790. cyclo(*Glycylglycyl*-DL-*prolyl*).

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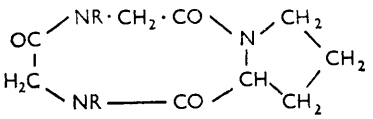
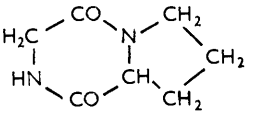
A crystalline product formed from a solution of glycyl-DL-prolylglycine ethyl ester has been shown to be *cyclo*(glycylglycyl-DL-prolyl). A modified chlorination method for the detection of certain cyclic peptides on paper chromatograms is described.

During work on the condensation–polymerisation of some peptide esters containing glycyl and prolyl residues,[1] a solution of glycyl-DL-prolylglycine ethyl ester in anhydrous ethanolic triethylamine was set aside at room temperature for a long time. A crystalline product slowly formed in low yield, and was removed periodically by filtration, during about two years. This product has now been shown to be *cyclo*(glycylglycyl-DL-prolyl) (I; R = H).

(I) 

 (II)

That the product did not give a ninhydrin reaction and yet yielded proline and glycine on acid hydrolysis suggested immediately that it was a cyclic peptide. After crystallisation from water and drying at room temperature the product appeared from analytical data to be a monohydrate, but drying at 60° failed to remove any water. Molecular-weight determination by the isopiestic method in trifluoroacetic acid [2] gave a value in only approximate agreement with the *cyclo*tripeptide structure, but larger cyclic peptide structures are obviously excluded.*

Other products which were slowly formed in the ethanolic solution, and were detected and identified by paper chromatography after a period of 2 years, were glycylprolylglycine, glycine, glycylproline anhydride [3 : 6-dioxopyrrolidino(1′ : 2′-1 : 2)piperazine] (II) and probably some hexapeptide ethyl ester. The anhydride (II) was in fact the main product isolated from heated solutions of the tripeptide ethyl ester.[1] None of these products was present in detectable amount when the first crop of cyclic product was removed.

When the behaviour of the compound (I; R = H) on paper-chromatography was investigated there was no reaction in the chlorination procedure used for its detection, either following the original method [3] or the modification described by Reindel and Hoppe,[4] under conditions which readily detected piperazine-2 : 5-diones and linear peptides. This surprising result cast doubt as to the nature of our compound. A specimen of *cyclo*(hexaglycyl) [5] [originally thought to be *cyclo*(triglycyl)] was therefore prepared from diglycylglycine hydrazide. This, too, gave no chlorination reaction. The chlorination of these cyclic peptides was therefore further investigated. An aqueous solution of piperazine-2 : 5-dione readily gave *NN*′-dichloropiperazine-2 : 5-dione on treatment with

* The referees have pointed out that the calculated molecular weights for the *cyclo*tripeptide hydrate and the *cyclo*hexapeptide dihydrate would be expected to be 229/2 and 458/3 respectively, the mole of water contributing a particle, *viz.* H_3O^+, in trifluoroacetic acid. A repetition of the control molecular-weight determination in the presence of a mole of added water gave a value of 135, in approximate agreement with the normal calculated value. Within the limits of the accuracy of the method, the water of crystallisation does not affect the determined molecular weight therefore, and must equilibrate throughout the system.

[1] Rydon and Smith, *J.*, 1956, 3642.
[2] Schwyzer, Iselin, Rittel, and Sieber, *Helv. Chim. Acta*, 1956, **39**, 872.
[3] Rydon and Smith, *Nature*, 1952, **169**, 922.
[4] Reindel and Hoppe, *Ber.*, 1954, **87**, 1103.
[5] Sheehan and Richardson, *J. Amer. Chem. Soc.*, 1954, **76**, 6329.

chlorine. The product could be crystallised from water or dioxan, but slight decomposition occurred (on one occasion decomposition was sudden and complete on attempted crystallisation from dioxan). Satisfactory analytical results were obtained on the specimen without crystallisation. The compound had the expected properties of a chloro-amide, liberating iodine from acidified potassium iodide solution and oxidising α-amino-acids to the corresponding aldehydes. Solutions of *cyclo*(hexaglycyl) and of the *cyclo*tripeptide (I; R = H) similarly gave hexa-*N*-chloro*cyclo*(hexaglycyl) and *NN'*-dichloro*cyclo*(glycylglycyl-DL-prolyl) (I; R = Cl), respectively. Detection of the cyclic peptides on chromatograms was then achieved by the modified procedure described below. As *cyclo*(glycyl-L-leucylglycyl-L-leucylglycyl) is reported to give a positive Reindel–Hoppe test,[6] the reason that *cyclo*(glycylglycylprolyl) and *cyclo*(hexaglycyl) react only to far more vigorous conditions of chlorination is not obvious. Molecular models of *cyclo*(glycylglycylprolyl) show that both chlorine atoms in the *NN'*-dichloro-compound can be accommodated quite readily. Some departure from planarity in the amide bonds is necessary in the formation of the *cyclo*tripeptide structure, however, if some distortion of normal bond angles is to be avoided.[7]

Attempts to confirm the structure (I; R = H) assigned to the product, by partial hydrolysis with N/15-lithium hydroxide,[8] were unsuccessful. Chromatograms of the partial hydrolysate showed two rather diffuse spots after being sprayed with ninhydrin; these were probably unresolved mixtures of several peptides. No spots having an R_F value less than that of a mixture of the three possible tripeptides were detected, indicating that larger peptides which would be expected to be formed from a *cyclo*hexapeptide ring were not present in the hydrolysate.

The infrared spectrum of the cyclic peptide is compatible with a *cyclo*peptide structure. It contains three strong bands due to carbonyl absorption, *i.e.* a doublet at 1675 and 1661 and a further band at 1621 cm.$^{-1}$. The last, the strongest in the spectrum, cannot be due to an ionised carboxyl group, the band for which occurs at 1603 cm.$^{-1}$ in the spectrum of glycyl-DL-prolylglycine. The linear tripeptide shows a weak band at 2632 cm.$^{-1}$ (NH_3^+?) not present in the cyclic peptide. Two possible " amide II " bands are shown by *cyclo*-(glycylglycylprolyl) (1546 and 1520 cm.$^{-1}$); this band occurs at 1541 cm.$^{-1}$ in the case of the linear tripeptide and is absent from the spectrum of glycylproline anhydride.

The formation of cyclic tripeptides has been claimed previously.[9,10] However the more usual product from the self-condensation of an active tripeptide derivative is the corresponding *cyclo*hexapeptide. Thus tripeptide azides,[11] cyanomethyl esters,[2] and *p*-nitrobenzenethiol esters [12] all yield *cyclo*hexapeptides, the formation of which may be due, in part, to the formation of bimolecular complexes, with pairs of molecules bonded together " head-to-tail." [13] In the present example, in which the tripeptide molecule has a centrally situated prolyl radical, the formation of such complexes is less likely; furthermore the molecule can readily take up a conformation favourable for intramolecular cyclisation to the cyclic tripeptide.

Experimental

All of the new compounds described decomposed when heated without showing any characteristic m. p.

Paper chromatography (5-μl. spots of solutions of about 5 mg./ml.) was on Whatman No. filter paper, butanol–acetic acid–water (4 : 1 : 5; upper phase) being used.

[6] Kenner and Turner, *Chem. and Ind.*, 1955, 602.
[7] Edward, *Research*, 1955, **8**, s38.
[8] Ballard, Bamford, and Weymouth, *Proc. Roy. Soc.*, 1954—1955, *A*, **227**, 155.
[9] Winitz and Fruton, *J. Amer. Chem. Soc.*, 1953, **75**, 3041.
[10] Brockmann, Tummes, and Metzsch, *Naturwiss.*, 1954, **41**, 37.
[11] Sheehan, Goodman, and Richardson, *J. Amer. Chem. Soc.*, 1955, **77**, 6391.
[12] Kenner, personal communication.
[13] Rees, Tong, and Young, *J.*, 1954, 662.

Analytical results were obtained in this Department by Miss J. Cuckney and her staff, and the infrared spectra by Mr. R. L. Erskine, B.Sc.

cyclo(*Glycylglycyl-*DL-*prolyl*).—Glycyl-DL-prolylglycine ethyl ester, liberated from the hydrochloride [1] (1·18 g.) with ammonia in chloroform, was dried at 30°/0·04 mm. for 30 min., dissolved in ethanol (50 ml.) containing triethylamine (6 ml.) and set aside at room temperature. After 4 months a first crop (40 mg.) of short rods was filtered off and crystallised from water, giving cyclo(*glycylglycyl-*DL-*prolyl*) *monohydrate* (28 mg., 3%), bipyramids (Found: C, 47·6, 47·0; H, 6·6, 6·7; N, 18·5, 18·7%; *M*, 177. $C_9H_{13}O_3N_3,H_2O$ requires C, 47·2; H, 6·6; N, 18·3%; *M*, 229).

NN′-*Dichloropiperazine-*2 : 5-*dione*.—Chlorine was passed for 15 min. into a solution of piperazine-2 : 5-dione (1 g.) in water (60 ml.). Next day, the *chloro-amide* (1·5 g., 94%) was collected, washed well with cold water, and dried (P_2O_5) (Found: Cl, 38·4. $C_4H_4O_2N_2Cl_2$ requires Cl, 38·7%). It gave no colour when boiled with aqueous ninhydrin, and when boiled with aqueous DL-valine gave *iso*butyraldehyde (characterised as the 2 : 4-dinitrophenylhydrazone). The following were similarly prepared: *hexa-*N-*chloro*cyclo(*hexaglycyl*) (35 mg.) from *cyclo*(hexaglycyl) [5] (25 mg.) in water (5 ml.) (Found: Cl, 37·1. $C_{12}H_{12}O_6N_6Cl_6$ requires Cl, 38·7%); and NN′-*dichloro*cyclo(*glycylglycyl-*DL-*prolyl*) (8 mg.) from the cyclic peptide (9 mg.) in water (2·5 ml.) (Found: Cl, 24·4. $C_9H_{11}O_3N_3Cl_2$ requires Cl, 25·3%).

Chlorination of Chromatograms: a Modified Procedure for Detection of Cyclic Peptides.—After the usual development of the chromatogram, solvent was removed at room temperature overnight followed by 30 min. at 50°. The chromatograms were then thoroughly moistened in steam for 3 min., treated with chlorine for 10 min., and dried at 50° for 15 min. Meanwhile,[4] a saturated solution of 4 : 4′-diamino-3 : 3′-dimethyldiphenyl in 2% aqueous acetic acid (1 part) was mixed with M/20-aqueous potassium iodide (1 part). The chlorinated chromatograms were dipped in this reagent until colour development was complete, rinsed in 2% aqueous acetic acid and blotted dry. Relative R_F values were: piperazine-2 : 5-dione, 1·00; glycylproline anhydride, 1·78; *cyclo*(hexaglycyl), 0·59; *cyclo*(glycylglycylprolyl), 1·53.

Molecular-weight Determination.—The method was a modification (Schwyzer *et al.*[2]) of Morton, Campbell, and Ma's [14] isopiestic method. Samples (3—4 mg.) of unknown and standard substances were weighed into " Polytop " specimen tubes (4 × 1 cm.) and dissolved in a weighed amount (*ca.* 700 mg.) of redistilled trifluoroacetic acid. The tubes were placed in a flat-bottomed desiccator bottle (10 × 3 cm.), together with a few drops of the solvent. The bottle was evacuated under controlled conditions [14] to *ca.* 150 mm. and totally immersed in a water-bath at room temperature, and the solutions were stirred electromagnetically. The weight of solvent in each tube was determined periodically.

Result were calculated from the expression $M = 114xW_2/yW_1$, and the conditions were: experiment 1, standard—piperazine-2 : 5-dione (2·54 mg. = W_1), " unknown "—glycylproline anhydride (II) (3·09 mg. = W_2); experiment 2, standard—piperazine-2 : 5-dione (3·30 mg. = W_1), " unknown "—cyclic peptide (4·28 mg. = W_2).

Solvent weights (*mg.*).

	Expt. 1			Expt. 2				
Time (hr.)	0	48	96	0	24	48	72	96
Standard (*x*)	871·9	790·7	734·7	744·4	653·5	590·5	414·4	382·0
" Unknown " (*y*) ...	752·8	610·4	558·8	749·1	609·5	538·8	346·8	318·9
x/*y*	1·158	1·295	1·315	0·994	1·072	1·096	1·195	1·198
M	—	—	189 [a]	—	—	—	—	177 [b]

[a] Calc. for $C_7H_{10}O_2N_2$: *M*, 154. [b] $C_9H_{13}O_3N_3$ requires *M*, 211.

Infrared Spectra (KBr *Discs*).—cyclo(*Glycylglycyl-*DL-*prolyl*). Max. at 3484, 3413, 3300, 3257, 2915 w, 1675 and 1661 vs, 1621 vs, 1546, 1520, 1458 and 1447, 1410, 1357 w, 1335, 1307 w, 1282, 1267, 1255, 1239, 1195, 1167 w, 1115 w, 1041 w, 1018 w, 963 w cm.$^{-1}$.

*Glycyl-*DL-*prolylglycine*. Max. at 3509 infl., 3390 infl., 3268, 3155 infl., 3067, 2941, 2857, 2755, 2632, 2545 infl., 2033 w, 1650 vs, 1603, 1575, 1541, 1477, 1460 infl., 1422, 1397 and 1383, 1361, 1319, 1282, 1269, 1238, 1205, 1183 w, 1170 w, 1156, 1139 w, 1068, 1046 w, 1024, 988 w, 955 w cm.$^{-1}$.

[14] Morton, Campbell, and Ma, *Analyst*, 1953, **78**, 722.

Glycyl-L-proline anhydride. Max. at 3190 and 3155, 3106, 2941, 2890, 1678 and 1650 vs, 1499, 1455, 1403, 1381, 1333, 1290, 1266, 1239, 1224, 1192, 1174, 1156, 1109, 1058 w, 1001, 966 w cm.$^{-1}$.

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