835. *Peptides. Part VIII.* Cyclic Peptides Derived from Leucine and Glycine.*

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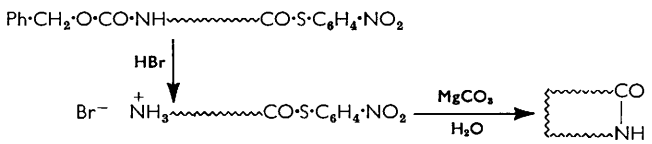
The *p*-nitrophenyl thiolesters of benzyloxycarbonylpeptides can be converted into cyclic peptides by removal of the benzyloxycarbonyl group with hydrogen bromide and subsequent cyclisation in neutral aqueous solution. The derivative of glycyl-L-leucylglycine yields the cyclic hexapeptide, identical with that prepared from the derivative of glycyl-L-leucylglycylglycyl-L-leucylglycine. No cyclic peptide could be obtained from the derivatives of glycyl-L-leucylglycyl-L-leucine and L-leucylglycyl-L-leucylglycine, but cyclic pentapeptides were formed in good yield. The yield was significantly higher from the derivative of glycyl-L-leucylglycyl-D-leucylglycine than from the L-L-diastereoisomer, and this difference has been correlated with the lower dielectric increment of the open-chain L-D-pentapeptide.

Cyclic peptides, of which the best known is gramicidin-S, form a well-recognised class of natural product,[1] but until recently there were only scattered, and not always convincing, reports of synthetic cyclic peptides.[2] In seeking a general method of synthesis, we adopted a principle already employed † by Winitz and Fruton;[3] in a synthesis of this pattern, a terminal amino-protecting group is removed from a peptide derivative possessing an activated carbonyl grouping at the other end of the chain, and then cyclisation can follow. The activating group must be comparatively stable in order to survive the removal of the amino-protecting group and the wait for the relatively infrequent approach of the terminal amino-group. Consequently the *p*-nitrophenyl thiolesters, which are easily prepared from amino-protected peptides,[5] are suitable derivatives, although the esterified amino-acid residue should then be glycine in order to avoid the substantial risk of racemisation.[5]



These thiolesters withstand completely the treatment with hydrogen bromide in acetic acid for removal of benzyloxycarbonyl, which is thus a convenient amino-protecting group. This method, which is depicted above, was tried first with a pentapeptide because this has the smallest ring which should be formed easily. Since the preliminary announcement of the successful outcome,[6] Schwyzer and his colleagues have published work along somewhat similar lines [7] and this has culminated in the synthesis of gramicidin-S.[8] Their improved technique for isolating the cyclic peptides, by passage through ion-exchange columns, has been incorporated in the experiments recorded below.

The aspect of cyclisation in the peptide series which has chiefly interested us, once the method had been developed, has been its stereochemistry. It is generally recognised that in open-chain peptides the amide group prefers a planar conformation and that the conformation in which the " carbonyl "-oxygen and the " imino "-hydrogen are *trans*- is

* Part VII, *J.*, 1957, 1407.

† There is some doubt [4] whether the material described by them was actually a cyclic peptide.

[1] Bricas and Fromageot, *Adv. Protein Chem.*, 1953, **8**, 1.
[2] Goodman and Kenner, *ibid.*, 1957, **12**, 494.
[3] Winitz and Fruton, *J. Amer. Chem. Soc.*, 1953, **75**, 3041.
[4] Heyns, Walter, and Müller, *Angew. Chem.*, 1956, **68**, 617.
[5] Farrington, Hextall, Kenner, and Turner, *J.*, 1957, 1407.
[6] Kenner and Turner, *Chem. and Ind.*, 1955, 602.
[7] Schwyzer, Iselin, Rittel, and Sieber, *Helv. Chim. Acta*, 1956, **39**, 872.
[8] Schwyzer and Sieber, *ibid.*, 1957, **40**, 624.

preferred to the other in which they are *cis*.[9] A natural presumption is that those cyclic peptides which can adopt conformations obeying these rules will be formed most easily, and, with the aid of molecular models particularly suitable for peptides,[10] it can be seen that five amino-acid residues are required by these conditions.* However dioxopiperazines, in which both amide groups are *cis*, are formed quite easily from many dipeptide esters, and therefore a small series of cyclisations was studied. For ease of handling, peptides with alternating glycine and L-leucine residues were chosen. A crystalline cyclic peptide was obtained from glycyl-L-leucylglycine *p*-nitrophenyl thiolester in more than 10% yield, but this was found to be the cyclic hexapeptide formed by junction of two units before cyclisation; it was identified with the substance produced by cyclisation of glycyl-L-leucylglycylglycyl-L-leucylglycine *p*-nitrophenyl thiolester. For determinations of molecular weight, the cyclic peptides were dissolved in trifluoroacetic acid and initially the apparatus of Morton, Campbell, and Ma [11] was used successfully for the isopiestic method. This technique was also used by Schwyzer and his colleagues,[7] but later we were unable to observe a steady state of equilibrium, possibly owing to a leakage of vapour. However, as no reactivity towards ninhydrin was generated from the cyclic peptides during half an hour in boiling trifluoroacetic acid, an ebullioscopic method [12] was a reliable alternative. Our failure to detect any cyclic tripeptide in the product from glycyl-L-leucylglycine *p*-nitrophenyl thiolester is not at all surprising because, even with three *cis*-amide groups, the molecular model is strained, mainly by the repulsion between one hydrogen atom on each of the methylene or methine groups. Formation of the same cyclic hexapeptide from derivatives of glycyl-L-leucylgylcine has been observed independently by Schwyzer, Sieber, and Gorup.[13] Similarly it has already been established [14] that the product [15] from glycylglycylglycine azide is *cyclo*hexaglycyl. On the other hand, *cyclo*glycylglycyl-DL-prolyl is formed gradually from gylcyl-DL-prolylglycine ethyl ester: [16] this may be regarded as an exceptional instance related to the pronounced tendency for formation of dioxopiperazines from derivatives of dipeptides containing proline.[17]

Our attempts to synthesise *cyclo*-L-leucylglycyl-L-leucylglycyl from either of the open-chain tetrapeptide *p*-nitrophenyl thiolesters were unsuccessful; traces of crystalline material were obtained but it was not characterised. Perhaps the most surprising feature of these results is the lack of the cyclic octapeptide; unfortunately the cyclisation of an octapeptide derivative has not been examined by us because our attempts to prepare the thiolester from benzyloxycarbonyl-L-leucylglycyl-L-leucylglycyl-L-leucylglycyl-L-leucylglycine were unsuccessful, presumably on account of its insolubility. It should be noted that the synthesis of *cyclo*tetraglycyl under different conditions is well authenticated.[7]

The cyclic pentapeptides were formed readily from glycyl-L-leucylglycyl-L-leucylglycine *p*-nitropheny thiolester and its L-D-diastereoisomer in yields of 41 and 57% respectively. Although the methods of isolation differed because the cyclic L-D-peptide separated immediately from the reaction mixture whereas the L-L-product remained in solution, we regard the difference as genuine and fundamentally significant. It might arise from reduced repulsion of the side-chains in the L-D-cyclic peptide, or rather the transition state leading to it; this effect has been invoked to account for the frequent occurrence of

* A model, which has planar *trans*-amide groups, of a cyclic tetrapeptide [7] can actually be assembled with some difficulty, but others in which one or more of the amide groups are *cis* are assembled much more easily.

[9] Pauling and Corey, *Fortschr. Chem. org. Naturstoffe*, 1954, **11**, 180.
[10] Hartley and Robinson, *Trans. Faraday Soc.*, 1952, **48**, 847.
[11] Morton, Campbell, and Ma, *Analyst*, 1953, **78**, 722.
[12] Wilson, *Analyst*, 1948, **73**, 585 (Sucharda–Bobranski apparatus).
[13] Schwyzer, Sieber, and Gorup, *Chimia*, 1958, **12**, 90.
[14] Sheehan, Goodman, and Richardson, *J. Amer. Chem. Soc.*, 1955, **77**, 6391; Bamford and Weymouth, *ibid.*, p. 6368.
[15] Sheehan and Richardson, *ibid.*, 1954, **76**, 6329.
[16] P. W. G. Smith, *J.*, 1957, 3985.
[17] E. L. Smith and Bergmann, *J. Biol. Chem.*, 1944, **153**, 627; Rydon and P. W. G. Smith, *J.*, 1956, 3642.

D-amino-acid residues in natural cyclic peptides,[18] but the interaction of neighbouring residues seems to have been envisaged originally and in the present instance the effect is not obvious with molecular models. A second possibility, which would correspond to a change in the entropy instead of the enthalpy of activation, is that the conformation of the open-chain L-D-compound is more frequently close to that of the transition state. While the amide groups have rigidly the planar *trans*-conformation, this situation could be caused by preference for certain angles of rotation about some or all of the bonds between the asymmetric carbon atoms and the amide groups. Although experimental evidence has been lacking, such preferences in the peptide series have been discussed [19] and we accept this explanation as a useful hypothesis.

Independent evidence of the conformation of the related open-chain peptides is furnished by dielectric data. The contributions made by these dipolar ions to the dielectric constants of their aqueous solutions (the dielectric increments) are approximately proportional to the squares of their dipole moments. We find that the dielectric increment of glycyl-L-leucylglycyl-L-leucylglycine is 186 whereas that of the L-D-diastereoisomer is 169. The conclusion [20] from earlier work, which did not include studies of diastereoisomers, either that there is free rotation of the peptide chain or that several preferred conformations are equally probable is apparently unjustified, but confirmation and theoretical explanation will require more sets of comparable data. We hope that greater differences will be encountered with more carefully selected diastereoisomers: the pair already studied was chosen for ease of manufacture and isolation of the cyclic peptides.

The difference between the dielectric increments of the diastereoisomeric pentapeptides is in the direction predicted by our hypothesis, but it might be considered too small to account for the difference in the yields of cyclic peptides. However, it must be realised that the ease of cyclisation will be determined by the contribution of only the conformations with smaller dipole moments and, therefore, although the two phenomena should vary in the same direction, close correspondence is not to be expected. Further it may not be legitimate to equate the free peptide with its thiolester.

Experimental

M. p.s are corrected. Evaporations were carried out under reduced pressure.

Tri-p-nitrophenyl Phosphorotrithioite.—Method (a) described in Part VII [5] was modified for work on a larger scale. A solution of lithium (2·21 g.) in methanol (100 c.c.) was mixed with a solution of *p*-nitrothiophenol (49·3 g.; prepared from either *p*-chloronitrobenzene [21] or bis-*p*-nitrophenyl disulphide [22]) in dimethylformamide (180 c.c.). The reaction with phosphorus trichloride (9·6 c.c.) was carried out as before, and the crude product was washed with dimethylformamide (10 c.c.) and ethylene dichloride (10 c.c.) before being recrystallised from ethylene dichloride, which did not dissolve a minor impurity. The yield of recrystallised phosphorotrithioite was 30 g. (55%).

Benzyloxycarbonyl-D-leucylglycine.—The same sequence of operations as in the L-series [5] led through *benzyloxycarbonyl-D-leucine* p-*nitrophenyl thiolester*, m. p. 106—107° (Found: C, 60·0; H, 5·6. $C_{20}H_{22}O_5N_2S$ requires C, 59·7; H, 5·5; N, 7·0%), to *benzyloxycarbonyl-D-leucylglycine*, m. p. 116—117°, $[\alpha]_D^{22}$ +25·9° (*c* 2 in EtOH) (Found: C, 59·2; H, 7·0; N, 8·8. $C_{16}H_{22}O_5N_2$ requires C, 59·6; H, 6·9; N, 8·7%).

Benzyloxycarbonyl-DL-leucylglycine.—This *compound*, m. p. 162° (Found: C, 59·3; H, 6·6; N, 8·9%), was likewise prepared through *benzyloxycarbonyl-DL-leucine* p-*nitrolphenyl thiolester*, m. p. 98° (Found: C, 60·0; H, 5·2; N, 7·3%).

Benzyloxycarbonylglycyl-L-leucylglycyl-D-leucylglycine p-*Nitrophenyl thiolester.*—Benzyloxycarbonyl-D-leucylglycine was treated with hydrogen bromide in acetic acid and then coupled

[18] Neuberger, *Adv. Protein Chem.*, 1948, **4**, 372.
[19] Pauling and Corey, *Proc. Nat. Acad. Sci. U.S.A.*, 1951, **37**, 729; Mizushima, *Adv. Protein Chem.*, 1954, **9**, 316.
[20] Cohn and Edsall, " Proteins, Amino Acids and Peptides," Reinhold Publ. Inc., New York, 1943, p. 152—154.
[21] Shirley, " Preparation of Organic Intermediates," Wiley, New York, 1951, p. 237.
[22] Zincke, *Annalen*, 1913, **400**, 1.

with benzyloxycarbonylglycyl-L-leucylglycine *p*-nitrophenyl thiolester, as in the preparation of benzyloxycarbonylglycyl-L-leucylglycyl-L-leucylglycine,[5] which has $[\alpha]_D^{27}$ −22·0° (*c* 2 in EtOH). The L-D-diastereoisomer was obtained as a resinous solid with $[\alpha]_D^{27}$ −4·0° (*c* 4·5 in EtOH), and it was converted by procedure (a) [5] into the p-*nitrophenyl thiolester*, a colourless amorphous powder having m. p. 150—152° after repeated precipitation by water from acetone (Found: C, 55·7; H, 6·5; N, 12·0. $C_{32}H_{42}O_9N_6S$ requires C, 56·0; H, 6·2; N, 12·2%).

Benzyloxycarbonylglycyl-L-leucylglycylglycyl-L-leucylglycine p-*Nitrophenyl Thiolester*.—The preparation of this *hexapeptide derivative* from benzyloxycarbonylglycyl-L-leucylglycine was analogous to that of the foregoing pentapeptide derivative. It was obtained as a colourless amorphous powder, m. p. 194—195°, after precipitation by ether from methanol (Found: C, 54·8; H, 5·8. $C_{34}H_{45}O_{10}N_7S$ requires C, 54·9; H, 6·1%).

cyclo*Glycyl-L-leucylglycyl-L-leucylglycyl*.—Benzyloxycarbonylglycyl-L-leucylglycyl-L-leucylglycine *p*-nitrophenyl thiolester [5] (0·686 g.) was shaken with acetic acid (3·5 c.c.) and 6N-hydrogen bromide in acetic acid (1 c.c.) until it had dissolved (1 hr.); the solution was then evaporated under nitrogen (1 mm.). The residue was thoroughly triturated with ether (50 c.c.) before being partitioned between water (50 c.c.) and ethyl acetate (30 c.c.), which was washed with water (15 c.c.). The material recovered by evaporation of the dried ethyl acetate was subjected to a second treatment with hydrogen bromide and subsequent separation. The combined aqueous solutions were added during 6 hr. (approximately 1 drop every 10 sec.) to a stirred suspension of magnesium carbonate (0·80 g.) in water (200 c.c.). Stirring was continued overnight and then 30% hydrogen peroxide (3 drops) was added. Filtration removed the excess of magnesium carbonate and the bis-*p*-nitrophenyl disulphide; a check that cyclic peptide did not remain in the solid was made by exhaustive extraction with hot 95% ethanol after the disulphide had been removed in boiling ethyl acetate (30 c.c.). The aqueous filtrate was passed successively through columns of Dowex-50 (hydrogen form; 50 g.) and Dowex-2 (hydroxide form; 50 g.) ion-exchange resins. The *cyclic peptide* (0·161 g., 41%) was obtained on concentration of the effluent in two crops of needles, decomp. 290—300°; recrystallised from 95% ethanol, it had $[\alpha]_D^{20}$ −8·7° (*c* 3 in trifluoroacetic acid) [Found: C, 54·4; H, 7·7; N, 17·6%; *M*, 389 (isopiestic [11]), 389 (ebullioscopic [12]). $C_{18}H_{31}O_5N_5$ requires C, 54·4; H, 7·9; N, 17·6%; *M*, 397]. Its infrared absorption had maxima at 3260, 3070, 2950, 2870, 1651 (s), 1537 (s), 1473, 1457, 1431, 1408, 1371, 1340, 1321, 1292, 1243, 1224, 1197, 1157, 1133, 1095, 1077, 1030, 1006, 963, 940, 877, and 852 cm.$^{-1}$ (mulls in Nujol of hexachlorobutadiene), or at 3279 (s), 3077, 2950, 2865, 1664 (s), 1546 (s), 1460, 1427, 1406, 1383, 1370, 1323, 1287, 1242, 1196, 1170, 1156, 1130, 1031, 1006, 966, 940, 922, 878, 852, 830 cm.$^{-1}$ (KBr disc).

Similar yields (between 38 and 44%) were obtained on several repetitions and also by an earlier technique in which the hydrobromide solution was added in one portion to an aqueous suspension of magnesium oxide (1 g. in 4 l. of water when starting with 0·2 g. of benzyloxycarbonylpeptide thiolester) and the product was isolated by countercurrent distribution; further details were given in our preliminary communication.[6] Satisfactory cyclisation of the phenyl thiolester (lacking the nitro-group) was achieved by treating its hydrobromide with potassium *tert.*-butoxide in boiling *tert.*-butyl alcohol during 48 hr.

cyclo*Glycyl-L-leucylglycyl-D-leucylglycyl*.—Treatment of benzyloxycarbonylglycyl-L-leucylglycyl-D-leucylglycine *p*-nitrophenyl thiolester with hydrogen bromide and cyclisation were carried out as in the foregoing experiment; during addition of the aqueous solution to the suspension of magnesium carbonate, a flocculent material precipitated and frothing occurred. After 30% hydrogen peroxide (3 drops) had been added, the mixture was neutralised with 10N-hydrochloric acid (0·7 c.c.) and filtered. Evaporation of the solution after passage through cation- and anion-exchange columns did not leave any product, but the insoluble *cyclic peptide* (0·217 g., 55%; on repetition, 0·235 g., 59%) remained when the solid was exhaustively extracted with boiling ethyl acetate. Being insoluble in hot water, hot methanol, concentrated hydrochloric acid, dilute aqueous sodium hydroxide, glacial acetic acid, and dimethylformamide, it was purified by precipitation with ether from trifluoroacetic acid, and formed a colourless amorphous powder, decomp. 300—310° [Found: C, 54·0; H, 8·1; N, 17·7%; *M* (ebullioscopic [12]), 376]. Its infrared absorption (mulls in Nujol or hexachlorobutadiene) had maxima at 3280, 3065, 2950, 1661 (s), 1643 (s), 1548 (s), 1473, 1423, 1390, 1377, 1327, 1295, 1277, 1242, 1215, 1173, 1147, 1087, 1020, 917, 851, and 787 cm.$^{-1}$.

cyclo*Glycyl-L-leucylglycylglycyl-L-leucylglycyl*.—(*a*) After treatment of benzyloxycarbonylglycyl-L-leucylglycylglycyl-L-leucylglycine *p*-nitrophenyl thiolester (0·700 g.) with hydrogen

bromide in acetic acid, cyclisation and isolation followed the same course as with the L-L-pentapeptide derivative. The crystalline *cyclic peptide* was obtained directly in two crops (total 0·095 g.) on concentration of the effluent from the second ion-exchange column; it was recrystallised from methanol–ether in prisms (0·087 g., 20%), decomp. 310—320° [Found: C, 53·0; H, 7·6; N, 18·8%; *M* (ebullioscopic [12]), 444. $C_{20}H_{34}O_6N_6$ requires C, 52·9; H, 7·5; N, 18·6%; *M*, 454]. Its infrared absorption (mulls in Nujol or hexachlorobutadiene) had maxima at 3305, 3060, 2920, 1679 (s), 1663 (s), 1640 (s), 1536 (s), 1469, 1437, 1420, 1407, 1385, 1375, 1370, 1336, 1289, 1255, 1244, 1160, and 1020 cm.$^{-1}$.

(*b*) When 6N-hydrogen bromide in acetic acid (1·75 c.c.) was added to a suspension of benzyloxycarbonylglycyl-L-leucylglycine *p*-nitrophenyl thiolester [5] (0·900 g.) in acetic acid (6·5 c.c.), a clear solution was formed at once. After this had been kept at room temperature during 40 min., it was worked up and the material still soluble in ethyl acetate was treated again with hydrogen bromide, as in the foregoing experiments. The combined aqueous solutions were added during 4 hr. to a stirred suspension of magnesium carbonate (1·4 g.) in water (400 c.c.). The cyclic hexapeptide (0·046 g., 11·5%) was isolated as in (*a*) above [Found: C, 53·3; H, 7·1; N, 18·6%; *M* (ebullioscopic [12]), 424], $\nu_{max.}$ 3305, 3060, 2920, 1679 (s), 1662 (s), 1640 (s), 1536 (s), 1465, 1436, 1420, 1407, 1385, 1370, 1335, 1288, 1256, 1245, 1164, 1020 cm.$^{-1}$.

In an earlier experiment on half the above scale, cyclisation was carried out by adding the whole hydrobromide solution to a suspension of magnesium oxide (1 g.) in 4 l. of water. The cyclic hexapeptide (16%) was isolated, without use of ion-exchange columns, by partition between butan-1-ol (100 c.c.), ethyl acetate (100 c.c.), and 2N-sulphuric acid (50 c.c.) followed, after washing of the organic layer with saturated sodium hydrogen carbonate solution (50 c.c.) and water (50 c.c.) and back-extractions, by countercurrent distribution (19 transfers) in the system ethyl acetate–methanol–water (10 : 1 : 9 volumes); the material in tubes 0—7 was distributed (14 transfers) in the system ethyl acetate–butan-1-ol–water (10 : 1 : 9 volumes), and the colourless crystalline product (*K* 0·65) was recovered from tubes 3—9 before being recrystallised from methanol–ether.

Measurement of Dielectric Increments.—The capacitance of a cell was measured at 30·5° with a Twin-T bridge (General Radio type 821-A), a communications receiver, and a signal generator maintained at 28 Mc. by frequent reference to a separate crystal-controlled oscillator. Like this circuit, the cell design was taken from the literature [23] apart from the addition of a capillary tube at the bottom; through this tube the cell could be filled, emptied, washed, and dried and it was not dismantled during a series of measurements. The cell was calibrated with solutions of glycine, 22·33 being used as its dielectric increment,[24] by plotting the difference between the readings on the dial of the precision condenser when the bridge was balanced with the solution and with water against the concentration of glycine. A straight line was obtained and this direct comparison was preferred to the evaluation of the *actual* capacitance in each case by allowance for conductance. Measurements were made with three concentrations of the peptides between 0·009M and 0·025M. The dielectric increment of tetraglycine was found to be 162·3 (±1·3), while 165·8 [23] and 159·2 [25] are recorded for 25°. The value for the L-L-pentapeptide was 186·0 (±0·9) and for the L-D-isomer 168·9 (±1·3).

The free peptides were prepared by hydrogenolysis of their benzyloxycarbonyl derivatives in 50% aqueous methanol, or in methanol containing a few drops of acetic acid, with charcoal bearing 10% of palladium. The peptides left on evaporation contained traces of salt, which gave their solutions too much conductance for balance of the Twin-T bridge, and these were removed by electrodialysis at 100 v. through a Cellophane sack. *Glycyl-L-leucylglycyl-L-leucylglycine* (Found: C, 49·3; H, 8·2; N, 15·4. $C_{18}H_{33}O_6N_5,1{\cdot}5H_2O$ requires C, 48·9; H, 8·2; N, 15·8%) and *glycyl-L-leucylglycyl-D-leucylglycine* (Found: C, 48·9; H, 8·2%) were obtained as sesquihydrates by precipitation with ethanol from aqueous solution.

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[23] Conner, Clarke, and Smyth, *J. Amer. Chem. Soc.*, 1942, **64**, 1379.
[24] Linquist and Schmidt, *Compt. rend. Trav. Lab. Carlsberg*, 1938, **22**, 307.
[25] Wyman and McMeekin, *J. Amer. Chem. Soc.*, 1933, **58**, 608.