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

Synthetic Studies on Arginine-vasopressin: Condensation of S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine and its O-Tosyl Derivative with S-Benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide[1]

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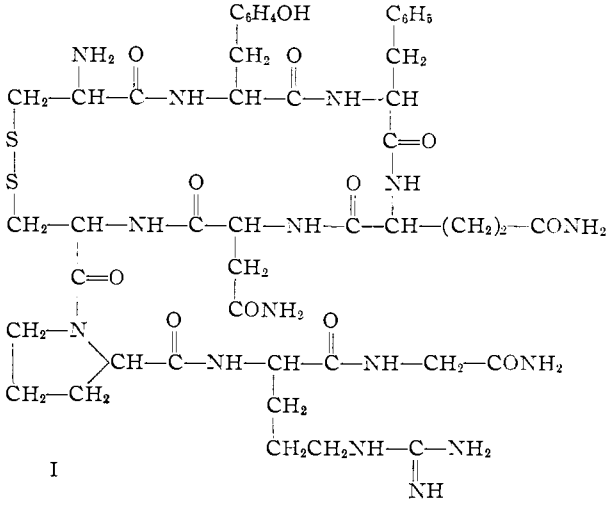
Two synthetic routes to arginine-vasopressin were investigated. The first approach involved the coupling of S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine with S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide monohydrobromide. Treatment of the product with sodium in liquid ammonia and subsequent oxidation of the sulfhydryl nonapeptide gave a biologically active product. Partial purification resulted in a product with a specific activity of 175 pressor units/mg. The synthetic product possessed biological properties associated with natural arginine-vasopressin and also many physical and chemical properties of the natural hormone. The second approach involved the coupling of S-benzyl-N-carbobenzoxy-L-cysteinyl-O-tosyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine with S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide monohydrobromide, routes to these intermediates differing from those used for the first approach. The resultant active product, after partial purification, assayed 220 pressor units/mg.

The isolation[3,4] in highly purified form of arginine-vasopressin, the pressor and antidiuretic hormone of the posterior pituitary gland of beef, followed by degradation studies[5-7] led to the postulation of structure I.[7,8] Synthetic studies were then undertaken. The ultimate objective of these synthetic studies was the preparation of a compound with the sequence L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-arginylglycinamide, the sulfhydryl and free amino groups of which were covered by protective groups which could be removed by treatment with sodium in liquid ammonia. It was expected that subsequent oxidation of this compound by aeration in aqueous solution would lead to a product of structure I. This is analogous to the work on synthetic oxytocin.[9]



I

The first approach undertaken in this endeavor[10] was the coupling of the pentapeptide, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine,[11] with the monohydrobromide of S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide,[12] by the tetraethyl pyrophosphite procedure.[13] The resultant crude product was isolated and treated with sodium in liquid ammonia. A dilute acetic acid solution of the reduction product was adjusted to *p*H 6.7 and aerated. Concentration on the flash evaporator[14] and lyophilization of the solutions from several runs gave material which on assay[15] showed a total of 58,000 units of pressor activity. After countercurrent distribution of this material in the solvent system 2-butanol–0.06 *M* *p*-toluenesulfonic acid, 520 mg. of a product with pressor activity of about 80 units/mg. was obtained. This material was purified further by electrophoresis at *p*H 4.0 in a pyridine–acetate buffer with a cellulose block[16] as the supporting medium. From the peak segments a solution was obtained which, after concentration and lyophilization, yielded a powder weighing 70 mg. which had a specific activity of about 175 pressor units/mg.

The distribution constant of this active material in 2-butanol–0.06 *M* *p*-toluenesulfonic acid (K = 0.84) was the same, within the limits of experimental duplication, as that for a highly purified sample of natural arginine-vasopressin[17] (K = 0.87). When equal quantities of the synthetic and natural products were mixed and chromatographed on an Amberlite IRC-50 (XE-64) ion-exchange column, a single peak was obtained. A com-

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(2) Lilly Postdoctoral Fellow in the Natural Sciences, administered by the National Research Council, 1953–1955.

(3) R. A. Turner, J. G. Pierce and V. du Vigneaud, *J. Biol. Chem.*, **191**, 21 (1951).

(4) E. A. Popenoe, J. G. Pierce, V. du Vigneaud and H. B. Van Dyke, *Proc. Soc. Exptl. Biol. Med.*, **81**, 506 (1952).

(5) E. A. Popenoe and V. du Vigneaud, *J. Biol. Chem.*, **205**, 133 (1953).

(6) E. A. Popenoe and V. du Vigneaud, *ibid.*, **206**, 353 (1954).

(7) V. du Vigneaud, H. C. Lawler and E. A. Popenoe, THIS JOURNAL, **75**, 4880 (1953).

(8) R. Acher and J. Chauvet, *Biochim. et Biophys. Acta*, **12**, 487 (1953).

(9) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, THIS JOURNAL, **76**, 3115 (1954).

(10) A preliminary report has been published [V. du Vigneaud, D. T. Gish and P. G. Katsoyannis, *ibid.*, **76**, 4751 (1954)].

(11) P. G. Katsoyannis and V. du Vigeaud, *ibid.*, **78**, 4482 (1956).

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

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

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

(15) J. Dekanski, *Brit. J. Pharmacol.*, **7**, 567 (1952).

(16) H. G. Kunkel in "Methods in Biochemical Analysis," Vol. 1, D. Glick, Ed., Interscience Publishers, Inc., New York, N. Y., p. 141.

(17) Specific activity, 425 units/mg., prepared in this Laboratory by Dr. D. N. Ward (see ref. 29).

parison of the behavior of the natural and synthetic preparations on a partition column of Hyflo Super-Cel also showed that the synthetic pressor activity occupied the same position in the chromatogram as did the natural hormone.[18] When the synthetic and natural products were spotted separately on a single strip of paper and subjected to electrophoresis at *p*H 4.0 in a pyridine–acetate buffer the two spots were indistinguishable with relation to mobility, size and shape and both spots consisted of material of uniform mobility under these conditions.

In addition to the assays against the United States Pharmacopeia Standard Posterior Pituitary Powder for pressor activity, the product was assayed for antidiuretic and avian vasodepressor activities. The ratios between pressor, antidiuretic[19] and avian vasodepressor[20] activities were the same as those found for natural arginine-vasopressin (1:1:0.15).

It was obvious, however, due to the low specific activity, that our synthetic preparation was not homogeneous. It was also apparent, assuming that the active material represented arginine-vasopressin, that the chemical structure of the contaminant differed from that of the natural hormone in such a subtle way that its behavior in countercurrent distribution, ion-exchange and partition chromatography and electrophoresis, under the experimental conditions used, was identical to that of the active material. An amino acid analysis of a hydrolysate by starch column chromatography[21] showed the presence of the expected amino acids in approximately the calculated ratio. However, it was noted that the cystine peak had a larger spread than usually found and in one chromatogram it appeared that a second peak had actually started to separate from the cystine peak. This indicated the possible presence of another amino acid, such as a dibasic amino acid, which might appear at this point on the chromatogram. The identity of this ninhydrin-positive material was not established.

Several possibilities of the cause of the difficulty were considered. Among those initially considered were the possibilities of racemization, instability of one of the intermediates, structural changes during treatment with sodium in liquid ammonia of the protected nonapeptide and subsequent oxidation of the reduction product, and inactivation of the active material once it had been formed. The latter is known to take place in the case of the natural hormone[22] and a steady loss in total activity is incurred during handling of this unstable hormone. Any of the possibilities outlined above could lead to slight structural changes which, while altering only slightly the physical and chemical properties of the desired material, might lead to complete loss of biological activity. Rendering the problem yet more difficult was the sparing solubility in almost all solvents of the crude protected nonapeptide and the tendency of this mixture to form a gel in aqueous solvents. This fact caused the failure of attempts to purify this crude product before conversion of the nonapeptide to the active hormone.

Although at this time the root of the difficulty had not yet become apparent, a second approach to the synthesis of arginine-vasopressin was undertaken which we hoped might render the intermediates more stable and alter their solubility. O-Tosyl-N-carbobenzoxy-L-tyrosine was coupled by the mixed anhydride procedure,[23] with isobutyl chlorocarbonate in tetrahydrofuran solution, to L-phenylalanyl-L-glutaminyl-L-asparagine[24] in aqueous solution containing an equivalent of triethylamine. Conversion of the O-tosyl-N-carbobenzoxy-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine to O-tosyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine by treatment with HBr in acetic acid[25] and coupling of this tetrapeptide with S-benzyl-N-carbobenzoxy-L-cysteine by the mixed anhydride procedure, as described for the preparation of the tetrapeptide, gave S-benzyl-N-carbobenzoxy-L-cysteinyl-O-tosyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-L-arginylglycinamide hydrobromide was prepared by a procedure analogous to that used for the preparation of the *p*-nitrobenzyloxycarbonyl derivative.[12] Treatment with sodium in liquid ammonia, rebenzylation and purification of the product by countercurrent distribution gave S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide dihydrohalide.[26] The tetrapeptide was then converted to the monohydrohalide and coupled with S-benzyl-N-carbobenzoxy-L-cysteinyl-O-tosyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine by the tetraethyl pyrophosphite method. The unreacted tetrapeptide was removed from the crude product by trituration with water. Treatment of the resulting product with sodium in liquid ammonia, followed by aeration in aqueous solution, gave 57,000 units of pressor activity. The solvent was removed by concentration and lyophilization and most of the inorganic salts were removed by an extraction procedure. The crude peptide material, which assayed 41,000 units, was distributed in the system 2-butanol–0.1% acetic acid. The material recovered from the active peak weighed 400 mg. and assayed 70 units/mg. Electrophoresis of 195 mg. of this material on a cellulose block at *p*H 4.0 in a pyridine–acetate buffer gave, as the most active fraction, 63 mg. of material which assayed 150 units/mg. This active material was then redistributed in the system 2-butanol–0.1% acetic acid. The most active fraction recovered was 23 mg. of a product which had a specific pressor activity of 220 units/mg.

It seemed to us that the activity obtained repre-

(18) P. G. Condliffe, *J. Biol. Chem.*, **216**, 455 (1955).

(19) The antidiuretic assays utilizing the hydrated normal dog were carried out by Professor H. B. Van Dyke, Dr. K. Adamsons, Jr., and Dr. S. L. Engel, to whom we express our appreciation.

(20) J. M. Coon, *Arch. Intern. pharmacodynamie*, **62**, 79 (1939).

(21) S. Moore and W. H. Stein, *J. Biol. Chem.*, **178**, 53 (1949).

(22) This has been the experience of the authors as well as that of others (refs. 3 and 5).

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

(24) E. A. Popenoe and V. du Vigneaud, *ibid.*, **76**, 4751 (1954).

(25) G. W. Anderson, J. Blodinger and A. D. Welcher, *ibid.*, **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).

(26) This product would be expected to be a mixture of the hydrobromide and hydrochloride salts.

sents unquestionably arginine-vasopressin because of all the biological and physical properties encountered with it. In view of the data on the authenticity of the protected pentapeptides and the tetrapeptide, it appeared that something had occurred during the condensation between the protected pentapeptides and the tetrapeptide yielding a by-product or by-products which we were unable to separate from arginine-vasopressin by the techniques available. Therefore, reactions leading to the formation of the peptide bond through the carboxyl group of asparagine have been subjected to closer scrutiny. As reported recently, evidence has now been derived that in the formation of asparaginyl peptides a mixture of products is obtained.[27] Means of avoiding the formation of by-products have been investigated and these results will be reported subsequently.

Experimental[28]

Coupling of S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine with S-Benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide Monohydrobromide and Conversion of the Product to Active Material.—S-Benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide dihydrobromide[12] was dissolved in about 4 parts of dimethylformamide and about a 10% excess of triethylamine was added with stirring. Chloroform was then added, dropwise at first, to redissolve the precipitate of triethylamine hydrobromide and to precipitate the tetrapeptide monohydrobromide as a semi-solid. The monohydrobromide was collected by filtration, washed with chloroform and then redissolved in dimethylformamide. Two or three drops of triethylamine was added and the material was again precipitated with chloroform, washed, and dried *in vacuo* over P_2O_5 and NaOH. The yield of the monohydrobromide was nearly quantitative.

In a typical experiment, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine[11] (114 mg., 0.16 millimole), S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide monohydrobromide (75 mg., 0.13 millimole), tetraethyl pyrophosphite[13] (0.09 g., 0.35 millimole) and diethyl phosphite (0.45 ml.) were heated at about 95° for 50 minutes. Ether was added to the cooled reaction mixture to precipitate the crude product which was collected by filtration, washed with ether and dried over P_2O_5 *in vacuo*. The crude product was treated with sodium in liquid ammonia.[9] The residue, after evaporation of the ammonia, was dissolved in 200 ml. of 0.1% acetic acid, the *p*H was adjusted to 6.7 with NH_4OH and the solution was aerated with a slow stream of air for one hour. Solutions from a number of runs were combined and a total activity of 66,000 pressor units[29] was obtained. The solution was concentrated in the rotary evaporator[14] (bath temperature 25°) and lyophilized to give about 4 g. of a mixture of peptide material and inorganic salt which assayed 58,000 units of pressor activity. This material was subjected to countercurrent distribution in the solvent system 2-butanol–0.06 *M* *p*-toluenesulfonic acid. The material was placed in the first 5 tubes of the all-glass countercurrent distribution machine.[30] The distribution was followed by developing the Folin color[31] and the Sakaguchi color,[32] and by pressor assays, on aliquots of selected tubes. After 690 transfers the solvent from the tubes containing the active peak was pooled and the two phases separated. The organic phase was extracted several times with water and the aqueous extract was combined with the aqueous phase. This solution was passed through a column of Amberlite IR-45 in the acetate form to remove the *p*-toluenesulfonic acid. The solution, which assayed 45,000 pressor units, was concentrated on the rotary evaporator and lyophilized to yield a powder, wt. 520 mg.

Electrophoresis on a Cellulose-supported Medium.—Essentially the procedure described by Kunkel[16] was followed. The supporting medium was a cellulose product, Solka-Floc, of Brown Co. of New York. The buffer used was a pyridine–acetic acid buffer of *p*H 4.0 prepared by diluting 24 ml. of pyridine and 91 ml. of acetic acid to 4 liters with distilled water. The above partially purified material (490 mg.) was dissolved in 3 ml. of the buffer and placed on the block along with two 0.25-ml. washes. The electrophoresis was carried out for 40 hours at 5° with a potential gradient of 9 volts/cm. The Folin color was developed on aliquots of the solutions from the various segments and pressor assays were obtained. The solutions from the three segments which exhibited the highest activity was lyophilized to give about 70 mg. of a powder which assayed 175 units/mg. of pressor activity. When a sample of this material was dissolved and the solution assayed for pressor, antidiuretic[19] and avian vasodepressor[20] activities, the ratio found was 1:1:0.15, the ratio found for natural arginine-vasopressin.[4]

Ion-exchange Chromatography.—The procedure described by Taylor,[33] which employed the resin Amberlite IRC-50 (XE-64) and a 0.2 *M* sodium phosphate buffer of *p*H 6.95, was followed. The preparation of the resin and operation of the column was carried out according to the procedures of Hirs, Moore and Stein[34] and Tallan and Stein.[35] One mg. each of the synthetic product and natural arginine-vasopressin[17] were mixed and chromatographed. A single peak was obtained and there was no evidence of any separation whatever.

Paper Electrophoresis.—The buffer used was the *p*H 4.0 pyridine–acetic acid buffer already described. Solutions of the synthetic product and of natural arginine-vasopressin[17] (0.1 ml. of a solution containing about 0.85 mg./ml.) were spotted, side by side, on Whatman No. 1 paper previously wet with the buffer, and the electrophoresis was carried out, with glass plates as described by Kunkel and Tiselius,[36] for 18 hours with a potential gradient of 9 volts/cm. and at a temperature of 5°. The material was stained on the paper with brom phenol blue as described by Durrum.[37] The spots were identical in appearance, each containing material of uniform mobility in this buffer, and the mobility of the synthetic product was the same as that of natural arginine-vasopressin.

Amino Acid Analysis.—Amino acid analysis by the starch column chromatographic method of Moore and Stein[21] of the synthetic material after hydrolysis showed the following molar ratios: phenylalanine, 1.2; tyrosine, 1.2; proline, 0.8; glutamic acid, 1.3; aspartic acid, 0.85; glycine, 1.0; ammonia, 3.7; arginine, 1.1; cystine, 0.85; unknown, 0.5.[38] The value for ammonia was somewhat high but is uncorrected for the liberation of ammonia due to the destruction of amino acids. In the example cited, sufficient separation of the unidentified peak, which emerged just following cystine, was obtained to permit its approximate quantification. In other chromatograms only partial separation of these two peaks was obtained.

O-Tosyl-L-tyrosine.[39]—To a solution of 18 g. (0.1 mole) of L-tyrosine in 200 ml. (0.2 mole) of *N* NaOH was added a solution of 12.5 g. (0.05 mole) of $CuSO_4 \cdot 5H_2O$ in 50 ml. of water. The mixture was stirred until solution was complete and then was added a solution of 19 g. (0.1 mole) of *p*-toluenesulfonyl chloride in 40 ml. of ether. The reaction mixture was shaken for 3.5 hours and the precipitated copper complex was isolated by filtration, washed with water and finally dissolved in 200 ml. of concd. HCl. After several

(27) D. T. Gish, P. G. Katsoyannis, G. P. Hess and R. J. Stedman, THIS JOURNAL, **78**, 5954 (1956).

(28) Corrected capillary melting points are reported.

(29) At the time the assays recorded in this paper were performed, a value of 0.47 unit/mg. was assigned to the United States Pharmacopeia Posterior Pituitary Standard Powder. A value of 0.40 unit/mg. has now been assigned to this standard.

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

(31) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(32) A. A. Albanese and J. E. Frankston, *ibid.*, **159**, 185 (1945).

(33) S. P. Taylor, Jr., *Proc. Soc. Exptl. Biol. Med.*, **85**, 226 (1954).

(34) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953).

(35) H. H. Tallan and W. H. Stein, *ibid.*, **200**, 507 (1953).

(36) H. G. Kunkel and A. Tiselius, *J. Gen. Physiol.*, **35**, 89 (1951).

(37) E. L. Durrum, THIS JOURNAL, **72**, 2943 (1950).

(38) It might be noted that there is a possibility that this unidentified peak might represent the presence of α,γ-diaminobutyric acid (see C. Ressler, *ibid.*, **78**, 5956 (1956)).

(39) The authors are indebted to Dr. F. H. C. Stewart for the description of the preparation of this compound.

hours cooling the precipitated crystalline hydrochloride of the product was collected by filtration, dissolved in 1 l. of water and the *p*H of the solution was adjusted to 6 with NH_4OH. After cooling, the precipitated crystalline product was filtered, washed with water and dried, wt. 15 g. Recrystallization from 1.5 l. of boiling water gave 12.5 g. (37%) m.p. 215–217°, $[\alpha]^{22}D$ +9.0° (*c* 3.0, *N* HCl); reported in literature[40] m.p. 213–214° uncor. and $[\alpha]^{20}D$ +9.5° (*c* 3.16, *N* HCl).

O-Tosyl-N-carbobenzoxy-L-tyrosine.—A mixture of 3.35 g. (0.01 mole) of O-tosyl-L-tyrosine, 10 ml. of *N* NaOH, 15 ml. of *N* Na_2CO_3 and 80 ml. of *N* $NaHCO_3$ (*p*H 9) was warmed for a few minutes and then cooled to 0°. To this suspension 2 g. (0.016 mole) of carbobenzoxy chloride was added in portions over a period of one hour with vigorous stirring and cooling. After the final addition the reaction mixture was stirred for a further 30 minutes, acidified with HCl and stirred for 15 minutes. The precipitated product was isolated by filtration, washed with water and dried. For purification it was dissolved in ethyl acetate and precipitated with hexane; wt. 3.8 g. (83%), m.p. 124–126°, $[\alpha]^{25}D$ −27° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{24}H_{23}O_7NS$: C, 61.4; H, 4.93; N, 2.98. Found: C, 61.3; H, 4.97; N, 3.05.

O-Tosyl-L-tyrosine from HBr–Acetic Acid Cleavage of N-Carbobenzoxy-O-tosyl-L-tyrosine.—Tosyl-N-carbobenzoxy-L-tyrosine (0.7 g., 0.0015 mole) was suspended in 15 ml. of acetic acid saturated with HBr and the mixture was allowed to stand at 25° for one hour with occasional stirring. The solution was concentrated *in vacuo* to dryness, the residue was mixed with 50 ml. of water and the *p*H of the mixture was adjusted to 6.0 with NH_4OH. The precipitated crystalline O-tosyl-L-tyrosine was filtered off, washed with water and dried; wt. 0.45 g. (90%), m.p. 214–216°.

O-Tosyl-N-carbobenzoxy-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine.—A solution of 1.88 g. (0.004 mole) of O-tosyl-N-carbobenzoxy-L-tyrosine and 0.4 g. (0.004 mole) of triethylamine in 12 ml. of tetrahydrofuran was cooled to −10° and 0.55 g. (0.004 mole) of isobutyl chlorocarbonate was added with stirring. After 10 minutes at −10° a cooled solution of 1.7 g. (5% excess) of L-phenylalanyl-L-glutaminyl-L-asparagine[24] and 0.43 g. of triethylamine in 9 ml. of water was added. The mixture was then allowed to come to room temperature over a period of 25 minutes and the triethylamine salt of the tetrapeptide was precipitated with ether. The product was then collected by filtration, washed with ether, suspended in 100 ml. of water and acidified with HCl. The tetrapeptide thus obtained was separated by filtration, washed with water, dried and triturated with ethyl acetate; wt. 2.7 g. (78.7%), m.p. 214–216°. A sample for analysis was precipitated from 50% acetic acid; m.p. 218–220°, $[\alpha]^{25}D$ −16.6° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{42}H_{46}O_{12}N_6S$: N, 9.78; S, 3.73. Found: N, 9.62; S, 3.62.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-O-tosyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine.—O-Tosyl-N-carbobenzoxy-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine (2.55 g.) was suspended in 50 ml. of 2 *N* HBr in acetic acid and the mixture was allowed to stand at 25° for one hour. The hydrobromide of the O-tosyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine was precipitated from the reaction mixture with ether, collected by filtration, washed with ether and reprecipitated twice from methanol–ether; wt. 1.92 g. (82%). The product (2.4 millimoles) was dissolved in 5 ml. of water plus 4.8 ml. (4.8 millimoles) of *N* NaOH and the mixture was cooled and added to the anhydride of S-benzyl-N-carbobenzoxy-L-cysteine and isobutylcarbonic acid, prepared according to the procedure described in the preceding section from 7.6 g. (2.34 millimoles) of S-benzyl-N-carbobenzoxy-L-cysteine[41] in 12 ml. of tetrahydrofuran, 0.24 g. (2.34 millimoles) of triethylamine and 0.32 g. (2.34 millimoles) of isobutyl chlorocarbonate. The sodium salt of the pentapeptide was precipitated from the reaction mixture with ether, separated by filtration, suspended in 200 ml. of water and acidified with HCl. The precipitated S-benzyl-N-carbobenzoxy-L-cysteinyl-O-tosyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine was filtered off, washed with water, dried and triturated with ethyl acetate; wt. 1.92 g. (83%), m.p. 214–216°. For analysis a sample was precipitated from 70% acetic acid; m.p. 217–219°, $[\alpha]^{25}D$ −33.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{62}H_{67}O_{13}N_7S_2$: C, 59.3; H, 5.46; N, 9.31. Found: C, 59.2; H, 5.56; N, 9.16.

S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-L-arginylglycinamide Hydrobromide.—S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-proline was prepared through the methyl ester as described for the *p*-nitrobenzyloxycarbonyl derivative.[12] The yields of the methyl ester and of the saponification product, neither of which could be crystallized, were 70 and 85%, respectively. The protected dipeptide (2.92 g., 0.0066 mole), L-arginylglycinamide monohydrobromide[12] (1.86 g., 0.003 mole), tetraethyl pyrophosphite (3.10 g., 0.006 mole) and diethyl phosphite (10 ml.) were heated at 100° for 30 minutes. The reaction mixture was cooled and the product, along with unreacted L-arginylglycinamide, was precipitated with ethyl acetate. The material was collected by filtration, washed with ethyl acetate and dried over P_2O_5 and NaOH. This crude product was purified by countercurrent distribution in the solvent system 2 butanol–1% acetic acid; 120 transfers were sufficient to separate the tetrapeptide (K = 1.2) from the dipeptide (K = 0.23). The Sakaguchi color reaction was used for the preparation of the distribution curve. The solvent from the tubes containing the tetrapeptide was concentrated and lyophilized to give 3.0 g. (68%) of an hygroscopic amorphous powder.

S-Benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide Dihydrohalide.[26]—S-Benzyl-N-carbobenzoxy-L-cysteinyl-L-prolyl-L-arginylglycinamide hydrobromide (2.87 g., 0.0039 mole) was treated with sodium (0.47 g.) in liquid ammonia (300 ml.). The sodium was added in small pieces until a permanent blue color persisted. Ammonium chloride was then added in an amount just sufficient to discharge the blue color, and benzyl chloride (0.5 ml., 10% excess) was added and the mixture was stirred for 30 minutes. Ammonium chloride (1.09 g.) was then added and the ammonia was allowed to evaporate and the last traces then removed *in vacuo*. The residue was extracted with 30 ml. of glacial acetic acid in 3 portions, the solution filtered, and the product was precipitated by addition of 400 ml. of ether. This material was reprecipitated from 70 ml. of acetic acid with ether. The amorphous peptide material was collected, washed with ether and dried *in vacuo* over P_2O_5 and NaOH. This material was purified by countercurrent distribution in the solvent system 2-butanol–0.1% acetic acid, 520 transfers being sufficient for the separation of the tetrapeptide (K = 0.18)[42] from a small peak which possessed a very low distribution constant. The Folin color reaction was used for preparation of the distribution curve. Concentration and lyophilization of the solvent from the tubes containing the tetrapeptide gave 1.9 g. (82%) of an amorphous hygroscopic powder. An analysis of this material indicated that it was a mixture of the hydrochloride and hydrobromide salt, that is, part of the acid bound was HBr and part was HCl. This fact rendered it difficult to interpret the analytical results, but the behavior of this tetrapeptide in countercurrent distribution indicated it to consist of a single component.

Preparation and Isolation of Active Material.—The coupling of the pentapeptide S-benzyl-N-carbobenzoxy-L-cysteinyl-O-tosyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine with the tetrapeptide S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide monohydrohalide by the tetraethyl pyrophosphite method was carried out as described above for the coupling without the presence of the O-tosyl group. In a typical experiment 1.05 g. (1 millimole) of pentapeptide, 0.56 g. (0.93 millimole) of tetrapeptide monohydrohalide, 4 ml. of diethyl phosphite and 0.56 g. (2.2 millimoles) of tetraethyl pyrophosphite was used. The reaction product was precipitated with water, filtered off and again triturated with water to remove the unreacted tetrapeptide, and dried. The treatment with sodium in liquid ammonia of this material and subsequent oxidation, in the manner described above, gave a solution which possessed an activity of 22,000 pressor units when assayed in the rat.[15] Solution from a number of runs with a total activity of 57,000 pressor units were combined, concentrated on the rotary evaporator and lyophilized to give about 6 g. of a mixture of peptide material and inorganic salt. This mixture was extracted with 50 ml. of glacial acetic acid, the

(40) E. L. Jackson, THIS JOURNAL, **74**, 837 (1952).

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

(42) This distribution constant is almost identical with that reported by Gish and du Vigneaud (see ref. 12).

solution was filtered and the peptide material was precipitated with 200 ml. of absolute, peroxide-free ether and collected immediately by filtration before crystallization of sodium acetate hydrate occurred. The peptide material thus obtained weighed 1.95 g. and had a specific activity of 21 pressor units/mg. For purification this material was placed in the first 8 tubes of the all-glass automatic countercurrent distribution apparatus and distributed in the system 2-butanol–0.1% acetic acid for 1100 transfers. The activity was concentrated in a single peak. The solvent from the tubes containing the active peak was pooled and the solution, which assayed 28,000 pressor units, was concentrated and lyophilized to give 400 mg. of a product which had a specific activity of about 70 units/mg. A portion of this material (195 mg.) was subjected to electrophoresis on a cellulose block at *p*H 4.0 in a pyridine–acetate buffer and yielded, as the most active fraction, 63 mg. of material which assayed 150 units/mg. This material was redistributed in the system 2-butanol–0.1% acetic acid for 2000 transfers to give as the most active fraction 23 mg. of a product which possessed a specific activity of 220 units/mg.

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE KON. NED. GIST- EN SPIRITUSFABRIEK]

Bottromycin. I. A New Sulfur-containing Antibiotic

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A new antibiotic bottromycin[1] has been isolated from fermentation broth by solvent extraction and purified either by chromatography on Florisil or by means of its salts with organic acids. On the basis of analysis of bottromycin and its crystalline derivatives, the antibiotic has been assigned an empirical formula $C_{53}H_{57-61}N_7O_{7-8}S$.

The antibiotic activity present in the fermentation broths of a soil actinomycete *Streptomyces bottropensis*[2] was found to be extractable at neutral or alkaline *p*H by a variety of organic solvents, such as diethyl ether, ethyl acetate and butyl acetate and at acid *p*H by chloroform.

Paper chromatographic methods gave evidence that two antibiotic substances were present in the culture broth, one of which was present in only minor quantities. This one was discarded during the isolation of bottromycin.

The main antibiotic was extracted from the filtered culture broth with butyl acetate. The extracts were concentrated to a small volume by distillation of the azeotropic mixture and thereafter re-extracted with phosphate buffer at *p*H 2. When the aqueous layer was adjusted to *p*H 9 with dilute sodium hydroxide, the crude bottromycin precipitated as an amorphous white product. This crude antibiotic produced a red color with concentrated mineral acids. The crude product was purified by chromatography on Florisil or by precipitation from its solutions in ether or butyl acetate with organic acids, such as salicylic, acetylsalicylic, *p*-aminosalicylic, 3,5-dinitrosalicylic, 3,5-dibromosalicylic, 3,5-dibromo-4-aminosalicylic, benzoic, *p*-aminobenzoic, 3,5-dinitrobenzoic, acetic, phenylacetic, picric, anthranilic acids and benzylpenicillin. Several of the amorphous salts thus obtained could be crystallized from ethyl acetate.

The water-soluble phosphate as well as the relatively insoluble sulfate and hydrochloride also have been prepared. The free bottromycin base could be obtained from its salts by suspending these salts in water–diethyl ether, adjusting the *p*H with dilute sodium hydroxide to 9 followed by repeated extraction of the alkaline water layer with ether. Evaporation of the dried ether extracts yielded the antibiotic as a glittering white amorphous powder The purified product, thus obtained, no longer produces a red color with concentrated mineral acids.

As to whether bottromycin is a chemically pure entity the following comments may be made. When highly purified bottromycin preparations derived from different production batches were compared, identical elementary analyses were always obtained. Also, the biological activities per mg. bottromycin, determined microbiologically, were identical and the value obtained could not be increased by further purification.

On the other hand some of these seemingly pure products gave a definite red coloration when treated with concentrated sulfuric acid whereas others did not. Such batches, after further purification, usually yielded products which no longer gave a positive test.

The molecular weight determined by the isothermic distillation method[3] was found to be about 743 ± 36. On the basis of analyses of bottromycin as well as of its crystalline salts, the antibiotic has been assigned the empirical formula $C_{53}H_{57-61}N_7O_{7-8}S$. The antibiotic is a very weak base (pK'_a = *ca.* 6.5); it is readily soluble in most organic solvents but is virtually insoluble in hexane, cyclohexane and petroleum ether. In ice-water it is more soluble (2.3 mg./ml.) than in water of about 30° (1.3 mg./ml.). The compound is optically active, $[\alpha]^{25}D$ −14.2° (*c* 0.5, in 96% ethanol).

Bottromycin is not adsorbed on alumina or cellulose but is completely adsorbed on activated carbon from which it can be recovered in part (*ca.* 65%) by elution with acetone containing 3% concentrated hydrochloric acid. "Magnesol" also adsorbs the antibiotic. In this case it can be eluted with benzene containing 5% of methyl alcohol.

Amorphous bottromycin or its crystalline salts are fully stable when stored for several months in the refrigerator. Aqueous solutions of the new antibiotic at *p*H 7 show no loss in microbiological activity after 8 days at 0°. However, a solution at *p*H 2 loses 50% of its activity when exposed for 1.5 hours at 100°. Alkaline solutions of *p*H 9 are completely inactivated when kept for the same

(1) Bottromycin is the generic name given to an antibiotic isolated from a culture of *Streptomyces bottropensis* n.sp.

(2) Dutch patent no. 79,749 (16-10-1955) B-mycin.

(3) E. P. Clark, *Ind. Eng. Chem., Anal. Ed.*, **13**, 820 (1941).