The Synthesis of Peptides Related to Gramicidin S and the Significance of Optical Configuration in Antibiotic Peptides

1. TRIPEPTIDES

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(Received 1 September 1949)

In a previous communication (Harris & Work, 1950) we reported that an amino-acid analogue which was not itself antibacterial might acquire antibacterial properties when joined in peptide linkage with other amino-acids.

Repeated demonstration of the occurrence of amino-acids of 'unnatural' (D) configuration in antibiotic polypeptides such as gramicidin (Lipmann, Hotchkiss & Dubos, 1941), tyrocidine (Gordon, Martin & Synge, 1943), gramicidin S (Synge, 1945), aerosporin (Jones, 1948), and bacitracin (Barry, Gregory & Craig, 1948), suggested that antibiotic

To test such a theory it did not seem sufficient to link any one of the D-amino-acids occurring in antibiotic peptides with a random selection of L-amino-acids, and the choice of a suitable series of synthetic peptides was governed by the knowledge that in only one antibiotic peptide, namely, gramicidin S, was the probable sequence of amino-acids known.

On the basis of degradative studies, Consden, Gordon, Martin & Synge (1947) have suggested that gramicidin S is a cyclic pentapeptide or a cyclic decapeptide with the following sequence of amino-acid residues: L-ornithyl-L-leucyl-D-phenylalanyl-

Fig. 1. Amino-acid sequence in gramicidin S.

activity might be dependent upon the occurrence in such peptides of amino-acid analogues (viz. D-amino-acids) which, although harmless in themselves, became antibacterial when incorporated in a suitable peptide sequence. The presence of D-penicillamine among the hydrolysis products of penicillin, and the subsequent demonstration by du Vigneaud, Carpenter, Holley, Livermore & Rachele (1946) that antibiotic penicillin could be synthesized from D-penicillamine, whereas the isomeric compound from L-penicillamine was inactive, seemed at first sight to lend support to this view.

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L-prolyl-L-valyl- (Fig. 1). As the only amino-acid of 'unnatural' configuration, phenylalanine is indicated in Fig. 1 by the projection of the benzyl side chain below the line of the peptide 'backbone'. We sought to examine the significance of optical configuration by synthesis, in the first instance, of the tripeptide fragment enclosed by the dotted lines in Fig. 1. The corresponding tripeptide with all three amino-acids in the L configuration was also synthesized as a control.

Synthetic methods

For the syntheses of the optically isomeric tripeptides, carbobenzyloxy derivatives of appropriate amino-acids were used according to the general pro-

cedure of Bergmann & Zervas (1932). Difficulties were encountered in the syntheses of dipeptide esters containing proline, and although *carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester* and *carbobenzyloxy-D-phenylalanyl-L-proline methyl ester* were obtained by the azide method, yields were low and the products were not crystallized. It is relevant to note that Synge (1948) also reported difficulty in the isolation of pure dipeptides containing proline; his products were amorphous and analytical figures departed appreciably from the expected values.

In view of the tendency of proline dipeptides to form diketopiperazines (Abderhalden & Nienburg, 1933; Smith & Bergmann, 1944), and as satisfactory characterization of intermediates was considered desirable, it was decided to modify the order of coupling. In the preparation of *carbobenzyloxy-L-leucyl-D-phenylalanine ester* the acid chloride and azide methods of coupling were compared; a higher yield and a purer product was obtained by the azide method and this was accordingly the method of choice whenever applicable. The azide from *carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide* coupled smoothly with *L-proline methyl ester* to give *carbobenzyloxy-L-leucyl-D-phenylalanyl-L-proline methyl ester* in 85% yield; the corresponding *carbobenzyloxy-L-L-L-tripeptide ester*, prepared by coupling *carbobenzyloxy-L-leucyl azide* with *L-phenylalanyl-L-proline ethyl ester*, was obtained in inferior yield as an amorphous solid. The identity and purity of the tripeptide esters was checked by elementary analyses, by paper chromatography of the tripeptide esters themselves, and by comparison of their acid hydrolysates with an artificial mixture of the component amino-acids.

EXPERIMENTAL

Amino-acids. Leucine and phenylalanine were synthesized by the acetamidomalonic ester synthesis of Albertson & Archer (1945), and resolved through the brucine salts of their formyl derivatives by the classical procedures of Fischer (1906). *L-Proline* was isolated from a protein hydrolysate by the rhodanilate method of Bergmann (1935).

Carbobenzyloxy-L-phenylalanyl hydrazide. *L-Phenylalanine methyl ester* was prepared according to the general method of Fischer (1901). The methyl ester (13.0 g.) in dry ether (100 ml.) was cooled to -10° and an ethereal solution of *carbobenzyloxy chloride* (6.2 g., 0.5 equiv.) was added dropwise, with stirring, during 45 min. The reaction mixture was left overnight at room temperature and the precipitated *L-phenylalanine methyl ester hydrochloride* recovered by filtration. *Carbobenzyloxy-L-phenylalanine methyl ester* was obtained as an oil (10.0 g.) after removal of solvent from the filtrate. This oil was mixed in methanol with an excess of 50% hydrazine hydrate and left for 24 hr. at room temperature. *Carbobenzyloxy-L-phenylalanine hydrazide* was precipitated on addition of water and collected by filtration. The pure hydrazide (7.9 g.) was obtained as needles, m.p. 168° , from methanol. (Found: C, 65.4; H, 6.2; N, 13.6. $C_{17}H_{19}O_3N_3$ requires C, 65.2; H, 6.1; N, 13.4%.)

Carbobenzyloxy-D-phenylalanine hydrazide. *D-Phenylalanine hydrochloride* (25 g.) yielded *carbobenzyloxy-D-phenylalanine methyl ester* (20 g.). The ester was converted to *carbobenzyloxy-D-phenylalanine hydrazide* by the method previously used for the *L*-isomer. The hydrazide crystallized from ethanol as needles, m.p. $169-170^{\circ}$ (16.5 g.). (Found: C, 65.6; H, 6.4; N, 13.5. $C_{17}H_{19}O_3N_3$ requires C, 65.2; H, 6.1; N, 13.4%.)

Carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester. *L-Phenylalanine hydrazide* (6.7 g.) in a mixture of glacial acetic acid (50 ml.) and 2*N*-HCl (100 ml.) was cooled to 0° and a solution of $NaNO_2$ (1.1 g.) in water (10 ml.) added dropwise to the stirred mixture during 10 min. The precipitated azide was extracted into cold ether (100 ml.) and washed with ice-cold water, with cold saturated $NaHCO_3$ solution and again with water. The azide solution was dried quickly over Na_2SO_4 and added during 20 min. to a cooled solution of *L-proline ethyl ester* (4.0 g.) in dry ether (100 ml.). After 24 hr. at 0° the solution was washed with dilute acid to remove proline ester, then with $NaHCO_3$ solution, and finally with water. Removal of the solvent *in vacuo* left a pale yellow solid (8.0 g.) which was not obtained in a crystalline form. (Found: C, 67.5; H, 6.3; N, 6.8. $C_{24}H_{28}O_5N_2$ requires C, 67.9; H, 6.6; N, 6.6%.)

L-Phenylalanyl-L-proline ethyl ester. Removal of the *carbobenzyloxy* group from *carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester* was readily accomplished by application of the catalytic hydrogenation procedure of Bergmann & Zervas (1932). *L-Phenylalanyl-L-proline ethyl ester hydrochloride* crystallized from ethanol as extremely hygroscopic needles, m.p. (sealed tube) 174° (decomp.). Owing to rapid uptake of water, no satisfactory analytical results could be obtained for this hydrochloride.

Carbobenzyloxy-L-leucyl-L-phenylalanyl-L-proline ethyl ester. *Carbobenzyloxy-L-leucyl hydrazide* was prepared from *L-leucine* by the method of Bergmann, Zervas, Fruton, Schneider & Schleich (1935). The hydrazide (2.0 g.) was converted to the azide and allowed to react with *L-phenylalanyl-L-proline ethyl ester* (2.7 g.). The method of handling was similar to that already employed in the synthesis of *carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester*. The product, a yellow gum (1.9 g.), was dissolved in ethyl acetate and precipitated with ligroin. Repetition of this operation four times yielded a product (1.5 g.) which was not obviously crystalline but which nevertheless melted rather sharply ($134-136^{\circ}$). Analysis and paper chromatography of a hydrolysate left no doubt that it was *carbobenzyloxy-L-leucyl-L-phenylalanyl-L-proline ethyl ester*. (Found: C, 67.2; H, 7.3; N, 8.0. $C_{30}H_{38}O_6N_3$ requires C, 67.1; H, 7.3; N, 7.8%.)

L-Leucyl-L-phenylalanyl-L-proline ethyl ester. The *carbobenzyloxy* derivative (1.2 g.) from the previous experiment was successfully hydrogenated in ethanol in the presence of 1 equiv. of H_2SO_4 . Removal of acid and solvent left an oil (0.95 g.) which could not be crystallized. Provisional identification of the product as *L-leucyl-L-phenylalanyl-L-proline ethyl ester* was achieved by paper chromatography.

Carbobenzyloxy-L-leucyl-D-phenylalanine ethyl ester. (*Acid chloride method.*) *Carbobenzyloxy-L-leucine* (6.2 g.) in dry ether (30 ml.) was allowed to react at 0° with PCl_5 (4.9 g.). When the reaction was complete, ether was removed and the residual oil washed with three 50 ml. portions of dry light petroleum. The petroleum-insoluble acid chloride was dissolved in dry $CHCl_3$ (25 ml.) and half of this solution was added, with stirring, to a cooled solution (-10°) of *D-phenylalanine ethyl ester* (4.5 g.) in dry $CHCl_3$ (25 ml.); after 5 min.

KHCO₃ (2.32 g.) was added in the minimum volume of water and followed by the remainder of the acid chloride. The mixture was stirred and the temperature held below 0° for 30 min. and at 15° for another hour. The CHCl₃ solution was washed successively with dilute acid and dilute bicarbonate and dried. Removal of solvent left carbobenzyloxy-L-leucyl-D-phenylalanine ethyl ester which crystallized from a mixture of ethyl acetate and light petroleum as needles (5.0 g.), m.p. 105°. (cf. Synge, 1948). (Found: C, 67.8; H, 7.2; N, 6.2. C₂₅H₃₃O₅N₃ requires C, 68.1; H, 7.3; N, 6.4%.)

L-Leucyl-D-phenylalanine ethyl ester. The carbobenzyloxy peptide as prepared above (1.0 g.) was hydrogenated in methanol containing 1 equiv. HCl. The carbobenzyloxy group was readily removed and L-leucyl-D-phenylalanine ethyl ester hydrochloride crystallized from ethyl acetate-ethanol as plates (0.8 g.), m.p. 216°. (Found: C, 59.6; H, 7.7; N, 8.0. C₁₇H₂₆O₃N₂·HCl requires C, 59.6; H, 7.9; N, 8.2%.)

Carbobenzyloxy-L-leucyl-D-phenylalanine methyl ester. (Azide method.) Carbobenzyloxy-L-leucyl hydrazide (18 g.) was converted to the azide and allowed to react with phenylalanine methyl ester (16.8 g.). The method was essentially that employed already in the synthesis of carbobenzyloxy-L-phenylalanyl-L-proline ethyl ester. The product, carbobenzyloxy-L-leucyl-D-phenylalanine methyl ester (20.8 g.) crystallized as needles, m.p. 110–112°, from ether. (Found:

of the amorphous solid confirmed this view. (Found: C, 65.9; H, 7.1; N, 8.5. C₂₅H₃₇O₅N₃ requires C, 66.5; H, 7.1; N, 8.1%.)

L-Leucyl-D-phenylalanyl-L-proline methyl ester. The carbobenzyloxy tripeptide ester (1.6 g.) from the previous experiment was hydrogenated with palladium black in methanol in the presence of 1 equiv. of HCl. Evolution of CO₂ was complete in 2 hr. L-Leucyl-D-phenylalanyl-L-proline methyl ester hydrochloride, white hygroscopic plates, m.p. 240° (decomp.), crystallized from a mixture of ethanol and ether; $[\alpha]_D^{20} = -38.9^\circ$ in methanol (c, 2.0). (Found: C, 58.9; H, 7.5; N, 10.0. C₂₁H₃₁O₄N₃·HCl requires C, 59.2; H, 7.5; N, 9.9%.)

MICROBIOLOGICAL RESULTS

The optically isomeric tripeptide ester hydrochlorides, together with the intermediate dipeptide esters, were tested *in vitro* against *Staphylococcus aureus*, *Streptococcus haemolyticus* and *Escherichia coli* (media: see Harris & Work, 1950), and the results obtained are given in Table 1. It was not considered necessary to remove the ester group before testing since esters are readily hydrolysed by living cells.

Table 1. Minimum inhibitory concentrations (M) of synthetic peptide esters as inhibitors of bacterial growth

Compound	<i>Esch. coli</i> (synthetic medium)	<i>Staph. aureus</i> (synthetic medium)	<i>Strep. haemolyticus</i> (broth)
D-Phenylalanyl-L-proline methyl ester	3×10^{-2}	2×10^{-2}	3×10^{-3}
L-Phenylalanyl-L-proline ethyl ester	2×10^{-2}	4×10^{-2}	1.5×10^{-2}
L-Leucyl-D-phenylalanine methyl ester	5×10^{-3}	2×10^{-3}	1×10^{-3}
L-Leucyl-D-phenylalanyl-L-proline methyl ester	7×10^{-3}	5×10^{-3}	1.5×10^{-3}
L-Leucyl-L-phenylalanyl-L-proline ethyl ester	$> 7 \times 10^{-3}$	6×10^{-3}	1×10^{-3}

C, 67.4; H, 7.1; N, 6.7. C₂₄H₃₀O₅N₃ requires C, 67.6; H, 7.1; N, 6.6%.) Comparison of this yield (77%) with that obtained by the acid chloride method (48%) indicated that the azide method was to be preferred.

Carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide. The methyl ester from the above preparation (19.0 g.) was converted to the hydrazide by treatment in methanol, with an excess of 90% hydrazine hydrate. Carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide (16.6 g.) crystallized from ethyl acetate as needles, m.p. 169. (Found: C, 64.5; H, 7.2; N, 13.2. C₂₃H₃₀O₄N₄ requires C, 64.8; H, 7.1; N, 13.2%.)

Carbobenzyloxy-L-leucyl-D-phenylalanyl-L-proline methyl ester. Carbobenzyloxy-L-leucyl-D-phenylalanyl hydrazide (16.0 g.) was converted to azide and reacted with proline methyl ester (12 g.) by the usual azide method, the only modifications being substitution of ethyl acetate as solvent in place of ether and allowance of 48 hr. at 0° for completion of the reaction. Excess proline ester was recovered as the hydrochloride (6.6 g.). Carbobenzyloxy-L-leucyl-D-phenylalanyl-L-proline methyl ester (19.0 g.) was a solid of doubtful crystallinity melting over the range 40–42° ($[\alpha]_D^{22} = -42.2^\circ$ in methanol, c, 2.7). Comparison of an acid hydrolysate of this peptide with an artificial mixture of the constituent amino-acids on a paper chromatogram, coupled with almost quantitative reduction of a sample of carbobenzyloxy tripeptide ester to a crystalline tripeptide ester hydrochloride, left no doubt that the desired product had been obtained in an almost pure state in a yield of 85% of theory; analysis

DISCUSSION

It is apparent from Table 1 that synthetic L-leucyl-D-phenylalanyl-L-proline ester is slightly antibacterial, but this activity is not peculiarly associated with the occurrence of D configuration, since the optically isomeric L-leucyl-L-phenylalanyl-L-proline ester is equally active.

This result is in general agreement with the findings of other investigators which have been published while the present work was in progress. In view of the isolation of D-leucine and D-valine from gramicidin (Dubos) hydrolysates, and since the intact gramicidin molecule contains no free primary amino groups, Fling, Minard & Fox (1947) synthesized prolyl derivatives of leucine and valine. No antipodal specificity was encountered despite the fact that the free D-amino-acids had shown some inhibition of bacterial growth under similar conditions (Fox, Fling & Bollenback, 1944; Fling & Fox, 1945). All four diastereoisomeric leucylleucines have also been synthesized and tested, but not one had antibacterial activity equal to that of D-leucine itself (Fox, Kobayashi, Melvin & Minard, 1948). The high content of D-leucine and L-tryptophan in gramicidin

led Fruton (1948) to synthesize the diketopiperazine from D-leucine and L-tryptophan, but no antibacterial activity was encountered.

These findings indicate that growth inhibitory properties cannot be correlated with the mere presence of D-amino-acids in peptide linkage. At the outset of the present investigation it was considered likely that, as an additional requirement, the D-amino-acid should be flanked by a suitable pair of amino-acids; it was supposed that when this requirement was met optically selective antibiotic action might appear. A tripeptide possessing the D-amino-acid present in gramicidin S, flanked by the same two amino-acids as in the natural antibiotic peptide has, however, failed to show selective toxicity.

All the available evidence now supports the view expressed by Work (1948) that peptide antibiotics containing D-amino-acids are active, not because they have this character in common, but rather by virtue of the individual structure of the peptide.

In order to eliminate as far as possible the complicating factors of molecular size, and number and type of polar groups per molecule, it seemed desirable

to extend the present investigation by the syntheses of appropriate pentapeptides containing the complete amino-acid sequence of gramicidin S; the results of this investigation will be reported in a future communication.

SUMMARY

1. Dipeptides and tripeptides containing D-phenylalanine have been synthesized and compared as growth inhibitors with optically isomeric peptides containing L-phenylalanine.

2. The amino-acids in these peptides were arranged in the same sequence as in gramicidin S.

3. Limited antibiotic activity was observed with synthetic peptides, but no difference in activity was found between a tripeptide containing D-phenylalanine and one containing L-phenylalanine.

4. The significance of these results in relation to the origin of antibiotic activity in peptides is discussed.

We are indebted to Dr A. T. Fuller for the biological results summarized in Table 1.

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The Steady State, a Test for Electrophoretic Homogeneity

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(Received 20 June 1949)

The distribution of the ion concentrations across the boundaries formed by a solution of a homogeneous protein changes during electrophoresis. The tendency is for the boundary in one limb of the U-tube to become narrower and for that in the other to become broader, mainly on account of the differences in conductivity on both sides of each boundary (Longworth & MacInnes, 1940), but also because the

mobility changes with the ionic strength. These effects may be enhanced or counteracted by changes in pH (Longworth, 1947). If the ascending boundary of a leading ion has the tendency to become narrower, and this is generally the case with proteins at a pH removed from the isoelectric point, its behaviour during migration may be followed for an unlimited period of time, provided a countercurrent of buffer