pyridine nucleotide dependent metallodehydrogenases (PMD). This group of PMD is now joined with other members of the respiratory metalloenzyme group: hemes, metalloflavoproteins, copper oxidases and carbonic anhydrase. The salient position of metals in oxidative catalysis is thereby extended and the major oxidative pathway thus seems to involve a metal in each enzymatic step.

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

Synthesis of a Protected Tetrapeptide Amide Containing the Carboxyl Terminal Sequence of Lysine–Vasopressin[1]

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The synthesis of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycine ethyl ester and related compounds starting from N$^{\epsilon}$-tosyl-L-lysine is described. This protected tetrapeptide ester was obtained by two different routes, one involving as the final step the coupling of S-benzyl-N-carbobenzoxy-L-cysteine with L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycine ethyl ester and the other, the coupling of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-proline with N$^{\epsilon}$-tosyl-L-lysylglycine ethyl ester. The product obtained after conversion of the protected tetrapeptide ester to the corresponding amide and removal of the carbobenzoxy group has been used as an intermediate in studies on the synthesis of lysine–vasopressin.

In connection with synthetic studies on the peptide hormone lysine–vasopressin, isolated from hog posterior pituitaries,[2,3] the tetrapeptide derivative S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L lysylglycinamide was desired. A primary consideration in the selection of this derivative was the protection of the ϵ-amino group of the lysine molecule by a group which would remain unaffected under the conditions of a peptide synthesis involving carbobenzoxy intermediates, but which could be conveniently removed later in the synthetic steps to vasopressin. The tosyl (*p*-toluenesulfonyl) group was chosen, since it is not attacked by any of the usual decarbobenzoxylating reagents except sodium in liquid ammonia, which cleaves it readily.[4] Since the employment of the latter reagent was already anticipated for effecting the final stage of the vasopressin synthesis, the tosyl group was particularly convenient for our purpose.

The series of reactions employed for the synthesis of the protected tetrapeptide amide is given in Fig. 1. N$^{\epsilon}$-Tosyl-L-lysine (I) was prepared in high yield by treatment of the copper complex of L-lysine with tosyl chloride in aqueous acetone and removal of the copper with hydrogen sulfide, essentially according to the procedure employed by Kurtz[5] for the preparation of analogous benzenesulfonyl derivatives of lysine and ornithine. Erlanger, Sachs and Brand[6] have recently prepared N$^{\delta}$-tosyl-L-ornithine by similar means and have used this compound in synthetic peptide work.

The α-carbobenzoxy derivative of N$^{\epsilon}$-tosyl-L-lysine was coupled with glycine ethyl ester according to the procedure of Young, *et al.*,[7] with ethylene chlorophosphite as the coupling reagent. Removal of the carbobenzoxy group was effected with hydrogen bromide in glacial acetic acid[8] at room temperature to give the crystalline hydrobromide of N$^{\epsilon}$-tosyl-L-lysylglycine ethyl ester (II) in 90% yield. This substance was used directly in the next synthetic step, but the tosyl dipeptide ester IIa could be obtained by treating the hydrobromide with potassium carbonate. This ester underwent slow decomposition in solution to give 3-(ω-tosylaminobutyl)-2,5-piperazinedione. A dipeptide ester identical with IIa in melting point and optical rotation was also obtained from N$^{\epsilon}$-tosyl-L-lysine-N$^{\alpha}$-carboxy anhydride and glycine ethyl ester essentially by the method of Bailey,[9] *i.e.*, by reaction in chloroform at low temperature.

II was coupled with N-carbobenzoxy-L-proline to give crystalline N-carbobenzoxy-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycine ethyl ester (III). The coupling reagent used with best results was *o*-phenylene chlorophosphite,[10] and it is noteworthy that in this case the use of this reagent resulted in higher yields (70–75%) than when either ethylene chlorophosphite or tetraethyl pyrophosphite was used (45–50%).

Decarbobenzoxylation of III with a 2 *M* solution of hydrogen bromide in glacial acetic acid gave the hydrobromide of L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycine ethyl ester, from which the tosyl tripeptide ester IV was obtained by treatment with triethylamine. The conversion of IV to S-benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycine ethyl ester (V) was accomplished by coupling with S-ben-

(1) A preliminary report of part of this work was made recently [M. F. Bartlett, A. Jöhl, R. Roeske, R. J. Stedman, F. H. C. Stewart, D. N. Ward and V. du Vigneaud, THIS JOURNAL, **78**, 2905 (1956)].

(2) E. A. Popenoe, H. C. Lawler and V. du Vigneaud, *ibid.*, **74**, 3713 (1952).

(3) V. du Vigneaud, H. C. Lawler and E. A. Popenoe, *ibid.*, **75**, 4880 (1953).

(4) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

(5) A. C. Kurtz, *ibid.*, **180**, 1253 (1949).

(6) B. F. Erlanger, H. Sachs and E. Brand, THIS JOURNAL, **76**, 1806 (1954).

(7) R. W. Young, K. H. Wood, R. J. Joyce and G. W. Anderson, Abstracts, 128th Meeting of the American Chemical Society, 73-O (1955).

(8) G. W. Anderson, J. Blodinger and A. D. Welcher, THIS JOURNAL, **74**, 5309 (1952); D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952); I. Schumann and R. A. Boissonnas, *Helv. Chim. Acta*, **35**, 2237 (1952).

(9) J. L. Bailey, *J. Chem. Soc.*, 3461 (1950).

(10) G. W. Anderson and R. W. Young, THIS JOURNAL, **74**, 5307 (1952).

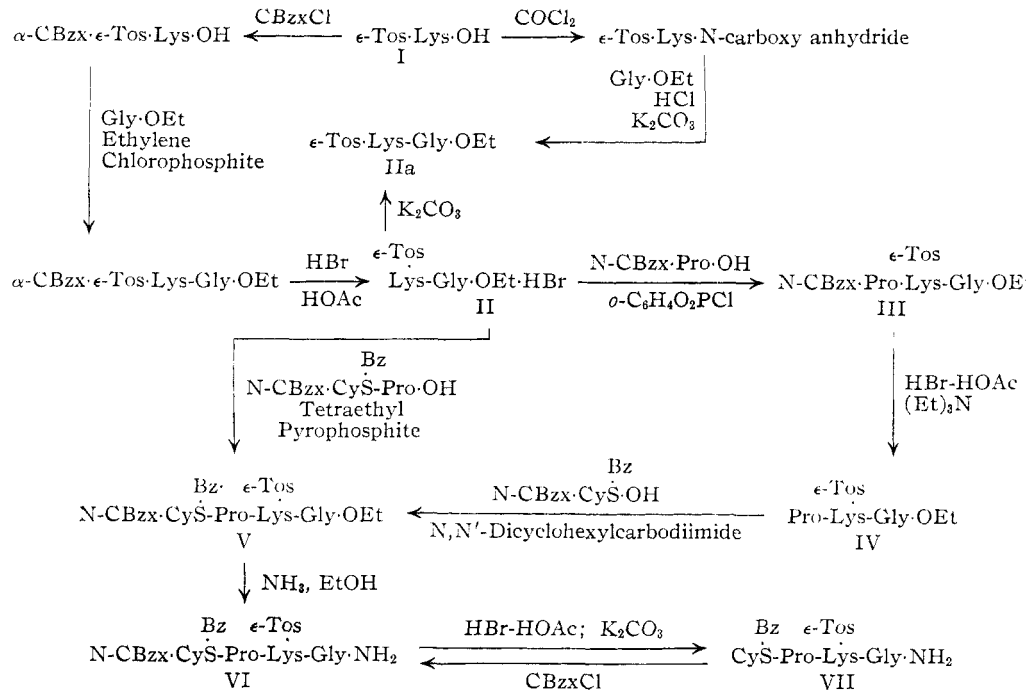

Fig. 1.—Synthesis of S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinamide from N$^{\epsilon}$-tosyl-L-lysine. The system of abbreviations for the amino acid residues follows generally that employed recently by Erlanger, Sachs and Brand.[6]

zyl-N-carbobenzoxy-L-cysteine[11] with the use of *o*-phenylene chlorophosphite,[10] or, more conveniently, by use of the method introduced by Sheehan and Hess[12] which employs N,N′-dicyclohexylcarbodiimide. The optical rotation and melting point of the crystalline protected tetrapeptide ester V obtained from either of these procedures were the same within experimental error.

The tosyl tripeptide ester IV was also coupled with S-benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteine according to the pyrophosphite method of Anderson, Blodinger and Welcher[8] to give the *p*-nitrobenzyloxycarbonyl derivative of S-benzyl-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycine ethyl ester.

An alternative route was developed for preparation of the protected tetrapeptide ester V, in which S-benzyl-N-carbobenzoxy-L-cysteinyl-L-proline was coupled as an oil with the tosyl dipeptide ester hydrobromide II by the pyrophosphite procedure. For this approach S-benzyl-N-carbobenzoxy-L-cysteine was first coupled with either the methyl or the benzyl ester of L-proline and the resulting protected dipeptide methyl or benzyl ester was hydrolyzed to give S-benzyl-N-carbobenzoxy-L-cysteinyl-L-proline, which was then converted into the tetrapeptide derivative V. The optical rotation and melting point of the product thus obtained indicated its identity with that prepared from the tosyl tripeptide ester IV.

In this connection it is noteworthy that an attempt to prepare the methyl ester of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-proline by the standard azide procedure from S-benzyl-N-carbobenzoxy-L-cysteinyl azide and L-proline methyl ester gave S-benzyl-N-carbobenzoxy-L-cysteinamide in 50% yield. Similar examples of this curious behavior in azide couplings have been observed by Prelog and Wieland,[13] Hegedüs,[14] and recently by Roberts.[15]

Treatment of the protected tetrapeptide ester V with saturated ethanolic ammonia gave a high yield of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysylglycinamide (VI), which was obtained as a microcrystalline powder. The desired tetrapeptide amide VII was prepared from VI by decarbobenzoxylation with hydrogen bromide in glacial acetic acid followed by treatment with potassium carbonate. It was obtained as a hygroscopic, amorphous material, which was characterized by reconversion to the crystalline carbobenzoxy derivative VI.

Experimental[16-18]

N$^{\epsilon}$-Tosyl-L-lysine (I).—A solution of 24 g. of L-lysine monohydrochloride in 1500 ml. of water was refluxed with 40 g. of cupric carbonate for 2 hours. The reaction mixture was filtered while hot and the solid was washed with 150 ml. of hot water. To the cooled filtrate were added 42 g. of $NaHCO_3$ and a solution of 37.8 g. of tosyl chloride in 1500 ml. of acetone, and the solution was stirred vigorously for 10 hours. The copper complex was collected and washed thoroughly with water, acetone and ether. After being dried in air the light blue powder from several experiments weighed 30–33

(11) C. R. Harington and T. H. Mead, *Biochem. J.*, **30**, 1598 (1936).

(12) J. C. Sheehan and G. P. Hess, THIS JOURNAL, **77**, 1067 (1955).

(13) V. Prelog and P. Wieland, *Helv. Chim. Acta*, **29**, 1128 (1946).

(14) B. Hegedüs, *ibid.*, **31**, 737 (1948).

(15) C. W. Roberts, THIS JOURNAL, **76**, 6203 (1954).

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

(17) The authors are indebted to Mr. Joseph Albert for carrying out the analyses.

(18) The specific rotations of the amino acids used as starting materials were as follows: L-cystine, $[\alpha]^{21}D$ −215° (*c* 1.02, 1 *N* HCl); L-proline, $[\alpha]^{21}D$ −84.3° (*c* 1, H_2O); L-lysine monohydrochloride, $[\alpha]^{21}D$ +11.1° (*c* 2.78, H_2O).

g. (average yield 74%) and melted at 238–240° dec. when the temperature was raised at the rate of 3° per minute.

The finely ground complex (30 g.) was suspended in 500 ml. of boiling water and a stream of H_2S was passed in with vigorous stirring until the complex was decomposed (*ca.* 0.5 hour). Boiling was continued until the excess H_2S was removed. Charcoal (5 g.) and 15 ml. of 6 *N* HCl were added and the solution was filtered through a fine paper. The filtrate was brought to *p*H 6 with 4 *N* NaOH and the product was collected after a few hours and washed with water and ethanol. The yield was 21.2 g. (78%) and the m.p. 233–234° dec. A sample was purified by precipitation from HCl solution with NaOH; m.p. 237–238° dec., $[\alpha]^{21}D$ +13.6° (*c* 3, 2 *N* HCl).

Anal. Calcd. for $C_{13}H_{20}O_4N_2S$: C, 52.0; H, 6.71. Found: C, 52.0; H, 6.91.

N^{α}-Carbobenzoxy-N^{ϵ}-tosyl-L-lysine.—ε-Tosyl-L-lysine (12 g.) was dissolved in 40 ml. of 1 *N* NaOH. The solution was cooled to 0° and stirred while a total of 8.5 g. of carbobenzoxy chloride and 50 ml. of 1 *N* NaOH was added in four equal portions at 15-minute intervals. Stirring was continued for 30 minutes and enough water was added to dissolve the oily precipitate of the sodium salt of the product. The solution was then washed with ether and acidified with 6 *N* HCl. The product was extracted with ethyl acetate and the extract was dried over magnesium sulfate. The solvent was then completely removed *in vacuo*, the product being obtained as a pale green, viscous oil. The yield of crude material was 18 g.

The compound was used in this state for the next step; it could, however, be obtained from ethyl acetate–hexane in crystalline form in 94% yield, m.p. 85–88°, $[\alpha]^{21}D$ −13.3° (*c* 1, 5% $NaHCO_3$).

Anal. Calcd. for $C_{21}H_{26}O_6N_2S$: N, 6.45; S, 7.37. Found: N, 6.24; S, 7.14.

N^{α}-Carbobenzoxy-N^{ϵ}-tosyl-L-lysylglycine Ethyl Ester.—A mixture of 18 g. of the crude N^{α}-carbobenzoxy-N^{ϵ}-tosyl-L-lysine, 4.2 g. of triethylamine, 4.3 g. of freshly distilled ethyl glycinate[19] and 5.3 g. of ethylene chlorophosphite[7] in 30 ml. of diethyl phosphite was heated for an hour on a boiling water-bath. It was then diluted with 500 ml. of water. The precipitated oil solidified in a short time and was filtered off and washed with 5% aqueous $NaHCO_3$ and water. The crude material was dried and then dissolved in 120 ml. of hot chloroform; the solution was filtered and diluted with 150 ml. of hexane, from which the product crystallized on cooling; yield 17.7 g. (85% based on N^{ϵ}-tosyl-L-lysine), m.p. 151.5–153.5°, $[\alpha]^{22}D$ −5.0° (*c* 1.7, $CHCl_3$).

Anal. Calcd. for $C_{25}H_{33}O_7N_3S$: C, 57.8; H, 6.4.0 Found: C, 57.5; H, 6.55.

N^{ϵ}-Tosyl-L-lysylglycine Ethyl Ester Hydrobromide (II).—N^{α}-Carbobenzoxy-N^{ϵ}-tosyl-L-lysylglycine ethyl ester (20 g.) was dissolved in 180 ml. of 2 *M* HBr in glacial acetic acid, and the solution was allowed to stand at room temperature for 1.5 hours. Dry ether (600 ml.) was then added with swirling, when the hydrobromide was precipitated as a crystalline solid. It was filtered off, washed with ether and dissolved in the minimum amount of hot ethanol. The filtered solution was diluted with ether until crystallization commenced and then cooled to 0° for several hours; yield 16.2 g. (90%), m.p. 175–177°, $[\alpha]^{21}D$ +16.7° (*c* 2, H_2O).

For analysis, the compound was again recrystallized from ethanol–ether and melted at 176.5–177.5°.

Anal. Calcd. for $C_{17}H_{27}O_5N_3S \cdot HBr$: C, 43.7; H, 6.05; N, 9.00. Found: C, 43.6; H, 6.14; N, 8.79.

N^{ϵ}-Tosyl-L-lysine-N^{α}-carboxy Anhydride.—N^{ϵ}-Tosyl-L-lysine (8 g.) was thoroughly dried and suspended in 175 ml. of freshly purified dioxane in a 500-ml., two-necked flask fitted with a gas inlet tube and a condenser protected with a drying tube. Phosgene was bubbled in slowly while the solution was stirred with a magnetic stirrer at 40°.[20] When all of the solid had dissolved, the passage of phosgene was discontinued and the slightly green solution was stirred at 40° for several hours. A stream of dry nitrogen was passed through the stirred solution for at least 24 hours, until the odor of phosgene could no longer be detected. The dioxane was evaporated *in vacuo* and the green residual oil was dissolved in 30 ml. of ethyl acetate; the solution was warmed to 50° and 25 ml. of hexane was added. The white, crystalline product was collected, washed with ethyl acetate–hexane, and dried *in vacuo* at room temperature; yield 7.5 g. (86%), m.p. 98–100°. After two recrystallizations from ethyl acetate–hexane the m.p. was 101–103°.

Anal. Calcd. for $C_{14}H_{18}O_5N_2S$: N, 8.59; S, 9.82. Found: N, 8.18; S, 9.85.

The compound is relatively unstable; it polymerizes within a month when stored in an evacuated desiccator.

N^{ϵ}-Tosyl-L-lysylglycine Ethyl Ester Hydrochloride.—N^{ϵ}-Tosyl-L-lysine-N^{α}-carboxy anhydride (3.26 g.) was dissolved in 50 ml. of freshly distilled tetrahydrofuran. The flask was protected from moisture and placed in a Dewar flask containing acetone at −30°. A solution of 2.03 g. of glycine ethyl ester and 1.1 g. of triethylamine in 50 ml. of chloroform at −30° was added. The bath temperature was kept at −30° for 3 hours and then allowed to warm to 0° overnight. The solution was kept at 40° for 15 minutes before being evaporated *in vacuo* at 35°. Dry toluene was added and evaporated several times. Approximately 10 ml. of a 15% solution of dry hydrogen chloride in ethanol was added and the flask was shaken vigorously; the hydrochloride precipitated immediately. It was crystallized from solution in 30 ml. of absolute ethanol by addition of 30 ml. of ether. The yield was 2.2 g. (52%), m.p. 200–203°. An additional 0.4 g., m.p. 197–198°, was obtained from the mother liquor. A sample recrystallized twice from ethanol–ether had m.p. 204–206°, $[\alpha]^{21}D$ +18.9° (*c* 3.4, H_2O).

Anal. Calcd. for $C_{17}H_{27}O_5N_3S \cdot HCl$: N, 9.94. Found: N, 9.86.

N^{ϵ}-Tosyl-L-lysylglycine Ethyl Ester (IIa).—A sample of N^{ϵ}-tosyl-L-lysylglycine ethyl ester hydrochloride (0.5 g.), prepared as indicated in the preceding section, was converted to N^{ϵ}-tosyl-L-lysylglycine ethyl ester by treatment with aqueous K_2CO_3 and extracted into chloroform. After crystallization from ethyl acetate–hexane, the tosyl dipeptide ester (0.4 g.) melted at 85.5–87°, $[\alpha]^{21}D$ −6.0° (*c* 3, $CHCl_3$).

For analysis, the compound was recrystallized from ethyl acetate–hexane; m.p. 86.5–88°.

Anal. Calcd. for $C_{17}H_{27}O_5N_3S$: C, 53.0; H, 7.06; S, 8.32. Found: C, 53.3; H, 7.17; S, 8.40.

IIa was also obtained from the tosyl dipeptide hydrobromide. N^{α}-Carbobenzoxy-N^{ϵ}-tosyl-L-lysylglycine ethyl ester (5 g.) was treated with HBr in glacial acetic acid by the procedure already described. The crude crystalline hydrobromide was dissolved in water (200 ml.) and the solution was washed with ether. A slight excess of 2 *M* aqueous K_2CO_3 was added and the precipitated tosyl dipeptide ester was extracted with ethyl acetate. The extract was dried over magnesium sulfate and evaporated to dryness *in vacuo*. The product remained as an oil which solidified in a short time and was recrystallized from ethyl acetate–hexane (1:1); yield 2.4 g. (65%), m.p. 85–87°, $[\alpha]^{21.5}D$ −6.0° (*c* 2, $CHCl_3$).

When a solution of IIa in ethyl acetate was allowed to stand for 2 weeks, crystals of 3-(ω-tosylaminobutyl)-2,5-piperazinedione were deposited, m.p. 180–185°. After recrystallization from ethanol the compound melted at 185–187°.

Anal. Calcd. for $C_{15}H_{21}O_4N_3S$: N, 12.4. Found: N, 12.2.

This substance was also obtained when the tosyl dipeptide ester was treated with saturated ethanolic ammonia for 2 days at room temperature.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-proline. A. *Via* the Methyl Ester.—A mixture of 17.3 g. of S-benzyl-N-carbobenzoxy-L-cysteine,[11] 7.8 g. (1.2 equiv.) of L-proline methyl ester, 7.6 g. (1.2 equiv.) of ethylene chlorophosphite, 6.06 g. (1.2 equiv.) of triethylamine and 35 ml. of diethyl phosphite was heated for 40 minutes on a boiling water-bath. Water (700 ml.) was added and the mixture was allowed to stand at 0° overnight. The supernatant liquid was decanted and the residual oil was triturated with 5% $NaHCO_3$ solution. The oil was dissolved in 200 ml. of ethyl acetate and the solution was washed with 5% $NaHCO_3$, 2 *N* HCl and water, and dried over magnesium sulfate. Evaporation of the solvent *in vacuo* left a viscous sirup (23.5 g.). The theoretical yield is 22.8 g. This crude ester was dissolved in ethanol to give 200 ml. of solution, and 65 ml. of 1 *N* NaOH was added. The solution was stirred at 25° until hydrolysis was complete (65 minutes). The alcohol was evaporated at 30°

(19) G. Hillmann, *Z. Naturforsch.*, **1**, 682 (1946).

(20) For a leading reference on this method of preparing N-carboxy anhydrides, see A. C. Farthing, *J. Chem. Soc.*, 3213 (1950).

in vacuo and replaced by 200 ml. of water. The solution (*p*H 8) was washed with 50 ml. of ethyl acetate and made acid to congo red paper. The precipitated oil was taken up in ethyl acetate–ether (1:1), extracted with 5% $NaHCO_3$ and re-extracted from the acidified solution with ethyl acetate–ether. The solution was dried over magnesium sulfate and evaporated *in vacuo*. The residue, a glass, weighed 18.3 g. (83% from S-benzyl-N-carbobenzoxy-L-cysteine); neut. equiv. calcd. 443; found 437, 432, 438.

B. *Via* the Benzyl Ester.—A solution of S-benzyl-N-carbobenzoxy-L-cysteine (3.45 g.) and L-proline benzyl ester hydrochloride (2.66 g., 1.1 equiv.) in 20 ml. of diethyl phosphite was treated with triethylamine (1.67 ml., 1.2 equiv.) and tetraethyl pyrophosphite (4.9 ml., 2 equiv.). It was heated for 30 minutes on a steam-bath, poured into water (300 ml.) and allowed to stand at 0° for 1 hour. The supernatant liquid was poured off and the residual gum was triturated three times with 5% aqueous $NaHCO_3$. It was dissolved in ethyl acetate–ether (2:1, 70 ml.), washed three times with dilute HCl, four times with 5% $NaHCO_3$ and once with water. The solution was dried over sodium sulfate and evaporated *in vacuo* to a colorless gum. The yield was 4.69 g. (88%).

The benzyl ester (2.13 g.) was dissolved in 30 ml. of ethanol and 1 *N* NaOH (4.4 ml., 1.1 equiv.) was added. The mixture was allowed to stand for 1.75 hours at 25°; the *p*H was then adjusted to 7 with dilute HCl and the solution was evaporated *in vacuo*. The residue was treated with 30 ml. of 1 *N* HCl and extracted with ethyl acetate. The peptide derivative was extracted from the ethyl acetate with 5% aqueous $NaHCO_3$, washed with ethyl acetate and reprecipitated by acidification to *p*H 2 with concentrated HCl. The resulting oil was extracted four times with ethyl acetate and the solution was dried over sodium sulfate and evaporated *in vacuo*, leaving the protected dipeptide as an oil which was converted to a dry foam when dried for several hours at 2 mm.; yield 1.60 g. (80% from S-benzyl-N-carbobenzoxy-L-cysteine).

Before measurement of the neutral equivalent and optical rotation, a specimen was dried for several days as a foam at 2 mm.; $[\alpha]^{22}D$ −80.8° (*c* 3, $CHCl_3$); neut. equiv. 442.

N^{ϵ}-Tosyl-L-lysine Ethyl-Ester Hydrochloride.—Dry HCl was passed into a mixture of 5 g. of N^{ϵ}-tosyl-L-lysine and 35 ml. of ethanol until the solution became clear. Ethanol (50 ml.) was added and the solution was heated under reflux for several hours. It was then evaporated to a small volume *in vacuo* and diluted with ether. The resultant oil solidified on cooling. One recrystallization from ethanol–ether gave 5.0 g. of product (83%), m.p. 125–127°. After recrystallization from ethyl acetate, the m.p. was 128–130°, $[\alpha]^{23.5}D$ +8.5° (*c* 1, H_2O).

For analysis the compound was recrystallized from ethanol–ether; m.p. 129–131°.

Anal. Calcd. for $C_{15}H_{24}O_4N_2S \cdot HCl$: N, 7.67; S, 8.77. Found: N, 7.67; S, 8.72.

N-Carbobenzoxy-L-proline.—This compound was prepared essentially as described by Abderhalden and Hanson.[21] The oily product was crystallized from ethyl acetate–hexane; m.p. 74.5–75.5°, $[\alpha]^{21}D$ −57.5° (*c* 1, 1 *N* NaOH).

N-Carbobenzoxy-L-prolyl-N^{ϵ}-tosyl-L-lysine Ethyl Ester.—To a solution of 2.65 g. of N-carbobenzoxy-L-proline in 12 ml. of diethyl phosphite was added 3.87 g. of N^{ϵ}-tosyl-L-lysine ethyl ester hydrochloride, 2.17 g. of triethylamine and 1.9 g. of *o*-phenylene chlorophosphite. The mixture was heated for an hour at 100° and diluted with 200 ml. of water. The resultant oil was extracted with warm ethyl acetate, washed with 5% aqueous $NaHCO_3$, 2 *N* HCl and water, and dried over magnesium sulfate. The solution was evaporated *in vacuo* to small volume and hexane was added. The product thus obtained was a white, crystalline solid, m.p. 118–119°, and weighed 4.0 g. (68%). It was recrystallized twice from ethyl acetate; m.p. 122.5–123.5°, $[\alpha]^{22}D$ −29.1° (*c* 1, $CHCl_3$).

Anal. Calcd. for $C_{28}H_{37}O_7N_3S$: C, 60.1; H, 6.67; N, 7.50. Found: C, 60.3; H, 6.90; N, 7.40.

N-Carbobenzoxy-L-prolyl-N^{ϵ}-tosyl-L-lysine.—A solution of 2.0 g. of N-carbobenzoxy-L-proline and 0.8 g. of triethylamine in 16 ml. of tetrahydrofuran was cooled to −10° and 1.1 g. of isobutyl chlorocarbonate[22] was added with stirring. The solution was stirred for 5 minutes, when a solution of 2.4 g. of N^{ϵ}-tosyl-L-lysine in 8 ml. of 1 *N* NaOH was added and stirring was continued for 30 minutes at −10°. The solution was allowed to come to room temperature and extracted with ethyl acetate. The extract was washed with dilute HCl and water, and dried over magnesium sulfate. It was evaporated to a small volume *in vacuo* and diluted with ether with vigorous shaking. The resultant thick oil was washed by decantation with ether and dissolved in ethyl acetate. Removal of the solvent *in vacuo* left the product as an amorphous, crisp mass. The yield was 1.0 g. (47%), $[\alpha]^{21}D$ −22.6° (*c* 1, $CHCl_3$).

Anal. Calcd. for $C_{26}H_{33}O_7N_3S$: N, 7.90. Found: N, 7.73.

The substance was characterized by conversion to the ethyl ester through treatment with a 5% ethanolic solution of HCl for 2 days at room temperature followed by evaporation *in vacuo*. After two recrystallizations from ethyl acetate the ester melted at 121–122°; mixed m.p. with authentic ester prepared as described in the preceding section, 121–122°; $[\alpha]^{21}D$ −28.3° (*c* 1, $CHCl_3$).

N-Carbobenzoxy-L-prolyl-N^{ϵ}-tosyl-L-lysylglycine Ethyl Ester (III).—A mixture of 5.3 g. of N-carbobenzoxy-L-proline, 4.4 g. of triethylamine, 10.0 g. of N^{ϵ}-tosyl-L-lysylglycine ethyl ester hydrobromide and 3.8 g. of *o*-phenylene chlorophosphite in 30 ml. of diethyl phosphite was heated for 1 hour on a boiling water-bath and the reaction mixture was then diluted with 500 ml. of water. The oil which was precipitated solidified in a short time and was filtered and washed thoroughly with 5% aqueous $NaHCO_3$ and water. It was dissolved in the minimum amount of boiling ethanol and filtered after addition of charcoal. Water was added until the solution was slightly turbid. On cooling of the solution slowly the product crystallized as long needles of a monohydrate which had m.p. 129–130° when heated rapidly. The anhydrous tripeptide derivative was obtained by drying this hydrate at 110° for 2 hours *in vacuo;* yield 9.9 g. (75%), m.p. 151–151.5°, $[\alpha]^{21}D$ −56.0° (*c* 1, glacial acetic acid).

The anhydrous substance reverted to the monohydrate on exposure to the atmosphere.

Anal. Calcd. for $C_{30}H_{40}O_8N_4S \cdot H_2O$: C, 56.7; H, 6.66; N, 8.82; S, 5.05. Found: C, 56.8; H, 6.70; N, 8.64; S, 5.00.

N-Carbobenzoxy-L-prolyl-N^{ϵ}-tosyl-L-lysylglycinamide.—N-Carbobenzoxy-L-prolyl-N^{ϵ}-tosyl-L-lysylglycine ethyl ester (4 g.) was dissolved in 150 ml. of warm ethanol and the solution was cooled to 0°. It was saturated with ammonia and allowed to stand, tightly stoppered, at room temperature for 2 days. The ethanol was then removed *in vacuo*. The residual oil solidified on being cooled and triturated with ethanol and was crystallized from acetonitrile as a microcrystalline powder; yield 2.6 g. (70%), m.p. 181–183°.

For analysis it was recrystallized from acetonitrile; m.p. 183–185°.

Anal. Calcd. for $C_{28}H_{37}O_7N_5S$: C, 57.2; H, 6.34; N, 11.9. Found: C, 57.0; H, 6.45; N, 11.8.

L-Prolyl-N^{ϵ}-tosyl-L-lysylglycine Ethyl Ester (IV).[23]—N-Carbobenzoxy-L-prolyl-N^{ϵ}-tosyl-L-lysylglycine ethyl ester (III) (10 g.) was dissolved in 95 ml. of 2.1 *M* hydrogen bromide in glacial acetic acid (95 ml.). After being allowed to stand for 1.5 hours at room temperature the solution was diluted with dry ether and the resultant oil was washed by decantation with ether. It was then dissolved in a little ethanol and precipitated with ether and this operation was repeated. Finally it was dissolved again in ethanol and the latter was removed *in vacuo*. The crude hydrobromide weighed 8.3 g. (91%).

IV was obtained from the hydrobromide by suspension of the latter in ethyl acetate (100 ml.), addition of triethylamine (3 ml.) and vigorous shaking of the mixture. Triethylamine hydrobromide was removed by filtration and the ethyl acetate solution was washed with water and dried over sodium sulfate. The solvent was evaporated and the oily residue crystallized from ethanol–ether as fine needles; yield 6.1 g. (78% based on III), m.p. 81–84°, $[\alpha]^{20}D$ −32.5° (*c* 2.49, ethanol).

(21) E. Abderhalden and H. Hanson, *Fermentforschung*, **15**, 382 (1937).

(22) J. R. Vaughan, Jr., and J. A. Eichler, THIS JOURNAL, **75**, 5556 (1953).

(23) The authors are indebted to Dr. Albert Jöhl of this Laboratory for the preparation of this compound.

Anal. Calcd. for $C_{22}H_{34}O_6N_4S$: C, 54.8; H, 7.10; N, 11.6. Found: C, 54.9; H, 7.00; N, 11.6.

S-Benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteinyl-L-prolyl-N$^\epsilon$-tosyl-L-lysylglycine Ethyl Ester.—A mixture of IV (802 mg.) and S-benzyl-N-*p*-nitrobenzyloxycarbonyl-L-cysteine[24] (651 mg.) was dissolved in 7 ml. of diethyl phosphite and treated with tetraethyl pyrophosphite (0.62 ml.). The mixture was heated for 50 minutes on the steam-bath and 90 ml. of water was added. The supernatant liquid was decanted after the solution had stood at 0° for 1 hour and the residual gum was triturated with three portions of 5% aqueous $NaHCO_3$. It was taken up in 50 ml. of ethyl acetate and washed three times with 25 ml. of 1 *N* HCl, nine times with 25 ml. of 5% aqueous $NaHCO_3$ and twice with 25 ml. of water. The solution was dried over sodium sulfate and evaporated under reduced pressure to a foam (1.18 g.), which was dissolved in hot ethanol (12 ml.) and crystallized by addition of water (4 ml.) and seeding with crystals initially obtained from ethanol–water on standing at room temperature. After 3 hours at room temperature, the product was collected, washed with aqueous ethanol and dried *in vacuo;* yield 851 mg. (59.7%), m.p. 110–113°. One recrystallization from ethanol (10 ml.) gave the product (772 mg.) as small elongated prisms, m.p. 114–117°, $[\alpha]^{24}D$ −20.1° (*c* 3, $CHCl_3$). For analysis, the compound was dried at 85° *in vacuo*.

Anal. Calcd. for $C_{40}H_{50}O_{11}N_6S_2$: C, 56.2; H, 5.89; N, 9.83; S, 7.50. Found: C, 56.3; H, 6.12; N, 9.63; S, 7.54.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-N$^\epsilon$-tosyl-L-lysylglycine Ethyl Ester (V). Method A.—A mixture of 8 g. of L-prolyl-N$^\epsilon$-tosyl-L-lysylglycine ethyl ester hydrobromide (oil), 4.8 g. of S-benzyl-N-carbobenzoxy-L-cysteine,[11] 3.1 g. of triethylamine and 2.6 g. of *o*-phenylene chlorophosphite in 30 ml. of diethyl phosphite was heated on a boiling water-bath for 1 hour and then diluted with a tenfold excess of water. The precipitated oil was extracted with ethyl acetate and the extract was washed thoroughly with 5% aqueous $NaHCO_3$ and water. After the solution was dried over magnesium sulfate the ethyl acetate was removed *in vacuo* and the crude product was obtained as a viscous oil. A sample was first crystallized from ethyl acetate–hexane, and by use of these crystals as seeds the main portion of the product was crystallized from ethanol. After two recrystallizations the product had m.p. 102–106° and was used for the next step; yield 6.5 g. (59%), $[\alpha]^{21}D$ −24.9° (*c* 2, $CHCl_3$).

For analysis, the compound was recrystallized repeatedly from ethanol and dried at 85° for 24 hours *in vacuo;* m.p. 110–112°, $[\alpha]^{21.5}D$ −25.5° (*c* 3, $CHCl_3$).

Anal. Calcd. for $C_{40}H_{51}O_9N_5S_2$: C, 59.3; H, 6.34; N, 8.64; S, 7.91. Found: C, 59.4; H, 6.47; N, 8.42; S, 7.78.

Method B.—L-Prolyl-N$^\epsilon$-tosyl-L-lysylglycine ethyl ester hydrobromide (oil) (9 g.) was triturated with 1.6 g. of triethylamine and *ca.* 20 ml. of tetrahydrofuran until the tosyl tripeptide ester hydrobromide had been replaced completely by crystalline triethylamine hydrobromide. The mixture was then filtered and the filter was washed with sufficient tetrahydrofuran to bring the total volume up to 50 ml. To the resultant clear solution was added 5.4 g. of S-benzyl-N-carbobenzoxy-L-cysteine and 3.6 g. of N,N′-dicyclohexylcarbodiimide.

The solution was allowed to stand at room temperature for 6 hours and the precipitated N,N′-dicyclohexylurea was filtered off. The solvent was removed *in vacuo* and the residue was diluted with water and then extracted with ethyl acetate. The extract was washed with 5% aqueous $NaHCO_3$, dilute HCl and water. It was then dried over magnesium sulfate and the solvent was removed *in vacuo*. The crude product thus obtained was recrystallized from ethanol; yield 10.2 g. (81%), m.p. 104–106°, $[\alpha]^{17.5}D$ −25.0° (*c* 1, $CHCl_3$).

Crystalline L-prolyl-N$^\epsilon$-tosyl-L-lysylglycine ethyl ester (IV) could also be used directly in this reaction and gave the tetrapeptide derivative, m.p. 108–110°. However, for practical purposes the use of the hydrobromide was preferred.

(24) V. du Vigneaud, D. T. Gish and P. G. Katsoyannis, THIS JOURNAL, **76**, 4751 (1954). The compound was kindly supplied by Dr. Gish.

Method C.—To a mixture of 6.8 g. of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-proline and 7.13 g. of N$^\epsilon$-tosyl-L-lysylglycine ethyl ester hydrobromide in 35 ml. of diethyl phosphite was added 2.12 ml. of triethylamine and 4.05 ml. (20% excess) of tetraethyl pyrophosphite. The mixture was heated on a boiling water-bath for 1 hour; 400 ml. of water was added and the mixture was allowed to stand overnight. The supernatant liquid was decanted and the oily residue was triturated with 5% aqueous $NaHCO_3$ to remove diethyl phosphite. The product was then dissolved in ethyl acetate and washed with 5% aqueous $NaHCO_3$, dilute HCl and water. Finally the solution was dried over magnesium sulfate and evaporated *in vacuo*. After recrystallization of the crude product from 40 ml. of ethanol, it melted at 98–103°. It was again recrystallized from ethanol (35 ml.) and melted at 106–109°, yield 6.0 g. (48%), $[\alpha]^{21.5}D$ −26.0° (*c* 2.96, $CHCl_3$).

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-N$^\epsilon$-tosyl-L-lysylglycinamide (VI).—A solution of 3.4 g. of V in 55 ml. of warm ethanol was cooled to 0°, saturated with ammonia and allowed to stand at room temperature for 2 days, when it was evaporated to dryness *in vacuo*. The product was obtained as an oil, a sample of which was crystallized from ethyl acetate–hexane. The main portion of the product was recrystallized from ethyl acetate with seeding. The compound formed a white, microcrystalline powder, m.p. 101–104°, yield 2.9 g. (90%), $[\alpha]^{21}D$ −27.6° (*c* 1, $CHCl_3$).

For analysis, the compound was recrystallized twice from ethyl acetate and dried at 75° over P_2O_5 *in vacuo* for 24 hours; m.p. 101–104°, $[\alpha]^{21}D$ −29.3° (*c* 1, $CHCl_3$).

Anal. Calcd. for $C_{38}H_{48}O_8N_6S_2$: C, 58.5; H, 6.19; N, 10.8; S, 8.21. Found: C, 58.3; H, 6.34; N, 10.6; S, 8.10.

S-Benzyl-L-cysteinyl-L-prolyl-N$^\epsilon$-tosyl-L-lysylglycinamide (VII).—S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-N$^\epsilon$-tosyl-L-lysylglycinamide (0.5 g.) was dissolved with gentle stirring in 5 ml. of 2 *M* hydrogen bromide in glacial acetic acid. The mixture was allowed to stand at room temperature for 1 hour and then diluted with *ca.* 100 ml. of dry ether. The hydrobromide of VII was thereby precipitated as a white, hygroscopic, amorphous solid. The supernatant solution was decanted and the product was washed by decantation with chloroform. It was then shaken vigorously with chloroform and a slight excess of aqueous K_2CO_3 solution until it had completely dissolved. The chloroform layer was separated and dried over sodium sulfate. The solvent was then removed *in vacuo* at a temperature below 50° leaving VII as a crisp, amorphous mass, which was hygroscopic and collapsed to a gum on standing; yield 0.38 g. (95%), $[\alpha]^{21}D$ −21.3° (*c* 1, 95% ethanol), $[\alpha]^{21}D$ −13.6° (*c* 2, $CHCl_3$). The rotation in chloroform varied appreciably from this value from one preparation to another and the variation was presumably attributable to the presence of traces of moisture.

Anal. Calcd. for $C_{30}H_{42}O_6N_6S_2$: S, 9.88. Found: S, 9.12.

This compound was characterized by reconversion to the crystalline carbobenzoxy derivative VI. To a solution of 0.5 g. of VII in 3 ml. of chloroform were added 0.1 g. of magnesium oxide and 3.0 ml. of 5% aqueous $NaHCO_3$. The mixture was cooled in ice and 0.27 ml. of carbobenzoxy chloride was added with vigorous shaking. The resultant solution was filtered and extracted with chloroform, and the extract was dried over sodium sulfate. The solvent was then removed *in vacuo* and the residual oil was washed with hexane. It was then dissolved in 5–6 ml. of ethyl acetate, filtered in the presence of charcoal and cooled. The solution was inoculated with a trace of crystalline VI and allowed to stand overnight at 0°, when the product separated in crystalline form; yield 0.45 g. (75%), m.p. 99–103°, $[\alpha]^{22}D$ −29.3° (*c* 1, $CHCl_3$). After another recrystallization from ethyl acetate it melted at 98–101°, $[\alpha]^{21}D$ −29.2° (*c* 1, $CHCl_3$).

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