ness under an infrared lamp with the aid of a current of warm air, and counting under a thin-window Geiger counter of conventional design.

The total amount of P^{32} added to each vessel was 500,000–1,000,000 c.p.m.

BERKELEY, CALIFORNIA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

A New Method for the Synthesis of Macrocyclic Peptides

BY JOHN C. SHEEHAN AND WALLACE L. RICHARDSON[1]

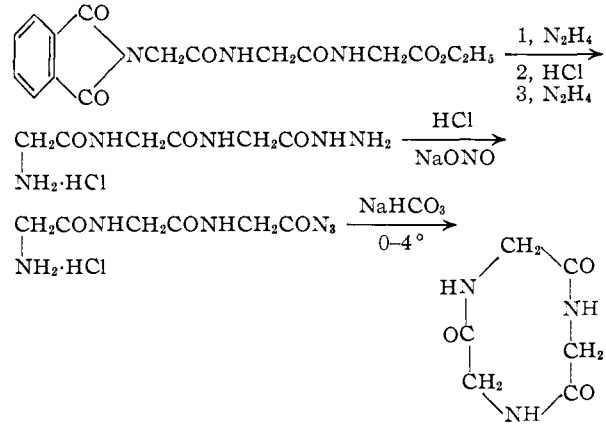
RECEIVED AUGUST 10, 1954

A synthesis of the crystalline macrocyclic peptide cyclotri-(glycyl) by a convenient new method is reported. Triglycine azide hydrochloride, prepared by selective diazotization of triglycine hydrazide, is cyclized in 42% yield by neutralization under dilution conditions.

Recently the syntheses of several cyclic tripeptides have been claimed. In two instances only amorphous products were obtained.[2,3] In a third case the cyclic peptide product was stated to be crystalline, although it consisted presumably of four stereoisomeric forms (two racemates), since it was obtained by heating glycyl-DL-alanyl-DL-phenylalanine methyl ester under reflux in a methanolic solution containing ammonia.[4] The cyclic peptide–hormone oxytocin, as prepared by du Vigneaud and co-workers,[5] constitutes a special case since the cyclization step involved formation of a disulfide linkage by the controlled oxidation of two sulfhydryl groups present as cysteine residues in the peptide chain.

This communication reports a new and convenient method for the synthesis of cyclic peptides containing more than two amino acid units (macrocyclic peptides). Triglycine azide hydrochloride is cyclized by neutralization in homogeneous aqueous solution at 0–4° under dilution conditions, producing 42% of crystalline cyclo-(triglycyl).

In 1949 Hofmann and Magee[6] reported that in acidic media triglycine hydrazide could be diazotized selectively (without appreciable deamination) to the corresponding azide, from which they obtained a polyglycine by basification in concentrated solution. Since our aim was to accomplish the opposite result, namely, to promote cyclization and to inhibit polymerization, we selected conditions of relatively high dilution for the neutralization. The reaction sequence employed is illustrated by the accompanying equations.



Since limited solubility in the common solvents handicaps molecular weight determinations based on colligative properties, X-ray unit cell measurements are being undertaken. Cyclotriglycyl samples are homogeneous as determined by paper chromatography, and the general behavior of the compound leaves little doubt that it is not a dimer or higher molecular weight compound.

The generality of this new peptide cyclization technique is being investigated, and extensions involving very slow addition of peptide azide salts to buffered solutions are being explored.

Phthaloyltriglycine ethyl ester, a precursor in this sequence, was prepared by a modification of the general method for the preparation of phthaloyl peptide esters as recommended by Sheehan, Chapman and Roth.[7] Best results were obtained when phthaloylglycyl chloride was added to a mixture of diglycine ethyl ester hydrochloride and triethylamine in methylene chloride at low temperature.

Cleavage of the phthaloyl group from phthaloyltriglycine ethyl ester was effected at room temperature and the product was isolated as triglycine ethyl ester hydrochloride. It was not feasible to convert the phthaloyl peptide ester directly to the corresponding peptide hydrazide by use of an excess of hydrazine hydrate.

Triglycine hydrazide was readily prepared by storing triglycine ethyl ester hydrochloride three days with excess hydrazine hydrate and then passing the reaction products through a column packed with an anion-exchange resin. On concentration, the effluent yielded the very hygroscopic triglycine hydrazide, which was selectively diazotized. The triglycine azide hydrochloride solution thus produced was diluted with a large volume of ice-water and several equivalents of sodium bicarbonate was added. After storage of the solution for two days at 0–4°, cyclo-(triglycyl) was isolated in 42% yield by crystallization from water as colorless rods. Analysis showed this material to be a hemihydrate.

(1) Public Health Service Research Fellow of the National Institutes of Health, 1952–1954. Taken from a dissertation submitted by W. L. R. in partial fulfilment of the requirements for the Ph.D. degree, January 13, 1954.

(2) R. A. Boissonas, *Helv. Chim. Acta*, **35**, 2229 (1952).

(3) M. Winitz and J. S. Fruton, THIS JOURNAL, **75**, 3041 (1953).

(4) V. H. Brockmann, H. Tummes and F. A. v. Metzsch, *Naturwis.*, **41**, 37 (1954).

(5) V. du Vigneaud, *et al.*, THIS JOURNAL, **75**, 4879 (1953).

(6) K. Hofmann and M. Magee, *ibid.*, **71**, 1515 (1949).

(7) J. C. Sheehan, D. W. Chapman and R. W. Roth, *ibid.*, **74**, 3823 (1952).

On storage for several days in water the solvate slowly changed to fine, colorless needles, which analyzed correctly for cyclo-(triglycyl). Both the hydrated and the anhydrous forms were insoluble in acid and base and gave a negative ninhydrin test.[3]

Experimental[8]

Phthaloyltriglycine Ethyl Ester.—Phthaloylglycyl chloride[9] (25 g., 0.112 mole) dissolved in 100 ml. of methylene chloride was added during one-half hour to a rapidly stirred mixture of 23.2 g. (0.23 mole) of triethylamine and 21.9 g. (0.112 mole) of diglycine ethyl ester hydrochloride[10] in 300 ml. of methylene chloride. The temperature was maintained between −45 and −40° during the addition and for an additional one-half hour. The cooling bath was then removed and stirring continued 2 hours at room temperature. The colorless precipitate was removed by filtration, dried, pulverized and washed with 500 ml. of 0.5 *N* hydrochloric acid and then with water. The product weighed 34.2 g. (88%); m.p. 232–232.5°. Recrystallization from aqueous dimethylformamide yielded 32.6 g. (84%) of cotton-like colorless needles; m.p. 232.5–233° (reported[11] 228–230°).

Triglycine Ethyl Ester Hydrochloride.—A suspension of 17.5 g. (50.4 mmoles) of phthaloyltriglycine ethyl ester in 500 ml. of ethanol was heated to reflux and 5 g. (0.1 mole) of hydrazine hydrate (100%) was added. The resulting clear solution was stored 18 hours at 25°, then evaporated to dryness under reduced pressure. The residue was subjected to 0.01 mm. pressure for 16 hours to remove the last trace of hydrazine hydrate. Phthalhydrazide precipitated on addition of 150 ml. of 0.5 *N* hydrochloric acid. After cooling for 2 hours at 4° the solution was filtered to remove the precipitated phthalhydrazide and the filtrate was freeze-dried to a white powder. Crystallization from absolute ethanol yielded a total of 10.6 g. (84%) of colorless needles; m.p. 214–217° dec. (reported[12] m.p. 214–219° dec.).

Triglycine Hydrazide.—Triglycine ethyl ester hydrochloride (0.95 g., 3.75 mmoles) was suspended in 50 ml. of hot ethanol and 0.7 ml. (14 mmoles) of hydrazine hydrate (100%) was added. A clear solution resulted which, after storage three days at 25°, yielded a white precipitate. The mixture was evaporated to dryness under reduced pressure and stored *in vacuo* overnight. The colorless residue, dissolved in 25 ml. of water, was passed slowly through a 15 × 400 mm. column packed with Amberlite IRA-400 anion-exchange resin. The chloride-free effluent was evaporated to dryness under reduced pressure, and the residue was recrystallized from ethanol yielding 0.61 g. (80%) of hygroscopic, colorless needles which sintered around 100°, slowly decomposed, and completely charred by 205°. An analytical sample, recrystallized twice from ethanol, still decomposed over a similar wide temperature range.

Anal. Calcd. for $C_6H_{13}N_5O_3$: C, 35.46; H, 6.46; N, 34.47. Found: C, 35.23; H, 6.76; N, 34.26.

Cyclo-(triglycyl).—To a solution of 0.812 g. (4 mmoles) of triglycine hydrazide in 38.4 ml. (8 mmoles) of 0.2083 *N* hydrochloric acid, cooled to 0°, was added 0.276 g. (4 mmoles) of sodium nitrite in 2 ml. of water. After swirling gently for 15 minutes, the solution was poured into 2 l. of ice-water. Sodium bicarbonate (5 g.) was added and the solution stored 40 hours at 4°. Following adjustment to *p*H 5 with 2 *N* hydrochloric acid, the solution was concentrated to approximately 50 ml. Acetone (350 ml.) was added and the colorless precipitate was collected and triturated with 25 ml. of cold water. The water-insoluble material was separated by filtration, weight 0.542 g.

Some polymeric material was removed by boiling the product in 20 ml. of water and filtering while hot. Long colorless rods were recovered from the filtrate; weight 0.290 g. (42%). The product gave a negative ninhydrin test,[3] was insoluble in dilute acid or base, and charred slowly above 300°, becoming completely charred by 350° without melting. Recrystallization from hot water yielded a hemihydrate.

Anal. Calcd. for $C_6H_9N_3O_3 \cdot {}^1/_2H_2O$: C, 40.00; H, 5.60; N, 23.32. Found: C, 40.27; H, 5.96; N, 23.42.

Further recrystallization from hot water yielded long colorless rods which slowly changed to very fine needles on storing several days in water. This material gave a negative ninhydrin test[3] and charred without melting between 350 and 365°.

Anal. Calcd. for $C_6H_5N_3O_3$: C, 42.10; H, 5.30; N, 24.55. Found: C, 41.87; H, 5.23; N, 24.54.

CAMBRIDGE 39, MASSACHUSETTS

(8) All melting points are corrected unless otherwise stated. We are indebted to Dr. S. M. Nagy and his associates for the microanalytical data.

(9) J. C. Sheehan and V. S. Frank, THIS JOURNAL, **71**, 1856 (1949).

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

(11) K. Hofmann, A. Lindenmann, M. Z. Magee and N. H. Khan, THIS JOURNAL, **74**, 470 (1952).

(12) E. Fischer, *Ber.*, **36**, 2985 (1903).

[CONTRIBUTION FROM THE RESEARCH DEPARTMENT OF CIBA PHARMACEUTICAL PRODUCTS, INC.]

A Study of the Kinetics of Potato Phenoloxidase Inhibition

BY HANS HEYMANN, ZOSIA ROGACH AND R. L. MAYER

RECEIVED JUNE 25, 1954

The effect of 4-chlororesorcinol and of N-phenylthiourea on the rate of oxidation of catechol by crude potato phenoloxidase has been observed by means of Miller and Dawson's chronometric method. The data suggest that both inhibitors act by a largely competitive mechanism. Manometric measurements of the oxidation of *p*-cresol and its inhibition by phenylthiourea, 4-chlororesorcinol and *m*-hydroxybenzoic acid failed to demonstrate a difference between the response of the catecholase activity and that of the cresolase activity. 4-Chlororesorcinol progressively inactivates both catecholase and cresolase; this explains its great potency as an inhibitor of melanin formation. 2,4-Dihydroxyphenylalanine also acts as an inhibitor with progressive inactivating effect, as shown manometrically. The results are discussed.

The literature contains many reports on inhibitors of phenoloxidase; references are cited in reviews by Dawson and Tarpley,[1] Massart,[2] and Lerner.[3] The last named author presents a classification of compounds known to interfere with melanin formation according to their presumed mode of action, *e.g.*, compounds known to form complexes with copper, metals that may replace copper, substances resembling the substrate and thus probably acting competitively, and other classes. The large amount of work that has been done on the inhibition of phenoloxidase includes only a few papers in which velocity studies are reported that apply current theories of enzyme action to phenoloxidase systems. Baur[4] included potato phenoloxidase in a manometric study of reaction rates and their inhibition but did not evaluate his findings with refer-

(1) C. R. Dawson and W. B. Tarpley in J. B. Sumner and K. Myrbäck, "The Enzymes," Vol. II, Academic Press, Inc., New York, N. Y., 1951, p. 472.

(2) L. Massart, *ibid.*, Vol. I, 1950, p. 337.

(3) A. B. Lerner, *Advances in Enzymol.*, **14**, 74 (1953).

(4) E. Baur, *Helv. Chim. Acta*, **22**, 810 (1939).