radioactivities of the various materials are shown in Table I. Chemical decarboxylations of radioactive samples of stipitatonic acid were carried out as described previously by Bentley;[28] the results are shown in Table II.

To study the ultilization of methyl triacetic lactone, a *P. stipitatum* culture was grown on 25 ml of media contained in a 125-ml Erlenmeyer flask. Additions of radioactive methyl triacetic lactone were made on days 5 and 6, and the culture was filtered on the eleventh day of growth. Metabolites were isolated as in the prior experiments. The results are given in Table III.

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(28) R. Bentley, *J. Biol. Chem.*, **238**, 1889 (1963).

Synthesis of Secretin. I. The Protected Tetradecapeptide Corresponding to Sequence 14–27

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Abstract: Synthesis of the protected tetradecapeptide, *t*-butyloxycarbonylnitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-leucyl-L-valinamide (XIV), is described. The chain was built up stepwise from its C-terminal moiety, L-valinamide. The nitrophenyl ester method was applied in all chain-lengthening steps.

The intestinal hormone (porcine) secretin was isolated in pure form by Jorpes and Mutt,[1] who, with their collaborators, also established the amino acid composition[2] and partial structure[3] of the hormone. Through a personal communication from Professor Jorpes and Dozent Mutt, we learned the tentative sequence of the 27 amino acids which constitute the single-chain molecule of secretin. The present paper reports the synthesis of the C-terminal half of the chain, a protected tetradecapeptide.

```
     NO2 OBZL BZL      NO2      NH2 NO2           NH2
      |    |           |         |   |             |
BOC-Arg-Asp---Ser-Ala-Arg-Leu-Glu-Arg-Leu-Leu-Glu-
                                          Gly-Leu-Val-NH2
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The synthesis starts with the ammonolysis of benzyloxycarbonyl-L-valine *p*-nitrophenyl ester.[4] The resulting benzyloxycarbonyl-L-valinamide (I) was treated with hydrobromic acid in acetic acid to afford L-valinamide hydrobromide which was acylated, in the presence of triethylamine, with benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester[4] to give benzyloxycarbonyl-L-leucyl-L-valinamide (II). The protecting group was removed from this dipeptide derivative in the same manner and the third amino acid residue, glycine, was attached. This stepwise chain lengthening by the nitrophenyl ester method[5] was continued, and the protected tri-, tetra-, penta-, and hexapeptide intermediates were all secured in excellent yield and in crystalline form. The next amino acid, arginine, was introduced as N^{α}-benzyloxycarbonyl-nitro-L-arginine 2,4-dinitrophenyl ester.[6] This new active ester was used also in the preparation of the decapeptide intermediate X.

From the dodecapeptide stage on, the *t*-butyloxycarbonyl group rather than the benzyloxycarbonyl group was applied as amino protecting group to avoid partial O-acetylation of the serine residue during the removal of benzyloxycarbonyl groups with hydrobromic acid in acetic acid. Removal of the *t*-butyloxycarbonyl group with trifluoroacetic acid from the protected dodecapeptide XII and acylation with *t*-butyloxycarbonyl-β-benzyl-L-aspartic acid *p*-nitrophenyl ester[4] led to the desired tridecapeptide derivative XIII. The last moiety of the tetradecapeptide, L-arginine, was added in the form of N^{α}-*t*-butyloxycarbonyl-nitro-L-arginine 2,4-dinitrophenyl ester which was prepared as described for the corresponding benzyloxycarbonyl derivative.[6]

The protected tetradecapeptide derivative N^{α}-*t*-butyloxycarbonyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminylnitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (XIV) is a suitable intermediate both for the continuation of the synthesis by the stepwise approach and for the synthesis of the hormone by fragment condensation.

It may be interesting to note that the synthetic hexapeptide amide L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VIb) and the tridecapeptide, L-aspartyl-L-seryl-L-alanyl-L-arginyl-L-leucyl-L-glutaminyl-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide, are indistinguishable on paper chromatograms from the corresponding tryptic and thrombic fragments of porcine secretin.[7]

(1) J. E. Jorpes and V. Mutt, *Acta Chem. Scand.*, **15**, 1790 (1961).
(2) J. E. Jorpes, V. Mutt, S. Magnusson, and B. B. Steele, *Biochem. Biophys. Res. Commun.*, **9**, 275 (1962).
(3) V. Mutt, S. Magnusson, J. E. Jorpes, and E. Dahl, *Biochemistry*, **4**, 2358 (1965).
(4) This and all other active esters used in the synthesis of the tetradecapeptide were prepared according to the general procedure described in "Biochemical Preparations," Vol. 9, John Wiley and Sons, Inc., New York, N. Y., 1962, p 110.
(5) (a) M. Bodanszky, *Nature*, **175**, 685 (1955); (b) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959); (c) M. Bodanszky, *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).
(6) M. Bodanszky and M. A. Ondetti, *Chem. Ind.* (London), 26 (1966)
(7) Personal communication from Dr. Mutt, *cf.* also ref 3.

The high over-all yield in which the protected intermediates were secured can be mentioned as a characteristic feature of the stepwise synthesis by the nitrophenyl ester method,[5] especially since the intermediates could be used without purification other than washing with solvents in the subsequent steps. However, the protected hendecapeptide XI can be converted into the crystalline hydrobromide of L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide, and small amounts of impurities present in XI are removed by this crystallization. The protected tetradecapeptide XIV requires purification by countercurrent distribution, probably because the last acylating agent, *t*-butyloxycarbonyl-nitro-L-arginine 2,4-dinitrophenyl ester was used as a crude and impure material. After this purification and removal of all the protecting groups the tetradecapeptide travels as a single band in electrophoresis and on amino acid analysis gives the expected ratios of constituent amino acids. All the protected intermediates were secured in crystalline form, and the reactions have been carried out on a fairly large scale.

Experimental Section[8]

Benzyloxycarbonyl-L-valinamide (I). Benzyloxycarbonyl-L-valine *p*-nitrophenyl ester[4] (37.3 g, mp 67–68°, lit.[9] 63°) was dissolved in methanol (200 ml). On the addition of a methanolic solution of ammonia (50 ml, *ca.* 9 *N*), a clear yellow solution resulted and crystallization of the product started after a few minutes. After 1 hr at room temperature, the crystals were filtered and washed with methanol (100 ml) and with ethyl acetate (100 ml). The air-dried product (17.0 g) melts at 206–208°; from the mother liquor more material (2.4 g) was secured, mp 206–209°, total yield 19.4 g (77.5%). A sample (3.5 g) was recrystallized from hot ethyl acetate (450 ml). The purified amide (3.0 g) melts at 206–208°, $[\alpha]^{22}D$ +22° (*c* 2, dimethylformamide). The melting point is unchanged after sublimation at 190° (0.05 mm).

Anal. Calcd for $C_{13}H_{18}N_2O_3$: C, 62.4; H, 7.2; N, 11.2. Found: C, 62.4; H, 7.3; N, 11.2.

Benzyloxycarbonyl-L-leucyl-L-valinamide (II). Benzyloxycarbonyl-L-valinamide (I) (25.0 g) was suspended in acetic acid (100 ml) and treated with hydrobromic acid in acetic acid (*ca.* 4 *N*, 100 ml). A clear solution was obtained, and evolution of gas and the deposition of crystals was observed. After 1.5 hr at room temperature, ether (600 ml) was added, and the crystalline hydrobromide was filtered, washed with ether (400 ml), and dried over sodium hydroxide *in vacuo*. The yield was quantitative (19.6 g), mp 248–253°. The amide hydrobromide was dissolved in dimethylformamide (200 ml); triethylamine (20 ml) was added to the solution followed by benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester[4] (42.5 g). The mixture was cooled to room temperature. After a few minutes it showed no reaction with ninhydrin and soon turned into a semisolid mass of crystals. After 2 hr at room temperature, the mixture was cooled with ice water, diluted with chloroform (500 ml), and filtered, and the crystals were washed with chloroform (300 ml). The air-dried product (32.6 g, 90%) sinters at 233° and melts at 235–236°. Recrystallization of a sample from hot 95% ethanol did not raise the melting point, $[\alpha]^{30}D$ −28° (*c* 2, acetic acid).

The combined mother liquors and washings were treated with dimethylaminopropylamine[10] (6 ml) and after a few hours were washed (two 250-ml portions) with 0.5 *N* HCl, water (250 ml), 0.5 *N* ammonium hydroxide (four 250-ml portions), and water (250 ml). The chloroform was removed *in vacuo* to a small volume. The precipitate which formed was filtered and washed with chloroform. The air-dried product (3.5 g, mp 216–220°) was recrystallized from hot 95% ethanol (200 ml). The purified material (2.2 g, 6%) melts at 234–236°. The protected dipeptide amide sublimed unchanged at 220° (0.05 mm).

Anal. Calcd for $C_{19}H_{29}N_3O_4$: C, 62.8; H, 8.0; N, 11.6. Found: C, 62.9; H, 8.1; N, 11.6.

Benzyloxycarbonylglycyl-L-leucyl-L-valinamide (III). To a suspension of the protected dipeptide amide II (14.6 g) in acetic acid (40 ml), hydrobromic acid in acetic acid (*ca.* 4 *N*, 40 ml) was added. After 1.5 hr at room temperature, the mixture was diluted with ether (400 ml). The hydrobromide was triturated with fresh ether, washed with ether, and dried *in vacuo* over sodium hydroxide. It was dissolved in dimethylformamide (100 ml); triethylamine (14 ml) and benzyloxycarbonylglycine *p*-nitrophenyl ester[4] (16.5 g) were added to the solution. The mixture was cooled to room temperature where it was kept for about 3 hr. The precipitated triethylammonium bromide was filtered and washed with dimethylformamide (50 ml). Ethyl acetate (500 ml) and 1 *N* HCl (400 ml) were added to the filtrate, and the organic layer was washed with 1 *N* HCl (400 ml) and with water (twice with 400 ml). The aqueous washes were extracted in the same order with ethyl acetate (300 ml). From the combined ethyl acetate solutions, a precipitate slowly formed. Most of the solvent was removed by evaporation and the protected tripeptide amide was washed with ethyl acetate. The dry product weighed 16.8 g, mp 170–175°, with some sintering at 160°. This crude product was extracted with boiling ethyl acetate (*ca.* 200 ml) to give 14.5 g (86%) of a product with a melting point of 186–187°. A sample (0.60 g) was recrystallized from hot water (300 ml); the crystals were washed with water (100 ml) and dried. The recovered material (0.50 g) melts at 187–190°, $[\alpha]^{30}D$ −30° (*c* 2, acetic acid).

Anal. Calcd for $C_{21}H_{32}N_4O_5$: C, 60.0; H, 7.7; N, 13.3. Found: C, 60.2; H, 7.7; N, 13.1.

Benzyloxycarbonyl-L-glutaminylglycyl-L-leucyl-L-valinamide (IV). The benzyloxycarbonyl group was removed from the protected tripeptide amide III (12.7 g) with hydrobromic acid in acetic acid as described in the previous paragraph. To the solution of the hydrobromide in dimethylformamide (90 ml), triethylamine (11.2 ml) and benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[11] (13.25 g) were added. The mixture, which was cooled with water to keep it at room temperature, soon turned into a semisolid mass of crystals. After standing overnight at room temperature, the mass was disintegrated with the aid of chloroform (600 ml). The product was filtered and washed on the filter with chloroform (200 ml), with warm ethyl acetate (200 ml), warm ethanol (200 ml), and again with warm ethyl acetate (300 ml). The protected tetrapeptide amide was dried at 50° *in vacuo*; weight 16.0 g (95% yield), mp 239–241° dec, $[\alpha]^{30}D$ −27° (*c* 2, acetic acid). In subsequent preparations 96–97% yields were obtained; mp 240–242° dec.

Anal. Calcd for $C_{26}H_{40}N_6O_7$: C, 56.9; H, 7.3; N, 15.3. Found: C, 56.9; H, 7.3; N, 15.5.

Benzyloxycarbonyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (V). The protected tetrapeptide amide IV (30.2 g) was suspended in acetic acid (180 ml) and treated with hydrobromic acid in acetic acid (*ca.* 4 *N*, 180 ml). After 1.5 hr at room temperature, the reaction mixture was diluted with ether (1.4 l.), and the hydrobromide was filtered, washed with ether, dried *in vacuo* over sodium hydroxide and dissolved in dimethylformamide (220 ml). Triethylamine (32 ml) was added to the solution followed by benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester[4] (25.5 g). After about 30 min, crystals of the protected pentapeptide amide started to separate and the reaction mixture turned into a semisolid mass. After 3 hr the mixture was diluted with 95% ethanol (1 l.), and the product was filtered and washed on the filter with 95% ethanol (500 ml). The protected pentapeptide amide was dried first in air, then at 50° *in vacuo*; weight 33.8 g (93%), mp 270–272° dec (with sintering at 260°), $[\alpha]^{30}D$ −35° (*c* 2, acetic acid).

Anal. Calcd for $C_{32}H_{51}N_7O_8$: C, 58.1; H, 7.8; N, 14.8. Found: C, 58.1; H, 7.8; N, 14.6.

L-Leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide Hydrobromide (Va). Compound V (3.3 g) was dissolved in hot acetic acid (20 ml); the solution was cooled to room temperature and hydrobromic acid in acetic acid (*ca.* 4 *N*, 10 ml) was added. After

(8) Melting points were taken in capillary tubes and are uncorrected. R_f^A refers to the system 1-butanol–acetic acid–water, 4:1:5 (S. M. Partridge, *Biochem. J.*, **42**, 238 (1948)); R_f^B refers to the system 1-butanol–pyridine–acetic acid–water, 30:20:6:24 (S. G. Waley and G. Watson, *ibid.*, **55**, 328 (1953)); both on Whatman No. 1 paper in descending chromatography.

(9) B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, 40, 373 (1957).

(10) M. Löw and L. Kisfaludy, *Acta Chim. Acad. Sci. Hung.*, **44**, 61 (1965).

(11) Prepared according to the procedure described for the corresponding ester of asparagine, "Biochemical Preparations," Vol. 10, John Wiley and Sons, Inc., New York, N. Y., 1963, p 122; *cf.* also M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

1 hr at room temperature the solution was diluted with ether (250 ml), and the gummy precipitate triturated with fresh ether, washed with ether on the filter, and dried in a desiccator over sodium hydroxide overnight. It was dissolved in methanol (50 ml) and precipitated with ether (150 ml). The gummy precipitate was dissolved in absolute ethanol (*ca.* 200 ml). A crystalline hydrobromide separated slowly from the solution. Triethylamine (*ca.* 1 ml) was added to neutralize the solution and to complete the crystallization. The crystals were washed with ethanol (100 ml) and were dried over P_2O_5 *in vacuo* at room temperature; weight 0.50 g, mp 215–216° dec after sintering at 210°. Evaporation of the mother liquor to dryness and trituration of the residue with chloroform (50 ml) gave a second crop, 1.75 g, the melting point of which was similar to the first crop. A small third crop (0.35 g) was obtained from the mother liquors of the second, mp 220–222° dec. On paper chromatograms a single spot, R_f^A 0.60, is shown by all the fractions; total yield 2.60 g (86%). A sample (0.50 g) was dissolved in hot 95% ethanol (25 ml). On standing slow crystallization occurs; a few days later ethyl acetate (25 ml) was added and two more days later the crystals were filtered and washed with a 1:1 mixture of ethanol–ethyl acetate (20 ml) and with ethyl acetate (20 ml). After drying the crystals weighed 0.40 g, mp 228° dec (sintering at 220°), $[\alpha]^{30}D$ −10° (*c* 2, acetic acid). For analysis a sample was dried at 110° *in vacuo*.

Anal. Calcd for $C_{24}H_{46}N_7O_6Br$: C, 47.2; H, 7.7; N, 16.1; Br, 13.2. Found: C, 47.2; H, 7.7; N, 15.9; Br, 13.0. Amino acid analysis: Glu, 0.9; Gly, 1.1; Val, 1.0; Leu, 2.3; NH_3, 0.9.

L-Leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (Vb). A solution of the pentapeptide amide hydrobromide Va (0.30 g) in methanol (50 ml) was treated with Amberlite IR 400, OH cycle, until the solution gave a negative test with silver nitrate. The resin was removed by filtration and washed with methanol, and the solvent was removed *in vacuo*. The residue was treated with chloroform (5 ml) in which it partially dissolved. Ethyl acetate (50 ml) was added. The product was filtered and washed with ethyl acetate (25 ml) and dried in air, weight 0.20 g, mp 260–261° dec. The product gives a single spot, R_f^A 0.60, on paper chromatograms.

Anal. Calcd for $C_{24}H_{45}N_7O_6$: C, 54.6; H, 8.6; N, 18.6. Found: C, 55.0; H, 8.6; N, 18.6.

Benzyloxycarbonyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VI). A. The pentapeptide amide hydrobromide Va (1.22 g) was suspended in dimethylformamide (10 ml), in which it partially dissolved. Triethylamine (0.45 ml) and benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester[4] (1.55 g) were added to the mixture which was shaken vigorously for a few minutes, then diluted with dimethylformamide (10 ml) and shaken again. After 2 hr at room temperature, the mixture was diluted with ethyl acetate (150 ml), filtered, and washed with ethyl acetate (20 ml). The precipitate was suspended in absolute ethanol (30 ml) and washed on the filter with absolute ethanol (20 ml) and with ethyl acetate (20 ml). The dried protected hexapeptide amide weighed 1.55 g (100%); the product sinters at 255°, melts with decomposition at 264–267°, $[\alpha]^{28}D$ −47° (*c* 2, acetic acid).

Anal. Calcd for $C_{38}H_{62}N_8O_9$: C, 58.9; H, 8.1; N, 14.4. Found: C, 58.3; H, 7.9; N, 14.4.

B. The pentapeptide amide Vb (prepared from 50 mmoles of protected pentapeptide) was dissolved in hot dimethylformamide (650 ml) and treated with benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester[4] (29 g). After 3 hr the thick reaction mixture was diluted with ethanol (2 l.), and the crystalline precipitate was washed with ethanol (1.5 l.) and with ethyl acetate (0.5 l.). Both the product (23 g) and a second crop from the mother liquor (7.4 g) melt at 266–269° dec.

L-Leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VIa). A. The protected hexapeptide amide VI (0.39 g) was suspended in acetic acid (5 ml) and treated with hydrobromic acid in acetic acid (4 *N*, 5 ml). Soon a clear solution resulted. After 1.5 hr at room temperature, ether (200 ml) was added; the precipitate was filtered, washed with ether, and dried *in vacuo* over sodium hydroxide. The salt was hygroscopic and was not analyzed. It gave a single spot (R_f^A 0.78) with ninhydrin on paper chromatograms and was converted to the free base as described for Vb. The product (0.29 g) separated in crystalline form; mp 239–241° dec, $[\alpha]^{27}D$ −28° (*c* 2, acetic acid).

Anal. Calcd for $C_{30}H_{56}N_8O_7$: C, 56.2; H, 8.8; N, 17.4. Found: C, 56.1; H, 8.9; N, 16.9.

A sample was hydrolyzed with constant boiling hydrochloric acid in an evacuated, sealed ampoule at 110° for 24 hr; it gave 5.1% NH_3 (calcd 5.3%). Quantitative amino acid analysis gave the following molar ratios: Leu, 2.7; Glu, 1.0; Gly, 1.0; and Val, 1.0.

B. The protected hexapeptide amide VI (3.9 g) was dissolved—with gentle warming—in acetic acid (100 ml). The solution was cooled to room temperature and hydrogenated in the presence of a 10% palladium on charcoal catalyst (1.0 g) until the evolution of carbon dioxide ceased. Removal of the catalyst by filtration was followed by evaporation of the acetic acid from the frozen state. The hexapeptide amide acetate gives a single spot, R_f^A 0.80, on paper chromatograms.

N^{α}-Benzyloxycarbonyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VII). A solution of N^{α}-benzyloxycarbonyl-nitro-L-arginine (3.53 g) and of 2,4-dinitrophenol (2.0 g) in tetrahydrofuran (60 ml) was cooled with ice water during the addition of dicyclohexylcarbodiimide (2.1 g). After about 1 hr at room temperature, the precipitate (dicyclohexylurea) was filtered off and was washed with tetrahydrofuran (40 ml). The combined filtrate and washings were evaporated to dryness *in vacuo;* the residue was dissolved in ethyl acetate (*ca.* 10 ml) and precipitated with ether (*ca.* 50 ml). The ester was washed with ether (*ca.* 50 ml) and dried *in vacuo*.

The hexapeptide amide VIa (from 3.9 g of protected hexapeptide amide by procedure B) was dissolved in hot (*ca.* 80°) dimethylformamide (150 ml) and was mixed with a solution of the above active ester in the same solvent (50 ml). The mixture was cooled to room temperature and after about 4 hr it was diluted with ether (1 l.). The precipitate was filtered, washed with ether (0.6 l.) and ethyl acetate (0.3 l.), and dried in air, 4.5 g (92%), mp 245–247° dec, $[\alpha]^{25}D$ −40° (*c* 2, acetic acid). A sample, 0.20 g, was recrystallized from hot 50% ethanol (22 ml); the crystals were washed with ethanol and ethyl acetate; 0.12 g was recovered, mp *ca.* 252° dec, λ_{max}^{EtOH} 269 mμ ($E_{1\%}^{1cm}$ 144).

Anal. Calcd for $C_{44}H_{73}N_{13}O_{12}$: C, 54.2; H, 7.5; N, 18.7. Found: C, 54.3; H, 7.6; N, 18.3.

Treatment of a small sample with hydrobromic acid in acetic acid, precipitation of the resulting amine–hydrobromide with ether, and paper chromatographic examination of this material revealed a single spot (R_f^A 0.83).

Benzyloxycarbonyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VIII). The protected heptapeptide VII (9.8 g) was dissolved in warm acetic acid (50 ml), cooled, and treated with hydrobromic acid in acetic acid (*ca.* 4 *N* 50 ml). After 1 hr at room temperature, ether (1.2 l.) was added to the solution; the hydrobromide was filtered, washed with ether, and dried *in vacuo* over sodium hydroxide for a short time. It was then dissolved in dimethylformamide (100 ml), and was made alkaline with triethylamine (10.4 ml). Benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[11] (5.0 g) was added to the mixture which was left at room temperature overnight; crystals separated, forming a semisolid mass. The mixture was diluted with ethyl acetate (1 l.), the precipitate was filtered, washed with ethyl acetate (500 ml), ethanol (500 ml), and with ethyl acetate, hot ethyl acetate (250 ml), and hot chloroform (250 ml). The product was dried first on air and then at 50° *in vacuo*, weight 11.0 g (100%), darkens from 250° dec at 262–264°. A sample (1.0 g) was dissolved in hot 80% ethanol (10 ml), cooled, and diluted with 95% ethanol (30 ml). Slow crystallization took place. After 3 days the crystals were filtered and washed with ethanol, chloroform, and ethyl acetate. The dried material weighed 0.85 g, mp 250° dec, $[\alpha]^{20}D$ −32° (*c* 2, dimethyl sulfoxide).

Anal. Calcd for $C_{49}H_{81}N_{15}O_{14}$: C, 53.3; H, 7.4; N, 19.0. Found: C, 53.4; H, 7.5; N, 19.2.

Benzyloxycarbonyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (IX). The protected octapeptide VIII (11.1 g) was powdered and added with stirring to acetic acid (50 ml). Hydrobromic acid in acetic acid (*ca.* 4 *N*, 50 ml) was slowly added to the suspension. Stirring was continued until all the material dissolved and then for 30 min more; a total of 3 hr was required. The amine hydrobromide was precipitated with ether (1 l.), filtered, washed with ether, and dried *in vacuo* over sodium hydroxide for a short time. The hydrobromide was dissolved in dimethylformamide (100 ml), and triethylamine (10.4 ml) was added to the cooled solution, followed by the benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester[4] (5.0 g). After standing overnight at room temperature, the mixture was diluted with ethyl acetate (2 l.). The precipitate which formed was filtered and washed with 200-ml portions of ethyl acetate, chloroform, hot chloroform, and hot ethyl acetate. The product was dried *in vacuo* at 50°; weight 12.1 g (99%), darkens from 255° dec at about 265°. Treatment of a sample of IX with hydrobromic acid in acetic acid, precipitation of the free nonapeptide amide hydrobromide with ether,

and paper chromatographic examination of the product revealed a single ultraviolet absorbing, ninhydrin-positive spot (R_f^A 0.77).

A sample of IX (0.50 g) was dissolved in a hot mixture of ethanol (30 ml) and water (15 ml). The solution was filtered and allowed to stand at room temperature. After a few days the crystals were collected and washed with liberal quantities of 95% ethanol, ethyl acetate, and hot ethyl acetate. The air-dried material (0.25 g) darkens from 260°, melts at 267–269° dec. For analysis it was dried at 110° *in vacuo*, $[\alpha]^{25}D$ −41° (*c* 2, acetic acid).

Anal. Calcd for $C_{55}H_{92}N_{16}O_{15}$: C, 54.3; H, 7.6; N, 18.4. Found: C, 54.2; H, 7.8; N, 18.2.

N^{α}-Benzyloxycarbonyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (X). The protected nonapeptide amide IX (24.2 g) was suspended in acetic acid (100 ml), and hydrobromic acid in acetic acid (*ca.* 4 *N*, 100 ml) was slowly added to the suspension. A homogeneous solution was obtained in about 1 hr, and the solution was kept at room temperature for an additional hour. Ether (2 l.) was added to the solution. The precipitated hydrobromide was filtered, washed with ether, and dried *in vacuo* over sodium hydroxide for a short time. It was dissolved in dimethylformamide (250 ml); the solution was cooled while being made alkaline with triethylamine (14 ml). Benzyloxycarbonyl-nitro-L-arginine 2,4-dinitrophenyl ester[6] (25.8 g) was added followed by more triethylamine (5 ml). After 3 hr at room temperature the solution gave no reaction with ninhydrin. The next day it was diluted with ethyl acetate (2 l.); the precipitate was collected and washed with ethyl acetate (500 ml), chloroform (500 ml), hot chloroform (1.5 l.), and hot ethyl acetate (500 ml). The product was dried at 50° *in vacuo* (25.1 g, 88%). It darkens from 250° and melts at 255–264° dec; recrystallization from 90% ethanol–ethyl acetate did not raise the melting point; $[\alpha]^{25}D$ −36° (*c* 2, acetic acid); $\lambda_{max}^{95\%\ EtOH}$ 270 mμ ($E_{1\%}^{1cm}$ 232).

Anal. Calcd for $C_{61}H_{103}N_{21}O_{18}$: C, 51.7; H, 7.3; N, 20.7. Found: C, 51.9; H, 7.6; N, 21.2.

The benzyloxycarbonyl group was removed from a small sample with hydrobromic acid in acetic acid; the resulting free amine gave a single spot on paper chromatograms with R_f^A 0.60 and R_f^B 0.82.

Benzyloxycarbonyl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (XI). To a solution of the protected decapeptide amide X (25.5 g) in acetic acid (120 ml), a solution of hydrobromic acid in acetic acid (*ca.* 4 *N*, 120 ml) was slowly added. After 1.5 hr at room temperature the hydrobromide was precipitated with ether (*ca.* 2 l.), washed with ether, and dried briefly *in vacuo* over sodium hydroxide. It was dissolved in dimethylformamide (180 ml); triethylamine (18 ml) was added to the solution followed by benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester[4] (9.3 g). The reaction was allowed to proceed overnight, then the mixture was filtered, the triethylammonium bromide washed with dimethylformamide (50 ml), more active ester (3.1 g) and triethylamine (1 ml) were added, and the solution was concentrated to a small volume. After 2 hr it was diluted with chloroform (2 l.). The crystalline precipitate was washed with chloroform and dried in air. The dry product (25.6 g, 95%) melts at 258–264° dec, $[\alpha]^{25}D$ −38° (*c* 2, 80% acetic acid).

Anal. Calcd for $C_{61}H_{103}N_{22}O_{16}$: C, 51.6; H, 7.3; N, 20.7. Found: C, 51.0; H, 7.5; N, 20.3.

N-*t*-Butyloxycarbonyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminyl-glycyl-L-leucyl-L-valinamide (XII). To a suspension of the protected hendecapeptide XI (24.0 g) in acetic acid (160 ml), a 4 *N* solution of hydrobromic acid in acetic acid (160 ml) was slowly added. After about 2 hr at room temperature, the solution was diluted with ether (2 l.) and the precipitate filtered, washed with ether, and dried *in vacuo* over sodium hydroxide. The hydrobromide was dissolved in methanol (235 ml); the solution was cooled in an ice–water bath and neutralized[12] with triethylamine (25 ml). A thick mass of crystals appeared. The suspension was diluted with chloroform (500 ml); the crystals were filtered, washed with chloroform (1 l.), and dried in air and finally *in vacuo* at 40°. The monohydrobromide (18.4 g, 80%. *Anal.* Calcd for N: 21.4. Found: 21.1) melts at 245–248° dec (softens at 240°); R_f^A 0.42, R_f^B 0.80. From the mother liquor an additional amount (2.2 g) of less pure material was obtained.

The monohydrobromide (18.0 g) in dimethylformamide (800 ml) was treated in the presence of triethylamine (1.5 ml) with N-*t*-butyloxycarbonyl-O-benzyl-L-serine *p*-nitrophenyl ester. This active ester was prepared from the corresponding acid (9.4 g, obtained from Cyclo Chemical Corp.), *p*-nitrophenol (5.5 g), and dicyclohexylcarbodiimide (6.6 g) in ethyl acetate (40 ml). The N,N′-dicyclohexylurea was filtered off and the solvent removed from the filtrate *in vacuo*. The oily residue, which failed to crystallize, was used without purification. After 1 day at room temperature the mixture was concentrated *in vacuo* to about 50 ml. Ethyl acetate (1.5 l.) was added and the precipitate filtered and washed with ethyl acetate (500 ml), chloroform (200 ml), and once more with ethyl acetate (500 ml). During the treatment with chloroform the material turned into a crystalline mass. The protected dodecapeptide XII was dried at 40° *in vacuo*, weight 19.1 g (93% calculated on the hendecapeptide hydrobromide). The product darkens from 250° and melts at about 300° dec, $[\alpha]^{25}D$ −33° (*c* 0.8, 80% acetic acid).

Anal. Calcd for $C_{71}H_{121}N_{23}O_{21}$: C, 52.2; H, 7.5; N, 19.7. Found: C, 52.8; H, 8.1; N, 19.7.

***t*-Butyloxycarbonyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (XIII).** The protected dodecapeptide amide XII (18.8 g) was dissolved in trifluoroacetic acid (120 ml). After about 15 min, most of the trifluoroacetic acid was evaporated *in vacuo* and the syrupy residue diluted with ether (500 ml). The precipitate was collected, washed with ether, and dried *in vacuo* over sodium hydroxide. The free amine, trifluoroacetate (19.0 g), has no well-defined melting point; it softens at about 135°, darkens from 240°, and decomposes at 304°. On paper chromatograms a single component (R_f^A 0.66) was revealed by ultraviolet absorption and by ninhydrin. This material (12.35 g) was dissolved in dimethylformamide (500 ml); triethylamine (0.70 ml) and *t*-butyloxycarbonyl-β-benzyl-L-aspartic acid *p*-nitrophenyl ester[4,13] (3.35 g) were added to the solution and 2 hr later a second portion (3.35 g) of the active ester and triethylamine was added (0.35 ml). The mixture was allowed to stand overnight at room temperature. After the addition of more active ester (3.35 g) and triethylamine (0.35 ml), the mixture was concentrated *in vacuo* to about 100 ml. A final portion of the active ester (3.35 g) and triethylamine (0.14 ml) were added, and the mixture was left at room temperature overnight. Most of the solvent was removed *in vacuo* and the residue diluted with ethyl acetate (160 ml). The protected tridecapeptide was filtered, washed with ethyl acetate, and dried first in air and then *in vacuo* at 40°. The product (13.05 g, 94.5%) softens at 140°, darkens from 240°, and melts at 308° dec. From ethanol, crystals were obtained which darken from 255° and melt at 315° dec, $[\alpha]^{25}D$ −27° (*c* 2, acetic acid).

Anal. Calcd for $C_{82}H_{132}N_{24}O_{24}$: C, 53.6; H, 7.6; N, 18.3. Found (on the crude product): C, 54.3; H, 7.2; N, 18.5.

A sample of the fully protected tridecapeptide amide XIII (40 mg) was dissolved in acetic acid (4 ml); a 10% palladium on charcoal catalyst (40 mg) was added and the mixture hydrogenated for 2 days. The catalyst was filtered off and the acetic acid removed by evaporation from the frozen state. The residue was dissolved in acetic acid (1 ml), and a solution of hydrogen chloride in acetic acid (2.6 *N*, 0.5 ml) was added. Soon an oily precipitate formed. After about 0.5 hr the mixture was diluted with ether, and the precipitate, which solidified, was washed with ether. The white solid material (hydrochloride) was shown to be homogeneous on paper chromatograms; R_f^A 0.32, R_f^B 0.28. The spots were revealed with ninhydrin and with the Sakaguchi reagent. On paper electrophoresis in collidine–acetate (pH 7) the material travels as a single band toward the cathode. It is indistinguishable from the C-terminal thrombic fragment of porcine secretin,[7] and the three peptides obtained on hydrolysis of the free tridecapeptide with trypsin show the same behavior on paper chromatograms as the corresponding tryptic fragments of the natural compound.[7]

N^{α}-*t*-Butyloxycarbonyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (XIV). The protected tridecapeptide XIII (13.0 g) was dissolved in trifluoroacetic acid (100 ml). After about 15 min at room temperature most of the trifluoroacetic acid was removed *in vacuo*, and the residue was triturated with ether (900 ml). The free amine–trifluoroacetate was filtered, washed with ether, and dried *in vacuo* over sodium hydroxide (13.3 g); R_f^A 0.78 (yellow with ninhydrin).

(12) An excess of 11 equiv of hydrobromic acid per mole of peptide was present in the precipitate. *Cf.* ref 5b, footnote 23.

(13) Crystals from ethanol, mp 80–81°. On recrystallization from ethyl acetate–hexane, a higher melting form, mp 104–106°, was obtained; $[\alpha]^{25}D$ −36° (*c* 2, dimethylformamide containing 1% acetic acid). *Anal.* Calcd for $C_{22}H_{24}N_2O_8$: C, 59.4; H, 5.4; N, 6.3. Found: C, 59.1; H, 5.4; N, 6.2.

To a solution of the trifluoroacetate in dimethylformamide (145 ml), *t*-butyloxycarbonyl-nitro-L-arginine 2,4-dinitrophenyl ester (6.0 g, prepared in the same manner as the corresponding benzyloxycarbonyl derivative) was added followed by triethylamine (1.0 ml). In the following 7 hr more triethylamine (a total of 1.1 ml) was added in several portions to maintain the slight alkalinity of the mixture. After about 24 hr at room temperature more active ester (2.0 g) and triethylamine (0.7 ml) were added, and after an additional day most of the solvent was removed *in vacuo*. The residue was triturated with ethyl acetate (about 1 l.) and the solid material filtered and washed with ethyl acetate and ether. After drying in air and *in vacuo* at 40°, the crude protected tetradecapeptide (13.75 g) softens at 150°, darkens from 250°, and melts at about 300° dec.

The crude material (0.75 g) was purified by countercurrent distribution in the solvent system of *n*-butyl alcohol–pyridine–acetic acid–water (4:2:1:7). The solution containing the crude product was placed in the first three tubes of a Craig apparatus (10-ml phases) and the distribution was carried out through 100 transfers. Essentially all the material was found in a band corresponding to a distribution coefficient of 11, and the experimental curve was found to be practically identical with the curve calculated for this K value. The recovered material (0.54 g)[14] softens and darkens as the crude product; its decomposition point is about 305°. On slow evaporation of the solvents used in the distribution, crystals were obtained; $[\alpha]^{24}D$ −39° (*c* 2, acetic acid); $\lambda_{max}^{95\%\ EtOH}$ 269 mμ ($E^{1cm}_{1\%}$240).

Anal. Calcd for $C_{88}H_{143}N_{29}O_{27}$: C, 51.8; H, 7.1; N, 19.9. Found: C, 52.2; H, 7.8; N, 19.9; and in a second preparation: C, 51.4; H, 7.8; N, 19.9.

A solution of XIV (8.2 g) in trifluoroacetic acid (40 ml) was kept at room temperature for 15 min. The trifluoroacetic acid was removed *in vacuo* and the residue triturated with ether. The trifluoroacetate was washed with ether and dried *in vacuo* over sodium hydroxide, weight 8.1 g. It softens at 140° and melts at 250° dec. *Anal.* Calcd for NF: N, 19.8; F, 2.8. Found: N, 20.4; F, 3.3. In the system *n*-butyl alcohol–acetic acid–water (4:1:5), a single spot is detected by its ultraviolet absorption and with ninhydrin, R_f 0.70.

A sample of the fully protected tetradecapeptide amide XIV (200 mg) was dissolved in acetic acid (10 ml) and the mixture hydrogenated in the presence of 10% palladium on charcoal catalyst (200 mg) for 2 days. The catalyst was removed by filtration and the acetic acid removed by evaporation from the frozen state. The fluffy white residue was dissolved in trifluoroacetic acid (4 ml) and left at room temperature for 15 min. Ether was added and the precipitate (trifluoroacetate) was collected by filtration and dried *in vacuo* to give material which traveled as a single band on paper electrophoresis (pH 1.9, formic acid–acetic acid). After hydrolysis with constant boiling hydrochloric acid at 110° for 19 hr *in vacuo*, a sample gave the following ratios of amino acids: Arg, 2.7; Asp, 1.1; Ser, 0.9; Ala, 1.0; Leu, 4.0; Glu, 2.2; Gly, 1.1; and Val, 1.2.

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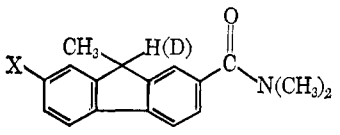
(14) In the similar purification of 12 g of the crude protected tetradecapeptide XIV, 10.75 g of purified material was recovered.

Communications to the Editor

A Kinetic Model for Mechanisms of Base-Catalyzed Hydrogen–Deuterium Exchange between a Carbon Acid and Deuterated Medium[1]

Sir:

Previous papers reported four stereochemical courses for the base-catalyzed hydrogen–deuterium exchange reaction at asymmetric carbon in systems I or II.[2]



I, X = H
II, X = NO_2

Values of k_e/k_α (one-point rate constant for isotopic exchange over that for racemization) indicated that electrophilic substitution can occur with net retention ($k_e/k_\alpha > 1$), total racemization ($k_e/k_\alpha = 1$), net inversion ($0.5 < k_e/k_\alpha < 1.0$), or net isoracemization (net racemization without exchange, $k_e/k_\alpha < 0.5$).[3] In this and the following communication,[4] we report two independent methods for determining the contributions of the individual stereochemical pathways which compose the classes of k_e/k_α values.

The first dissection of the over-all stereochemical result of an exchange reaction of a protio carbon acid with a deuterated solvent (greater than 99% deuterated) utilizes a kinetic scheme which assumes only that, at low concentrations of starting protio carbon acid, the generated deuterio carbon acid never becomes reprotonated. Symbols such as H_+ and D_- indicate the isotope attached to carbon and the sign of rotation of the particular enantiomer. All possible courses of reaction are defined by the scheme's four rate constants, k_1 (exchange with retention), k_2 (exchange with inversion), k_3 (inversion without exchange, defined as *isoinversion*), and k_4 (inversion of exchanged material). Solution of the four simultaneous first-order rate

$$\begin{array}{ll} H_+ \xrightarrow{k_1} D_+ & H_- \xrightarrow{k_2} D_+ \\ H_+ \xrightarrow{k_2} D_- & H_- \xrightarrow{k_3} H_+ \\ H_+ \xrightarrow{k_3} H_- & D_+ \xrightarrow{k_4} D_- \\ H_- \xrightarrow{k_1} D_- & D_- \xrightarrow{k_4} D_+ \end{array}$$

equations of the scheme with H_+ as starting material provides eq 1–4, where $[H_+]$ and $[D_-]$ represent mole fractions at time t, $\beta = k_1 + k_2$, and $\gamma = k_1 + k_2 + 2k_3$. Substitution of eq 1–4 into an expression for

(1) This research was supported by the U. S. Army Research Office, Durham, N. C. The authors extend their thanks.

(2) (a) D. J. Cram and L. Gosser, *J. Am. Chem. Soc.*, **86**, 5445 (1964); (b) D. J. Cram and L. Gosser, *ibid.*, **86**, 2950 (1964).

(3) For a review, see D. J. Cram, "Fundamentals of Carbanion Chemistry," Academic Press Inc., New York, N. Y., 1965, Chapter 3.

(4) W. T. Ford, E. W. Graham, and D. J. Cram, *J. Am. Chem. Soc.*, **89**, 690 (1967).