The Synthesis of Some Dipeptides Related to Gramicidin S

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This paper describes the synthesis of five dipeptides required for study of the structure of gramicidin S (Consden, Gordon, Martin & Synge, 1947). For all the syntheses carbobenzoxy derivatives were employed according to Bergmann's general procedure; coupling was effected in each case through the acid chloride. Fair yields were obtained at all stages, and no special difficulties were encountered. This appears to be the first time that peptides of valine or of ornithine have been synthesized by the carbobenzoxy procedure. For synthesizing α-(L-valyl)-L-ornithine it was necessary to block the δ-amino group of ornithine with the carbobenzoxy radical. This was done by both the procedures that have been used for homologous preparations with lysine, i.e. (1) through the *NN'*-dicarbobenzoxy derivative, its acid chloride and the cyclic α-*N*-carboxy anhydride (Bergmann, Zervas & Ross, 1935); (2) through the copper complex of the free amino-acid (Neuberger & Sanger, 1943; cf. Kurtz, 1938). Sanger (1946) has already employed the latter procedure for the preparation of δ-*N*-2:4-dinitrophenylornithine. Both procedures gave similar yields, but the copper method involved somewhat simpler manipulations. The fact that the monocarbobenzoxyornithine obtained reacted with ninhydrin to yield one molecule of CO_2 provided further evidence that it was the δ-carbobenzoxy derivative. Van Slyke, Dillon, MacFadyen & Hamilton (1941) have demonstrated that ε-carbobenzoxylysine yields one molecule of CO_2 with ninhydrin.

The advantages of using dilute anhydrous HCl in cold methanol for esterifying proteins and other compounds somewhat readily split by acid, such as *N*-acylamino-acids, have been demonstrated by Fraenkel-Conrat & Olcott (1945). The present work has shown that the method is applicable to carbobenzoxy derivatives of amino-acids (δ-carbobenzoxyornithine, carbobenzoxyvaline) and to free amino-acids (valine, leucine).

The purity of the resulting dipeptides has been checked in every case by paper-strip chromatograms with a suitable solvent system (cf. Consden, Gordon & Martin, 1944). In some preliminary experiments the products, when tested in this way, showed, in addition to the desired compound, a complex array of contaminants that coloured with ninhydrin. Such results occurred in the syntheses of valylornithine and leucylphenylalanine, if the coupling was effected with acid chloride that had been purified in the usual way by washing with ligroin; in these cases the treatment appears inadequate to remove phosphorus compounds that may have deleterious effects. It was found that washing of the acid chlorides with water, as in the preparation of carbobenzoxyleucyl chloride by Bergmann, Zervas & Fruton (1936), gave much purer products. Good results were also obtained in this way in the synthesis of prolylvaline. It appears, as pointed out to me by Dr C. R. Harington, that these acid chlorides are much less sensitive to moisture than would appear from the literature. It was also found that the coupling reactions, in the syntheses of valylornithine and leucylphenylalanine, proceeded much more rapidly, giving better yields, in organic solvents shaken with saturated aqueous $KHCO_3$, than in dry mixtures of organic solvents. This procedure also effects a valuable economy in the amount of ester that has to be used.

A point worth re-emphasizing is the importance of the general practice of employing amino-acid esters rather than free amino-acids in the coupling. In the present work, in view of the reported instability of proline ester to moisture (Abderhalden & Sickel, 1926), and following the procedure of Bergmann, Zervas, Schleich & Leinert (1932) for making peptides of proline, free proline was employed for the synthesis of phenylalanylproline. The separation of the desired carbobenzoxydipeptide from carbobenzoxyamido-acid resulting from decomposition of the acid chloride with water is then difficult. Furthermore, the free carboxyl groups of free amino-acid and/or carbobenzoxydipeptide may react with unchanged acid chloride to form mixed anhydrides, which in turn can react with more amino-acid, and so on, to form a complicated mixture of peptide derivatives. Both these difficulties are avoided by adhering to the use of the esters for coupling, and, in view of the impurity associated with the product of the phenylalanylproline synthesis described here, it would be worth searching for a derivative of proline for such syntheses in which the carboxyl group is blocked by a suitable radical. A similar formation of mixed anhydrides of acetic acid with acetamido-acids may explain the difficulty of quantitatively converting the mixture of amino-acids in a protein hydrolysate to acetamido-acids for chromatographic analyses (Gordon, Martin & Synge, 1943;

Tristram, 1946). Some of the 'artefacts' observed in such analyses, on this view, might be acetylpeptides.

EXPERIMENTAL

General technical notes

Unless otherwise stated, compounds were recrystallized before analysis until the recorded m.p. and/or optical rotation (usually both) were substantially constant. M.p.'s are given uncorrected. Optical rotations were determined in a 0·5 dm. tube. Evaporations *in vacuo* were conducted below 40° unless otherwise stated.

One-dimensional paper partition chromatograms were done according to Consden *et al.* (1944). The R_F values observed for the dipeptides are given by Consden *et al.* (1947).

Nitrogen determinations were made by a micro-Kjeldahl procedure. For the estimation of the acid equivalent weight (AEW), the sample was titrated in aqueous ethanol with 0·01 N-$Ba(OH)_2$, employing phenolphthalein as internal indicator. The titration mixture was subsequently used for N determination. C, H and Cl (Carius) determinations were by Dr G. Weiler, Oxford.

Catalytic reduction of carbobenzoxy compounds was effected in the usual way; 50–250 mg. of the carbobenzoxy compound were dissolved in 1 ml. glacial acetic acid, 10 ml. methanol and 1–2 ml. water, and hydrogenated at atmospheric pressure and room temperature in the presence of 0·1–0·2 g. active Pd (Willstätter & Waldschmidt-Leitz, 1921). In the case of the carbobenzoxy derivatives of the ornithine peptides, 2–3 mol. equiv. of HCl were added in the water, and in the preparation of ornithylleucine 10 ml. acetic acid and no methanol were arbitrarily employed in the solvent mixture. In every case CO_2 evolution was complete after 0·5–1 hr.

Starting materials. The amino-acids employed for the present syntheses were prepared in this laboratory, and tested for the absence of expected contaminant amino-acids with suitable solvents on paper partition chromatograms. Stereochemical purity was checked in each case by determinations of optical rotation of the amino-acid or its formyl derivative.

The racemic synthetic amino-acids were resolved through their formyl derivatives by the methods of Fischer (1906). After characterization, the formyl derivatives were hydrolyzed with hydrochloric acid (the strength of acid and duration of refluxing being as given by Fischer), and the hydrolysate was evaporated to dryness *in vacuo* up to 100° several times with water. The resulting amino-acid hydrochlorides were used directly for the syntheses.

L-*Ornithine monohydrochloride.* L-Arginine monohydrochloride was prepared from a HCl hydrolysate of gelatin (Cox, 1928). From this, L-ornithine monohydrochloride ($[\alpha]_D^{18°}$ +10·8° (water, $c=5$)) was prepared by the arginase method of Hunter (1939).

L-*Proline.* The above gelatin hydrolysate, freed from arginine by flavianic acid, was subjected to the rhodanilate procedure of Bergmann (1935) for the isolation of L-proline. The product, dried to constant weight at 60° over P_2O_5 *in vacuo* had $[\alpha]_D^{19°}$ −81·9° (water, $c=7·0$).

L-*Valine.* *iso*Propanol was converted to *iso*propyl bromide (Norris, 1907) and this, by malonic ester synthesis (Marvel & du Vigneaud, 1943) to α-bromo*iso*valeric acid, which was aminated (Marvel, 1940) to DL-valine. This was resolved (Fischer, 1906) yielding formyl-L-valine, m.p. 151–152°, $[\alpha]_D^{18°}$ −16·6° (water, $c=5·0$).

L-*Leucine.* *iso*Butyl bromide, by malonic ester synthesis (Marvel & du Vigneaud, 1943) yielded α-bromo*iso*caproic acid which was aminated (Marvel, 1941) to DL-leucine. This was resolved (Fischer & Warburg, 1905) yielding formyl-L-leucine, m.p. 137–140°, $[\alpha]_D^{18°}$ −18·6 (ethanol, $c=8·7$).

D-*Phenylalanine.* DL-Phenylalanine was prepared from hippuric acid and benzaldehyde (Gillespie & Snyder, 1943). It was resolved (Fischer & Schoeller, 1907) to yield formyl-D-phenylalanine, m.p. 164–166°, $[\alpha]_D^{18°}$ −74·5° (ethanol, $c=3·3$).

Substitution products of ornithine used as intermediates

(*a*) δ-*Carbobenzoxy*-L-*ornithine methyl ester through the* Cu *derivative of ornithine.* A solution of L-ornithine monohydrochloride (5 g.) in 50 ml. water was saturated with $CuCO_3$ at 100°, cooled and filtered. The resulting deep-blue solution was treated with 2 N-NaOH and benzyl chloroformate, added alternately in 6 equal portions over a period of 20 min. at 0°, whilst the flask was vigorously shaken. In all, 30 ml. 2 N-NaOH and 6 ml. benzyl chloroformate were added. The mixture was then shaken mechanically at room temperature for 30 min. The pale-blue precipitate was then filtered off and thoroughly washed on the filter, first with water, and then with ethanol. The air-dry precipitate was suspended in 150 ml. water, and treated with H_2S overnight in a closed vessel. Water (550 ml.) was then added, the mixture was boiled for a few minutes and rapidly filtered from CuS through a pre-heated filter. The CuS was further washed with 300 ml. of boiling water. On cooling the combined filtrate and washings, δ-*carbobenzoxy*-L-*ornithine* (3 g.) crystallized out in needles. The mother liquors yielded on concentration *in vacuo* only 0·3 g. of inferior material which was not utilized. $[\alpha]_D^{16°}$ +17° (in 1 : 1 v/v aqueous acetone in the presence of 2 mol. HCl, $c=2·9$). Found: C, 58·8; H, 7·3; N, 10·5. $C_{13}H_{18}O_4N_2$ requires C, 58·6; H, 6·8; N, 10·5%. A ninhydrin-CO_2 determination (Van Slyke *et al.* 1941) at a total volume of 1·25 ml., pH 2·5, with 50 mg. ninhydrin gave carboxyl N, 5·2%. (Theory requires 5·3%.)

2 g. δ-Carbobenzoxy-L-ornithine were dissolved in 100 ml. anhydrous methanol, N in respect of HCl, and kept for 24 hr. at room temperature. The mixture was evaporated to dryness *in vacuo*. After two further such treatments with methanolic HCl, the residue crystallized rapidly and completely. It was recrystallized from hot acetone, ether being added to increase the completeness of recovery. The yield of δ-*carbobenzoxy*-L-*ornithine methyl ester hydrochloride* was 2·35 g. For characterization see (*c*) below.

(*b*) α*δ*-*Dicarbobenzoxy*-L-*ornithyl chloride, and* δ-*carbobenzoxy*-L-*ornithine methyl ester therefrom.* 1·25 g. L-ornithine monohydrochloride were dissolved in 8 ml. 2 N-NaOH and to the mixture, cooled to 0°, were added alternately, with shaking, four 4·5 ml. portions of 2 N-NaOH and four 0·9 ml. portions of carbobenzoxy chloride. After some minutes the mixture was acidified with 10 N-HCl (5·5 ml.). The oil which was precipitated was taken up in 25 ml. ether, and the ether extract was counter-extracted with four successive 50 ml. portions of 7% (w/v) $KHCO_3$ solution. The combined

$KHCO_3$ extracts were acidified with 25 ml. 10N-HCl, and extracted with ether containing some ethyl acetate. The organic solvent layer was after drying evaporated to dryness *in vacuo*. The syrupy residue (2·7 g.) was crystallized from ether-light petroleum. The product was filtered off, washed with light petroleum and dried (2·47 g.). *αδ-Dicarbobenzoxy-L-ornithine* was recrystallized for analysis from ether-light petroleum, and had m.p. 112–114°, $[\alpha]_D^{20°} - 4°$ (ethanol, $c=3$). Found: C, 62·6; H, 6·1; N, 6·8; AEW, 376. $C_{21}H_{24}O_6N_2$ requires C, 63·0; H, 6·0; N, 7·0%; AEW, 400.

0·64 g. αδ-Dicarbobenzoxy-L-ornithine and 0·35 g. PCl_5 were pulverized separately and then shaken together in dry ether (12 ml.) in a glass-stoppered vessel at 5–10°. Before all of the reactants had dissolved, a fluffy crystalline precipitate of *αδ-dicarbobenzoxy-L-ornithyl chloride* had begun to form. When no further obvious reaction was occurring, the mixture was cooled to 0° for 1 hr., with intermittent shaking. Dry ligroin (8 ml.) was then added, and the mixture was kept at 0° for a further hour. The crystals were then filtered off and dried. Yield, 0·63 g. of material having m.p. (sealed tube) 54–56°. The product was not analyzed, and was used immediately in the synthesis of ornithyl-leucine.

For the preparation of δ-carbobenzoxyornithine derivatives, as in the preparation of ε-carbobenzoxylysine derivatives (Bergmann *et al.* 1935), it was not necessary to isolate the acid chloride. A preparation was made as above from 0·84 g. αδ-dicarbobenzoxy-L-ornithine. Instead, however, of adding ligroin to the reaction mixture, some dry ethyl acetate was added, whereupon the crystals dissolved. The mixture was evaporated to dryness at room temperature, yielding a crystalline residue. On heating *in vacuo* to 60° the crystals melted, and the resulting syrup gassed freely. It was dissolved in dry ethyl acetate and evaporated to dryness *in vacuo* twice (odour of benzyl chloride) and further heated for 1 hr. at 40–50°. The syrupy residue was then dissolved in ethyl acetate and brought to crystallization by addition of ether and light petroleum. After one recrystallization, 0·45 g. of product resulted. *δ-Carbobenzoxy-L-ornithine-α-N-carboxy anhydride* had m.p. 86–88°, $[\alpha]_D^{19°} - 18°$ (ethyl acetate, $c=4$). Found: C, 57·2; H, 6·0; N, 9·3. $C_{14}H_{16}O_5N_2$ requires C, 57·5; H, 5·5; N, 9·6%. The compound appeared more stable than the corresponding lysine derivative (Bergmann *et al.* 1935), since after storage in a desiccator for 6 months the appearance and m.p. were unchanged.

0·4 g. of the anhydride was dissolved in 15 ml. anhydrous methanol, N in respect of HCl. Immediate evolution of gas was noticed. The mixture was warmed to 50°, and then kept overnight at room temperature. On evaporation *in vacuo* the product crystallized. Recrystallization from acetone-ether yielded 0·34 g. of *δ-carbobenzoxy-L-ornithine methyl ester hydrochloride*. The properties and analytical data are given below.

(*c*) *Comparison of δ-carbobenzoxy-L-ornithine methyl ester hydrochloride prepared by the two methods.* A comparison of specimens obtained by procedures (*a*) and (*b*) above gave the following results. M.p. (*a*) 132–134°; (*b*) 132–134°; mixture, 132–134°. $[\alpha]_D^{19}$ (*a*) +14°, +15°, (*b*) +13° (methanol, $c=4$). Found: C, (*a*) 52·6, (*b*) 52·9; H, (*a*) 6·6, (*b*) 6·5; N, (*a*) 8·8, (*b*) 8·5; Cl, (*a*) 13·1, (*b*) 11·1%. $C_{14}H_{20}O_4N_2$. HCl requires C, 53·1; H, 6·6; N, 8·8; Cl, 11·2%.

α-(L-Valyl)-L-ornithine

The evaporated hydrolysate from 1·9 g. formyl-L-valine (see above) was dissolved in 2N-NaOH (16 ml.) and carbobenzoxylated as above using in all 8·25 ml. 2N NaOH and 2·6 ml. benzyl chloroformate. The mixture was shaken at room temperature for a further 20 min. and acidified with 4 ml. 10N-HCl. The oil which precipitated was extracted into about 20 ml. ether, and the ether extract was counter-extracted with five successive 16 ml. portions of 7% (w/v) $KHCO_3$ solution. The combined $KHCO_3$ extracts were acidified with 10N-HCl, and extracted with an equal volume of ether. The ether extract was evaporated *in vacuo* (50°) to a clear syrup (2·6 g.). After some weeks of storage in a desiccator, the syrup crystallized, and after recrystallization from ether-light petroleum, *carbobenzoxy-L-valine* was found to have m.p. 64–65°, $[\alpha]_D^{20°} + 4°$ (ethanol, $c=2·9$). Found: C, 62·6; H, 7·0; N, 5·5; AEW, 251. $C_{13}H_{17}O_4N$ requires C, 62·2; H, 6·8; N, 5·6%; AEW, 251. The methyl ester was prepared from carbobenzoxy-L-valine by treatment with methanolic HCl. 0·38 g. of syrupy carbobenzoxy-L-valine was dissolved in 15 ml. methanol N in respect of HCl, and kept at room temperature for 24 hr. At the end of this time the mixture was evaporated to dryness *in vacuo*: the whole treatment was repeated and the residue was dissolved in ether and water. The ether layer was separated, washed with $KHCO_3$ solution, then with water, and evaporated to dryness *in vacuo*. The product (0·39 g.) crystallized readily, and was purified by recrystallization from ether-light petroleum. *Carbobenzoxy-L-valine methyl ester* was found to have m.p. 56–57°, $[\alpha]_D^{17°} - 16°$ (ethanol, $c=3·1$). Found: C, 63·6; H, 7·2; N, 5·1. $C_{14}H_{19}O_4N$ requires C, 63·4; H, 7·2; N, 5·3%.

Carbobenzoxy-L-valyl chloride was frequently obtained crystalline on evaporating *in vacuo* the product of reaction of equimolecular quantities of carbobenzoxy-L-valine and PCl_5 in dry ether. It was not, however, characterized, and was best used for the coupling with δ-carbobenzoxy-L-ornithine methyl ester without isolation, as follows. 0·77 g. carbobenzoxy-L-valine was dissolved in 8 ml. ether (dried over Na) and 0·75 g. powdered PCl_5 was added. The temperature was maintained initially at 0°, but as solution of the PCl_5 occurred rather slowly, the mixture was allowed to approach room temperature. When the PCl_5 was completely or nearly dissolved, the mixture was diluted with a further 8 ml. ether and quickly washed twice with equal volumes of ice water. The ether layer was quickly treated with Na_2SO_4 and the solution was then mixed with 30 ml. of a solution of δ-carbobenzoxy-L-ornithine methyl ester in ethyl acetate. (The ester was liberated from 1·25 g. of its hydrochloride with K_2CO_3 in the usual way into ether; after evaporation *in vacuo* the syrupy ester was dissolved in ethyl acetate.) To the reaction mixture were added 7 ml. of a saturated solution of $KHCO_3$. The mixture was kept at room temperature and shaken at intervals over a period of 2 hr. Water was then added, and the organic solvent layer was washed successively with water, N-HCl, water, 7% (w/v) $KHCO_3$, and water, partially dried with Na_2SO_4, and evaporated to dryness *in vacuo*, giving 0·55 g. of a colourless, crystalline residue smelling of benzyl chloride. Recrystallization from chloroform-light petroleum yielded 0·41 g. of material satisfactory for further work. *α-(Carbobenzoxy-L-valyl)-δ-carbobenzoxy-L-ornithine methyl ester* had

m.p. 155–156°, $[\alpha]_D^{18°} \pm 0°$ (B.P. chloroform, $c = 2{\cdot}5$). Found: C, 62·7; H, 6·9; N, 8·1. $C_{27}H_{35}O_7N_3$ requires C, 63·2; H, 6·8; N, 8·2%.

0·20 g. of this compound was saponified by dissolving in 3 ml. dioxan and adding 2 ml. N-NaOH. Crystals precipitated, but these dissolved on gently shaking for 10 min. After a further 30 min. the mixture was diluted with water to about 50 ml. On acidification with 0·4 ml. 10 N-HCl an oil was precipitated, which crystallized rapidly. Yield, 0·19 g. *α-(Carbobenzoxy-L-valyl)-δ-carbobenzoxy-L-ornithine* was recrystallized from 20 parts of methanol by gradual addition of water and had m.p. 193–195°, $[\alpha]_D^{20°} - 11°$ (glacial acetic acid, $c = 0{\cdot}9$). Found: C, 62·5; H, 6·6; N, 8·3; AEW, 504. $C_{26}H_{33}O_7N_3$ requires C, 62·5; H, 6·6; N, 8·4%; AEW, 499.

0·15 g. was hydrogenated as described above. The syrupy residue was kept in a high vacuum in a desiccator over soda lime, dissolved in aqueous ethanol, and neutralized to bromothymol blue with NH_3. The mixture was evaporated *in vacuo* to dryness and treated with ethanol. Crystallization resulted, yielding *α-(L-valyl)-L-ornithine monohydrochloride (hydrated)* (0·08 g.) which was recrystallized from small volumes of water by addition of much ethanol. The optical rotation before and after recrystallization was respectively $[\alpha]_D^{19°} + 18°$ and $+19°$ (water, $c = 2$). The recrystallized material showed two very faint contaminants on a paper-strip chromatogram developed with *s*-collidine, one moving faster, and the other slower than the principal zone. The product was deliquescent, and was stored in a sealed tube. The rotation determination and analyses are referred to material dried in a vacuum desiccator over H_2SO_4-soda lime. Found: C, 43·8; H, 8·1; N, 15·0; amino N (Van Slyke, 0·5 hr. reaction time), 10·8; Cl, 13·9. $C_{10}H_{21}O_3N_3.HCl.\frac{1}{2}H_2O$ requires C, 43·4; H, 8·3; N, 15·2; amino N, 10·1; Cl, 12·8%.

L-*Ornithyl*-L-*leucine*

L-Leucine methyl ester hydrochloride (Abderhalden & Spinner, 1919; cf. Smith & Brown, 1941) was prepared in nearly theoretical yield from L-leucine by the same method as used for the preparation of δ-carbobenzoxy-L-ornithine methyl ester. 0·58 g. of the ester hydrochloride was decomposed with K_2CO_3 in the usual way. To the dry ethereal solution of the free ester (20 ml.) at 0° was added a solution of 0·63 g. αδ-dicarbobenzoxy-L-ornithyl chloride in 5 ml. ethyl acetate. Immediate separation of needles of leucine methyl ester hydrochloride resulted. The mixture was stored overnight at 0°; the crystals were then filtered off, and the filtrate was washed successively with water, N-HCl, 7% (w/v) $KHCO_3$ solution (twice) and water, and evaporated to dryness *in vacuo*. The syrupy residue gave crystals from chloroform-light petroleum (0·66 g.). *αδ-Dicarbobenzoxy-L-ornithyl-L-leucine methyl ester* was recrystallized from ethyl acetate-ether-light petroleum, and had m.p. 83–84°, $[\alpha]_D^{20°} - 18°$ (ethanol, $c = 2{\cdot}8$). Found: C, 63·7; H, 7·1; N, 7·9. $C_{28}H_{37}O_7N_3$ requires C, 63·8; H, 7·0; N, 8·0%. 0·57 g. of this compound was dissolved in 2·1 ml. dioxan and saponified by addition of 2·1 ml. N-NaOH. The mixture was kept for 0·5 hr. after becoming homogeneous and was then diluted with water to 35 ml. On acidifying with 10 N-HCl oil was precipitated, which soon crystallized, yielding 0·535 g. of crystalline material, having m.p. 60–61°, and suitable for the next step. The material was *αδ-dicarbobenzoxy-L-ornithyl-L-leucine* crystallizing, according to the analytical data, with 0·5 mol. dioxan, not removable by drying *in vacuo* at temperatures below the m.p. Recrystallization from methanol-H_2O yielded hydrated material from which the water could likewise not be removed at temperatures below the m.p. This material, dried in a desiccator at room temperature, had m.p. 63–64°, $[\alpha]_D^{17°} - 14°$ (methanol, $c = 4{\cdot}2$). Found: C, 59·4; H, 6·8; N, 7·6; AEW, 527. $C_{27}H_{35}O_7N_3.2H_2O$ requires C, 59·0; H, 7·1; N, 7·7%; AEW, 549.

0·5 g. (with dioxan of crystallization) was hydrogenated as described above. The product, a colourless gum, was dissolved in water (slight turbidity) and the solution was neutralized to bromothymol blue by addition of ammonia. The mixture was again evaporated to dryness *in vacuo*, and on treatment with ethanol yielded 0·125 g. of crystals, which were recrystallized from very concentrated aqueous solution by addition of ethanol. The analytical data suggest that the product was *L-ornithyl-L-leucine monohydrochloride* incorporating half a molecule of water of crystallization and somewhat contaminated with the dihydrochloride. It was not deliquescent, and showed on a paper chromatogram developed with *s*-collidine a very faint trace of contaminant material running just ahead of the main component. $[\alpha]_D^{18°} + 2°$ (water, $c = 2{\cdot}1$). Found: C, 45·2; H, 8·3; N, 14·2; amino N (Van Slyke, 0·5 hr. reaction time), 9·5; Cl, 14·3. $C_{11}H_{23}O_3N_3.HCl.\frac{1}{2}H_2O$ requires C, 45·4; H, 8·6; N, 14·5; amino N, 9·7; Cl, 12·2%.

L-*Leucyl*-D-*phenylalanine*

From carbobenzoxy-L-leucine was prepared the acid chloride (Bergmann *et al.* 1936) by treating 0·25 g. with 0·25 g. PCl_5 in dry ether at 0°. When reaction was complete, the mixture was twice washed rapidly with equal volumes of cold water, and dried over Na_2SO_4. To this solution at 0° was added D-phenylalanine ethyl ester (prepared from 0·25 g. of its hydrochloride) in 4 ml. ethyl acetate. 3 ml. saturated aqueous $KHCO_3$ were added, and the mixture was kept at 0° with intermittent shaking for 1 hr. Water was added, and the organic solvent layer was separated and washed successively with N-HCl and 7% (w/v) $KHCO_3$ solution, dried, and concentrated *in vacuo*. The residue crystallized; recrystallization from ether-light petroleum yielded 0·22 g. *carbobenzoxy-L-leucyl-D-phenylalanine ethyl ester* suitable for further work. The product was recrystallized from chloroform-light petroleum, and had m.p. 103–105°, $[\alpha]_D^{17°} - 19°$ (ethanol, $c = 2{\cdot}7$). Found: C, 67·9; H, 7·1; N, 6·3. $C_{25}H_{32}O_5N_2$ requires C, 68·2; H, 7·3; N, 6·4%.

0·2 g. of this compound was dissolved in 1·5 ml. dioxan, and 1·5 ml. N-NaOH were added. The mixture, which became homogenous on gentle shaking, was kept for 45 min. at room temperature, and was then largely diluted with water and acidified. The oil which was precipitated was isolated by extraction with chloroform. The chloroform extract was evaporated to dryness *in vacuo*, yielding a syrupy residue of *carbobenzoxy-L-leucyl-D-phenylalanine*. This did not readily crystallize and was hydrogenated as described above. The gummy product was dissolved in 95% (v/v) aqueous ethanol; the solution was concentrated *in vacuo* until crystallization began, and ether was then added. In this way was obtained 0·12 g. of *L-leucyl-D-phenylalanine dihydrate*. A paper chromatogram with *n*-butanol showed only one band colouring with ninhydrin.

The product was recrystallized from warm 50% (v/v) aqueous ethanol. The water of crystallization was not readily lost in a vacuum desiccator at room temperature, but was removed on drying at 100° over P_2O_5 *in vacuo*. The hydrated material had $[\alpha]_D^{17°}$ +25° (glacial acetic acid, $c=2{\cdot}4$) and was employed for analysis. Found: C, 57·3; H, 7·9; N, 8·7; loss of weight on drying, 11·7. $C_{15}H_{22}O_3N_2.2H_2O$ requires: C, 57·3; H, 8·3; N, 8·9; loss of weight on drying, 11·5%. An amino-N determination (Van Slyke, 4 min. reaction time) on the material dried at 100° gave amino N, 5·0%; $C_{15}H_{22}O_3N_2$ requires 5·0%. Thus the water lost was solvent of crystallization, and cyclization to the diketopiperazine had not occurred.

D-*Phenylalanyl*-L-*proline*

0·4 g. of carbobenzoxy-D-phenylalanine was prepared from D-phenylalanine and converted to the acid chloride according to Smith & Brown (1941). The product was washed by decantation with dry ligroin and allowed to react with 0·3 g. L-proline dissolved in 4·4 ml. N-NaOH (cf. Bergmann *et al.* 1932). After 20 min. shaking at room temperature the still alkaline mixture was filtered, and the filtrate acidified (thymol blue) with 10N-HCl. An oil was precipitated, which hardened on keeping at 0° for a few hours. This material, presumed to be largely *carbobenzoxy*-D-*phenylalanyl*-L-*proline*, was filtered off and washed with water. Yield, 0·13 g. On attempting recrystallization, only rather greasy crystals were obtained. Accordingly, the whole was hydrogenated as described above. The reaction product was dissolved in ethanol. On addition of ether a flocculent precipitate resulted, which was filtered off, washed with ether, dried, and reprecipitated from the same solvents. The preparation had approximately the same optical rotation ($[\alpha]_D^{18°}$ −93° and −96°, respectively, (acetic acid, $c=2{\cdot}4$)) before and after reprecipitation. The final amorphous product (0·07 g.) proved, however, on paper chromatography with *n*-butanol, to be complex, giving a well marked faster moving band in addition to the stronger band corresponding to D-*phenylalanyl*-L-*proline*. The elementary analyses likewise depart considerably from the expected values; some of this deviation may, however, be due to retention of ethanol by the amorphous material dried at room temperature in a vacuum desiccator. Found: C, 62·4; H, 7·0; N, 8·3. $C_{14}H_{18}O_3N_2$ requires C, 64·1; H, 6·9; N, 10·7%. An attempt was made to prepare the acetyl derivative (cf. Behrens, Doherty & Bergmann, 1940) by acetylation with acetic anhydride and aqueous NaOH, in the usual way. Extraction of the acidified reaction mixture with *n*-butanol in chloroform (17% v/v), did not, however, yield satisfactory crystals.

L-*Prolyl*-L-*valine*

L-*Valine methyl ester hydrochloride* was prepared in nearly theoretical yield from L-valine by the methanolic HCl procedure used above. The reaction product crystallized on evaporation to dryness *in vacuo* and was recrystallized first from acetone-ether and subsequently from methanol-ether. The product, resulting in nearly theoretical yield, had m.p. 146–149°, $[\alpha]_D^{20°}$ +14° (water, $c=3{\cdot}0$) after drying in a vacuum desiccator at room temperature. Lower, and less sharp m.p.'s were observed with air-dry material, indicating the presence of solvent of crystallization. That some HCl, present as such, was incompletely removed even in the desiccator, is indicated by the analytical data for desiccator-dried material. Found: C, 41·8; H, 8·3; N, 8·2; Cl, 24·7. $C_6H_{13}O_2N.HCl$ requires C, 43·0; H, 8·4; N, 8·4; Cl, 21·2%.

Carbobenzoxy-L-proline was prepared according to Abderhalden & Nienburg (1933) from L-proline in 75% of the theoretical yield. 0·5 g. of the syrupy carbobenzoxy-L-proline was dissolved in 5 ml. dry ether and 0·5 g. PCl_5 was added to this solution at 0°. After shaking gently for 15 min. most of the PCl_5 had dissolved. The ethereal solution was decanted from undissolved PCl_5, and washed twice, quickly, with ice water and partially dried. To this solution was added an ethyl acetate solution of L-valine methyl ester, liberated from 0·4 g. of its hydrochloride by aqueous K_2CO_3. Saturated aqueous $KHCO_3$ was added to the mixture which was shaken intermittently during 20 min. After a further 40 min. water was added and the organic solvent layer was washed successively with N-HCl and 7% (w/v) aqueous $KHCO_3$, partially dried, and evaporated *in vacuo*, yielding 0·55 g. of colourless syrup. Attempts to bring this material, *carbobenzoxy*-L-*prolyl*-L-*valine methyl ester*, to crystallization were unsuccessful. 0·45 g. was saponified in 3 ml. dioxan with 3 ml. N-NaOH. The mixture quickly became homogeneous, and after 0·5 hr. at room temperature, was diluted with water. On acidifying (HCl), an oil was precipitated. This crystallized within a few hours. Yield, 0·33 g. of material suitable for subsequent use. *Carbobenzoxy*-L-*prolyl*-L-*valine* was recrystallized from methanol-water, and had m.p. 134–135°, $[\alpha]_D^{18°}$ −58° (methanol, $c=2{\cdot}8$). Found: C, 62·5; H, 6·9; N, 8·0; AEW, 350. $C_{18}H_{24}O_5N_2$ requires C, 62·1; H, 6·9; N, 8·0%; AEW, 348.

0·18 g. was hydrogenated as described above. 0·10 g. of crystalline material resulted. A paper-strip chromatogram developed with *n*-butanol revealed, on colouring with ninhydrin, a single yellow band. The material was recrystallized from water by addition of ethanol. Analysis showed the product to be L-*prolyl*-L-*valine hydrate*. The water of crystallization was lost very slowly in a vacuum desiccator at room temperature. $[\alpha]_D^{20°}$ −61° (water, $c=1{\cdot}7$) (calc. for the hydrate). The air-dry hydrate was used also for the analyses. Found: C, 52·1; H, 8·5; N, 12·6; amino N (Van Slyke, 4 min. reaction time), 0·0. $C_{10}H_{18}O_3N_2.H_2O$ requires C, 51·7; H, 8·6; N, 12·1%; amino N, nil.

SUMMARY

1. Syntheses are described of the dipeptides α-(L-valyl)-L-ornithine, L-ornithyl-L-leucine, L-leucyl-D-phenylalanine, D-phenylalanyl-L-proline and L-prolyl-L-valine. Carbobenzoxy derivatives were employed as intermediates, coupling being effected in each case through an acid chloride.

2. Some factors affecting the purity of the product in such syntheses are discussed.

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REFERENCES

Abderhalden, E. & Nienburg, H. (1933). *Fermentforschung*, **13**, 573.
Abderhalden, E. & Sickel, H. (1926). *Hoppe-Seyl. Z.* **152**, 95.
Abderhalden, E. & Spinner, H. (1919). *Hoppe-Seyl. Z.* **107**, 5.
Behrens, O. K., Doherty, D. G. & Bergmann, M. (1940). *J. biol. Chem.* **136**, 61.
Bergmann, M. (1935). *J. biol. Chem.* **110**, 471.
Bergmann, M., Zervas, L. & Fruton, J. S. (1936). *J. biol. Chem.* **115**, 593.
Bergmann, M., Zervas, L. & Ross, W. F. (1935). *J. biol. Chem.* **111**, 245.
Bergmann, M., Zervas, L., Schleich, H. & Leinert, F. (1932). *Hoppe-Seyl. Z.* **212**, 72.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Consden, R., Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1947). *Biochem. J.* **41**, 596.
Cox, G. J. (1928). *J. biol. Chem.* **78**, 475.
Fischer, E. (1906). *Ber. dtsch. chem. Ges.* **39**, 2320.
Fischer, E. & Schoeller, W. (1907). *Liebigs Ann.* **357**, 1.
Fischer, E. & Warburg, O. (1905). *Ber. dtsch. chem. Ges.* **38**, 3997.
Fraenkel-Conrat, H. & Olcott, H. S. (1945). *J. biol. Chem.* **161**, 259.
Gillespie, H. B. & Snyder, H. R. (1943). *Organic Synth.* Coll. Vol. **2**, 489.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 79.
Hunter, A. (1939). *Biochem. J.* **33**, 27.
Kurtz, A. C. (1938). *J. biol. Chem.* **122**, 477.
Marvel, C. S. (1940). *Organic Synth.* **20**, 106.
Marvel, C. S. (1941). *Organic Synth.* **21**, 74.
Marvel, C. S. & du Vigneaud, V. (1943). *Organic Synth.* Coll. Vol. **2**, 93.
Neuberger, A. & Sanger, F. (1943). *Biochem. J.* **37**, 515.
Norris, J. F. (1907). *Amer. chem. J.* **38**, 627.
Sanger, F. (1946). *Biochem. J.* **40**, 261.
Smith, C. S. & Brown, A. E. (1941). *J. Amer. chem. Soc.* **63**, 2605.
Tristram, G. R. (1946). *Biochem. J.* **40**, 721.
Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.
Willstätter, R. & Waldschmidt-Leitz, E. (1921). *Ber. dtsch. chem. Ges.* **54**, 113.

A Method for Estimating Peptic Activity in Gastric Contents

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An accurate simple method of estimating the activity of proteolytic enzymes is clearly desirable. To achieve this end, not only must a precise method of measuring proteolysis be devised, but a reproducible substrate must also be found. These two requirements were first met in the method of estimating pepsin described by Anson & Mirsky (1932) and Anson (1938). Their method, in which the substrate is carboxyhaemoglobin, has been widely used for estimating pure enzymes on account of its accuracy. In modified forms it has proved valuable in the determination of the activity of digestive juices (Beazell, Schmidt, Ivy & Monoghan, 1938; Bucher, Grossman & Ivy, 1945). However, without large scale apparatus, the preparation of sufficient quantities of the substrate of carboxyhaemoglobin is time-consuming and the method is correspondingly restricted in its applicability.

This paper describes the use of another reproducible but more readily available substrate. The modified reagents and procedure are given in full. A method of standardization is described and the adoption of a new pepsin unit is suggested.

METHOD

The substrate

General remarks. Dehydrated human plasma and serum, rejected as unfit for transfusion purposes, have been used exclusively for this work. The dried plasma or serum is of variable colour and is freely soluble in distilled water. Two samples have been encountered which immediately formed a gel on addition to water. These were discarded.

The substrate solution. Substrate solution is made up to contain 5·6 g. dried citrated plasma or dried serum in 100 ml. of distilled water with sufficient HCl to give an acidity of pH 2·1 as determined with a glass electrode at 18–22°. The substrate solution is stirred and then filtered through a Green's 309¼ Agar filter paper or cotton wool. Considerable variations in the rate of filtration have been encountered but this had no apparent significance in the method.

Other reagents are: 0·350 N-trichloroacetic acid (approximately 6 g./100 ml.), 0·250 N-NaOH, HCl solution, pH 2·1, Folin & Ciocalteu's (1927) phenol reagent, phenol solution (5·0 mg./100 ml.).