390. *Syntheses of Peptidyl-nucleotidates including* L-*Arginyl*-L-*alanyl*-L-*arginyl*-L-*alanyl Uridine*-5′ *Phosphate.*

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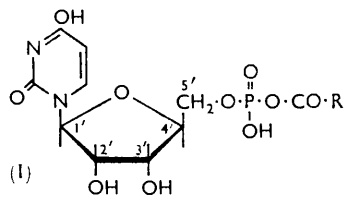
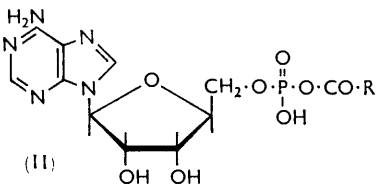
Condensation of various peptides with representative nucleoside-5′ phosphates by means of dicyclohexylcarbodi-imide yields mixed anhydrides of the peptides and nucleotides. Application of this reaction to uridine-5′ phosphate and L-arginyl-L-alanyl-L-arginyl-L-alanine (prepared by condensation of N^{α}-benzyloxycarbonyl-N^{ϵ}-nitro-L-arginyl-L-alanine with N^{ϵ}-nitro-L-arginyl-L-alanine benzyl ester followed by removal of the protecting groups by hydrogenation) gave L-arginyl-L-alanyl-L-arginyl-L-alanyl uridine-5′ phosphate, identical with a product isolated from brewer's yeast.

THE existence in brewer's yeast of nucleotide–peptide compounds containing active carboxyl groups (as judged by the formation of peptide hydroxamates) has recently been demonstrated.[1,2,3] One such compound was isolated [3] and shown by degradative studies to be arginyl-alanyl-arginyl-alanyl uridine-5′ phosphate (as I), and in view of the possible importance of materials of this nature as intermediates in protein synthesis the preparation of representative members of the series was undertaken.

Reactions were first carried out by methods already available for the synthesis of the related adenine compounds (II; R = amino-acid residue). For instance, application of Berg's method,[4] as modified by Kingdon, Webster, and Davie,[5] to condensation of adenosine-5′ phosphate with L-leucylglycine by means of dicyclohexylcarbodi-imide



(I)



(II)

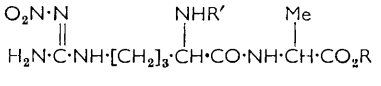
yielded a nucleotide–peptide anhydride (II; R = CH_2·NH·CO·CHBu^i·NH_2) in small yield. The same product was obtained in rather better, though still small, yield by condensing adenosine-5′ phosphate with the protected dipeptide, *N*-(benzylthio)carbonyl-leucylglycine, in the same way and then removing the protecting group with ice-cold perbenzoic acid.[6] In the same manner the corresponding uridine derivative (I) was obtained and the reaction was extended to the tripeptide leucylglycylglycine to yield both compounds (I) and (II). Further, the dipeptide, L-arginyl-L-alanine,[7] closely related to the tetrapeptide obtained by degradation of the natural peptide–nucleotide anhydride of yeast, was readily condensed with uridine-5′ phosphate.

All the peptide–nucleotide anhydrides (I and II) prepared gave a characteristic red colour with ninhydrin reagent on paper and a strong red-brown colour due to the formation of a ferric hydroxamate on treatment with hydroxylamine followed by ferric chloride under controlled conditions (cf. ref. 8). The products of degradation of the leucylglycine derivatives (I and II) and the arginylalanine derivative (I) by hydroxylamine were (*a*) the peptide portion bearing the hydroxamate group and (*b*) the respective nucleotides.

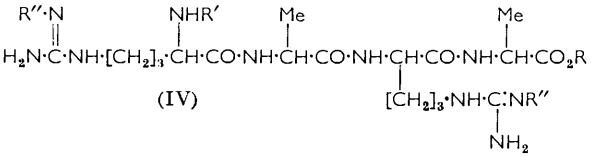
[1] Harris, Davies, and Parsons, *Nature*, 1958, **182**, 1565.
[2] Harris and Davies, *Nature*, 1959, **184**, 788.
[3] Davies and Harris, *Proc. Roy. Soc.*, 1960, *B*, **151**, 537.
[4] Berg, *Fed. Proc.*, 1957, **16**, 151; *J. Biol. Chem.*, 1958, **233**, 608.
[5] Kingdon, Webster, and Davie, *Proc. Nat. Acad. Sci. U.S.A.*, 1958, **44**, 757.
[6] McCorquodale and Mueller, *Arch. Biochem. Biophys.*, 1958, **77**, 13.
[7] Hofmann, Peckham, and Rheiner, *J. Amer. Chem. Soc.*, 1956, **78**, 238.
[8] Koningsberger, van der Grinten, and Overbeek, *Biochim. Biophys. Acta*, 1957, **26**, 483.

Analysis of the anhydrides by complete hydrolysis showed them to contain equimolar proportions of nucleotide and amino-acids. As, moreover, they bore a net positive charge at pH 4·0, migrating towards the cathode like the simple amino-acyl adenosine phosphates on electrophoresis at this pH, had ultraviolet absorption of the same type as the corresponding nucleotides, and reacted with periodate through the *cis*-2′- and 3′-hydroxyl groups,[9] it follows that they have the structures shown. The 3′-phosphates of adenosine and uridine also condensed with peptides to give analogous phosphoric amino-acyl anhydrides which, however, failed to react with periodate.

With this experience the synthesis of the tetrapeptide compound mentioned above was undertaken. The parent tetrapeptide had not previously been prepared.[10] The precursor, arginylalanine, had been prepared[7] from N^{α}-benzyloxycarbonyl-N^{ϵ}-nitro-L-arginyl-L-alanine methyl ester (III; R = Me, R′ = CO·O·CH_2Ph), but in the present work the more readily accessible benzyl ester (III; R = CH_2Ph, R′ = CO·O·CH_2Ph) was preferred. Condensing L-alanine benzyl ester with N^{α}-benzyloxycarbonyl-N^{ϵ}-nitro-



(III)



(IV)

L-arginine[11] by means of dicyclohexylcarbodi-imide[12] gave the new ester (III; R = CH_2Ph, R′ = CO·O·CH_2Ph) in better yield than was given by the mixed anhydride method.[7] After hydrolysis of the ester group by alkali the benzyloxycarbonyl group was smoothly and selectively removed by treatment with dry hydrogen chloride in carbon tetrachloride,[11] to give N^{ϵ}-nitro-L-arginyl-L-alanine benzyl ester (III; R = R′ = H). Combination of the acid (III; R = H, R′ = CO·O·CH_2Ph) with the ester (III; R = CH_2Ph, R′ = H) by means of dicyclohexylcarbodi-imide afforded crystalline N^{α}-benzyloxycarbonyl-N^{ϵ}-nitro-L-arginyl-L-alanyl-N^{ϵ}-nitro-L-arginyl-L-alanine benzyl ester (IV; R = H, R′ = CO·O·CH_2Ph, R″ = NO_2) and thence by hydrogenation the required tetrapeptide (IV; R = R′ = R″ = H). Direct condensation of this tetrapeptide with uridine-5′ phosphate as in the reactions above (cf. ref. 4) gave a complex mixture from which a compound was isolated having properties identical with those of the naturally occurring material from brewer's yeast.[3]

Experimental

Adenine-5′ L-Leucylglycyl Phosphate.—(*a*) Adenosine-5′ phosphate (190 mg.) and L-leucylglycine (1·04 g.) were dissolved in a mixture of N-hydrochloric acid (5·5 ml.), pyridine (44 ml.), and water (7·6 ml.) at 0°, and dicyclohexylcarbodi-imide (2·0 g.) was added with stirring. The mixture was kept for 3 hr. at 0°, then ice-cold acetone (500 ml.) was added. The precipitate was immediately filtered off, dried for 1 hr. in a vacuum, and extracted with 0·1M-acetate buffer at pH 5·0 (10 ml.). The extract was evaporated in a vacuum at room temperature. The residue was dissolved in a small amount of water and placed on two Whatman 3MM papers, each 10 cm. wide, for electrophoresis in acetate buffer at pH 4·0 with a voltage gradient of 10—15 v/cm. overnight. The material which combined the properties of reacting with hydroxylamine to form a hydroxamate,[8] giving a red colour with ninhydrin reagent, and migrating towards the cathode, as located by guide strips, was eluted from the papers by means of cold water, and the solution was dried lyophilically. The resulting solid was dissolved in a little water and chromatographed on Whatman 3MM paper in butanol–acetic acid–water (4 : 1 : 1). The required material was located by its reactions with hydroxylamine and ninhydrin in a band

[9] Dixon and Lipkin, *Analyt. Chem.*, 1954, **26**, 1092.
[10] Goodman and Kenner, *Adv. Protein Chem.*, 1957, **12**, 466.
[11] Katchalsky and Paecht, *J. Amer. Chem. Soc.*, 1954, **76**, 6042.
[12] Sheehan, Goodman, and Hess, *J. Amer. Chem. Soc.*, 1956, **78**, 1367.

having R_F 0·27 in the above solvent and R_F 0·20 in ethyl acetate–propan-1-ol–water (2 : 7 : 1) (containing 5 ml. of acetic acid per l. to prevent decomposition of the product). It was eluted by means of cold water, and the solution was freeze-dried.

(*b*) Adenosine-5′ phosphate (30 mg.) and *N*-(benzylthio)carbonyl-DL-leucylglycine (135 mg., see below) were dissolved in pyridine (4·2 ml.) and water (0·45 ml.) at 0°, whereafter dicyclohexylcarbodi-imide (1·8 g.) was added. The mixture was cooled to −10° and kept for 1 hr. Ether (30 ml.) was added and the precipitate collected and dried in a vacuum. It was then extracted with 0·01N-hydrochloric acid (1 × 10 ml.; 2 × 5 ml.), and the solution was treated at 0° with perbenzoic acid (100 mg.) in water (12 ml.) and kept at 0° for 20 min. The mixture was extracted with cold chloroform, and the aqueous phase freeze-dried. Further working up by electrophoresis and chromatography was effected as under (*a*).

Analysis of the product was carried out as described in detail [3] for the nucleotide–peptide compound from yeast. The molar ratios found were adenosine-5′ phosphate : leucine : glycine = 1·0 : 1·3 : 1·0.

DL-*Leucylglycyl Uridine-5′ Phosphate*.—Uridine-5′ phosphate (30 mg.) and *N*-(benzylthio)-carbonyl-DL-leucylglycine (135 mg.) in pyridine (4·2 ml.) and water (0·28 ml.) were condensed as above by means of dicyclohexylcarbodi-imide (1·8 g.), and the product, presumably consisting of a mixture of diastereoisomers, was worked up as for the corresponding adenosine compound. Analysis of the product gave the molar ratios uridine-5′ phosphate : leucine : glycine = 0·92 : 1·0 : 0·80.

Similar condensations with DL-leucylglycylglycine and its *N*-(benzylthio)carbonyl derivative (see below) together with either adenosine-5′ or uridine-5′ phosphate gave analogous products; uridine-5′ phosphate reacted with glycine or leucine to give the respective amino-acid derivatives. Degradation with hydroxylamine and characterisation of the products was effected as described earlier.[3]

N-(*Benzylthio*)*carbonylglycine*.—Glycine (0·6 g.) and sodium hydrogen carbonate (1·68 g.) in water (12 ml.) at 0° were treated dropwise with stirring with *S*-benzylthioformyl chloride [13] (1·1 ml.) in dioxan (8 ml.). After 1 hr. the mixture was acidified with 5N-hydrochloric acid, concentrated, and kept at 0°. The product separated as plates, m. p. 135—136° (Found: N, 6·0; S, 14·3. Calc. for $C_{10}H_{11}NO_3S$: N, 6·2; S, 14·2%). McCorquodale and Mueller [6] give m. p. 153·5° for needles, presumably polymorphic with the product described here.

N-(*Benzylthio*)*carbonyl*-DL-*leucylglycine*.—DL-Leucylglycine (5·0 g.) and sodium hydrogen carbonate (6·1 g.) in water (70 ml.) were treated, as above, with *S*-benzylthioformyl chloride (5·6 g.). The upper oily layer was separated and the aqueous solution extracted with ether, after which the clear aqueous layer was acidified to pH 2 and kept at 0° overnight after seeding. The *product* (5·0 g.) (m. p. 175—176°), together with a further crop (0·25 g.) from the mother-liquor, recrystallised from ethanol as needles, m. p. 177° (Found: C, 56·4; H, 6·8; N, 8·4; S, 9·1. $C_{16}H_{22}N_2O_4S$ requires C, 56·8; H, 6·5; N, 8·3; S, 9·5%).

N-(*Benzylthio*)*carbonyl*-L-*leucylglycine*.—This *compound* was obtained in the same manner as the DL-isomer and recrystallised from aqueous ethanol as needles, m. p. 109—111° (Found: N, 7·8. $C_{16}H_{22}N_2O_4S$ requires N, 8·3%).

N-(*Benzylthio*)*carbonyl*-DL-*leucylglycylglycine*.—Prepared by the above procedure, this *peptide* recrystallised from aqueous ethanol as needles, m. p. 148—150° (Found: N, 10·1; S, 7·8. $C_{18}H_{25}N_3O_5S$ requires N, 10·6; S, 8·1%).

N^{ϵ}-*Nitro*-L-*arginine*.—This was prepared by the method of Hofmann, Peckham, and Rheiner [7] and recrystallised (yield 60%) from hot water. It had m. p. 253—254°, $[\alpha]_D^{20}$ +23° in 2N-HCl (lit.,[7] m. p. 262°, $[\alpha]_D^{23}$ +24·3° in 2N-HCl).

N^{α}-*Benzyloxycarbonyl*-N^{ϵ}-*nitro*-L-*arginine*.—N^{ϵ}-Nitro-L-arginine (4·5 g.) was treated with benzyloxyformyl chloride [14] (9 g.). The product, recrystallised from aqueous ethanol (3·8 g., 52%), had m. p. 127—128°, $[\alpha]_D^{27}$ −4° in MeOH (lit., m. p. 134—136°, $[\alpha]_D^{27}$ −3·5° in MeOH,[7] m. p. 126° [14]).

L-*Alanine Benzyl Ester*.—The hydrochloride was obtained [15] as needles (78%) from methanol–ether. It had m. p. 137—138°, $[\alpha]_D^{22}$ −13° in MeOH (Erlanger and Hall [15] give m. p. 140°, $[\alpha]_D$ −14·8°). The free base was prepared by dissolving the hydrochloride in water, adjusting the pH of the solution to 9 with sodium carbonate solution, extracting the mixture with ether,

[13] Kollonitsch, Gabor, and Hajos, *Chem. Ber.*, 1956, **89**, 2293.
[14] Bergmann, Zervas, and Rinke, *Z. physiol. Chem.*, 1934, **224**, 40.
[15] Erlanger and Hall, *J. Amer. Chem. Soc*, 1954, **76**, 5781.

evaporating the ether layer to dryness, and dissolving the residual syrup in tetrahydropyran (see below).

N$^{\alpha}$-*Benzyloxycarbonyl*-N$^{\epsilon}$-*nitro*-L-*arginyl*-L-*alanine Benzyl Ester* (III; R = CH_2Ph, R′ = $CO{\cdot}O{\cdot}CH_2Ph$).—The preceding ester (1·70 g.) in tetrahydropyran (20 ml.) was mixed with N^{α}-benzyloxycarbonyl-N^{ϵ}-nitro-L-arginine (1·29 g.) in the same solvent (10 ml.). Dicyclohexylcarbodi-imide (1·0 g.) was added and the mixture shaken at room temperature for 5 hr. and kept overnight at 0°. Acetic acid (1 ml.) was added, the mixture shaken and left aside for 0·5 hr., and the precipitate of dicyclohexylurea filtered off and washed with ethyl acetate. The solvent was removed from the filtrate and the resulting residue redissolved in ethyl acetate (100 ml.), washed successively with N-hydrochloric acid, water, N-sodium hydrogen carbonate, and water, and dried (Na_2SO_4). The *peptide* (2·0 g., 81%) crystallised from aqueous ethanol as plates, m. p. 163°, $[\alpha]_D$ −11° in MeOH (Found: C, 55·7; H, 6·0; N, 16·1. $C_{24}H_{30}N_6O_7$ requires C, 56·0; H, 5·8; N, 16·3%).

N$^{\alpha}$-*Benzyloxycarbonyl*-N$^{\epsilon}$-*nitro*-L-*arginyl*-L-*alanine* (III; R = H, R′ = $CO{\cdot}O{\cdot}CH_2{\cdot}Ph$).—The preceding dipeptide ester (0·8 g.) was shaken with 0·5N-sodium hydroxide (10 ml.) for 1 hr. at room temperature, and the solution was then neutralised with hydrochloric acid and extracted with ethyl acetate (3 × 50 ml.). The extracts were washed with water (2 × 25 ml.), dried, and evaporated to dryness. The residue crystallised from aqueous ethanol (0·59 g., 88%) and had m. p. 206—207°, $[\alpha]_D^{20}$ −4° in pyridine (Hofmann *et al.*[7] give m. p. 207—208°, $[\alpha]_D^{20}$ −5·9°) (Found: N, 22·7. Calc. for $C_{11}H_{23}N_5O_5$: N, 22·9%).

L-*Arginyl*-L-*alanine* (III; R = R′ = H). L-Alanine benzyl ester (0·2 g.) in methanol containing 10% of acetic acid was hydrogenated over 10% palladium–charcoal as described by Hofmann *et al.*[7] After removal of the catalyst and solvents the dipeptide was isolated as the acetate and was recrystallised from aqueous ethanol. It had m. p. 170—171°, $[\alpha]_D^{20}$ +7·8°. Hofmann *et al.*[7] found m. p. 173—174°, $[\alpha]_D^{23}$ +9·7° in H_2O.

L-*Arginyl*-L-*alanyl Uridine*-5′ *Phosphate*.—L-Arginyl-L-alanine (60 mg.) and uridine-5′ phosphate (52 mg.) were dissolved in water (1·2 ml.), N-hydrochloric acid (0·8 ml.), and pyridine (8 ml.) and cooled to 0°. Dicyclohexylcarbodi-imide (300 mg.) in pyridine (4 ml.) was added and the mixture was treated as described above for L-leucylglycyl adenosine-5′ phosphate. After separation from unchanged uridine-5′ phosphate by electrophoresis on paper, the product was isolate by freeze-drying, as a glass (40 mg.). It migrated to the cathode during electrophoresis in acetate buffer at pH 4 and with hydroxylamine gave a hydroxamate.[8] The following molar ratios were found: uracil : ribose : phosphate : arginine : alanine = 1·18 : 1·02 : 1·0 : 0·85 : 0·92. With sodium metaperiodate under our usual conditions[9] immediate uptake of the reagent was observed.

N$^{\epsilon}$-*Nitro*-L-*arginyl*-L-*alanine Benzyl Ester* (III; R = CH_2Ph, R′ = H).—N^{α}-Benzyloxycarbonyl-N^{ϵ}-nitro-L-arginyl-L-alanine benzyl ester (0·5 g.) was dissolved in carbon tetrachloride (20 ml.), and dry hydrogen chloride was passed through the solution for 0·5 hr. The solution was then poured into dry ether (100 ml.) to precipitate the *product* as hydrochloride. This separated from methanol–ether as needles (0·25 g., 61%), m. p. 171—173°, $[\alpha]_D$ −10° in MeOH (Found: C, 46·6; H, 5·5; N, 20·1. $C_{16}H_{25}ClN_6O_5$ requires C, 46·2; H, 5·8; N, 20·2%).

N$^{\alpha}$-*Benzyloxycarbonyl*-N$^{\epsilon}$-*nitro*-L-*arginyl*-L-*alanyl*-N$^{\epsilon}$-*nitro*-L-*arginyl*-L-*alanine Benzyl Ester* (IV; R = CH_2Ph, R′ = $CO{\cdot}O{\cdot}CH_2Ph$, R″ = NO_2).—N^{α}-Benzyloxycarbonyl-N^{ϵ}-nitro-L-arginyl-L-alanine (0·21 g.) and the preceding ester (0·19 g.) in tetrahydropyran (10 ml.) were shaken for 5 hr. with dicyclohexylcarbodi-imide (0·2 g.) and treated as above. The *tetrapeptide ester* separated from aqueous ethanol as plates (0·31 g., 79%), m. p. 212—213°, $[\alpha]_D^{19}$ −5·6° in MeOH (Found: C, 50·7; H, 5·8; N, 20·9. $C_{33}H_{46}N_{12}O_{11}$ requires C, 50·4; H, 5·9; N, 21·4%).

L-*Arginyl*-L-*alanyl*-L-*arginyl*-L-*alanine* (IV; R = R′ = R″ = H).—The last-mentioned product (0·2 g.) was hydrogenated as described above for L-arginyl-L-alanine. The solvent was removed by evaporation to leave the free *tetrapeptide* as an amorphous powder (0·11 g., 81%), $[\alpha]_D$ +7·8° in H_2O (Found: C, 45·1; H, 7·5; N, 24·1. $C_{18}H_{36}N_{10}O_5{,}2CH_3{\cdot}CO_2H$ requires C, 44·6; H, 7·5; N, 23·7%).

L-*Arginyl*-L-*alanyl*-L-*arginyl*-L-*alanyl Uridine*-5′ *Phosphate*.—The above free tetrapeptide (56 mg.) in N-hydrochloric acid (0·5 ml.) was mixed with uridine-5′ phosphate (26 mg.) in water (0·5 ml.) and pyridine (5 ml.) and cooled to 0°. Dicyclohexylcarbodi-imide (0·2 g.) in pyridine (1 ml.) was added. The mixture was treated as described for L-leucylglycyl adenosine-5′ phosphate and then subjected to electrophoresis in acetate buffer at pH 4 for 4 hr. at 10 mA (see above). A zone on the electrophoregram containing materia which migrated *ca.* 2 cm.

towards the cathode and with hydroxylamine formed a product yielding a ferric salt was separated and eluted from the papers with 50% aqueous ethanol. After removal of the solvent under reduced pressure at 10°, a glass (10 mg.), $[\alpha]_D$ +11·2° in H_2O, was obtained which analysis [3] showed to contain uracil, ribose, phosphate, arginine, and alanine in the ratio 1·0 : 0·9 : 1·1 : 1·9 : 1·8. The material behaved as a homogeneous substance when subjected to electrophoresis as above or to chromatography in acidic solvents [butanol–acetic acid–water (4 : 1 : 1); acetone–30% acetic acid (1 : 1); [16] or ethyl acetate–propan-1-ol–water (2 : 7 : 1)], and had the same mobilities as the natural material. Treatment with aminopeptidase gave the products [3] obtained also from the natural compound.

The authors thank Dr. A. H. Cook, F.R.S., for his advice and encouragement throughout the investigation.

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[*Received, April 4th,* 1960.]

[16] Davies and Harris, *Biochim. Biophys. Acta*, 1961, **45**, 39.