RING FORMATION IN A PENTAPEPTIDE WITH ALTERNATING L AND D RESIDUES: AN ANALOGY TO CYCLIZATION IN THE BIOSYNTHESIS OF PEPTIDE ANTIBIOTICS

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Acetylation of L-isoleucyl-D-alanyl-D-alanyl-L-valyl-D-leucine with acetic anhydride followed by methylation with diazomethane yielded the expected acetylpentapeptide methyl ester with molecular weight 541, but also resulted in the formation of a by-product with molecular weight 555. The incorporation of the mass corresponding to CH_2 seems to be due to ring closure—*via* a mixed anhydride—and methylation of the cyclol derivative thus formed. A preferred, ring-like conformation stabilized by intramolecular hydrogen bonds that in turn are the consequences of the alternation of D- and L- residues in the sequence, is invoked as explanation for the unexpected cyclization. This assumption is supported by the conversion of the pentapeptide methyl ester to desthiomalformin in molten imidazole.

The structure of malformin[1] was recently revised[2,3], and the revised structure (Fig. 1a) corroborated by a study of its mass spectrum[4]. The homodetic cyclic character of malformin and the presence of a disulfide bridge contributed to the considerable complexity of the spectrum. For the sake of unambiguous interpretation the spectra of the related simpler compounds, synthetic desthiomalformin[5] (Fig. 1b) and the open chain pentapeptide L-isoleucyl-D-alanyl-D-alanyl-L-valyl-D-leucine[5] (**I**, Fig. 2) were also included in the study. A sample of compound **I** was acetylated with acetic anhydride and the acetylpentapeptide **II** converted to the methyl ester **III** by the action of diazomethane. The electron impact mass spectrum of **III** showed the expected molecular ions and the predictable fragments, but early scans of the chemical (isobutane) ionization spectrum (Fig. 3) in addition to the mass of the protonated molecule of **II** (*m/e* 542) revealed also a strong peak with mass 556, corresponding to the ion (M+14+H). No such product was found in a preparation of **III** secured through partial deprotection of benzyloxycarbonyl-L-isoleucyl-D-alanyl-D-alanyl-L-valyl-D-leucine methyl ester[5] with hydrobromic acid in acetic acid followed by acetylation with *p*-nitrophenyl acetate.

A species with a molecular weight 555 can be explained if we assume that during acetylation of **I** also some mixed anhydride of the acetylpentapeptide **II** with acetic acid formed, and that this reactive intermediate (compound **IV** in Fig. 2) was attacked not only by methanol used as the solvent in the esterification step, but also by the NH of the N-terminal amide group of the pentapeptide chain. Amides, while poor nucleophiles, do participate in intramolecular acylation reaction if held in the proximity of the activated acyl group by an appropriate geometry of the molecule. Such intramolecular acylation would yield in this case the cyclol **V**,[6] the monoamide of an orthocarboxylic acid. Methylation of **V** with diazomethane would then lead to the dimethyl ester **VI** with molecular weight 555.

To test this hypothesis the acetylation product of **I** was examined by its IR spectrum. The characteristic bands at 1835 and about 1740 cm^{-1} confirmed the presence of a mixed anhydride intermediate (**IV**). In a subsequent experiment the acetylation product of **I** (a mixture of **II** and **IV**) was dissolved in CH_3OD

Fig. 1. The structure of malformin (a) and desthiomalformin (b).

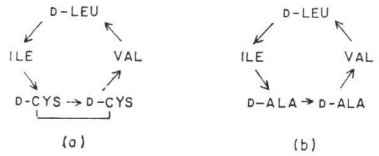
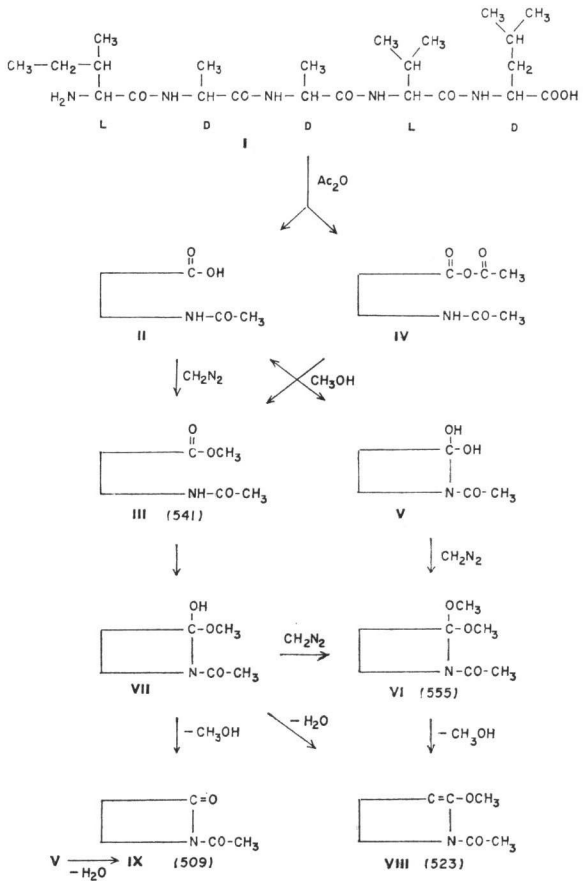
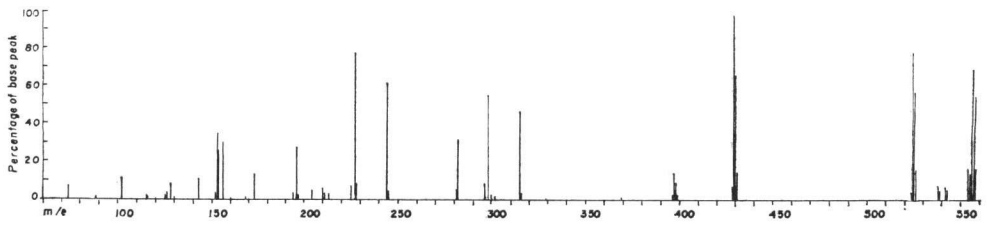


Fig. 2. Formation of cyclic derivatives from a pentapeptide with the sequence of desthiomalformin.



rather than CH_3OH and treated with an ethereal solution of CD_2N_2. The cyclic product had, as expected, a molecular weight of 565, due to the exchange of four NH protons and to the incorporation of two CD_3 groups.* Further support for the assumed cyclic product (**VI**) could be found in the fragment ion with *m/e* values 429. This can be readily derived from structure **VI** (cf. Fig. 3) but not from **III**. The ion with *m/e* 429 was abundant in early scans which showed the protonated molecular ion with mass 556, but absent in later scans where the molecule of **III** (*m/e* 542) was the dominant species. This fragment ion (*m/e* 429) appeared with the appropriately higher masses in the spectra of deuterated samples.* The fact that a compound with a molecular weight of 555 is more volatile than a closely related one with a molecular weight of 541 (**III**) suggests lower polarity in the former. This is in harmony with the structure (**VI**) proposed for the species with higher molecular weight. Sublimation of **III** resulted in the appearance of a 524 *m/e* ion (protonated **VIII**) and a 510 *m/e* ion (protonated **IX**) in the chemical ionization spectra. Interestingly, some cyclization of **II** is indicated by the presence of a molecular ion with the mass of the cyclol dimethyl ester in the spectra of samples of **III** treated with diazomethane. Numerous attempts,

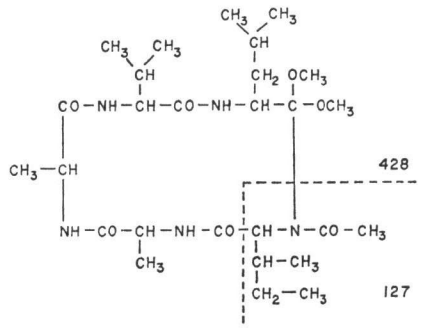
Fig. 3. Chemical ionization mass spectrum of the product obtained by acetylation and methylation of compound **I**.



* Since the CD_2N_2 reagent was not isotopically pure and the exchange of NH protons was also incomplete, the mass spectrum showed in addition to the expected peak at 566 also peaks corresponding to incomplete replacement of amide-hydrogens by deuterium.

however, to obtain **VI** in amounts sufficient for further studies, *e. g.* by nmr or IR spectra, failed so far.

Fig. 4. The formation of fragment ion *m/e* 428 from compound **VI**.



Spontaneous formation of cyclol derivatives was reported by SHEPPARD and his associates[7~9]*, TITLESTAD[10] and ROTHE,[11] but always with compounds in which the cyclols were stabilized by already existing rings. In our case no covalently bonded ring was present, but a ring-like structure stabilized by hydrogen bonds. The ready cyclization of the open chain precursor of desthiomalformin and its very small tendency for cyclodimerization were already interpreted[5] as indications of a ring-like conformation. Further support for this assumption was found in the conversion of the methyl ester of **I** to desthiomalformin in molten imidazole[12]. Our new observations by mass spectra provide additional evidence for the existence of such a quasi-cyclic architecture. This kind of geometry was predicted by RAMACHANDRAN and his associates[13] for peptides in which alternating D and L residues occur. The ring portions of bacitracin[14], stendomycin[15] and of longicatenamycin[16] are examples of naturally occurring compounds with this characteristic feature. This seems to be one of the devices (cf. ref. 13) by which ring formation on the specific surfaces operating in the biosynthesis of microbial peptides can readily occur. Whether or not cyclols are indeed intermediates of the cyclization steps remains to be demonstrated.

Experimental

Capillary melting points are uncorrected. Dimethylformamide was dried over a molecular sieve, Linde Type 4A, MeOH on Linde Type 3A. Spots on thin-layer chromatograms (tlc) were revealed by *tert*-butyl hypochlorite KI-starch reagent and by charring. For tlc the solvent system $CHCl_3$–MeOH (9: 1) was used.

Acetyl-L-Isoleucyl-D-Alanyl-D-Alanyl-L-Valyl-D-Leucine Methyl Ester (III)

A. In a 40-ml centrifuge tube, provided with a 24/40 standard tapered joint[17] benzyloxycarbonyl-L-isoleucyl-D-alanyl-D-alanyl-L-valyl-D-leucine methyl ester[5] 159.6 mg (0.25 mmol) was suspended in glacial acetic acid (1.25 ml) and treated with 5 M HBr in acetic acid (1.25 ml). After 1.5 hours at room temperature, the partially protected pentapeptide hydrobromide was precipitated with ether (30 ml). The solid was centrifuged, the solution decanted and the hydrobromide disintegrated under and washed with ether. It was air dried, then *in vacuo* over P_2O_5 and NaOH for 2 hours. The product was dissolved in DMF (2.5 ml), diisopropylethylamine (0.04 ml, 0.25 mmol) was added followed by *p*-nitrophenyl acetate (Aldrich, 70 mg, 0.375 mmol). The reaction mixture was stirred overnight: it became ninhydrin negative. The solvent was removed *in vacuo*, the residue triturated with H_2O (30 ml), centrifuged, and the supernate decanted. The solid was washed with ether (30 ml), dried *in vacuo* over NaOH; 108 mg (80%); m.p., 297~298°C dec.; Rf 0.34. A sample (45 mg) was recrystallized from hot 95% EtOH (10 ml); recovery, 32 mg; m.p. >300°C.

Anal. Calcd for $C_{26}H_{47}N_5O_7$ (541.7): C, 57.7; H, 8.8; N, 12.9.
Found: C, 57.9; H, 8.8; N, 12.9.

B. The free pentapeptide L-isoleucyl-D-alanyl-D-alanyl-L-valyl-D-leucine (**I**)[5] (6.0 mg, 0.012

* Subsequently, however, evidence was found in this case against a cyclol intermediate (JONES, D. S.; G. W. KENNER, J. PRESTON & R. C. SHEPPARD: Peptides. XIX. The isomerization of some oxazolones derived from tripeptides. Tetrahedron 21: 3209~3218, 1965).

mmol) was dissolved in glacial acetic acid (0.5 ml) and acetic anhydride (0.05 ml) was added. After 2 hours, the solvent was removed with a stream of N_2, and the product dried *in vacuo*. The acetyl derivative was dissolved in MeOH (10 ml) and freshly prepared ethereal diazomethane (~0.25 M, 6 ml) was added. The yellow solution was allowed to stand for 1 hour at room temperature. The solvent was removed with the help of a stream of N_2 and the product dried *in vacuo*. m.p., >300°C. For analysis a sample was sublimed at 210~260°C and 0.05 mm.

Anal. Found: C, 58.5; H, 8.7; N, 12.8.

In a separate experiment the methylation was carried out by adding an ethereal solution of deutero-diazomethane, prepared from deutero-Diazald (Aldrich), to a solution of the acetylation product in CH_3OD (Aldrich).

L-Isoleucyl-D-Alanyl-D-Alanyl-L-Valyl-D-Leucine Methyl Ester

A sample of benzyloxycarbonyl-L-isoleucyl-D-alanyl-D-alanyl-L-valyl-D-leucine methyl ester (0.32 g) was suspended in AcOH (3 ml) and treated with a *ca* 5 molal solution of HBr in AcOH (3 ml). After 1.5 hours at room temperature the solution was added dropwise to ether (100 ml) with stirring. The precipitate was filtered, washed with ether (100 ml) and dried. The hydrobromide was dissolved in MeOH (30 ml) and treated with Amberlite IR 400 in OH cycle (*ca* 4.0 g). The ion-exchange resin was removed by filtration and washed with methanol. The combined filtrate and washings were evaporated to dryness with a stream of N_2. The residue, 0.22 g gave a negative BEILSTEIN test. M.p. 232°C, resolidifies on cooling. The m.p. was unchanged after sublimation *in vacuo* at 180°C and 0.03 mm. The IR spectrum shows an ester band at 1745 cm^{-1}.

Anal. Calcd. for $C_{24}H_{45}N_5O_6$ (499.6): C, 57.7; H, 9.1; N, 14.0.
Found: C, 57.9; H, 8.9; N, 13.7.

Cyclization in Molten Imidazole

A sample of the pentapeptide methyl ester (50 mg) was dissolved in molten imidazole (1.0 g) and heated in a steam bath for 4 hours. After cooling EtOAc (10 ml) was added. Next day the precipitate that separated was collected on a filter, washed with EtOAc (20 ml) and ether (10 ml). The dried material (13 mg) did not melt up to 300°C. On tlc—although probably contained some diastereoisomer—it was indistinguishable from synthetic desthiomalformin[5] (the spots were revealed by spraying with H_2O).

The crude product was sublimed at *ca* 0.05 mm and 250~280°C, leaving a very small residue. M.p. >300°C.

Anal. Calcd. for $C_{23}H_{41}N_5O_5$: C, 59.1; H, 8.8; N, 15.0.
Found: C, 59.3; H, 9.0; N, 14.9.

Acknowledgement

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