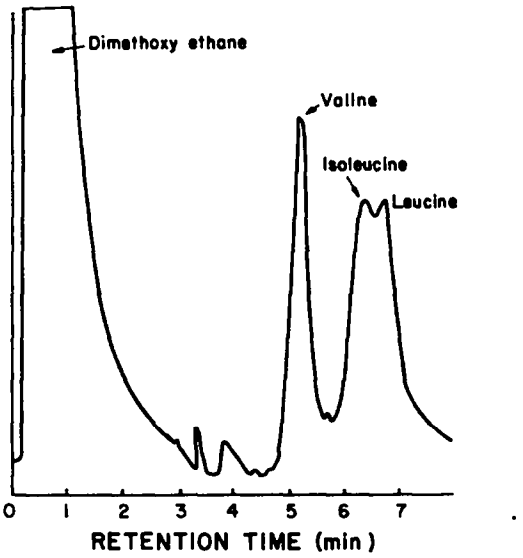
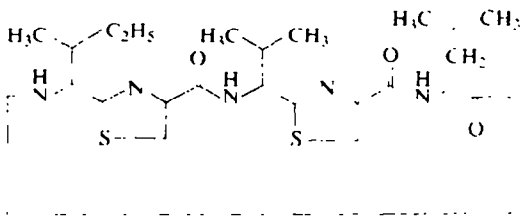


FIG. 1. GAS CHROMATOGRAPHY OF VALINE, ISOLEUCINE, AND LEUCINE FROM MALFORMIN *A*.

malformin *A*. Maximum formation of thiazoline-containing malformin *A* from thiolmalformin *A* in concentrated HCl occurred after 3 days, a period of such length that some hydrolysis of the peptide had occurred. It was, therefore, impossible to obtain completely racemized valine. Nonetheless, valine racemized 22 per cent by this method. This value was in close agreement with that reported for racemization of isoleucine (26 per cent).[5] When malformin *A* itself was treated similarly only 2 per cent racemization of valine occurred. These results confirm the presence of a valyl-cysteinyl linkage in the molecule.

Racemization of isoleucine and valine during hydrolysis of thiazoline-containing malformin *A* in itself limits the amino acid sequence to two possibilities, cyclo-valyl-cysteinyl-isoleucyl-cysteinyl-leucyl and cyclo-isoleucyl-cysteinyl-valyl-cysteinyl-leucyl. The latter coincides with the sequence proposed earlier and the former is inconsistent with the experimental results.[5] We concluded that the proposed sequence was correct.

[8] J. J. PISANO, W. J. A. VANDENHEUREL and E. C. HORNING, *Biochem. Biophys. Res. Communs.* 7, 82 (1962).
[9] W. KONIGSBERG and L. C. CRAIG, *J. Am. Chem. Soc.* 81, 3452 (1959).

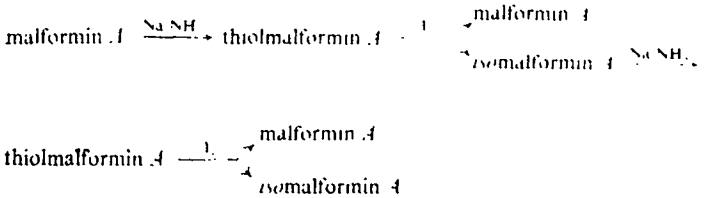


V

Structure surrounding the sulfur. The presence of an S—S bond in malformin *A* was not postulated because (1) D-half-cystine was racemized to DL-cysteic acid during performic acid oxidation and hydrolysis whereas authentic cystine was not racemized by performic acid oxidation, (2) the far i.r. spectrum of malformin *A* was essentially the same as that of desthiomalformin *A* and showed no appreciable absorption in the region of S—S bonds, and (3) two cysteine-containing peptides, leucyl-isoleucyl-cysteine and valyl-cysteinyl-leucine, were among the partial hydrolysis products of malformin *A*.[5] We have shown that racemization of cysteic acid occurred during hydrolysis and need not be considered.

The far i.r. spectra of malformin *A* (480 and 580 cm^{-1}), thiolmalformin *A* (470 and 590 cm^{-1}), and dibenzylmalformin *A* (470 and 590 cm^{-1}) were essentially the same in the region of disulfide absorption (400–500 cm^{-1}).[8] We attempted to show an S—S bond in the corresponding Raman line by the method of Ferraro *et al.*[10] Because of the low solubility of malformin *A*, the Raman spectrum was made in the solid state. Whereas cystine has a strong Raman line at 500 cm^{-1}, malformin *A* did not. These results will be discussed later.

While it is theoretically possible to obtain cysteine-containing peptides as partial hydrolysis products of thiolmalformin *A*, their occurrence in partial hydrolysates of malformin *A* is unusual and inexplicable without intramolecular oxidation-reduction. An S—S bond of sufficient energy, effected by peculiarities in third-dimensional structure, might split into radicals, dismutate, and produce cysteine residues. Dismutation of an aromatic disulfide was suggested by McClelland and Warren.[11] To obtain disulfide malformin *A*, thiolmalformin *A* was oxidized in dimethylsulfoxide by iodine, consuming an equivalent amount of iodine. Two substances were isolated from the reaction mixture. The first was difficultly soluble in numerous organic solvents and less active (approx. 5 ppm optimum) in inducing corn-root curvatures than thiolmalformin *A* (1 ppm). The molecular weight of this compound, determined by Signer's method, was 570 (mol. wt. required: 530), indicating that it was a monomer. Although the optical rotation of this compound ($[\alpha]_D^{23} = +15$, trifluoroacetic acid) and malformin *A* ($[\alpha]_D^{23} = -18$, trifluoroacetic acid) were different, their i.r. spectra



SCHEME I

[10] J. R. FERRARO, J. S. ZIOMEIC and G. MACK, *Spectrochim. Acta* **17**, 802 (1961).
[11] E. W. MCCLELLAND and L. A. WARREN, *J. Chem. Soc.* 1095 (1930)

were almost identical. Some difference was noted in the region of hydrogen-bonded amide absorption (3300 cm^{-1}). We believe this compound is a stereoisomer of malformin *A* and named it isomalformin *A*. Reduction of isomalformin *A* by sodium in liquid ammonia yielded thiolmalformin *A*, as judged by identical $[\alpha]_D$ values, i.r. spectra, and biological activity. Thiolmalformin *A* obtained by reduction of isomalformin *A* was also oxidizable to isomalformin *A* and malformin *A* (Scheme I). Because the second compound was more readily soluble in dimethylsulfoxide, dimethylformamide, and ethyl acetate it could be separated from isomalformin *A*. The mol. wt. (found, 550; required, 530), biological activity (optimum activity for corn-root curvatures, 0·1 ppm), optical rotation ($[\alpha]_D^{23} = -18°$, $c = 1$, trifluoroacetic acid), i.r. spectrum, and summit potential in A.C. polarography ($E_s = -0{\cdot}375$ V in 0·1 N HCl) of this compound were identical with that of malformin *A*. The polarograms (Fig. 2) indicated that malformin *A* was more easily reduced than cystine.

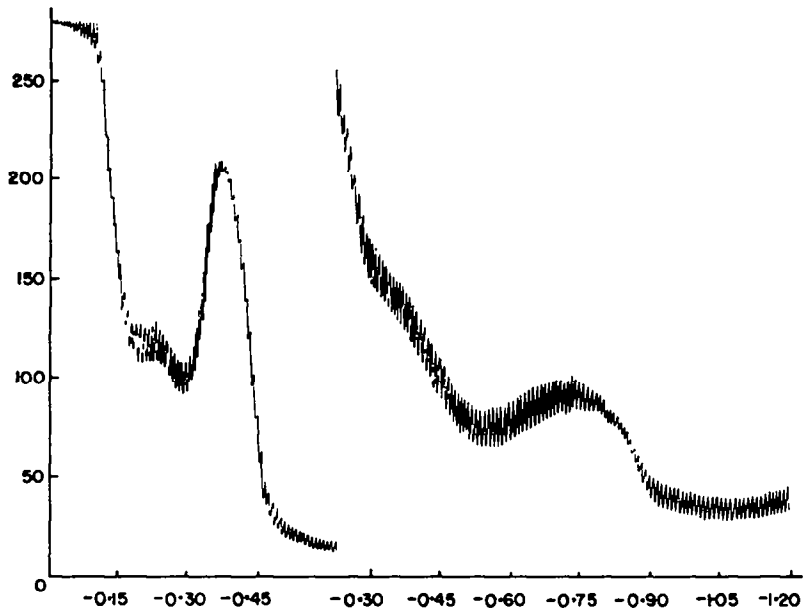


FIG. 2. POLAROGRAPHY OF MALFORMIN *A* (LEFT) AND CYSTINE (RIGHT).

Attempts to obtain malformin *A* directly from isomalformin *A* by solvent treatment, i.e. heating in dimethylsulfoxide or dissolution in trifluoroacetic acid for 20 days, were not successful. Ferric chloride was also capable of oxidizing thiolmalformin *A* to malformin *A*.

Steric Configuration of Malformin A

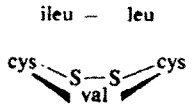
Molecular models showed that four stereoisomers (VI–IX) of malformin *A* are possible. They correspond to the four possible directions of two accessing sulfur atoms which form a disulfide linkage during oxidation of thiolmalformin *A*. One pair of isomers has *cis*-configuration and the other has *trans*-configuration. They are not interconvertible without breaking the disulfide bond.

One *cis*-isomer (VI) is quite flexible and has numerous opportunities for intramolecular hydrogen bonding between carbonyl and amido groups within the ring. Opportunities for

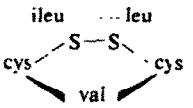
hydrogen bonding directed toward the outside of the ring are possible by breaking the intramolecular hydrogen bonds. In this case, intermolecular hydrogen bonding is possible. The angle between the two C—S bonds can be shifted by transformation of the peptide ring while maintaining *cis*-configuration of the disulfide bond. A Raman line of the S—S bond in this isomer would be expected to be considerably weaker than that of cystine. The other *cis*-isomer (VII) is less flexible with fewer opportunities for intramolecular hydrogen bonding. Carbonyl and amido groups tend to be directed to the outside of the ring and rotation of C—S bonds would not be expected in this isomer.

The structure of one *trans*-isomer (VIII) is quite rigid. The S—S bond is perpendicular to the ring plane and is close to the symmetrical center of the molecule. A strong Raman line in the disulfide region would be expected. The other *trans*-isomer (IX) is difficult to construct, depending on the type of molecular model, and has strong steric hindrance of the S—S bond.

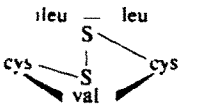
The energy level in the S—S bonds of the four isomers of malformin *A* is probably higher than that in the S—S bond of cystine. In cystine, the cysteine residues rotate freely around the disulfide bond, and the energy level in this bond would be relatively low. In malformin *A*, however, free rotation around the S—S bond is restricted and the energy level of this bond is probably increased. This suggestion is supported by the different polarographic behavior of the two compounds and agrees with the results of Nygård and Schott[12] regarding polarography of disulfide bonds in five- to seven-membered ring systems.



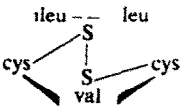
(VI)



(VII)



(VIII)



(IX)

We have considered the structure of malformin *A* only as a mono-cyclic pentapeptide. Theoretically, bi-cyclic peptide rings are possible which would not be inconsistent with the experimental results. We calculated approximately thirty-five possible bi-cyclic structures. While construction of their models was possible in the thiol form, in most cases the structures were too rigid to permit S—S linkage without rupture of the ring. In a few cases, S—S bonds could be formed but the formation of stereoisomers, such as isomalformin *A* was impossible.

EXPERIMENTAL

Isolation of Cystine from Malformin A

Malformin *A* (400 mg) was dissolved in glacial acetic acid (40 ml) and conc. HCl (40 ml) was added. After heating at 110° for 24 hr the hydrolysate, containing cystine precipitate, was dried and the residue (440 mg) dried *in vacuo* over NaOH. Cystine was separated from the other amino acids by cellulose column chromatography using a 3·8 × 50 cm column delivering 3 drops per min and collecting 8 ml fractions. The solvent, ethanol : H_2O : NH_3, was varied as follows: fractions 1–20, 85:10:5; 21–73, 80:15:5; 74–117, 75:20:5. Cystine was eluted with tailing in fractions 70–115. These fractions were combined, dried, and the residue dissolved in 0·1 N HCl. Trace impurities were removed by filtration and the filtrate was dried. The residue, which gave a single spot by paper chromatography, was recrystallized from water and

[12] B. NYGÅRD and L. SCHOTT, *Acta Chem. Scand.* **10**, 469 (1956).

ethanol. Weight 45 mg, $[\alpha]_D^{23} = +40°$ ($c = 1$, 1 N HCl). Racemization of D-cystine was calculated as 81 per cent. When malformin *A* was hydrolyzed at 120° for 60 hr and cystine isolated as described above, racemization was 97 per cent; $[\alpha]_D^{23} = +6{\cdot}5°$ ($c = 1$, 1 N HCl).

Leucine, isoleucine, and valine were eluted without complete separation in fractions 6–50. These fractions were combined, dried, and the amino acids separated by cellulose column chromatography using butanol:acetic acid:H_2O = 4:1:5. Valine, 29 mg. $[\alpha]_D^{22} = +19{\cdot}8°$ ($c = 1{\cdot}5$, 6 N HCl), was racemized 2 per cent as judged by the specific rotation of authentic L-valine, $[\alpha]_D^{22} = +20{\cdot}1°$, ($c = 1{\cdot}5$, 6 N HCl). Leucine and isoleucine were isolated as a mixture. Weight 70 mg, $[\alpha]_D^{22} = +8{\cdot}3°$ ($c = 3{\cdot}5$, 6 N HCl).

Racemization of Cystine and Cysteic Acid

Authentic L-cystine, $[\alpha]_D^{23} = -209{\cdot}4°$ ($c = 1$, 1 N HCl), was heated under various conditions (Table 1). Per cent racemization was calculated from the decrease in specific rotation. Similarly, authentic L-cysteic acid, $[\alpha]_D^{20} = +8{\cdot}59°$ ($c = 6$, 0·1 N HCl), was heated at 110° for 24 hr in acetic acid:conc. HCl (1:1) or in 20% HCl. The $[\alpha]_D^{20}$ ($c = 6$, 0·1 N HCl) after heating in acetic acid: conc. HCl (1:1) was +1·08° (88 per cent racemization) and in 20% HCl was +3·63° (58 per cent racemization).

TABLE 1. RACEMIZATION OF L-CYSTINE IN STRONG ACID SOLUTION

Treatment of L-cystine, $[\alpha]_D^{23} = -209{\cdot}4°$ ($c = 1$, 1 N HCl)	$[\alpha]_D^{23}$ after heating	Per cent racemization
Acetic acid:conc. HCl (1:1), 110°, 24 hr	−15·6°	92·5
Acetic acid:conc. HCl (1:1), 120°, 60 hr	−3·5°	98·3
20% HCl, 110°, 24 hr	−91·0°	56·5
20% HCl, 120°, 60 hr	−1·5°	99·3
Acetic acid:20% HCl (1:1), 110°, 24 hr	−128·0°	38·9

Hydrolysis of Thiazoline-containing Malformin A

To determine the optimum length of time for maximum production of thiazoline-containing malformin *A*, thiolmalformin *A* (10 mg) was suspended in conc. HCl, 10 ml, and shaken at 27° for varying intervals. By u.v. absorption, maximum thiazoline formation occurred after 3 days (Table 2).

TABLE 2. FORMATION OF THIAZOLINE-CONTAINING MALFORMIN *A* IN CONC. HCl

Days	0	2	3	5	50
ϵ at 270 mμ	965	3460	3680	2450	820

Thiolmalformin *A* (500 mg) was suspended in conc. HCl (50 ml) and shaken for 3 days at 27°. After drying in an air stream at 80°, the residue was dissolved in glacial acetic acid, 100 ml, and divided into ten 10 ml aliquots. Conc. HCl (10 ml) was added to each aliquot and the samples hydrolyzed in sealed tubes at 110° for 24 hr. The combined hydrolysates were

dried, the residue dried *in vacuo* over KOH, and chromatographed by cellulose column (2×70 cm) chromatography using butanol:acetic acid:H_2O (4:1:5). Leucine, isoleucine, and allo-isoleucine were isolated as a mixture, 103 mg, $[\alpha]_D^{24} = +6{\cdot}1^\circ$ ($c = 4{\cdot}7$, 6 N HCl). Valine (40 mg), $[\alpha]_D^{23} = +22{\cdot}4^\circ$ ($c = 1{\cdot}8$, 2 N HCl) was racemized 22 per cent via thiazoline-containing malformin *A*, as judged by comparison with authentic DL-valine, $[\alpha]_D^{23} = +28{\cdot}7^\circ$ ($c = 1{\cdot}8$, 2 N HCl). When malformin *A* and authentic L-valine were hydrolyzed similarly, racemization was only 2 and 4 per cent, respectively.

Molar Ratio of Valine, Isoleucine and Leucine by Gas Chromatography

Methyl esters of DNP valine, isoleucine, and leucine were prepared[8] from hydrolysates of malformin *A*. Most of the cystine, which precipitated from the neutralized hydrolysate, was excluded by filtration before dinitrophenylation. Gas chromatography employed a hydrogen flame detection system, Aerograph HY-FI model 600, a 3 ft stainless steel coil, $\frac{1}{8}$ in. o.d., packed with SE-30, 21 ml per min nitrogen as carrier. Retention times for authentic DNP-amino acid methyl esters were valine, 5·5 min, isoleucine, 6·3 min, and leucine, 6·8 min, at 225°. Although separation of leucine and isoleucine from malformin *A* was not complete, the results supported a 1:1:1 ratio for valine, leucine, and isoleucine (Fig. 1).

Oxidation of Thiolmalformin A *by Iodine*

A solution of thiolmalformin *A* (300 mg) in dimethylsulfoxide (100 ml) was treated with iodine in dimethylsulfoxide (25·4 mg/ml) until the solution colored slightly with excess iodine (0·55 ml, 0·96 equivalent). Ethyl acetate (1 l.) was added and the mixture extracted with 1% sodium thiosulfate to remove excess iodine. A precipitate, isomalformin *A*, which gradually increased after standing overnight at room temperature, was removed by filtration and washed with water and ethanol. Weight 60 mg, m.p. over 300°, $[\alpha]_D^{23} = +15$ ($c = 1$, trifluoroacetic acid), mol.wt. 570 (required for monomer, 530) by Signer method in trifluoroacetic acid. Compared to malformin *A* and thiolmalformin *A*, isomalformin *A* was less soluble in dimethylsulfoxide and dimethylformamide. The optimum concentration for induction of corn-root curvatures by isomalformin *A* (approx. 5 ppm) was considerably greater than that required for malformin *A* (0·1 ppm) or thiolmalformin *A* (1·0 ppm). Reduction of isomalformin *A* by sodium in liquid ammonia gave thiolmalformin *A*.

After removal of isomalformin *A* precipitate, the solvent was washed repeatedly with water, concentrated to 100 ml, filtered to remove a trace of precipitate, and evaporated to dryness. Weight 140 mg, m.p. over 300°, white powder. This material was purified by alumina chromatography.[4] $[\alpha]_D^{23} = -18$ ($c = 1$, trifluoroacetic acid), $E_{\frac{1}{2}} = -0{\cdot}375$ V (polarography in 0·1 N HCl), mol. wt. 550 (required: 530) by Signer method in trifluoroacetic acid, optimum concentration for induction of corn-root curvatures 0·1 ppm. These values are identical with those of malformin *A*.

Malformin *A* itself was not reduced by treatment with sodium thiosulfate as described above.

Oxidation of Thiolmalformin A *by Ferric Chloride*

When a solution of ferric chloride (0·02 M) in dimethylsulfoxide was added to an aqueous solution of cysteine HCl (0·01 M), a deep blue formed instantly and rapidly changed to colorless. When cysteine was also dissolved in dimethylsulfoxide the yellow color of ferric chloride persisted up to 1 hr at room temperature or after heating at 100° for 5 min. Ferric chloride did not react with cysteine in formic or acetic acid.

Although the solubility of thiolmalformin *A* in water is very low we attempted oxidation as described above. Ferric chloride (0·02 M in dimethylsulfoxide), 50 ml, and thiolmalformin *A* (2·5 mg/ml in dimethylsulfoxide), 20 ml, were mixed and diluted with glacial acetic acid, 50 ml, and water, 300 ml. The slightly turbid solution was shaken for 2 hr at room temperature and extracted three times with ethyl acetate, 100 ml. The ethyl acetate was repeatedly washed with water and evaporated to dryness. Weight 30 mg, white powder. To remove isomalformin *A* the sample was dissolved in dimethylsulfoxide, 10 ml, treated with ethyl acetate, 50 ml, and filtered to remove white precipitate. The filtrate was washed with water and evaporated to dryness. Weight 5 mg, m.p. over 300°, $[\alpha]_D^{23} = -18°$ ($c = 1$, trifluoroacetic acid), optimum concentration for induction of corn-root curvatures 0·1 ppm. These values are identical with those of malformin *A*.

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