drying over $CaCl_2$ and NaOH the solid weighed 11.75 g. It was dissolved in dimethylformamide (20 ml.) and triethylamine (6.7 ml.) was added followed by VI (3.86 g.). Soon a solid started to separate. After 18 hours at room temperature the semi-solid mass was triturated with ethyl acetate (300 ml.), washed on the filter with ethanol (200 ml.) and with ethyl acetate (100 ml.); wt. 8.0 g. (92%), m.p. 227–229° dec.

B.—Compound XIII (7.75 g.) was treated with HBr in acetic acid as described above. The resulting hydrobromide was dissolved in methanol (200 ml.) and treated with Amberlite IRA-400 (in OH cycle). Removal of the solvent left a residue (8.3 g. probably not completely dry), which was dissolved in dimethylformamide (20 ml.) and VI (3.86 g.) was added. Isolation of the product was carried out as described under A; wt. 7.8 g. (90%), m.p. 233–235° dec., $[\alpha]^{20}D$ −50° (*c* 1, dimethylformamide). This product was used in the next step. A sample (0.20 g.) was recrystallized from 28 ml. of 80% hot ethanol; 0.14 g. was recovered, m.p. 241–243° dec.

Anal. Calcd. for $C_{46}H_{66}O_{12}N_{10}S$: C, 57.1; H, 6.88; N, 14.5. Found: C, 56.9; H, 6.96; N, 14.4.

N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (XV). A.—Compound XIV (9.7 g.) dissolved in acetic acid (50 ml.) was treated with HBr in acetic acid (3.3 *N*, 50 ml.). After 1.5 hours at room temperature dry ether (400 ml.) was added. The solid which separated was filtered off and washed with ether. It was dissolved in dimethylformamide (85 ml.) and triethylamine (9 ml.) was added to the filtrate followed by VII (6.0 g.). After 20 hours at room temperature ethyl acetate (450 ml.) was added to the thick mass, the solids were washed on the filter with ethyl acetate (100 ml.), ethanol (400 ml.) and with ethyl acetate (150 ml.) again; wt. 11.3 g. (92.5%), m.p. 230–234° dec.

B.—Compound XIV (1.3 g.) was treated with HBr in acetic acid as described in section A. The HBr was removed from the resulting salt with the anion exchange resin as described with similar compounds. Only 0.92 g. (82%) of the base was recovered, probably on account of its low solubility in methanol. This amount of the free heptapeptide amide was dissolved in dimethylformamide (10 ml.) and VII (0.70 g.) was added. After isolation in the usual manner 1.35 g. of a white powder was obtained, m.p. 245–247° dec., $[\alpha]^{20}D$ −41° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{62}H_{81}O_{13}N_{11}S$: C, 61.0; H, 6.69; N, 12.6. Found: C, 60.8; H, 6.75; N, 12.5.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (XVI).[26]—Compound XV (7.3 g.) was suspended in acetic acid (40 ml.) and HBr in acetic acid (3.3 *N*, 60 ml.) was added. After two hours at room temperature dry ether was added to the orange solution, the precipitated hydrobromide filtered and washed with ether. After short drying over $CaCl_2$ and NaOH the hydrobromide was dissolved in dimethylformamide (60 ml.) and triethylamine (5.6 ml.) was added followed by III (3.35 g.). After a day at room temperature the reaction mixture turned into a thick mass which after a further day was mixed with ethyl acetate (400 ml.). The precipitate was washed on the filter with more ethyl acetate (200 ml.), with ethanol (400 ml.) and again with ethyl acetate (50 ml.); wt. 7.3 g. (92%), m.p. 237–241°.

If the octapeptide with the free amino group was liberated from its hydrobromide, considerable loss was observed, probably due to the low solubility of the base in methanol and incomplete removal from the anion exchange resin. On the other hand, from the base obtained, on reaction with III, a quantitative yield of XVI was obtained, m.p. 245–248° dec., $[\alpha]^{20}D$ −50.5° (*c* 1, dimethylformamide) $[\alpha]^{20}D$ −64.5° (*c* 2.5, acetic acid); lit. m.p. 224–245°,[3] 241°,[4] 243–245°,[8] $[\alpha]^{20}D$ −51.5° (*c* 2.5, acetic acid),[4] $[\alpha]^{22}D$ −43° (*c* 2, dimethylformamide).[8]

Anal. Calcd. for $C_{65}H_{86}N_{12}O_{14}S_2$: C, 59.0; H, 6.55; N, 12.7. Found: C, 58.6; H, 6.52; N, 12.6.

Oxytocin (XVII).—Compound XVI was reduced with sodium in liquid ammonia as described earlier. The total avian depressor activity obtained from 50 mg. of XVI varied from 12,500 to 15,000 units. Calculated on the basis that the activity of pure oxytocin is 500 units/mg. the maximum obtainable activity would be 19,000 units.

Reduction on a larger scale (1.3 g. of XVI) led to less favorable results: a total of 168,000 units was obtained. Isolation of highly purified oxytocin was accomplished as described earlier.[8]

A series of assays[27] on the isolated material gave values of 500, 440, 500, 560, 500 U./mg.

Acknowledgments.—The authors wish to thank Mr. Joseph Albert for the microanalyses, Mr. David N. Reifsnyder for his assistance in the experiments, and Miss Dade Tull and Miss Maureen O'Connell for the biological assays.

(26) Treatment with HBr in acetic acid removed the O-benzyl group from the octapeptide intermediate.

(27) Assayed by direct comparison with the standard solution.

NEW YORK 21, N. Y.

[CONTRIBUTION FROM THE RESEARCH DIVISION OF THE CLEVELAND CLINIC FOUNDATION AND THE FRANK E. BUNTS EDUCATIONAL INSTITUTE]

The Use of p-Nitrobenzyl Esters in Peptide Synthesis[1]

BY HANS SCHWARZ[2] AND KIKUO ARAKAWA[3]

RECEIVED APRIL 23, 1959

The *p*-nitrobenzyl esters of some amino acids and peptides were prepared in good yields and without racemization. They proved to be more stable to cleavage by dry hydrobromic acid than the corresponding unsubstituted benzyl esters. This fact was shown to facilitate the preferential removal of a protecting carbobenzoxy group, and to increase yield and purity of the resulting ester hydrobromide. The *p*-nitrobenzyl group was readily removed by catalytic hydrogenation.

The use of carbobenzoxy[4] groups combined with benzyl esters as protective groups in peptide syntheses has several advantages.[5] Catalytic reduction removes both protective groups in one operation, eliminating alkaline hydrolysis. Carbobenzoxy, on the other hand, is removed preferentially by cleavage with dry hydrobromic acid.[6] Benzyl esters are quite stable to this treatment, but small amounts of ester are always hydrolyzed even if the reaction time is kept to a minimum.[7] The result is a mixture of a benzyl ester hydrobromide and a small amount of the corresponding peptide. Their separation is often quite easy, but may amount to

(1) This work was supported in part by The National Heart Institute, U. S. Public Health Service, Grant No. H-96 (C 7).

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(4) Carbobenzoxy = benzyloxycarbonyl = cbzo.

(5) For references see M. Goodman and G. W. Kenner, *Advances in Protein Chem.*, **12**, 465 (1957).

(6) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

(7) G. W. Anderson, J. Blodinger and A. D. Welcher, THIS JOURNAL, **74**, 5309 (1952).

a major problem as with histidyl-prolyl-phenylalanine benzyl ester described in this paper.

It seemed desirable, therefore, to find an ester which combined the advantages of the benzyl ester with greater stability to cleavage by dry hydrobromic acid. It was mentioned to us that the nitrobenzyl ester might fulfill these requirements.[8] In some preliminary experiments the applicability of *p*-nitrobenzyl esters in peptide syntheses was tested. *p*-Nitrobenzyl esters were prepared by the method of Schwyzer and co-workers.[9] Carbobenzoxyamino acid refluxed with an excess of *p*-nitrobenzyl chloride and triethylamine yielded carbobenzoxy-amino acid *p*-nitrobenzyl ester in over 90% yield. By treatment of this intermediate with hydrobromic acid the desired amino acid *p*-nitrobenzyl ester hydrobromide was obtained.

By the same method *p*-nitrobenzyl esters of dipeptides were obtained in equally good yield. With histidine or histidine-containing peptides, the reaction was complicated by the presence of the imidazole ring. Additional experiments showed that imidazole treated with *p*-nitrobenzyl chloride and triethylamine gave mainly di-*p*-nitrobenzylimidazolium chloride. In the absence of a tertiary base a mixture of imidazole, *p*-nitrobenzylimidazole and the quaternary compound was obtained. The *p*-nitrobenzyl groups could not be removed by catalytic hydrogenation. Instead the di-*p*-aminobenzylimidazolium chloride dihydrochloride was isolated in almost quantitative yield.

The behavior of *p*-nitrobenzyl esters toward hydrobromic acid or catalytic hydrogenation was shown on 3 dipeptides synthesized from amino acid *p*-nitrobenzyl ester and carbobenzoxyamino acid by the mixed anhydride or azide method, respectively. The reactions were followed by paper chromatography. Carbobenzoxy-glycyl-glycine *p*-nitrobenzyl ester showed no trace of glycylglycine after standing for 1 hour in hydrobromic acid 2.5 *N* in glacial acetic acid. A more quantitative result was obtained with carbobenzoxy-glycyl-phenylalanine *p*-nitrobenzyl ester. The amounts of glycylphenylalanine were estimated as: 1 hour treatment, 3%; 1.5 hours, 6%; 2 hours, 15%. Catalytic hydrogenation furnished the expected dipeptides. Carbobenzoxy-L-histidyl-L-leucine *p*-nitrobenzyl ester was treated with hydrobromic acid in acetic acid for 20 minutes. The *p*-nitrobenzyl ester hydrobromide was obtained in 95% yield and was chromatographically pure. On catalytic hydrogenation the ester group was removed quantitatively within 20 minutes. These results seemed to indicate that *p*-nitrobenzyl esters were, indeed, superior to benzyl esters.

In order to test this further a direct comparison was made by synthesizing benzyl ester and *p*-nitrobenzyl ester of the tripeptide L-histidyl-L-propyl-L-phenylalanine, a sequence which is contained as amino acids 6–8 in the angiotensin molecule.

L-Phenylalanine benzyl ester was prepared by two methods, by way of the carbobenzoxy-L-phenylalanine benzyl ester[6] and directly from L-phenylalanine.[10] The yield in either case was about 80%. Carbobenzoxy-L-proline was condensed with L-phenylalanine benzyl ester by the mixed anhydride method. The L-prolyl-L-phenylalanine benzyl ester hydrobromide formed by treatment with dry hydrobromic acid could not be obtained in crystalline form and was condensed, without isolation, with cbzo-L-histidine azide. The tripeptide carbobenzoxy-L-histidyl-L-prolyl-L-phenylalanine benzyl ester was purified by counter-current distribution but again was not crystalline. It did not give a positive reaction with diazotized sulfanilic acid but was visible as a faint yellow spot on paper. Since no isatin-positive (prolyl-) nor Pauly-positive spot was visible, it was considered to be chromatographically pure.[11]

After removal of the carbobenzoxy group a crystalline product was isolated. It showed three spots on paper, a main component L-histidyl-L-prolyl-L-phenylalanine benzyl ester, and two minor components, one the corresponding free tripeptide. Recrystallization did not separate the three compounds and the free ester was insoluble in ethyl acetate and chloroform. Separation was possible on paper with the one-phase system acetonitrile–water. An identical separation was obtained on a cellulose column using the same solvent system. The small capacity of cellulose powder, however, forbade any large scale purification attempts.

L-Phenylalanine *p*-nitrobenzyl ester was condensed with carbobenzoxy-L-proline by the mixed anhydride method. The same dipeptide derivative, carbobenzoxy-L-prolyl-L-phenylalanine *p*-nitrobenzyl ester, was obtained by refluxing carbobenzoxy-L-prolyl-L-phenylalanine with *p*-nitrobenzyl chloride and triethylamine. The identity of the two products showed that no racemization had taken place when esterifying the dipeptide.

L-Prolyl-L-phenylalanine *p*-nitrobenzyl ester hydrobromide was obtained chromatographically pure on removal of the carbobenzoxy group. Reaction with carbobenzoxy-L-histidine azide furnished the non-crystalline tripeptide derivative, carbobenzoxy-L-histidyl-L-prolyl-L-phenylalanine *p*-nitrobenzyl ester. This was again treated with hydrobromic acid in glacial acetic acid, and the desired tripeptide *p*-nitrobenzyl ester was obtained chromatographically pure after one recrystallization. None of the difficulties encountered with the benzyl ester was present.

Several observations and conclusions could be made from the foregoing results. *p*-Nitrobenzyl esters of amino acids or peptides can be obtained in good yield and without racemization. Histidine or histidine containing peptides form an exception since the imidazole >NH is quaternized under the reaction conditions employed. *p*-Nitrobenzyl esters are considerably more stable to cleavage by dry hydrobromic acid than benzyl esters. This inertness facilitates the preferential removal of the carbobenzoxy group and increases yield and purity of the resulting ester hydrobromides. Catalytic hy-

(8) Private communication from R. Schwyzer, Ciba Pharmaceuticals, Basel, Switzerland.

(9) R. Schwyzer, B. Iselin and M. Feurer, *Helv. Chim. Acta*, **38**, 69 (1955).

(10) N. Izumiya, and S. Makisumi, *J. Chem. Soc. Japan*, **78**, 662 (1957).

(11) The negative Pauly reaction is probably due to steric hindrance; after removal of the cbzo group a red color is again obtained.

drogenation removes the *p*-nitrobenzyl group in a very short time.

Experimental[12]

All melting points were taken on a Kofler hot-stage and are corrected. Microanalyses were done by Micro-tech Laboratories, Skokie, Ill. All samples submitted had been dried for 3 hours at 78° over P_2O_5 in high vacuum, except otherwise stated. Two solvent systems were employed for paper chromatography: a, 1-butanol–acetic acid–water 4:1:5 (BAW); b, acetonitrile–water 3:1 (AW) the latter system has never been described before. It showed especially good separation between peptides or amino acids and ester hydrobromides. Other advantages of this one-phase system were short time of equilibration and fast development. Results were usually obtained within 3 hours. The chromatograms were sprayed with ninhydrin (isatin–ninhydrin for proline) and/or diazotized sulfanilic acid (Pauly reagent).

Cbzo-glycine p-Nitrobenzyl Ester.—A solution of 4.18 g. (0.02 mole) of cbzo-glycine, 5.15 g. (0.03 mole) of *p*-nitrobenzyl chloride and 4.2 cc. (0.03 mole) of triethylamine in 35 cc. of ethyl acetate was refluxed overnight. Triethylamine hydrochloride started to crystallize after 30 minutes. The reaction mixture was filtered hot, and 5% of methanol was added to the filtrate. After cooling, the filtrate was washed with cold H_2O (1 x), 1 *N* HCl (3 x), H_2O (1 x), 1 *M* $KHCO_3$ (3 x) and satd. NaCl solution (3 x). It was then dried over Na_2SO_4 and concentrated *in vacuo*. Cbzo-glycine *p*-nitrobenzyl ester crystallized from ethyl acetate–ligroin; yield 6.32 g., (92%), m.p. 107–109.5°.

A sample was recrystallized twice from the same mixture of solvents and dried for analysis, m.p. 107–109.5°.

Anal. Calcd. for $C_{17}H_{16}N_2O_6$: C, 59.30; H, 4.68; N, 8.14. Found: C, 59.54; H, 4.91; N, 8.03.

Cbzo-L-phenylalanine p-nitrobenzyl ester was prepared as the cbzo-glycine *p*-nitrobenzyl ester. It crystallized from ethyl acetate–ligroin; yield 94%, m.p. 113.5–115.5°. A sample was recrystallized twice from methanol and dried for analysis; m.p. 115–116.5°, $\alpha^{22}D$ −8.1° (*c* 2, pyridine).

Anal. Calcd. for $C_{24}H_{22}N_2O_6$: C, 66.35; H, 5.11; N, 6.45. Found: C, 66.55; H, 5.27; N, 6.68.

Cbzo-L-leucine p-nitrobenzyl ester was prepared as described for the glycine ester. It was crystallized from ether–petroleum ether; yield 70%, m.p. 63.5–65°. A sample was recrystallized twice from ethyl acetate–ligroin, and dried for 3 hours at 36° over P_2O_5 in high vacuum; m.p. 64–65°, $\alpha^{22}D$ −3.4° (*c* 4, ethyl acetate).

Anal. Calcd. for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 6.99. Found: C, 63.14; H, 6.16; N, 6.94.

Glycine p-Nitrobenzyl Ester Hydrobromide.—Cbzo-glycine *p*-nitrobenzyl ester (6.22 g.) was dissolved in 30 cc. of dry glacial acetic acid, and 20 cc. of HBr 4.7 *N* in glacial acetic acid was added. Crystals appeared after 5 minutes at room temperature, and after 15 minutes the solid mass was diluted with 100 cc. of absolute ether and filtered; yield, 4.50 g., m.p. 192–196°. After recrystallization from abs. ethanol–ethyl acetate 4.25 g. (81%) of glycine *p*-nitrobenzyl ester hydrobromide was obtained, m.p. 196–202°.

A sample, twice recrystallized from the same solvent mixture, and dried, melted at 196–202°, $R_{f\ AW}$ 0.71.

Anal. Calcd. for $C_9H_{10}N_2O_4$.HBr: C, 37.13; H, 3.81; N, 9.63; Br, 27.46. Found: C, 37.14; H, 3.99; N, 9.60; Br, 27.45.

L-Phenylalanine p-nitrobenzyl ester hydrobromide was prepared as preparation of the glycine ester. It was recrystallized from ethanol–ethyl acetate; yield 90%, m.p. 213.5–216.5°.

A sample recrystallized again from the same solvents and dried had m.p. 213–216° and $\alpha^{22}D$ −1.1° (*c* 4, methanol); $R_{f\ AW}$ 0.81.

Anal. Calcd. for $C_{16}H_{16}N_2O_4$.HBr: C, 50.40; H, 4.50; N, 7.35; Br, 20.96. Found: C, 50.42; H, 4.46; N, 7.47; Br, 21.00.

L-Leucine p-Nitrobenzyl Ester Hydrobromide.—To a solution of 3.17 g. of cbzo-L-leucine *p*-nitrobenzyl ester in 20 cc. of glacial acetic acid 40 cc. of HBr 2.5 *N* in glacial acetic acid was added. After 20 minutes at room temperature 300 cc. of abs. ether and 150 cc. of petroleum ether were added. The oily precipitate crystallized on cooling. It was collected by filtration, washed with abs. ether and recrystallized from ethyl acetate–ligroin; yield, 2.48 g. (92%), m.p. 123–125°/130–134°.

A sample was recrystallized twice from the same mixture of solvents and dried for analysis. It still showed the double melting point: 124–125°/132.5–134°, $\alpha^{22}D$ + 2.6° (*c* 4, ethanol), $R_{f\ AW}$ 0.81.

Anal. Calcd. for $C_{18}H_{18}N_2O_4$.HBr: C, 44.97; H, 5.52; N, 8.07; Br, 23.02. Found: C, 44.97; H, 5.56; N, 8.04; Br, 22.82.

Cbzo-glycyl-glycine p-Nitrobenzyl Ester.—The mixed anhydride was prepared at 0° from 2.09 g. (0.01 mole) of cbzo-glycine, 1.4 cc. of triethylamine and 0.96 cc. of ethyl chloroformate in 20 cc. of dry tetrahydrofuran. After 15 minutes the precipitated triethylamine hydrochloride was removed by filtration, and the mixed anhydride added to a solution of 2.9 g. (0.01 mole) of glycine *p*-nitrobenzyl ester hydrobromide and 2.4 cc. of tri-*n*-butylamine in 20 cc. of tetrahydrofuran-dimethylformamide 1:1. The reaction mixture was stirred for 2 hours at room temperature and then evaporated to dryness. The oily residue was dissolved in ethyl acetate and washed with H_2O, cold 0.25 *N* HCl, cold 1 *M* K_2CO_3, and H_2O. It was dried over Na_2SO_4 and crystallized from ethyl acetate–ligroin; yield, 3.13 g., m.p. 107–109°, 78%. A sample was recrystallized from abs. ethanol and dried for analysis, m.p. 110.5–111.5°.

Anal. Calcd. for $C_{19}H_{19}N_3O_7$: C, 56.85; H, 4.77; N, 10.47. Found: C, 56.95; H, 4.91; N, 10.52.

Cbzo-L-phenylalanyl-glycine p-nitrobenzyl ester was prepared as described for preparation of the glycyl-glycine ester. It was crystallized from abs. ethanol; yield 63%, m.p. 145–146°, $\alpha^{22}D$ −8.7° (*c* 2, acetic acid). These constants were not changed by further recrystallizations.

Anal. Calcd. for $C_{28}H_{25}N_3O_7$: C, 63.54; H, 5.13; N, 8.50. Found: C, 63.66; H, 5.08; N, 8.66.

Cbzo-L-histidyl-L-leucine p-Nitrobenzyl Ester. A.—A solution of 1.76 g. (0.0058 mole) of cbzo-L-histidine hydrazide in 8.7 cc. of 2 *N* HCl was cooled to 0°. The azide was prepared by the addition of 0.4 g. of $NaNO_2$. After 3 minutes 7 cc. of 50% K_2CO_3 solution was added, and the oily azide extracted into three 20-cc. portions of cold ethyl acetate. The combined extracts were dried for a short time over Na_2SO_4 and then filtered into a cold solution of 2.01 g. (0.0058 mole) of L-leucine *p*-nitrobenzyl ester hydrobromide and 1.38 cc. of tri-*n*-butylamine in 15 cc. of ethyl acetate. The reaction mixture was kept for 7 hours at 0° and overnight at room temperature. After washing with water and drying over Na_2SO_4 the solvent was evaporated *in vacuo*. The residue was crystallized from ethyl acetate–ligroin; yield 2.17 g. (70%), m.p. 131–133.5°.

A sample was recrystallized from the same solvents and dried for analysis; m.p. 132–133°, $\alpha^{20}D$ −19.3° (*c* 2, abs. ethanol), $R_{f\ BAW}$ 0.95.

Anal. Calcd. for $C_{27}H_{31}N_5O_7$: C, 60.32; H, 5.81; N, 13.03. Found: C, 60.29; H, 5.77; N, 13.06.

B.—An attempt was made to prepare the same *p*-nitrobenzyl ester starting from cbzo-L-histidyl-L-leucine,[13] *p*-nitrobenzyl chloride and triethylamine as described below for cbzo-L-prolyl-L-phenylalanine p-nitrobenzyl ester (A). No product could be isolated.

Di-*p*-nitrobenzylimidazolium Chloride.—A solution of 0.68 g. (0.001 mole) of imidazole, 3.44 g. (0.002 mole) of *p*-nitrobenzyl chloride and 1.44 cc. (0.001 mole) of triethylamine in 20 cc. of acetonitrile was refluxed overnight. After cooling, 3.03 g. of crystalline material, m.p. 220–230°, was isolated. Several more fractions were obtained from the mother liquor by the addition of ethyl acetate. Most of them were triethylamine hydrochloride. All others were combined with the first crop to yield 3.2 g. (85%) of crude di-*p*-nitrobenzylimidazolium chloride. This was recrystallized from acetonitrile; yield 2.16 g. (58%), m.p. 232–236°. Electrometric titration confirmed the quaternary structure of the compound. A sample was recrystallized twice from 95% ethanol and dried for analysis, m.p. 234–238°.

(12) We wish to thank Jennifer King and Jefferson Jones for their valuable technical assistance.

(13) R. W. Holley and E. Sondheimer, THIS JOURNAL, **76**, 1326 (1954).

Anal. Calcd. for $C_{17}H_{15}N_4O_4^+ Cl^-$: C, 54.48; H, 4.03; N, 14.95; Cl, 9.46. Found: C, 54.27; H, 4.05; N, 14.95; Cl, 9.49.

The same product in 20% yield was obtained when imidazole and *p*-nitrobenzyl chloride in equimolar amounts and, in the absence of a tertiary base, were refluxed in ethyl acetate for 4 hours (20% of the *p*-nitrobenzylchloride was recovered unchanged). The mono-*p*-nitrobenzylimidazole hydrochloride, a possible intermediate, could not be separated from unchanged imidazole hydrochloride.

Di-*p*-aminobenzylimidazolium Chloride Dihydrochloride.—Di-*p*-nitrobenzylimidazolium chloride (1.88 g.) in 20 cc. of glacial acetic acid was hydrogenated with 200 mg. of PtO_2 as catalyst for 4 hours and under 15 lb. of pressure. The catalyst was removed by filtration and 30 cc. of 1 *N* HCl was added to the filtrate. At 0° overnight 1.82 g. (94%) of crystalline material was obtained. It was recrystallized from water–ethanol–acetonitrile; yield 1.22 g. (63%), m.p. 201° dec. The mother liquor was analyzed by paper chromatography. No trace of imidazole could be detected.

A sample was recrystallized twice from the solvent mixture mentioned above and dried for 6 hours at 110° over P_2O_5 *in vacuo* just before analysis, m.p. *204–211°* dec.

Anal. Calcd. for $C_{17}H_{19}N_4Cl{\cdot}2\,HCl$: C, 52.66; H, 5.46; N, 14.45; Cl, 27.73. Found: C, 52.49; H, 5.51; N, 14.62; Cl, 27.17.

Stability of *p*-Nitrobenzyl Esters to Hydrobromic Acid in Glacial Acetic Acid.—One hundred mg. of cbzo-glycyl-glycine *p*-nitrobenzyl ester was dissolved in 4 cc. of 2.5 *N* HBr in glacial acetic acid; 10-mg. aliquots were removed at 0, 10, 20, 30, 45 and 60 min. of reaction time. After the addition of 5 cc. of abs. ether each sample was centrifuged, and the solvent decanted. The insoluble part was dissolved in abs. ethanol and spotted on paper. The chromatograms were developed in the AW system and sprayed with ninhydrin. No trace of glycyl-glycin was visible in any of the samples.

Cbzo-L-phenylalanyl-L-glycine *p*-nitrobenzyl ester was incubated as described above. Samples were removed at 0, 10, 20, 30, 45, 60, 90 and 120 min. and analyzed in the same manner. Traces of L-phenylalanyl-glycine showed in the last 3 samples. They were approximated against known amounts of L-phenylalanyl-glycine, as: 60 min., 3%; 90 min., 6%; 120 min., 15%.

L-Histidyl-L-leucine *p*-Nitrobenzyl Ester Dihydrobromide.—Cbzo-L-histidyl-L-leucine *p*-nitrobenzyl ester (1.72 g.) was dissolved in 6 cc. of dry glacial acetic acid; 18 cc. of 2.5 *N* HBr in glacial acetic acid was added, and the reaction mixture was left for 20 min. at room temperature. The product was precipitated by the addition of 300 cc. of cold abs. ether, and, after cooling, collected by filtration. All attempts of crystallization were unsuccessful. The product was dissolved in abs. ethanol, reprecipitated with ether and dried; yield 1.73 g., 98%, m.p. 123–131°. Paper chromatography showed it to be homogeneous (one spot only with ninhydrin or Pauly reagent); $R_{f\,AW}$ 0.73, $R_{f\,BAW}$ 0.49.

Reductive Removal of the *p*-Nitrobenzyl Ester Group.—L-Histidyl-L-leucine *p*-nitrobenzyl ester hydrobromide (0.5 g.) was hydrogenated in 10 cc. of water with 10% Pd-on-charcoal as catalyst. The reaction was followed by paper chromatography of aliquots removed at different time intervals. The resulting chromatograms showed that the spot assigned to L-histidyl-L-leucine *p*-nitrobenzyl ester had completely disappeared after 20 minutes of shaking; L-histidyl-L-leucine, $R_{f\,AW}$ 0.18.

Glycyl-glycine.—A solution of 0.65 g. of cbzo-glycyl-glycine *p*-nitrobenzyl ester in 4 cc. of tetrahydrofuran and 4 cc. of methanol was added to a suspension of 10% Pd-on-charcoal in 2.64 cc. of 1 *N* HCl. Hydrogen was bubbled through the mixture, and samples were removed at 1-hour intervals and chromatographed in the AW-system. After 2 hours the intensity of the glycyl-glycine spot became constant. Glycyl-glycine *p*-nitrobenzyl ester did not seem to be an intermediate. After 4 hours the catalyst was removed by filtration. The filtrate after addition of 0.37 cc. of triethylamine was evaporated to dryness and the residue was dissolved in a small amount of hot water. Five volumes of abs. ethanol were added and leaflets began to form. They were collected after cooling; yield 0.18 g., decomposition point[14] 216–222° (does not melt), $R_{f\,BAW}$ 0.25.

(14) E. Fischer and E. Fourneau, *Ber.*, **34**, 2868 (1901).

L-Phenylalanyl-glycine Hydrate.—Cbzo-L-phenylalanyl-glycine *p*-nitrobenzyl ester was reduced as described above. Again paper chromatography indicated the hydrogenation to be complete after 2 hours. The reaction was stopped after 4 hours, the catalyst removed by filtration, triethylamine added, and the filtrate evaporated to dryness. The residue was dissolved in 95% ethanol and acetone added. The amorphous precipitate obtained on cooling was collected and crystallized from H_2O plus 10 volumes of abs. ethanol; m.p. 259–263°, $\alpha^{20}D$ + 99.6° (*c* 2, water).[15]

L-Phenylalanine Benzyl Ester *p*-Toluenesulfonate.[16]—A suspension of 23.13 g. (0.14 mole) of L-phenylalanine in 500 cc. of benzene containing 31.98 g. (0.17 mole) of *p*-toluenesulfonic acid hydrate and 140 cc. of benzyl alcohol was refluxed and the water of reaction removed azeotropically. L-Phenylalanine slowly dissolved during the first hour of heating. At the end of 5 hours all the benzene was removed *in vacuo*. Ether (500 cc.) added to the resulting benzyl alcohol solution caused spontaneous crystallization of the product. After cooling for several hours it was collected by filtration and recrystallized from ethanol–ether; yield 56.6 g. (95%), m.p. 164–165.5°, $\alpha^{20}D$ + 7.8° (*c* 2, dimethylformamide).

L-Phenylalanine Benzyl Ester Hydrobromide. A.—A solution of 18.1 g. (0.06 mole) of cbzo-L-phenylalanine, 9.6 g. of benzyl alcohol and 1.0 g. of *p*-toluenesulfonic acid hydrate in 90 cc. of benzene was refluxed for 4 hours while the water of reaction was removed azeotropically. After cooling, the benzene solution was washed with cold 5% $KHCO_3$ and H_2O, dried over Na_2SO_4 and concentrated *in vacuo*. The oil thus obtained was dissolved in 30 cc. of glacial acetic acid and treated with 90 cc. of 2.5 *N* HBr in glacial acetic acid. After 20 min. at room temperature most of the L-phenylalanine benzyl ester hydrobromide had crystallized out. The addition of 600 cc. of cold abs. ether completed the crystallization. The product was collected by filtration and washed thoroughly with abs. ether. After recrystallization from abs. ethanol–ethyl acetate 18.3 g. (90%) of m.p. 205–209° was obtained, $\alpha^{20}D$ −21.8° (*c* 2, methanol–1 *N* HBr 1:1), $R_{f\,AW}$ 0.85[17].

B.—L-Phenylalanine benzyl ester *p*-toluenesulfonate (56.4 g.) was distributed between 400 cc. of cold 1 *M* $KHCO_3$–1 *M* K_2CO_3 1:1 and 200 cc. of cold ether. The aqueous phase was extracted with 4 additional portions of ether and the combined and dried ether extracts were saturated with HBr gas. The crystalline precipitate was collected by filtration, washed thoroughly with cold abs. ether, and recrystallized from abs. ethanol–ethyl acetate; yield 37.12 g., 83%, m.p. 206–209.5°, $\alpha^{20}D$ −22.1° (*c* 2, methanol −1 *N* HBr 1:1).

Cbzo-L-prolyl-L-phenylalanine Benzyl Ester.—The mixed anhydride of 24.9 g. (0.10 mole) of cbzo-L-proline (used as a sirup) dissolved in 65 cc. of dioxane was prepared by addition of 23.8 cc. (0.10 mole) of tri-*n*-butylamine and 9.53 cc. (0.10 mole) of ethyl chloroformate under cooling in an ice-salt-bath. L-Phenylalanine benzyl ester hydrobromide (33.62 g., 0.10 mole) was dissolved in 300 cc. of hot dioxane containing 23.8 cc. (0.10 mole) of tri-*n*-butylamine, and cooled quickly. The two solutions were combined and stirred magnetically for 4 hours at 0° and for an equal time at room temperature. Evaporation of the solvent *in vacuo* left a viscous oil which, dissolved in ethyl acetate, was washed with cold satd. Na_2CO_3 solution, H_2O, 1 *N* HCl, and satd. NaCl solution. After drying over Na_2SO_4 at 40° and concentration *in vacuo* an equal volume of ligroin was added. Crystallization was complete after cooling for several hours; crude product 44.7 g. (92%), m.p. 124–126°.

Two treatments with active charcoal and two recrystallizations from 95% ethanol resulted in a white product of good melting point; yield 38.4 g. (82%), m.p. 126–128°, $\alpha^{20}D$ −15.9° (*c* 2, pyridine).

A sample was recrystallized twice from 95% ethanol and dried for 3 hours at 65° over P_2O_5 in high vacuum; m.p. 128–129°, $\alpha^{20}D$ −15.3° (*c* 2, pyridine).

Anal. Calcd. for $C_{29}H_{30}N_2O_5$: C, 71.58; H, 6.22; N, 5.76. Found: C, 71.68; H, 6.18; N, 6.07.

(15) J. R. Vaughan, Jr., and J. A. Riehler, THIS JOURNAL, **75**, 5556 (1953), give $\alpha^{22}D$ +95.6° (*c* 2, water). All earlier rotations are lower.

(16) Reference 10 gives m.p. 165°, $\alpha^{23}D$ +7.2° (*c* 2, dimethylformamide). Since the original preparation is in Japanese it is repeated here.

(17) D. Ben-Ishai, *J. Org. Chem.*, **19**, 62 (1954) (m.p. 209°).

L-Prolyl-L-phenylalanine Benzyl Ester Hydrobromide.—Cbzo-L-prolyl-L-phenylalanine benzyl ester (24.33 g.) was dissolved in 80 cc. of HBr 2.5 *N* in glacial acetic acid. After 20 min. at room temperature 1.5 l. of abs. ether and 0.4 l. of petroleum ether were added. The resulting suspension cleared within 3 hours at −10°. The solvent was decanted and the oily product dissolved in ethyl acetate. All attempts for crystallization were unsuccessful. The L-prolyl-L-phenylalanine benzyl ester hydrobromide was converted into the free base by washing its ethyl acetate solution with cold carbonate buffer until all Br^- was removed. This solution dried over Na_2SO_4 was used directly for the following condensation.

Cbzo-L-histidyl-L-prolyl-L-phenylalanine Benzyl Ester.—An ethyl acetate solution of cbzo-L-histidine azide, prepared as described above, from 15.16 g. (0.050 mole) of cbzo-L-histidine hydrazide, was combined with the above mentioned solution of L-prolyl-L-phenylalanine benzyl ester (above). This reaction mixture was kept at 0° for 1 day and at room temperature for 2 days. It was then filtered, washed several times with water, dried and evaporated to constant weight; crude yield 22.5 g. (72%). The product which could not be crystallized was purified by counter-current distribution. The solvent system was made up of methanol–carbon tetrachloride–chloroform–water 10:10:5:4; 12.23 g. of crude material was dissolved in lower phase and added to the first tube of an 18-tube 35-cc. counter-current apparatus. After 92 transfers by the single withdrawal method the peak of tripeptide material was found to be in tube 5 (K 0.05).

Samples of random fractions were analyzed by paper chromatography in the AW system. Material from tubes 3–18 showed one spot only with Pauly reagent and none with ninhydrin. All withdrawn fractions showed several spots with either reagent and were discarded. The single spot with Pauly reagent was only light yellow, but total hydrolysis showed the product to contain an equivalent amount of histidine. Fractions 3–18 combined yielded 10.50 g. (62%) of pure cbzo-L-histidyl-L-prolyl-L-phenylalanine benzyl ester.

L-Histidyl-L-prolyl-L-phenylalanine Benzyl Ester Dihydrobromide.—To a solution of 8.05 g. of cbzo-L-histidyl-L-prolyl-L-phenylalanine benzyl ester in 20 cc. of glacial acetic acid 50 cc. of 2.5 *N* HBr in glacial acetic acid was added. The reaction mixture was left at room temperature for 20 minutes. The addition of 550 cc. of cold abs. ether produced a voluminous white precipitate which was collected after cooling and washed thoroughly with abs. ether. On crystallization from abs. ethanol–benzene, 4 fractions of varying purity were isolated; crude yield 6.36 g. (68%). After several recrystallizations of the first fraction from ethanol an almost pure product was usually obtained in 30–40% yield, m.p. 186–192°, $\alpha^{22}D$ −54.5° (*c* 2, water). Paper chromatography in the AW system showed one major spot and one very faint spot visible with Pauly reagent only.[18] Repeated recrystallizations of other fractions gave products that contained one major and two minor components; $R_{f\ AW}$ 0.74, 0.46, 0.29. Since they were easily separated on paper in the AW system, a preparative separation was attempted. The capacity of cellulose was determined at 1 g. per 200 g. of powder. A column was prepared from 200 g. of prewashed cellulose powder and acetonitrile–water 3:1; 1.16 g. of crude L-histidyl-L-prolyl-L-phenylalanine dihydrobromide was separated on it. The main fraction, R_f 0.74, contained 1.01 g. of material from which 0.72 g. of chromatographically pure tripeptide dihydrobromide was isolated after two recrystallizations from ethanol. The disadvantages of the column were its low capacity and the fact that some of the HBr was adsorbed and had to be readded to the eluate.

A sample was twice recrystallized from absolute ethanol and dried for analysis; m.p. 188–192°, $\alpha^{22}D$ −54.3° (*c* 2, water), $R_{f\ AW}$ 0.74.

Anal. Calcd. for $C_{27}H_{32}N_5O_4 \cdot 2HBr$: C, 49.70; H, 5.25; N, 10.74; Br, 24.50. Found: C, 49.36; H, 5.28; N, 10.79; Br, 24.30.

Cbzo-L-histidyl-L-prolyl-L-phenylalanine.—To a solution of 14.63 g. (0.023 mole) of cbzo-L-histidyl-L-prolyl-L-phenylalanine benzyl ester in 40 cc. of tetrahydrofuran 34.5 cc. of 1*N* NaOH was added. After one hour at room temperature, the tetrahydrofuran was removed *in vacuo* at low temperature. The aqueous solution was extracted with two 50-cc. portions of ether and acidified to *p*H ~ 5 by the addition of 34.5 cc. of 1*N* HCl and a few drops of acetic acid. After cooling, the liquid was decanted and the semi-solid mass crystallized from ethanol–ethyl acetate; yield 8.33 g., m.p. 191–194°; 2.77 g., m.p. 188–191° (total 89%).

A sample of the first fraction was recrystallized twice from ethanol–ethyl acetate and dried for analysis; m.p. 193–195°, $\alpha^{20}D$ −22.7 (*c* 4, dimethylformamide), $R_{f\ AW}$ 0.75.

Anal. Calcd. for $C_{28}H_{31}N_5O_6$: C, 63.02; H, 5.86; N, 13.13. Found: C, 63.06; H, 6.03; N, 12.98.

Cbzo-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester. A.—A solution of 3.5 g. (0.0088 mole) of cbzo-L-prolyl-L-phenylalanine,[19] 2.27 g. (0.0132 mole) of *p*-nitrobenzyl chloride and 1.85 cc. (0.0132 mole) of triethylamine in 15 cc. of ethyl acetate was refluxed overnight. Triethylamine hydrochloride was removed by filtration, and the filtrate was washed with cold 1 *N* HCl, water, cold 1 *M* $KHCO_3$ and satd. NaCl solution. After drying over Na_2SO_4 the solvent was removed *in vacuo* and the resulting oil crystallized from tetrahydrofuran–ligroin; yield 3.74 g. (80%), m.p. 92–93.5°, $\alpha^{20}D$ −43.8° (*c* 2.7, ethyl acetate).

B.—The same compound was obtained from the mixed anhydride of cbzo-L-proline and L-phenylalanine *p*-nitrobenzyl ester. The condensation was accomplished exactly as described for the benzyl ester; yield 76%, m.p. 91–93.5°, $\alpha^{20}D$ −43.0° (*c* 2.1, ethyl acetate).

A and B were combined and a sample was recrystallized twice from tetrahydrofuran–ligroin. After drying the constants were: m.p. 92–94°, $\alpha^{20}D$, −45.5° (*c* 2, ethyl acetate).

L-Prolyl-L-phenylalanine *p*-Nitrobenzyl Ester Hydrobromide.—A solution of 28.03 g. of cbzo-L-prolyl-L-phenylalanine *p*-nitrobenzyl ester in 260 cc. of 2.5 *N* HBr in glacial acetic acid was kept at room temperature for 20 minutes. The addition of 2 l. of abs. ether and 0.6 l. of petroleum ether resulted in a milky suspension that cleared within 10 hours at −10°. The supernatant was discarded and the oil dissolved in abs. methanol. The solution was evaporated several times after the addition of benzene to remove every trace of acetic acid. Crystals were obtained from absolute ethanol–ethyl acetate–ether; yield 21.21 g. (84%), m.p. 142–144°, $R_{f\ AW}$ 0.82. A sample was recrystallized twice from the same mixture of solvents and dried; m.p. 143–145°, $\alpha^{20}D$ −26.7° (*c* 2, ethanol).

Anal. Calcd. for $C_{22}H_{23}N_3O_5 \cdot HBr$: C, 52.73; H, 5.06; N, 8.78; Br, 16.71. Found: C, 52.79; H, 5.04; N, 8.82; Br, 16.72.

Cbzo-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester.—A solution of cbzo-L-histidine azide in 500 cc. of ethyl acetate, prepared from 13.32 g. (0.0044 mole) of cbzo-L-histidine hydrazide as described above, was filtered into 21.05 g. (0.0044 mole) of L-prolyl-L-phenylalanine *p*-nitrobenzyl ester hydrobromide and 10.45 cc. (0.0044 mole) of tri-*n*-butylamine suspended in 100 cc. of ethyl acetate. The mixture became clear after 10 minutes of stirring at 0°. The stirring was continued for 5 hours at 0° and overnight at room temperature. The solution was washed with cold water, cold 1 *N* HCl, water, cold 1 *M* K_2CO_3 and satd. NaCl solution. After drying over Na_2SO_4 it was evaporated *in vacuo* to constant weight; yield 26 g. (88%). The compound could not be crystallized; it was, however, chromatographically pure (no isatin-positive material, one spot with Pauly reagent), $R_{f\ AW}$ 0.91.

L-Histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester Dihydrobromide.—Cbzo-L-histidyl-L-prolyl-L-phenylalanine *p*-nitrobenzyl ester (26 g.) was dissolved in 50 cc. of warm glacial acetic acid. To the cooled solution 200 cc. of 2.5 *N* HBr in glacial acetic acid was added. After 25 minutes at room temperature the product was precipitated by the addition of 2.5 l. of abs. ether. It was collected by filtration and crystallized from abs. ethanol–ethyl acetate; yield 22.03 g. (79%), m.p. 155–159°, $\alpha^{20}D$ −55.1° (*c* 2, water). A sample was recrystallized twice from the same solvents and dried for analysis; m.p. 155–159°, $\alpha^{20}D$ −53.9° (*c* 2, water), $R_{f\ AW}$ 0.86.

Anal. Calcd. for $C_{27}H_{30}N_6O_6 \cdot 2HBr \cdot H_2O$: C, 45.39; H, 4.80; N, 11.76; Br, 22.37. Found: C, 45.69; H, 4.77; N, 11.84; Br, 22.57.

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(18) This is due to the greater sensitivity of this reagent.

(19) R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel and H. Zuber, *Helv. Chim. Acta*, **41**, 1273 (1958).