BIOORGANIC CHEMISTRY 4, 212–218 (1975)

Synthesis and Properties of the Cyclopentapeptide Desthiomalformin

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Received January 10, 1975

Desthiomalformin, *cyclo*-D-alanyl–D-alanyl–L-valyl–D-leucyl–L-isoleucyl, was synthesized by conventional methods. Cyclization of the open chain intermediate through the azide produced the cyclopentapeptide in short reaction time and in high yield and was not accompanied by cyclodimerization. The extreme readiness of the pentapeptide azide to form a cyclic structure can be attributed to the presence of alternating D and L residues in the sequence, a feature that should result in reverse turns stabilized by intramolecular hydrogen bonds. Thus the open chain intermediate could have a preferred cyclic conformation.

Desthiomalformin has high thermal stability; it can be sublimed *in vacuo* without decomposition. It lacks, however, the biological activity of its parent compound, malformin.

INTRODUCTION

The structure of the microbial peptide malformin (*1*) was recently revised (*2*, *3*) and the revised structure (I) confirmed by synthesis (*2*, *3*). In our continuing studies on the different stable conformations of malformin (*4*) and their relationship to biological activity it became desirable to prepare desthiomalformin (*5*), *cyclo*-D-alanyl–D-alanyl–L-valyl–D-leucyl–L-isoleucyl (II). In the last step of the synthesis, cyclization of the open chain intermediate by the azide method,[2] the remarkably facile formation of the cyclic pentapeptide (II) was noted. No decapeptide could be detected in the product; thus, closure of the ring remained unaccompanied by cyclodimerization (*6*).

Small peptide rings, with less than six residues, could be prepared only exceptionally. Such exceptions, apart from diketopiperazines,[3] are cyclopeptides that contain proline and sometimes also glycine (*7*), that is, residues which favor or permit cyclic conformations in the open chain intermediates. Cyclopentapeptides were obtained when grami-

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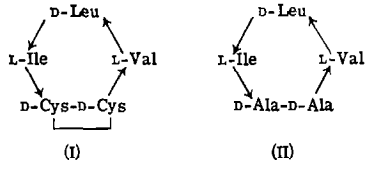
[2] The azide method might favor cyclization of a monomer over cyclodimerization (cf. M. A. Ruttenberg and B. Mach, *Biochemistry* 5, 2864 (1966)).

[3] Diketopiperazine formation is enhanced if the two residues are not of the same configuration. Here, however, the fact that in DL-diketopiperazines the side chains are on the opposite side of the plane of the ring and, therefore, interfere less with each other is sufficient to explain the facile formation of diketopiperazines.

cidin S and certain analogs of gramicidin S were prepared via cyclodimerization of reactive pentapeptide intermediates at *high dilution*. However, even under such conditions the major product was usually the cyclodecapeptide (*8*). The reason for the prevalence of cyclodimerization was recognized (*6*) in the conformation of the linear precursors, in their association in β-sheetlike structures, which are similar to the conformation of the cyclic decapeptide gramicidin S.



Malformin (I) and Desthiomalformin (II)

An enhanced tendency for ring formation in peptides that contain alternating D and L residues in their sequence was explained by Ramachandran and Chandrasekaran (*9*) by the presence of hydrogen-bond-stabilized reverse turns. They pointed to microbial peptides, such as stendomycin (*10*) or depsipeptides like the enniatins (*11*), as characteristic examples. In the synthesis of malformin (*2*, *3*) and of enantio-5-valine malformin (*12*), cyclization through the partially protected pentapeptide azides produced S,S′-dibenzyl-cyclopentapeptides in about 50% yield, considered fair for this step. The satisfactory formation of these cyclopentapeptides at moderately high (5×10^{-3} *M*) dilution was attributed to the alternation of D and L residues. Yet, since no clear evidence could be found for the absence of cyclodimerization products, this point was not discussed in the respective papers (*2*, *3*, *12*). We expected, however, that the synthesis of desthiomalformin, unencumbered by S-benzyl groups, would provide a better opportunity for the observation of the outcome of cyclization. This expectation was fulfilled.

The cyclopentapeptide (II) was obtained via the azide of a corresponding open chain pentapeptide (III) in a surprisingly short time and in high yield and exceptional purity. It gave a single spot on thin layer chromatograms and *sublimed* at about 270–290°C and 0.05 mm without decomposition or racemization as a homogeneous material and with no residue. Its molecular weight, determined by mass spectrometry, was found to be that of the monomer, 467 atomic mass units.

The ir spectrum of II in KBr revealed no "free" NH groups. This extensive hydrogen bonding must be in part intermolecular. An inspection of a molecular model of II shows that no more than two intramolecular hydrogen bonds are possible. A study of the ir spectra in solution was impractical because of the extreme insolubility of II in the numerous solvents tried. The same insolubility hampered a study of the nmr spectra, which could provide evidence for the postulated intramolecular hydrogen bonds.[4] Some support for our contention about a preferred cyclic conformation in the open chain precursor was found in the comparison of the ORD-CD spectra of II with those of L-isoleucyl–D-alanyl–D-alanyl–L-valyl–D-leucine (III). The conformation of this

[4] Nmr studies of II and III with a more sophisticated approach and instrumentation are being undertaken by Dr. Dinshaw Patel of Bell Laboratories as part of his investigation on the conformations of malformin.

pentapeptide might be influenced by trifluoroethanol, the solvent in which the spectra were recorded, yet this was the only useful solvent found for II. The observed weak Cotton effects (Fig. 1) do not lend themselves to an interpretation in terms of molecular geometry. Still, the similarity of the spectra suggests some conformational analogy between II and III. Of course, this is only modest support for cyclic character in III. Furthermore, cyclization of its azide was carried out not in trifluoroethanol but in dimethylformamide, a solvent unsuited for the examination of the ORD-CD spectra in the region of the observed Cotton effects. Thus, at this time no direct experimental evidence is available to prove a cyclic conformation in the open chain precursor. Nevertheless the remarkable rate of ring closure, the rapid separation of II from the reaction mixture, convinced us that the cyclization reaction is exceptionally favored. The absence of dimers was even more impressive and led us to attempt cyclization at the unusually high concentration of about 0.1 *M*; again the cyclopentapeptide was the sole product.[5] We regard this experience as vindication of the predictions of Ramachandran and Chandrasekaran (*9*). The low energy content calculated by these authors for cyclic peptides with alternating D and L residues also finds experimental support in the thermal stability of II revealed by its sublimation without decomposition.

Desthiomalformin (II) exhibited no malformin-like activity on plants or on microorganisms. This shows that the bicyclic character of I is necessary for such effects. An answer to the question whether the disulfide bridge is indeed indispensable could be obtained by the examination of analogs in which the disulfide is replaced by $-S-CH_2-$ or $-CH_2-CH_2-$ bridge (*13*, *14*). Synthesis of such analogs of malformin is under consideration.

EXPERIMENTAL

Capillary melting points are uncorrected. DMF[6] was dried over a molecular sieve (Linde Type 4A). Spots on thin layer chromatograms (tlc) were revealed by tert-butyl hypochlorite–KI–starch reagent (*15*) or by charring (*16*). The solvent systems for tlc were (A), $CHCl_3$–MeOH (19:1); (B), $CHCl_3$–MeOH (9:1); (C), *n*-butanol–AcOH–H_2O (4:1:1); (D), TFE. For amino acid analysis, samples were hydrolyzed with constant-boiling hydrochloric acid in evacuated sealed ampoules at 110°C for 16 hr and analyzed by the Spackman–Stein–Moore (*17*) method on a Beckman Spinco 120C amino acid analyzer.

*Benzyloxycarbonyl-*D*-Alanyl-*L*-Valyl-*D*-Leucine Methyl Ester (IV)*

Benzyloxycarbonyl-L-valyl-D-leucine methyl ester (*3*) (3.78 g, 10.0 mmole) was dissolved in 95% EtOH (50 ml). Hydrochloric acid (1 *N*, 10 ml) was added dropwise to the stirred solution followed by a 10% Pd on charcoal catalyst (1.0 g) and the mixture hydrogenated. The catalyst was removed by filtration and the solvent by evaporation under reduced pressure. The residue was dissolved in DMF (20 ml); TEA (1.4 ml, 10 mmole) and benzyloxycarbonyl-D-alanine *p*-nitrophenyl ester (*18*) (4.14 g, 12 mmole)

[5] Note added in proof: On tlc the cyclopentapeptide appeared as a single spot. The mass spectra, however, revealed the presence of some dimer.

[6] The following abbreviations were used: DMF, dimethylformamide; TFE, trifluoroethanol; TEA, triethylamine; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid.

were added. When tested with moist indicator-paper, the vapor phase above the solution was alkaline. The reaction mixture was stirred overnight at room temperature. At that time, the mixture was ninhydrin negative. A small precipitate was removed by filtration, and the filtrate evaporated to dryness under reduced pressure, and the residue was dissolved in chloroform (50 ml). The solution was washed with 1.2 *N* HCl (2 × 50 ml) and water (2 × 50 ml), and each aqueous layer was reextracted with chloroform (25 ml). The chloroform layers were combined and evaporated under reduced pressure. The residue was triturated with ether, the solid collected, washed with ether and dried. The protected tripeptide methyl ester weighed 3.80 g (85%); mp, 146–148°C; tlc: R_fA, 0.47; R_fB, 0.74; R_fC 0.86; R_fD, 0.58. A sample (103 mg) was recrystallized from hot 95% EtOH (1.0 ml); 43.6 mg was recovered; mp, 148.5–149.5°C; $[\alpha]_D^{25}$ + 18.4 (*c* 1, DMF). Amino acid analysis: Ala, 1.00; Val, 1.07; Leu, 0.95.

Anal. Calcd for $C_{23}H_{35}N_3O_6$ (449.5): C, 61.5; H, 7.9; N, 9.4. Found: C, 61.7; H, 7.9; N, 9.1.

Benzyloxycarbonyl-D-*Alanyl*–D-*Alanyl*–L-*Valyl*–D-*Leucine Methyl Ester* (*V*)

Compound IV (2.24 g, 5 mmole) was dissolved in 95% EtOH (100 ml) with slight warming. Hydrochloric acid (1 *N*, 5 ml) was added to the stirred solution and the mixture was hydrogenated at room temperature in the presence of a 10% Pd on charcoal catalyst (0.50 g). The catalyst was removed by filtration and the solvent by evaporation *in vacuo*. The residue was dissolved in DMF (10 ml); TEA (0.70 ml, 5 mmole) was added followed by benzyloxycarbonyl-D-alanine *p*-nitrophenyl ester (*18*) (2.06 g, 6 mmole). The reaction mixture was stirred overnight at room temperature. At that time the mixture was ninhydrin negative. The product (0.36 g, 7%, mp, 207–207.5°C) was removed by filtration and the filtrate evaporated under reduced pressure. The residue was recrystallized from hot 95% EtOH. The white crystals were filtered, washed with EtOH and ether and dried: 1.23 g (58.8%); mp, 207–207.5°C. The filtrate was evaporated to dryness, the residue triturated with water, filtered, washed with water, dried, washed with ethyl acetate, and dried again: 0.53 g (25.4%); mp, 195–198°C; total yield, 2.2 g (91%); tlc: R_fA, 0.30; R_fB, 0.60; R_fC, 0.82; R_fD, 0.50. A sample (640 mg) was recrystallized from hot 95% EtOH (10 ml); recovery, 410 mg; mp, 207–207.5°C; $[\alpha]_D^{25}$ + 22.4° (*c* 1, DMF); amino acid analysis: Ala, 1.92; Val, 1.00; Leu, 0.91.

Anal. Calcd for $C_{26}H_{40}N_4O_7$ (520.6): C, 60.0; H, 7.7; N, 10.8. Found: C, 60.0; H, 7.7; N, 11.0.

Benzyloxycarbonyl-L-*Isoleucyl*–D-*Alanyl*–D-*Alanyl*–L-*Valyl*–D-*Leucine Methyl Ester* (*VI*)

A sample of compound V (1.56 g, 3 mmole) was hydrogenated in a mixture of glacial acetic acid (20 ml) and 1 *N* hydrochloric acid (3 ml) in the presence of 10% Pd on charcoal catalyst (0.48 g). The catalyst was removed by filtration and the solvent by evaporation under reduced pressure. The residue was dissolved in DMF (12 ml). DIEA (0.48 ml, 3 mmole) was added to the stirred solution followed by benzyloxycarbonyl-L-isoleucine *p*-nitrophenyl ester (*18*) (1.39 g, 3.6 mmole). Next day the solvent was removed by evaporation, the residue triturated with $CHCl_3$, filtered, washed with $CHCl_3$ and ether, and dried. The protected pentapeptide methyl ester (III) weighed

1.47 g (77%); mp, 239–242°C; tlc: R_fA, 0.27; R_fB, 0.58; R_fC, 0.85; R_fD, 0.48. A sample (148 mg) was recrystallized from hot EtOH; 100 mg was collected; mp, 247–248.5°C dec.; $[\alpha]_D^{25} + 39.8°$ (*c* 1, TFE); amino acid analysis: Ile, 1.03; Ala, 2.00; Val, 1.00; Leu, 1.02.

Anal. Calcd for $C_{32}H_{51}N_5O_8$ (633.8): C, 60.6; H, 8.1; N, 11.1. Found: C, 60.7; H, 8.0; N, 11.0.

Benzyloxycarbonyl-L-*Isoleucyl*–D-*Alanyl*–D-*Alanyl*–L-*Valyl*–D-*Leucine Hydrazide* (*VII*)

Compound VI (0.63 g, 1 mmole) was dissolved in warm MeOH (75 ml), and hydrazine (4 ml) was added. While the reaction mixture was stirred overnight, a white crystalline solid separated. The product was collected on a filter, washed with MeOH and ether, and dried. The protected pentapeptide hydrazide weighed 0.61 g (96%); mp, 266–267°C; $[\alpha]_D^{25} + 20.5°$ (*c* 1, DMF); tlc: R_fA, 0.06; R_fB, 0.27; R_fC, 0.73; R_fD, 0.22; amino acid analysis; Ile, 1.00; Ala, 1.90; Val, 1.00; Leu, 1.04.

Anal. Calcd for $C_{31}H_{51}N_7O_7$ (633.8): C, 58.8; H, 8.1; N, 15.5. Found: C, 59.0; H, 8.0; N, 15.2.

L-*Isoleucyl*–D-*Alanyl*–D-*Alanyl*–L-*Valyl*–D-*Leucine* (*III*)

A sample of VI (0.32 g, 0.5 mmole) was suspended in acetic acid (2.5 ml) and treated with 4.5 *M* HBr in acetic acid (2.5 ml). After 1.5 hr at room temperature, the partially protected pentapeptide hydrazide hydrobromide was precipitated with ether (80 ml), collected on a filter, washed with ether (40 ml) and dried *in vacuo* over NaOH. The dry material was dissolved in MeOH (6 ml) and *N* NaOH (1.2 ml) was added. After 2.5 hr at room temperature *N* HCl (1 ml) and a few drops of glacial acetic acid were added. Gradually, white needles formed; the crystals were collected and washed with a MeOH–water (6:4) mixture, finally with MeOH. The air-dried pentapeptide weighed 91 mg (41%); mp, 232–233°C dec.; R_fC, 0.51. For analysis two samples were dried at 90°C and about 0.1 mmHg for 3 hr.

Anal. Calcd for $C_{23}H_{43}N_5O_5$ (485.6): C, 56.89; H, 8.93; N, 14.42 for $C_{23}H_{43}N_5O_5 \cdot 2.5\ H_2O$ (530.7): C, 52.06; H, 9.12; N, 13.20; for $C_{23}H_{43}N_5O_5 \cdot 3\ H_2O$ (539.7): C, 51.19; H, 9.15; N, 12.98. Found: Sample A, C, 51.86 and 52.11; H, 8.63 and 8.65; N, 13.07 and 13.21; Sample B, C, 51.00 and 50.87; H, 8.67 and 8.71; N, 12.81 and 12.84. The discrepancy of values is probably due to the varying amounts of water the compound contains when analyzed.

Cyclo-L-*Isoleucyl*–D-*Alanyl*–D-*Alanyl*–L-*Valyl*–D-*Leucyl* (*II*)

A sample of VII (0.64 g, 1 mmole) was suspended in glacial acetic acid (5 ml), HBr in acetic acid (4.5 *M*, 5 ml) was added, and the solution was allowed to stand at room temperature for 1.5 hr. The partially protected pentapeptide hydrazide hydrobromide was precipitated with ether (150 ml), washed with ether, and dried *in vacuo* over NaOH. The dry material was dissolved in DMF (10 ml), concd, HCl (0.1 ml) was added, and the solution was cooled to –20°C in a Dry Ice–acetone bath. To the cooled solution, 1 *M* $NaNO_2$ (1 ml) was added dropwise and with swirling. After 15 min at -17 ± 3°C, precooled DMF (200 ml) was added. The solution was made alkaline with DIEA (1.4 ml) and allowed to stand for 1 day at 4°C and 1 day at room temperature. A white crystalline solid, brilliant in cross polarized light under the microscope, separated,

most of it already during the first 2 hr. The crystals were collected, washed with DMF, ethyl acetate, and dried. The cyclopentapeptide weighed 0.40 g (86%); mp, >300°C; $[\alpha]_D^{25}$ + 55.6° (*c* 1, TFA); tlc: R_fA, 0.19; R_fB, 0.47; R_fC, 0.79; R_fD, 0.39; amino acid analysis: Ile, 1.04; Ala, 2.00; Val, 1.00; Leu, 0.98. The ORD-CD spectra (Fig. 1) remained unchanged after sublimation *in vacuo*. The product was obtained with the

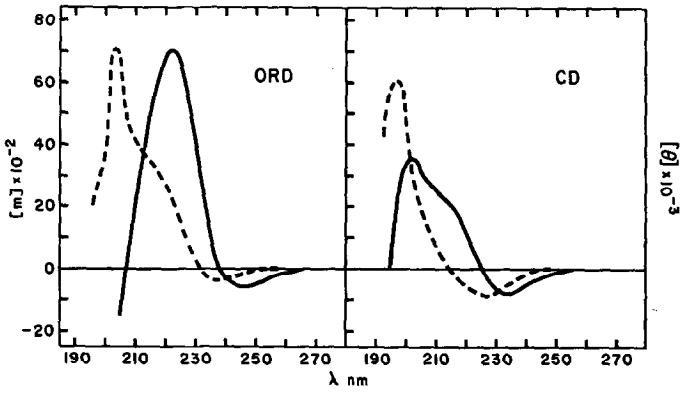


FIG. 1. ORD and CD spectra of desthiomalformin (II), (——), and L-isoleucyl-D-alanyl-D-alanyl-L-valyl-D-leucine (III), (– – – –), in trifluoroethanol.

same properties when dilution with DMF was omitted.[5] The cyclic peptide sublimed practically without residue.

Anal. Calcd for $C_{23}H_{41}N_5O_5$ (467.6); C, 59.1; H, 8.8; N, 15.0. Found: C, 59.1; H, 8.6; N, 14.9.

ACKNOWLEDGMENTS

The authors express their gratitude to Professor R. W. Curtis of Purdue University, West Lafayette, IN, for the examination of potential malformin-like activity in synthetic desthiomalformin, and to Professor D. Perlman of the University of Wisconsin for the assays of antimicrobial activity. The mass spectra were kindly provided by Dr. R. L. Foltz of Batelle Memorial Institute, Columbus, OH. Amino acid analyses were carried out by Mrs. Delores Gaut, elemental analyses by the Baron Consulting Co., Orange, CT, and Par-Alexander Labs, South Daytona, FL. This study was supported by a grant from the U.S. Public Health Service (No. NIH AI-07515).

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