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Synthesis and Biological Activity of *enantio*-[5-Valine]malformin, a Palindrome Peptide

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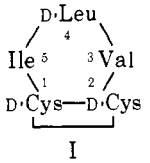
Abstract: The cyclic pentapeptide disulfide *enantio*-[5-valine]malformin (II) was prepared by stepwise synthesis in solution. The ring was closed by the azide method and the dithiol was oxidized to the disulfide. The product is the enantiomer of a palindrome peptide, [5-valine]malformin; it has one-tenth of the biological activity of natural malformin in causing curvatures on corn roots, inhibiting adventitious root formation, and stimulating the growth of etiolated bean cuttings. It induces malformations on bean seedlings which are identical with those induced by the parent compound.

Malformins, a family of metabolic products of *Aspergillus niger*, induce malformations on higher plants,[1] inhibit adventitious root formation,[2] have antibacterial[3] and cytotoxic properties, and under some conditions stimulate plant growth.[4] The initially proposed[5] structure for malformin A_1, a principal member of the family hereafter referred to as malformin, was recently revised.[6,7] The revised sequence, I, was confirmed by synthesis.[7]



I

The examination of structure-activity relationships in malformins, including a study of their different stable conformations,[8] necessitated additional efforts toward the synthesis of malformin analogs. This work, however, was hampered by the limited availability of one of the constituent amino acids, D-cystine. Therefore, we decided to prepare and study the enantio form of a malformin.

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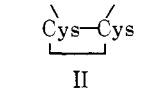
The decision to synthesize *enantio*-[5-valine]malformin[9] was based on the following considerations. In some biologically active members of the malformin family, isoleucine is replaced by valine.[10] Although the sequence of these constituents has not been determined, it seemed to be likely that there are no additional structural differences between malformin and other members of the family, or if there are differences, they do not affect the biological activity in any major way. Therefore, we expected that the enantio analog of [5-valine]malformin (II) would be a suitable model for a



II

study of the conformation of malformins and also hoped that the synthetic material would be biologically active. This hope was supported by the antibacterial properties of retro-enantio analogs of gramicidin S[11] and of enniatins.[12] The topochemical[13] similarity between a microbial peptide and its enantiomer is further enhanced if the direction of the chain is reversed.[14] Such retro enantiomers are closely

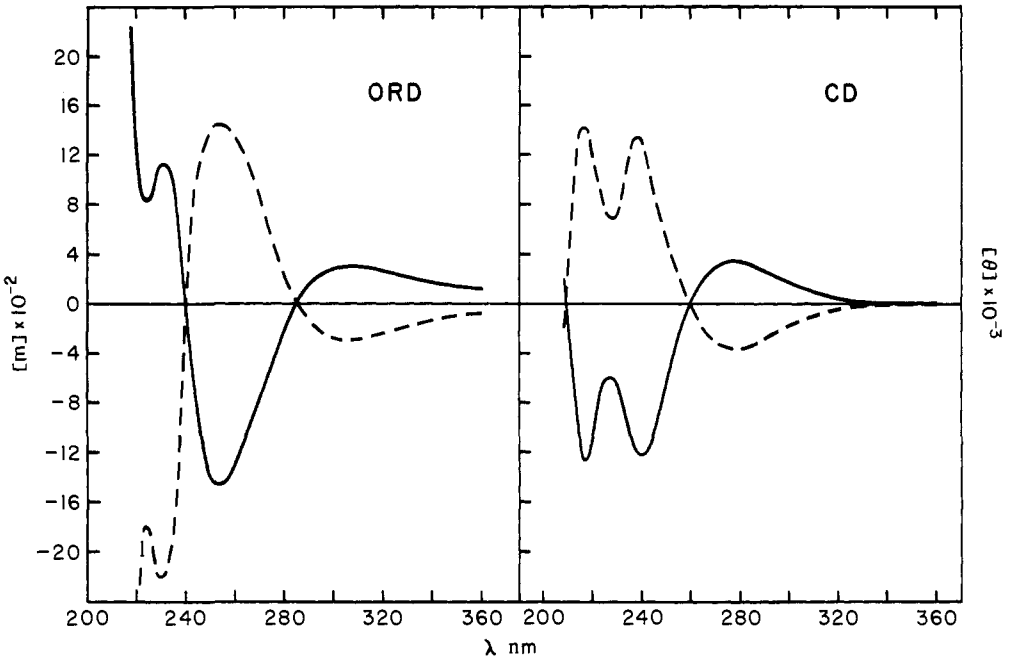


Figure 1. ORD and CD spectra of *enantio*-[5-valine]malformin (—) and of malformin A (- · -) in trifluoroethanol.

similar to their parent molecules and should behave similarly in many systems, e.g., in forming complexes with metal ions. The antibacterial potency of enantio and retro-enantio analogs of peptide antibiotics is probably related to their similar ionophore[15] properties. On the other hand, a cyclic peptide and its retro enantiomer are certainly not identical and they might be distinguished by some biological receptors. Thus, the expectation that on different plants *enantio*-[5-valine]malformin should exert the various effects of natural malformin had to be proved or disproved by experiments.

It was our intention to maintain topochemical similarity between the synthetic analog and malformin. In this respect it was not obvious whether L-isoleucine should be replaced by D-isoleucine or D-alloisoleucine in the enantiomer. Replacement of isoleucine by valine eliminated this problem and at the same time resulted in a *palindrome peptide*, a sequence which is identical with its retro sequence. It was not unreasonable to assume close topochemical similarity between malformin (I) and the planned symmetrical analog II.

The synthesis of II was carried out similarly to that of I[6,7] by stepwise chain lengthening from the C-terminal residue.[16] The protected linear intermediate, benzyloxycarbonyl-D-valyl-*S*-benzyl-L-cysteinyl-*S*-benzyl-L-cysteinyl-D-valyl-L-leucine methyl ester, was converted to the hydrazide, partially deprotected with HBr in acetic acid, and cyclized via the azide[17] to the *S,S'*-dibenzyl derivative of II. After removal of the *S*-benzyl protecting groups by reduction with sodium in liquid ammonia,[18] the dithiol was heated in dimethyl sulfoxide to convert it to the more active conformation[8] and then oxidized to the disulfide with iodine[19] or by diiodoethane.[20] After purification by chromatography on silica gel,[10] II was secured in crystalline and analytically pure form. It travelled as a single spot on thin-layer chromatograms and exhibited ORD-CD spectra (Figure 1) which were closely similar to the mirror image of the spectra of I (cf. ref 7).

The biological activity of malformin (I) and *enantio*-[5-valine]malformin (II) was compared in a series of tests and confirmed our expectations. In the corn root curvature assay,[1] the optimum concentration of malformin (I) and *enantio*-[5-valine]malformin (II) was 2×10^{-7} and 2×10^{-6} *M*, respectively (Table I). The concentration (10^{-6} *M*) of synthetic II required to stimulate the growth of etiolated cuttings of *Phaseolus aureus*[4] was tenfold greater than that required for similar responses to malformin (Table I). In addition, the concentration (10^{-6} *M*) of synthetic II required to inhibit adventitious root formation[2] of similar cuttings of *P. aureus* was tenfold greater than that required for similar responses to malformin. We concluded that synthetic II is about one-tenth as active as natural malformin. In a qualitative test using seedlings of *P. vulgaris*, the synthetic product induced malformations which could not be distinguished from those induced by malformin itself.[1]

Experimental Section

Capillary melting points are reported uncorrected. For thin-layer chromatography, the following systems were used: A, $CHCl_3$-CH_3OH (19:1); B, $CHCl_3$-CH_3OH (9:1); C, $CHCl_3$-TFE (85:15); D, water-saturated ethyl acetate. Spots on TLC were revealed with *tert*-butyl hypochlorite-KI-starch reagents.[21] For amino acid analysis, samples were hydrolyzed in a mixture of concentrated HCl and HOAc (1:1) at 110° for 16 hr and analyzed by the Spackman-Stein-Moore method[22] on a Beckman Spinco 120C amino acid analyzer. The following abbreviations are used: DMF, dimethylformamide; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; DIPEA, diisopropylethylamine; THF, tetrahydrofuran; TFE, 2,2,2-trifluoroethanol.

Benzyloxycarbonyl-D-valyl-L-leucine methyl ester (III) was prepared similarly to its enantiomer:[7] yield, 98%; mp 134–135°; $[\alpha]^{25}D$ −25.5° (*c* 2, DMF); TLC R_f^A 0.63 [lit.[7] mp 135–136°; $[\alpha]^{25}D$ +25.4° (*c* 2, DMF)].

***N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-D-valyl-L-leucine methyl ester (IV)** was prepared analogously to its enantiomer:[7] yield, 73%; mp 160–161°; $[\alpha]^{25}D$ −42.1° (*c* 2, DMF); TLC R_f^A 0.68 [lit.[7] mp 160–161°; $[\alpha]^{25}D$ +40.0° (*c* 2, DMF)].

***N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-*S*-benzyl-L-cysteinyl-D-valyl-L-leucine methyl ester (V)** was prepared by the method used for its enantiomer:[7] yield, 73%; mp 190–191°; $[\alpha]^{25}D$ −44.4° (*c* 2, DMF); TLC R_f^A 0.68 [lit.[7] mp 193–194°; $[\alpha]^{25}D$ +44.9° (*c* 2, DMF)]; amino acid analysis, Val 1.00; Leu 1.00, Cys(Bzl) 2.00.

Anal. Calcd for $C_{40}H_{52}O_7N_4S_2$ (765.0): C, 62.8; H, 6.8; N, 7.3. Found: C, 63.0; H, 6.9; N, 7.1.

Benzyloxycarbonyl-D-valyl-*S*-benzyl-*L*-cysteinyl-*S*-benzyl-L-cysteinyl-D-valyl-L-leucine Methyl Ester (VI). A sample of V (13.2

Table I. Biological Activity of *enantio*-[5-Valine]malformin and Malformin

Concn, *M*	*enantio*-[5-Valine]-malformin	Malformin
Corn root curvature assay[1]	% roots with >90° curvature	
2×10^{-5}	18.2	
2×10^{-6}	49.0	33.7
2×10^{-8}	15.2	77.9
2×10^{-8}		25.0
H_2O (control)	9.2	
Phaseolus aureus growth assay[4]	Growth increments, cm	
10^{-5}	2.84	
10^{-6}	3.42[a]	3.02
10^{-7}	2.72	3.36[a]
10^{-8}		2.60
H_2O (control)	3.04	

[a] Significantly different from controls at 5% level.

g, 17 mmol) was suspended in 3 *M* HBr in HOAc (32 ml). After 2 hr, some material remained undissolved and therefore more HBr in acetic acid (8 ml) was added. After 1 hr, the mixture was diluted with ether (450 ml); the precipitated hydrobromide was collected, washed with ether (100 ml), and dried in vacuo over NaOH. The partially deprotected peptide derivative was dissolved in DMF (50 ml), and benzyloxycarbonyl-D-valine *p*-nitrophenyl ester[23] was added, followed by DIPEA (3.1 ml). After 24 hr, the product began to crystallize. At the end of 48 hr, the reaction was complete. The crystals were filtered and washed with DMF (20 ml) and with methanol: yield, 10.2 g. The washings were pooled with the filtrate, and the solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (100 ml) and washed with water (50 ml) and 0.1 *M* HCl (10 ml). The solvent was removed in vacuo and the solid material triturated and washed with ether (100 ml): yield, 1.5 g. The two crops were pooled and washed with EtOAc: yield, 11.5 g (78%); mp 222–224°; $[\alpha]^{25}D$ −41.1° (*c* 2, DMF); TLC, a single spot at R_f^A 0.57; amino acid analysis, Val 2.05; Leu 1.00, Cys(Bzl) 1.95.

Anal. Calcd for $C_{45}H_{61}O_8N_5S_2$ (864.1): C, 62.5; H, 7.1; N, 8.1. Found: C, 62.3; H, 7.1; N, 7.9.

***cyclo*-(S-Benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-D-valyl-L-leucyl-D-valyl) (VII).** A sample of VI (1.0 g) was suspended in a mixture of peroxide-free THF (50 ml), hydrazine (5 ml), and methanol (6 ml). Dissolution occurred within 5 min; after 15 min, the product began to precipitate. After standing overnight at room temperature, the product was collected by filtration and washed with THF and methanol: yield, 0.84 g (84%); mp 251–252°; $[\alpha]^{25}D$ −16.2° (*c* 2, TFA); TLC R_f^B 0.54. A sample of the hydrazide (1.82 g, 2.1 mmol) was partially deprotected by treatment with 3 *M* HBr in acetic acid (20 ml) for 90 min. The hydrobromide was precipitated with ether (400 ml), washed with ether, and dried in vacuo over NaOH and P_2O_5: yield, 1.76 g. It was dissolved in DMF (20 ml) and cooled to −20°. Sodium nitrate (1 *M* solution, 2.1 ml) was added to the cooled mixture, which was stirred for 20 min, and then cold DMF (250 ml) was added. The solution was made alkaline with DIPEA (1.1 ml), set aside at 4° for 48 hr, and kept at room temperature for 24 hr. Most of the solvent was removed under reduced pressure, and the cyclic material was precipitated with water (200 ml), collected on a filter, washed with warm ethanol, and dried: yield, 0.64 g (0.93 mmol, 43%); mp >300°, slight decomposition above 250°; $[\alpha]^{25}D$ −62.7° (*c* 2, TFA); TLC R_f^C 0.51; amino acid analysis, Val 2.05, Leu 0.95, Cys(Bzl) 2.00.

Anal. Calcd for $C_{36}H_{51}O_5N_5S_2$ (697.9): C, 61.9; H, 7.4; N, 10.0. Found: C, 61.7; H, 7.2; N, 10.3.

***enantio*-[5-Valine]malformin (II).** A sample of VII (113 mg) was suspended in liquid NH_3 (ca. 100 ml), and small pieces of sodium[18] were added until the blue color persisted for 2 min. The color dispelled with NH_4Cl. The NH_3 was allowed to evaporate under N_2 and finally in vacuo. The residue was washed with 0.1% HOAc (2 ml) and dried: yield, 81.5 mg (98%). The dithiol was dissolved in DMSO (16 ml); the solution was warmed on a steam bath for 1 hr, cooled to room temperature, and titrated with an iodine solution (0.05 *M* in DMSO, 2.9 ml, calcd 3.1 ml). The small excess of iodine was reduced with $Na_2S_2O_3$ (0.5 ml, 1% in H_2O). The mixture was diluted with ethyl acetate (150 ml) and washed with water (300 ml). The precipitate that formed[8] was removed by centrifugation and the solvent by evaporation. The residue was triturated with ether. The product (33.5 mg, 41%) was purified by chromatography on silica gel.[10] The purified material (90% recovery) gave a single spot on TLC (R_f^D 0.44) and exhibited ORD-CD spectra that were quite similar to the mirror image of the spectra of malformin A (Figure 1): amino acid analysis, Cys 1.95, Val 2.05, Leu 1.00.

Anal. Calcd for $C_{22}H_{37}O_5N_5S_2$ (515.7): C, 51.2; H, 7.2; N, 13.6. Found: 51.4; H, 7.2; N, 13.6.

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