[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Synthesis of Two Protected Hexapeptides Containing the N-Terminal and C-Terminal Sequences of Arginine-Vasopressin[1]

BY PANAYOTIS G. KATSOYANNIS, DUANE T. GISH, GEORGE P. HESS AND VINCENT DU VIGNEAUD

RECEIVED DECEMBER 30, 1957

Two protected hexapeptides, one containing the N-terminal and the other the C-terminal sequence of arginine-vasopressin, have been prepared, namely, S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine and carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide hydrobromide. The synthesis of these two compounds in highly purified form was made possible by the removal of a by-product formed in the synthesis of the smaller asparaginyl peptides used as intermediates. This by-product appears to be an anhydro derivative of the expected peptides.

A recent paper from this Laboratory[2] described a synthetic approach to arginine-vasopressin through the coupling of S-benzyl-N-carbobenzoxy-L-cysteinyl - L - tyrosyl - L - phenylalanyl - L - glutaminyl-L-asparagine or its O-tosyl derivative with S - benzyl - L - cysteinyl - L - prolyl - L - arginylglycinamide, followed by sodium–liquid ammonia reduction and subsequent oxidation. In this work it appeared that the coupling yielded a by-product or by-products which upon subsequent reduction and oxidation formed a compound or compounds which could not be separated from arginine-vasopressin by the techniques available. Therefore, the products of reactions employing various coupling reagents to bring about formation of the peptide bond through the carboxyl group of asparagine have now been subjected to closer scrutiny.

It was of considerable interest to find[3] that some carboxyl group-activating reagents, namely, tetraethyl pyrophosphite[4] and N,N′-dicyclohexylcarbodiimide,[5] lead to what appear to be intramolecular dehydrations during the formation of asparaginyl peptide bonds[6] and, to a lesser extent, during the formation of glutaminyl peptide bonds. Coupling of carbobenzoxy-L-asparagine with S-benzyl-L-cysteine methyl ester by the tetraethyl pyrophosphite procedure leads to a product, from which two neutral crystalline compounds have been separated on the basis of different solubility properties. One of the compounds, m.p. 199–200°, obtained in 35% yield, is the expected product, carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteine methyl ester. The second compound, m.p. 128–129°, obtained in 27% yield, is, on the basis of elementary analysis, an anhydro compound of the protected dipeptide ester. Coupling of carbobenzoxy-L-asparagine with S-benzyl-L-cysteine methyl ester by the carbodiimide procedure gives results almost identical with those obtained when tetraethyl pyrophosphite is used.

The infrared spectrum of the anhydro compound indicates the presence of a nitrile grouping.[7] This suggests that the tetraethyl pyrophosphite and the N,N′-dicyclohexylcarbodiimide have dehydrated the amide group of asparagine. However, no further work has been carried out on the identification of this compound.

In contrast to the use of tetraethyl pyrophosphite and N,N′-dicyclohexylcarbodiimide, activation of the carboxyl group of carbobenzoxy-L-asparagine through formation of a mixed anhydride with isovaleryl chloride essentially according to the method of Vaughan and Osato[8] did not lead to the formation of the anhydro compound in the synthesis of carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteine methyl ester.[9]

The preparation of carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteine methyl ester has been reported independently from two other laboratories. Boissonnas, *et al.*,[10] report a m.p. of 189° for the product obtained when tetraethyl pyrophosphite was used and a m.p. of 196° for the product of the coupling with the use of diethyl chlorophosphite. Rudinger, *et al.*,[11] obtained a product melting at 194–195° when the coupling was effected by the phosphorazo method or by formation of the mixed anhydride with *sec*-butylchloroformate.

The coupling of tosyl-L-glutaminyl-L-asparagine with S-benzyl-L-cysteine benzyl ester with the use of N,N′-dicyclohexylcarbodiimide gave the desired tripeptide ester in 40% yield and the corresponding anhydro compound in 15% yield. Both the tripeptide ester and its anhydro compound could be converted to their respective crystalline hydrazides, which again differed in composition by one molecule of water.

Synthesis of carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine methyl ester by the tetraethyl pyrophosphite procedure afforded the desired product in 70% yield and only a small amount (5%) of the crystalline anhydro compound. Couplings of carbobenzoxy-L-glutamine with S-benzyl-L-cysteine methyl ester or L-asparaginyl-S-benzyl-L-cysteine methyl ester with the use of N,N′-dicyclohexyl-

(1) This work was supported in part by a grant (H-1675) from the National Heart Institute, Public Health Service, for which we wish to express our appreciation.

(2) P. G. Katsoyannis, D. T. Gish and V. du Vigneaud, THIS JOURNAL, **79**, 4516 (1957).

(3) D. T. Gish, P. G. Katsoyannis, G. P. Hess and R. J. Stedman, *ibid.*, **78**, 5954 (1956).

(4) G. W. Anderson, J. Blodinger and A. D. Welcher, *ibid.*, **74**, 5309 (1952).

(5) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

(6) Alteration of asparagine during peptide synthesis using tetraethyl pyrophosphite has been independently noted by C. Ressler, [THIS JOURNAL, **78**, 5956 (1956)].

(7) This interpretation of the infrared spectrum was made by Dr. R. C. Gore of the American Cyanamid Company on his examination of the infrared spectrum which we submitted to him.

(8) J. R. Vaughan, Jr., and R. L. Osato, THIS JOURNAL, **73**, 5553 (1951).

(9) This method had been shown to give a satisfactory yield of this protected dipeptide ester by Dr. R. J. Stedman in this Laboratory.

(10) R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud and J.-P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).

(11) J. Rudinger, J. Honzl and M. Zaoral, *Coll. Czechoslov. Chem. Communs.*, **21**, 202 (1956).

carbodiimide or through formation of the mixed anhydride with isobutyl chlorocarbonate resulted in high yields of the desired dipeptide or tripeptide ester, and the formation of detectable amounts of the corresponding anhydro compounds was not observed.

The preparation of these peptide derivatives, free of contamination by the corresponding anhydro compounds, has made possible the preparation, free of by-products, of several compounds of potential use in the synthesis of arginine-vasopressin. These peptides include L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine, S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine and carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide hydrobromide, and they were prepared in the following manner.

The L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine was obtained by treatment of tosyl-L-glutaminyl - L - asparaginyl - S - benzyl - L -cysteine benzyl ester with sodium in liquid ammonia, benzylation of the resulting sulfhydryl compound and purification of the product by countercurrent distribution.

S - Benzyl - N - tosyl - L - cysteinyl - L - tyrosine[12,13] was coupled with L-phenylalanine methyl ester by the carbodiimide procedure to give S-benzyl - N - tosyl - L - cysteinyl - L - tyrosyl - L-phenylalanine methyl ester in crystalline form in high yield. Treatment of the tripeptide methyl ester with hydrazine hydrate in methanol yielded the hydrazide and saponification of the ester gave S - benzyl - N - tosyl - L - cysteinyl - L - tyrosyl-L-phenylalanine. Conversion of the hydrazide to the azide and coupling of the latter compound with L - glutaminyl - L - asparaginyl - S - benzyl - L-cysteine gave the protected hexapeptide, S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine, in crystalline form.

Carbobenzoxy - L - glutaminyl - L - asparaginyl-S-benzyl-L-cysteine methyl ester was converted to the corresponding hydrazide[10] and then to the azide, which was coupled with the monohydrobromide of L-prolyl-L-arginylglycinamide.[14] Purification of the product by countercurrent distribution gave the protected hexapeptide amide, carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-propyl-L-arginylglycinamide hydrobromide, in 73% yield.

Experimental[15,16]

Carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteine Methyl Ester. A. Tetraethyl Pyrophosphite. (1) Standard Procedure.—S-Benzyl-L-cysteine methyl ester hydrochloride[17] (10.4 g., 0.04 mole) was suspended in 200 ml. of tetrahydrofuran and 6.15 ml. (10% excess) of triethylamine was added. The mixture was stirred and cooled and the triethylamine hydrochloride was filtered off and washed with tetrahydrofuran, the washings being added to the filtrate. After removal of the solvent *in vacuo* the ester remained as an oil, to which 10.7 g. (0.04 mole) of carbobenzoxy-L-asparagine,[18] 13.0 g. (0.05 mole) of tetraethyl pyrophosphite and 28 ml. of diethyl phosphite were added. The mixture was heated at 100°. Crystalline material began to separate after about 25 minutes and the mixture soon became a thick mass. After 40 minutes the mixture was cooled and the product was collected, washed successively with cold diethyl phosphite, *N* HCl, water, *N* $NaHCO_3$ and water and dried over P_2O_5 *in vacuo;* yield 7.52 g., m.p. 188–191°. Recrystallization from 50% acetic acid gave 6.62 g. (35%), m.p. 199–200°.

The addition of 75 ml. of *N* HCl to the diethyl phosphite filtrate from the crude product caused the separation of *the anhydro compound of carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteine methyl ester* in crystalline form. The material was collected, washed successively with *N* HCl, water, *N* $NaHCO_3$ and water and dried over P_2O_5 *in vacuo;* yield 5.4 g., m.p. 118–128°. Recrystallization from 50% acetic acid gave 4.94 g. (27%), m.p. 126–128°. A sample was recrystallized from 50% acetic acid for analysis, m.p. 128–129°; $[\alpha]^{24}D$ −42.1° (*c* 1, acetic acid).

Anal. Calcd. for $C_{23}H_{25}O_5N_3S$: C, 60.6; H, 5.53; N, 9.22; S, 7.04. Found: C, 60.6; H, 5.73; N, 9.11; S, 7.08.

(2) Amide Procedure.—S-Benzyl-L-cysteine methyl ester (4.5 g., 0.02 mole) in 14 ml. of diethyl phosphite was allowed to react with 6.5 ml. (0.025 mole) of tetraethyl pyrophosphite at 100° for 10 minutes. Carbobenzoxy-L-asparagine (5.33 g., 0.02 mole) was then added to the mixture and the temperature was maintained at 90° for 1 hr. The reaction products were then isolated as described in the preceding section; 3.3 g. of crystalline dipeptide ester, m.p. 192–195° (recrystallization from 50% acetic acid gave 3.0 g., m.p. 199–200°) and 3.0 g. of the corresponding anhydro compound, m.p. 126–128° (recrystallization from 50% acetic acid gave 2.6 g., m.p. 128–129°) were obtained.

B. Carbodiimide Procedure.—To a solution of 2.66 g. (0.01 mole) of carbobenzoxy-L-asparagine in 10 ml. of dimethylformamide were added 2.25 g. (0.01 mole) of S-benzyl-L-cysteine methyl ester in 60 ml. of tetrahydrofuran and 2.3 g. (0.011 mole) of N,N′-dicyclohexylcarbodiimide. The reaction was allowed to proceed for 5.5 hr. at 0°. The resulting precipitate was collected, washed with tetrahydrofuran and dried. The filtrate (F) was treated as described subsequently.

The precipitate (4.0 g.) was suspended in 35 ml. of dimethylformamide and approximately 2 g. of N,N′-dicyclohexylurea was removed by filtration. Addition of about 30 ml. of 0.5 *N* HCl to the filtrate gave a precipitate which was washed successively with water, 5% $KHCO_3$ and water and then dried and triturated with tetrahydrofuran; wt. 2.15 g., m.p. 189–192°. Recrystallization from 50% acetic acid gave 1.8 g. (39%) of carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteine methyl ester, m.p. 197–199°.

The filtrate (F) was diluted with 15 ml. of dimethylformamide and the tetrahydrofuran was removed *in vacuo*. The crystalline N,N′-dicyclohexylurea was removed by filtration. Addition of 0.5 *N* HCl to the filtrate yielded a precipitate which was washed successively with water, 5% $KHCO_3$ and water and dried. The crude product weighed 1.3 g., m.p. 123–125°. Recrystallization from 50% acetic acid gave 1.2 g. (26%) of the anhydro compound of carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteine methyl ester, m.p. 128–129°; $[\alpha]^{24}D$ −41.8° (*c* 1, acetic acid).

C. Mixed Anhydride Procedure.—To a suspension of 8.9 g. (0.033 mole) of carbobenzoxy-L-asparagine and 3.33 g. (0.033 mole) of triethylamine in 200 ml. of tetrahydrofuran, cooled at −5°, was added 4.04 ml. (0.033 mole) of isovaleryl chloride. The reaction mixture was stirred at −5° for 30 minutes and then mixed with a tetrahydrofuran solution of S-benzyl-L-cysteine methyl ester prepared from 8.75 g. of its hydrochloride with one equivalent of triethylamine. The mixture soon formed a thick mass which was partially broken up with a spatula and stirred for 2 hr. at −5°. The reaction mixture was then allowed to stand overnight in the refrigerator. The precipitate was sepa-

(12) J. Honzl and J. Rudinger, *Coll. Czechoslov. Chem. Communs.*, **20** 1190 (1955).

(13) V. du Vigneaud, M. F. Bartlett and A. Jöhl, THIS JOURNAL, **79**, 5572 (1957).

(14) D. T. Gish and V. du Vigneaud, *ibid.*, **79**, 3579 (1957).

(15) Capillary melting points were determined for all compounds and are corrected.

(16) The authors are indebted to Mr. Joseph Albert for carrying out the microanalyses.

(17) Prepared by the Fischer esterification procedure [*Ber.*, **34**, 433 (1901)]; yield 84%; m.p. 154°. Boissonnas, *et al.* (see ref. 10), report m.p. 150°.

(18) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

rated by filtration, washed successively with tetrahydrofuran, *N* $NaHCO_3$, water, *N* HCl and water and dried. On recrystallization from 300 ml. of 50% aqueous acetic acid, 8.75 g. (55%) of crystalline carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteine methyl ester, m.p. 199–200°, was obtained.

The tetrahydrofuran mother liquor of the crude dipeptide ester was concentrated *in vacuo* to dryness. The residue was washed with ether and then extracted twice with *N* $NaHCO_3$. On acidification of the combined extracts, 2.4 g. of crystalline carbobenzoxy-L-asparagine, m.p. 163–166°, was recovered. The yield of product and recovery of starting material accounted for almost 90% of the starting carbobenzoxy-L-asparagine and no material of m.p. 128–129° was isolated from the reaction mixture.

S-Benzyl-L-cysteine Benzyl Ester Hydrochloride.[19]—The method of Miller and Waelsch[20] was applied to the preparation of this ester. A mixture of 17.4 g. (0.11 mole) of benzenesulfonic acid, 50 ml. of redistilled benzyl alcohol and 21 g. (0.10 mole) of S-benzyl-L-cysteine[21] was heated in an oil-bath at 110° until solution occurred. The excess benzyl alcohol was removed by distillation *in vacuo* at 120–130°. The crystalline residue was cooled and washed thoroughly with ether. The procedure was repeated with 50 ml. of benzyl alcohol and 5.5 g. of benzenesulfonic acid. The residue after removal of the excess benzyl alcohol crystallized on the addition of ether. The S-benzyl-L-cysteine benzyl ester benzenesulfonate was recrystallized by dissolving it in 400 ml. of boiling acetone and adding 600 ml. of ether to the cooled solution; wt. 28 g., m.p. 134–135°.

For the preparation of S-benzyl-L-cysteine benzyl ester hydrochloride, 15.7 g. of the S-benzyl-L-cysteine benzyl ester benzenesulfonate was suspended in 40 ml. of chloroform, 3.95 ml. of triethylamine was added and the mixture was stirred until solution occurred. With the addition of 300 ml. of ether, triethylamine benzenesulfonate precipitated and was separated by filtration. To the filtrate was added 200 ml. of ether saturated with dry HCl, whereupon crystalline S-benzyl-L-cysteine benzyl ester hydrochloride precipitated and was separated by filtration; wt. 9.5 g. After recrystallization from acetone–ether the compound melted at 129–130°.

Anal. Calcd. for $C_{17}H_{19}O_2NS \cdot HCl$: C, 60.4; H, 5.97; N, 4.15. Found: C, 60.3; H, 6.07; N, 4.17.

Tosyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine Benzyl Ester.—To a solution of 7.5 g. (0.018 mole) of tosyl-L-glutaminyl-L-asparagine[22] in 40 ml. of redistilled diethylformamide were added a solution of 5.4 g. (0.018 mole) of S-benzyl-L-cysteine benzyl ester (obtained by evaporation of the chloroform–ether filtrate following removal of triethylamine benzenesulfonate, as described in the preceding section) in 40 ml. of dimethylformamide and 4 g. (10% excess) of N,N′-dicyclohexylcarbodiimide. The reaction mixture was stirred overnight, a few drops of acetic acid were added and the precipitate of N,N′-dicyclohexylurea was filtered off. The product was precipitated in amorphous form from the filtrate by the addition of water, separated by filtration, washed with water, triturated with aqueous $KHCO_3$, washed again with water and dried; wt. 9.2 g., m.p. 192–195°. The crude product was then dissolved in 50 ml. of dimethylformamide and the tosyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine benzyl ester was precipitated with 150 ml. of ether; wt. 5 g., m.p. 214°. On reprecipitation from dimethylformamide–ether 4 g. (40%) was obtained, m.p. 226–228°. For analysis, a sample was again precipitated from dimethylformamide–ether; m.p. 228–229°, $[\alpha]^{23}D$ −30.7° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{33}H_{39}O_8N_5S_2$: C, 56.8; H, 5.63; N, 10.0; S, 9.17. Found: C, 56.6; H, 5.75; N, 10.0; S, 9.04.

Anhydro Compound of Tosyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine Benzyl Ester.—The ether was removed from the dimethylformamide–ether mother liquor of the crude product from the preceding section by concentration *in vacuo* and the anhydro compound of tosyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine benzyl ester was precipitated by addition of water. The product was filtered off, washed with water and dried; wt. 3.8 g., m.p. 188–190°. The product crystallized from 75% aqueous acetone as needles (1.8 g. (15%)), m.p. 210–211°, $[\alpha]^{23}D$ −33.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{33}H_{37}O_7N_5S_2$: C, 58.0; H, 5.40; N, 10.3; S, 9.43. Found: C, 58.0; H, 5.56; N, 10.3; S, 9.14.

Tosyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine Hydrazide.—A mixture of 0.7 g. (0.001 mole) of tosyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine benzyl ester, 20 ml. of methanol and 1.6 ml. of hydrazine hydrate was heated under reflux for 2.5 hr. and cooled. The crystalline hydrazide was filtered off, washed with methanol and dried; wt. 0.52 g. (84%), m.p. 224–225°, $[\alpha]^{24}D$ −32.6° (*c* 1, dimethylformamide). For analysis a sample was recrystallized from dimethylformamide–water; m.p. 224–225°.

Anal. Calcd. for $C_{26}H_{35}O_7N_7S_2$: C, 50.2; H, 5.67; N, 15.8. Found: C, 50.1; H, 5.83; N, 15.4.

Hydrazide Prepared from the Anhydro Compound of Tosyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine Benzyl Ester.—In 15 ml. of warm methanol was suspended 0.7 g. (0.001 mole) of the anhydro compound of tosyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine benzyl ester. Hydrazine hydrate (1.6 ml.) was added and the mixture was allowed to stand at room temperature overnight. The crystalline hydrazide was separated by filtration, washed with 50% aqueous methanol and dried; wt. 0.42 g. (67%), m.p. 210–212°, $[\alpha]^{24}D$ −17.2° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{26}H_{33}O_6N_7S_2$: C, 51.7; H, 5.51; N, 16.2. Found: C, 51.7; H, 5.64; N, 16.0.

Carbobenzoxy-L-glutamine.[18]—L-Glutamine (14.6 g., 0.1 mole) was dissolved in 25 ml. of 4 *N* NaOH and 100 ml. of *N* $NaHCO_3$. The resultant *p*H was 9.5. The mixture was cooled in an ice-bath and stirred mechanically. Then 24.5 g. of carbobenzoxy chloride in purified dioxane (total volume, 60 ml.) and 30 ml. of 4 *N* NaOH were added alternately in five equal portions over approximately 1 hr. The *p*H was thus maintained between 9.0 and 9.7. After the final addition the cold mixture was stirred for an additional 45 minutes. The *p*H of the mixture was adjusted to 7.0 and the solution was extracted three times with ethyl acetate and then acidified to *p*H 1. The crystalline product was collected, washed with cold water and dried over P_2O_5 *in vacuo;* wt. 25.0 g., m.p. 125–133°. Recrystallization from water gave 21.7 g. (77%), m.p. 133–137°.

Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine Methyl Ester. A. Tetraethyl Pyrophosphite Method.—S-Benzyl-L-cysteine methyl ester hydrochloride (2.61 g., 0.01 mole) was converted to the free base according to the procedure already described. A mixture of this material, 2.8 g. (0.01 mole) of carbobenzoxy-L-glutamine, 3.14 g. (0.012 mole) of tetraethyl pyrophosphite and 7 ml. of diethyl phosphite was heated at 95°. After about 25 minutes a crystalline product began to precipitate. After 40 minutes the mixture was cooled, and the carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine methyl ester was separated by filtration, washed successively with cold diethyl phosphite, *N* HCl, water, *N* $NaHCO_3$ and water and dried; wt. 3.61 g., m.p. 196–198°. Recrystallization from 50% acetic acid gave 3.41 g. (70%), m.p. 203–204° (lit.[23] 204°), $[\alpha]^{23}D$ −28.0° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{24}H_{29}O_6N_3S$: C, 59.1; H, 5.99; N, 8.62. Found: C, 59.2; H, 6.07; N, 8.53.

A second product was precipitated on the addition of 25 ml. of *N* HCl to the diethyl phosphite mother liquor. The crude product was collected, washed successively with water, *N* $NaHCO_3$ and water and dried. After several recrystallizations of this material from ethyl acetate–hexane, 0.25 g. (5%) of the anhydro compound of carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine methyl ester was obtained; m.p. 103–104°, $[\alpha]^{23}D$ −35.0° (*c* 1, dimethylformamide).

(19) The compound was first prepared by this method in this Laboratory by Dr. J. M. Swan.

(20) H. K. Miller and H. Waelsch, THIS JOURNAL, **74**, 1092 (1952).

(21) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 110 (1939).

(22) J. M. Swan and V. du Vigneaud, THIS JOURNAL, **76**, 3110 (1954).

(23) R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud and J.-P. Waller, *Helv. Chim. Acta*, **39**, 1421 (1956).

Anal. Calcd. for $C_{24}H_{27}O_5N_3S$: C, 61.4; H, 5.75; N, 8.9. Found: C, 61.7; H, 5.90; N, 8.8.

B. Carbodiimide Procedure.—To a solution of 0.9 g. (0.004 mole) of S-benzyl-L-cysteine methyl ester in 20 ml. of tetrahydrofuran was added 1.12 g. (0.004 mole) of carbobenzoxy-L-glutamine and 0.83 g. (0.004 mole) of N,N′-dicyclohexylcarbodiimide. The reaction was allowed to proceed for 2.5 hr. at room temperature and for 0.5 hr. at 0°. The precipitated material was filtered off, washed three times with 15-ml. portions of tetrahydrofuran and once with 20 ml. of ether, and dried to a fine powder (2.85 g.). This product was suspended in 30 ml. of dimethylformamide and stirred for 1 hr., when the undissolved N,N′-dicyclohexylurea was filtered off. Addition of ether to the dimethylformamide solution afforded 1.48 g. (76%) of carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteine methyl ester, m.p. 201–202°. On recrystallization of a sample from 50% acetic acid a m.p. of 203–204° was obtained. None of the corresponding anhydro compound was found in the tetrahydrofuran mother liquor of the dipeptide.

C. Mixed Anhydride Procedure.—A solution of carbobenzoxy-L-glutamine (1.12 g., 0.004 mole) and triethylamine (0.40 g., 0.004 mole) in tetrahydrofuran was cooled to −10° and isobutyl chlorocarbonate (0.55 g., 0.004 mole) was added. After 10 minutes a solution of S-benzyl-L-cysteine methyl ester in 20 ml. of tetrahydrofuran cooled to −10° was added to the reaction mixture. The reaction was allowed to proceed for 1 hr. at −10° and for 1.5 hr. at room temperature. The crystalline precipitate was filtered off and washed with two 30-ml. portions of tetrahydrofuran and then with ether. The crystals were washed successively with *N* HCl, water, 5% $NaHCO_3$ and water, and dried to give 1.7 g. (89%) of the protected dipeptide ester, m.p. 199–202°. On recrystallization of a sample from 50% acetic acid a m.p. of 203–204° was obtained.

L-Asparaginyl-S-benzyl-L-cysteine Methyl Ester Hydrobromide.—Carbobenzoxy-L-asparaginyl-S-benzyl-L-cysteine methyl ester (7.0 g.) was suspended in 50 ml. of acetic acid which had been saturated with HBr, and the mixture was stirred at room temperature for 1 hr. For removal of the acetic acid and excess HBr, the mixture was concentrated *in vacuo*, the residual oil was dissolved in 20 ml. of methanol and the solvent was distilled *in vacuo*. The hydrobromide was crystallized and recrystallized from methanol–ether; wt. 5.8 g. (95%), m.p. 124–126°. For analysis, a sample was again recrystallized from methanol–ether, m.p. 125–126°, $[\alpha]^{23}D$ −22.5° (*c* 1, water).

Anal. Calcd. for $C_{15}H_{21}O_4N_3S \cdot HBr$: C, 42.9; H, 5.28; N, 10.0. Found: C, 42.9; H, 5.39; N, 9.90.

Carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine Methyl Ester. A. Carbodiimide Procedure.—L-Asparaginyl-S-benzyl-L-cysteine methyl ester hydrobromide (1.8 g., 0.0043 mole) was suspended in 12 ml. of tetrahydrofuran, and triethylamine (0.45 g., 0.0045 mole) was added. The mixture was stirred for 30 minutes and cooled, and the triethylamine hydrobromide was filtered off. To the filtrate were added a solution of carbobenzoxy-L-glutamine (1.22 g., 0.0043 mole) in 12 ml. of tetrahydrofuran and N,N′-dicyclohexylcarbodiimide (0.97 g., 0.0047 mole), and the reaction was allowed to proceed for 8 hr. at 0°. Water (2.5 ml.) was added and the reaction mixture was allowed to warm to room temperature. The precipitate was collected by filtration and suspended twice in 90% tetrahydrofuran, in which N,N′-dicyclohexylurea dissolved. The crystalline product was then filtered off, washed with ether and dried; wt. 2.2 g., m.p. 234–236°. Recrystallization from 50% acetic acid yielded 2.0 g. (78%), m.p. 239–240°, $[\alpha]^{23}D$ −38.4° (*c* 0.8, acetic acid). Boissonnas, *et al.*,[10] reported m.p. 239°, $[\alpha]^{20}D$ −38.6° (*c* 2.4, acetic acid); Rudinger, *et al.*,[11] reported m.p. 236–237°.

B. Mixed Anhydride Procedure.—A solution of carbobenzoxy-L-glutamine (1.96 g., 0.007 mole) and triethylamine (0.98 ml., 0.007 mole) in 20 ml. of tetrahydrofuran was cooled to −10° and isobutyl chlorocarbonate (0.92 ml., 0.007 mole) was added. The mixture was stirred for 10 minutes and L-asparaginyl-S-benzyl-L-cysteine methyl ester (prepared from 2.94 g. (0.007 mole) of the hydrobromide as already described) in 20 ml. of tetrahydrofuran was added. The resulting thick mass was stirred at −10° for 1 hr. and at room temperature for 1 hr. and then cooled. The crystalline product was collected and washed successively with tetrahydrofuran, ether and water and dried; wt. 3.7 g., m.p. 231–233°. Recrystallization from 50% acetic acid gave 3.4 g. (81%), m.p. 239–240°, $[\alpha]^{22}D$ −38.0° (*c* 1.2, acetic acid).

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine Methyl Ester.—To a suspension of L-phenylalanine methyl ester hydrochloride (2.4 g., 0.0115 mole) in 30 ml. of tetrahydrofuran was added 1.7 ml. of triethylamine (1.23 g., 0.0122 mole), and the mixture was stirred for 30 minutes before the triethylamine hydrochloride was filtered off. To the filtrate were added S-benzyl-N-tosyl-L-cysteinyl-L-tyrosine[13] (5.6 g., 0.0106 mole) in 30 ml. of tetrahydrofuran and N,N′-dicyclohexylcarbodiimide (2.42 g., 0.0117 mole). The reaction was allowed to proceed for 4 hr. at room temperature, the N,N′-dicyclohexylurea was filtered off and the tetrahydrofuran was replaced by ethyl acetate. The ethyl acetate solution was washed successively with *N* HCl, water, *N* $NaHCO_3$ and water, and dried over anhydrous magnesium sulfate. Concentration of the ethyl acetate solution and addition of petroleum ether afforded 6.8 g. of the crystalline protected tripeptide, m.p. 176–178°. A sample was recrystallized from methanol–water for analysis; m.p. 180–181°, $[\alpha]^{22}D$ −42.6° (*c* 1.2, methanol).

Anal. Calcd. for $C_{36}H_{39}O_7N_3S_2$: C, 62.7; H, 5.68; N, 6.09. Found: C, 62.6; H, 5.85; N, 6.10.

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine Hydrazide.—To a solution of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine methyl ester (2.5 g., 0.0036 mole) in 50 ml. of methanol was added 3.0 ml. of hydrazine hydrate. The solution was allowed to stand at room temperature overnight and then cooled to 0°. The crystalline hydrazide was filtered off, washed with four 10-ml. portions of cold methanol and dried; wt. 2.2 g. (89%), m.p. 240–241°. A sample was recrystallized from 90% hot aqueous tetrahydrofuran; m.p. 242–243°, $[\alpha]^{24}D$ +6.8° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{35}H_{39}O_6N_5S_2$: C, 61.0; H, 5.68; N, 10.1. Found: C, 60.8; H, 5.77; N, 9.90.

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine.—To a solution of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine methyl ester (1.0 g., 0.0015 mole) in 20 ml. of acetone was added, with stirring, 4.6 ml. of *N* NaOH over a period of 20 minutes at ice-bath temperature. The reaction mixture was allowed to stand at room temperature for 40 minutes, diluted with 18 ml. of water, filtered and acidified. On removal of the acetone *in vacuo* the product crystallized and was separated and washed with cold water. The material was purified by dissolving it in 130 ml. of water containing 3 equivalents of NaOH and extracting the solution with ethyl acetate. The crystalline product obtained upon acidification was washed with cold water, dried and recrystallized from ethyl acetate–petroleum ether; wt. 0.85 g. (88%), m.p. 205–206°, $[\alpha]^{23}D$ −37.5° (*c* 1, methanol).

Anal. Calcd. for $C_{35}H_{37}O_7N_3S_2$: C, 62.2; H, 5.49; N, 6.24. Found: C, 61.9; H, 5.55; N, 6.19.

L-Glutaminyl-L-asparaginyl-S-benzyl-L-cysteine.—N-Tosyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine benzyl ester (2.8 g., 0.004 mole) was dissolved in 100 ml. of liquid ammonia, and approximately 0.9 g. of sodium (0.04 mole) was added in portions until a permanent blue color was obtained. A few crystals of ammonium chloride were added to discharge the color and then 0.6 ml. of benzyl chloride (20% excess) was added over a period of 35 minutes with stirring. After the reaction mixture was stirred for another 30 minutes, 2.0 g. of ammonium chloride (0.04 mole) was added and the ammonia was removed by evaporation.

The residue was extracted with three 25-ml. portions of warm glacial acetic acid and inorganic salts were removed by filtration. Addition of 180 ml. of ether to the filtrate precipitated the crude product (1.77 g.). The material was purified by countercurrent distribution[24] with 2-butanol–0.5% acetic acid (400 transfers, *K* 0.41); wt. 1.23 g. (68%), $[\alpha]^{25}D$ −26° (*c* 1, water).

Anal. Calcd. for $C_{19}H_{27}O_6N_5S$: N, 15.4. Found: N, 15.0.

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl - L - asparaginyl - S - benzyl - L - cysteine.—S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine hydrazide (1.0 g., 0.0015 mole) was dissolved in a mixture of 50 ml. of water, 2.5 ml. of concd. HCl and 75 ml. of glacial

(24) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

acetic acid. The solution was cooled to 0°, and a solution of 0.12 g. of sodium nitrite in 2.5 ml. of water was added in 0.5-ml. portions over a 10-minute period. The reaction was allowed to proceed for 10 minutes at 0°, 100 ml. of ice-cold water was added to the reaction mixture and the tripeptide azide was filtered off and washed successively with cold water, 5% $NaHCO_3$ and water. A solution of the azide in 19 ml. of cold tetrahydrofuran was added to an aqueous solution (14 ml.) of L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine (0.45 g., 0.001 mole) and triethylamine (0.1 g., 0.001 mole) and stirred for 24 hr. at 4° and for 12 hr. at room temperature. After addition of 50 ml. of water to the reaction mixture, the precipitate was filtered off and washed with 25 ml. of hot ethyl acetate and 50 ml. of 0.5 *N* HCl and dried; wt. 0.9 g., m.p. 209–211°. A sample was recrystallized from 90% aqueous tetrahydrofuran–water; m.p. 213–214°, $[\alpha]^{24}D$ −24.4° (*c* 0.9, 90% aqueous tetrahydrofuran).

Anal. Calcd. for $C_{54}H_{62}O_{12}N_8S_3$: C, 58.4; H, 5.62; N, 10.1. Found: C, 58.3; H, 5.66; N, 9.79.

This protected hexapeptide also was prepared by coupling S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine with L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine by the mixed anhydride method with isobutyl chlorocarbonate in a mixture of tetrahydrofuran and dimethylformamide. The yield of purified product, m.p. 213–214°, was 59%.

Carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide Hydrobromide.—The dihydrobromide of L-prolyl-L-arginylglycinamide[14] (1.92 g., 0.004 mole) was dissolved in 4 ml. of dimethylformamide, and 0.67 ml. of triethylamine was added with stirring. The mixture was cooled and the triethylamine hydrobromide was removed by filtration and washed with 1.5 ml. of cold dimethylformamide in three portions, the washings being added to the main filtrate. The monohydrobromide was purified by two precipitations from dimethylformamide solution with chloroform and then washed with chloroform and ether and dried *in vacuo* over P_2O_5 and NaOH. This material was dissolved in 15 ml. of dimethylformamide, and carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteine azide (2.8 g., 0.0046 mole) in 10 ml. of dimethylformamide was added. The mixture was stirred at room temperature for 3 hr. The crude product was then precipitated with ethyl acetate, filtered off, washed with ethyl acetate and chloroform, and dried.

The material was purified by countercurrent distribution in 2-butanol–0.5% acetic acid (130 transfers), and the Sakaguchi color reaction[25] was used for preparation of the distribution curve. The solutions from the tubes containing the protected hexapeptide amide hydrobromide (*K* 0.88) were concentrated on the rotary evaporator[26] and then lyophilized to give 2.87 g. (73%) of white, amorphous powder, $[\alpha]^{22}D$ −34.8° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{40}H_{56}O_{10}N_{12}S \cdot HBr$: C, 49.2; H, 5.87; N, 17.2. Found: C, 48.8; H, 6.07; N, 17.0.

(25) A. A. Albanese and J. E. Frankston, *J. Biol. Chem.*, **159**, 185 (1945).

(26) L. C. Craig, J. C. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).

NEW YORK, N. Y.

[CONTRIBUTION FROM THE SHIONOGI RESEARCH LABORATORY, SHIONOGI & CO., LTD.]

A Partial Synthesis of Caranine[1]

BY KEN'ICHI TAKEDA, KATSUMI KOTERA AND SATOSHI MIZUKAMI

RECEIVED NOVEMBER 21, 1957

Reduction of the chlorohydrin IV, obtained by chlorination of lycorine (I), with zinc in acetic acid afforded three products: caranine (III), isocaranine (XII) and the diene derivative XIII. The dehydration reactions of caranine and isocaranine and the basic double-bond migrations of the diene derivative XIII were examined, and structures were established for these products. Lithium aluminum hydride was found to be the most effective reagent for conversion of the chlorohydrin to caranine.

Caranine is an alkaloid isolated from the bulbs of some *Amaryllidaceae plants* by W. C. Wildman and co-workers in 1955.[2,3] The structure III was proposed by them[4,5] recently. During the course of our investigations on the stereochemistry of dihydrolycorine, it was found that monodesoxydihydrolycorine (XIV) was identical with α-dihydrocaranine, obtained by the hydrogenation of caranine over platinum in acetic acid. This fact provided unequivocal evidence that caranine has the same carbon skeleton as lycorine.[6,7] Hence, it seemed of interest of synthesize caranine from lycorine (I).[8,9]

For this purpose, we first attempted to synthesize caranine through the reduction of the monotosylate II of lycorine as shown in Chart I, but neither the mono- nor the ditosylate was obtained under the various conditions. The next approach was the preparation of lycorine chlorohydrin by a procedure analogous to that used for the chlorination of dihydrolycorine.[10] Treatment of lycorine with phosphoryl chloride containing a trace of concentrated hydrochloric acid at 37° for 45 minutes gave the chlorohydrin IV, $C_{16}H_{16}ClNO_3$, m.p. 150° dec., in a 10% yield along with a large amount of anhydrolycorinium salt V.

The structure of chlorohydrin IV was deduced from its conversion to monoacetyllycorine (VI), m.p. 231–232°, with potassium acetate in acetone. This substance on acetylation or alkaline hydrolysis furnished diacetyllycorine (VII) or lycorine (I), respectively, and this monoacetyl derivative also was obtained by the direct acetylation of lycorine with acetyl chloride in pyridine at 0°, along with diacetyllycorine.

On the other hand, S. Uyeo and his co-workers[11] obtained on partial hydrolysis of diacetyllycorine

(1) The outline of this paper was read at the 16th International Congress of Pure and Applied Chemistry in Paris, France, July 19, 1957.

(2) L. H. Mason, E. R. Puschett and W. C. Wildman, THIS JOURNAL, **77**, 1253 (1955).

(3) H. M. Fales, E. W. Warnhoff and W. C. Wildman, *ibid.*, **77**, 5885 (1955).

(4) E. W. Warnhoff and W. C. Wildman, *Chemistry & Industry*, 348 (1956).

(5) E. W. Warnhoff and W. C. Wildman, THIS JOURNAL, **79**, 2192 (1957).

(6) K. Takeda and K. Kotera, *Chemistry & Industry*, 347 (1956).

(7) K. Takeda and K. Kotera, *Pharm. Bull. Japan*, **5**, 234 (1957).

(8) Configuration of the hydroxyl groups in lycorine was assigned according to that of dihydrolycorine (*cf.* refs. 6, 7).

(9) Caranine was obtained from lycorine by the action of sodium in amyl alcohol by W. C. Wildman (*cf.* ref. 5, footnote 14a).

(10) K. Takeda and K. Kotera, unpublished observation.

(11) Y. Nakagawa, S. Uyeo and H. Yajima, *Chemistry & Industry*, 1238 (1956).