was warmed occasionally to dissolve some of the starting material that had precipitated. After neutralization with an equivalent of lithium hydroxide, the solution was lyophilized to a colorless powder, which crystallized from methanol–ethyl acetate; 0.275 g. (83%), m.p. 270–272°. A sample was recrystallized twice from the same solvents for analyses; m.p. 271–272°, $[\alpha]^{23}D$ +93.9° (20.0 mg. in 1.3 ml. of water), reported[16] $[\alpha]^{21}D$ +93.3° (10% solution in water).

Anal. Calcd. for $C_7H_{14}N_2O_3$: C, 48.26; H, 8.10; N, 16.09. Found: C, 48.30; H, 8.09; N, 15.82.

Formyl-L-valyl-L-phenylalanine Methyl Ester.—A solution of 0.87 g. (6.0 millimoles) of formyl-L-valine, 1.07 g. (6.0 millimoles) of phenylalanine methyl ester and 1.30 g. (6.6 millimoles) of N,N′-dicyclohexylcarbodiimide in 40 ml. of purified methylene chloride was stirred overnight at room temperature. After removal of the precipitated urea by filtration, the filtrate, diluted with 50 ml. of methylene chloride, was washed with successive small portions of 5% hydrochloric acid, 5% sodium bicarbonate and water. Concentration of the dried methylene chloride layer at reduced pressure yielded 1.40 g. (80%) of a crystalline residue, which was recrystallized from water to give 1.13 g. of small needles, m.p. 148–149.5°, $[\alpha]^{28}D$ −43.2° (18.5 mg. in 1.2 ml. of methanol).

Anal. Calcd. for $C_{16}H_{22}O_4$: C, 62.72; H, 7.24; N, 9.13. Found: C, 62.49; H, 7.38; N, 9.23.

L-Valyl-L-phenylalanine Methyl Ester Hydrochloride.—A mixture of 0.80 ml. of 5% methanolic hydrochloric acid and 0.5 ml. of methanol containing 0.20 g. (0.66 millimole) of formyl-L-valylphenylalanine methyl ester was stored at room temperature for 48 hours. During this period the mixture was shaken occasionally; complete solution resulted at the end of 10 hours. After addition of an equal volume of anhydrous ether, the crystalline hydrochloride separated in fine, long needles; 0.185 g. (90%), m.p. 192–195°. One recrystallization from methanol–ether yielded 0.165 g. of an analytical sample, m.p. 196–196.5°, $[\alpha]^{28}D$ +26.6° (33.6 mg. in 1.2 ml. of water).

Anal. Calcd. for $C_{15}H_{23}N_2O_3Cl$: C, 57.23; H, 7.36; N, 8.90. Found: C, 57.45; H, 7.53; N, 8.99.

Formyl-L-valyl-L-phenylalanine.—A mixture of 0.42 g. (1.34 millimoles) of formyl-L-valyl-L-phenylalanine methyl ester, 3.2 ml. of dioxane and 1.44 ml. of 1.0 *N* sodium hydroxide was stored at room temperature for 1 hour. At the end of this period, an equivalent amount of *N* hydrochloric acid was added and the solution was lyophilized to a colorless powder. Crystallization of the powder from water yielded 0.37 g. (92%) of needles, m.p. 203–204°, $[\alpha]^{28}D$ −31.4° (9.9 mg. in 1.2 ml. of methanol).

Anal. Calcd. for $C_{15}H_{20}N_2O_4$: C, 61.62; H, 6.90; N, 9.59. Found: C, 61.82; H, 7.09; N, 9.52.

Formyl-L-valyl-L-phenylalanylglycine Ethyl Ester.—A suspension of the salt derived from the addition of 0.086 g. (0.83 millimole) of glycine ethyl ester in 30 ml. of methylene chloride to a solution of 0.242 g. (0.83 millimole) of formyl-L-valylphenylalanine in 15 ml. of dioxane was treated with 0.190 g. (0.92 millimole) of N,N′-dicyclohexylcarbodiimide in 5 ml. of methylene chloride. The mixture was stirred overnight at room temperature. As the salt dissolved slowly, crystalline urea separated. After removal of the urea by filtration and washing of the urea with more methylene chloride, the combined methylene chloride solution was extracted with small portions of 5% hydrochloric acid, 5% sodium bicarbonate and water. The residue, obtained from evaporation of the methylene chloride solution, was dissolved in hot water plus a small quantity of methanol. An additional amount of the insoluble urea could be removed by filtration. When most of the methanol was evaporated, the hot filtrate was allowed to cool gradually and the tripeptide separated as flocculated solid. Recrystallization of the solid in the same manner afforded 0.15 g. (40%) of fine needles, m.p. 187–188°, $[\alpha]^{28}D$ −12.5° (9.5 mg. in 1.1 ml. of glacial acetic acid).

Anal. Calcd. for $C_{19}H_{27}N_3O_5$: C, 60.46; H, 7.21; N, 11.14. Found: C, 60.65; H, 7.45; N, 10.91.

CAMBRIDGE 39, MASS.

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

A New Synthesis of Cysteinyl Peptides[1]

BY JOHN C. SHEEHAN AND DING-DJUNG H. YANG[2]

RECEIVED APRIL 29, 1957

Using a new protective system for the sulfhydryl and amino groups of cysteine, cysteinyl peptides and peptide derivatives have been prepared in good yield in crystalline form without detectable racemization. Cysteine was converted to a thiazolidine derivative by reaction with acetone; the nitrogen may be blocked by formylation. After formation of the new peptide bond the formyl group was removed by acid-catalyzed solvolysis and the thiazolidine ring was disrupted by mild acid hydrolysis or by mercuric chloride treatment. The new method permits the synthesis of cysteinyl peptides through the carboxyl function (L-cysteinylglycine) or through the amino group (γ-L-glutamyl-L-cysteine) or both.

In recent years, there has been a growing interest in the synthesis of cysteinyl peptides in connection with the studies of many peptides and proteins of biological importance, of which cysteine is an essential constituent. Due to the sensitivity of the cysteine molecule toward oxidation and elimination, it usually is necessary to protect β-sulfhydryl function in addition to the amino group or the carboxyl group during synthesis. Customarily this is accomplished by transformation of the β-sulfhydryl group to a S-benzyl thioether[3] or oxidation to the cysteinyl derivative[4,5] with the amino group blocked usually by the N-carbobenzoxy procedure. After the condensation is complete, both the carbobenzoxy group and the S-benzyl group or the disulfide linkage can be cleaved readily by reductive means.[5,6]

In this communication, a new[1] protective system for the synthesis of cysteinyl peptides which does not require the conventional reductive method, is reported. The synthesis consists of a thiazolidine

(1) The essential features of this method were reported in 1952 at the 122nd Meeting of the American Chemical Society and summarized in the Meeting Abstracts (J. C. Sheehan and W. A. Armstrong, Abs. No. 23, p. 15M) in a paper entitled, "A New Synthesis of Peptides Derived from Cysteine and Penicillamine." Since the preparation of this manuscript for publication, a paper has appeared (F. E. King, J. W. Clark-Lewis and R. Wade, *J. Chem. Soc.*, 880 (1957)) describing substantially our same 1952 scheme as, "A New Route to Cysteinyl-peptides," although our aforementioned meeting abstract was acknowledged. Compounds II, III and VI were reported in the Sheehan and Armstrong 1952 reference and also in the King, *et al.*, 1957 publication.

(2) James Flack Norris Memorial Fellow, 1953–1954. Aided in part by a contract from the Office of Naval Research.

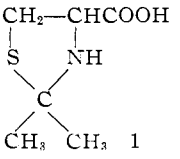
(3) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 109 (1939).

(4) N. W. Pirie, *Biochem. J.*, **25**, 614 (1931).

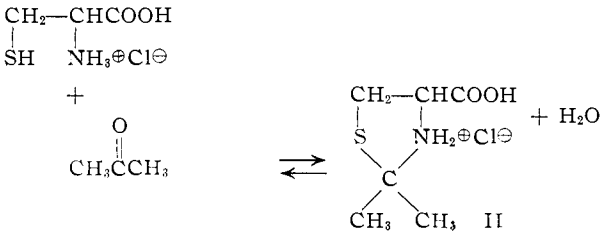
(5) C. R. Harington and T. H. Mead, *ibid.*, **29**, 1602 (1935).

(6) (a) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935); (b) H. S. Loring and V. du Vigneaud, *ibid.*, **111**, 385 (1935).

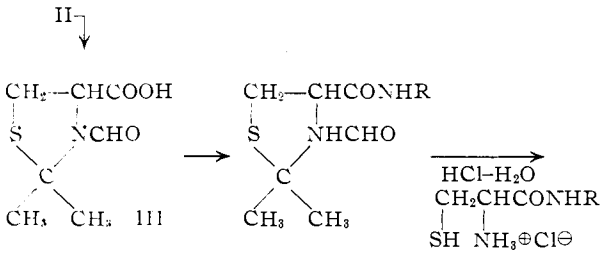
intermediate I through which extension of the peptide chain on the carboxyl group or through the amino group has been demonstrated.



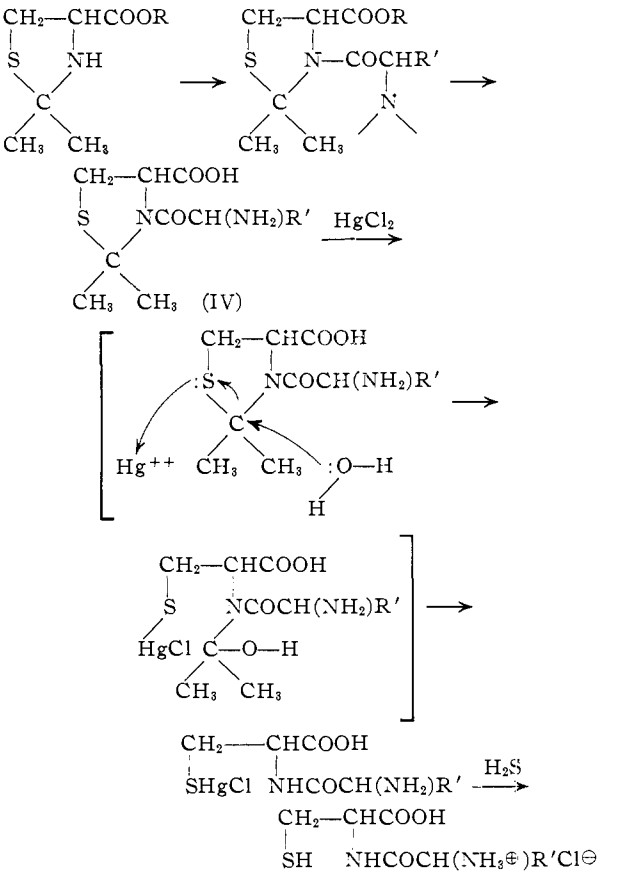
The isopropylidine group protects the sulfhydryl group from oxidation and interference during the acylation processes and tends to stabilize the cysteine moiety against β-elimination. Since the formation of the thiazolidine from cysteine and acetone involves a reversible equilibrium,[7] the isopropylidine group can be removed readily in the presence of water.



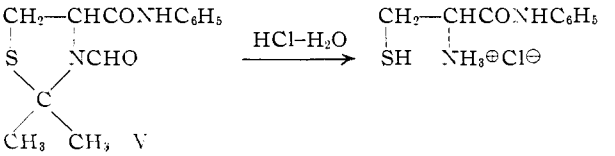
In order to prepare a peptide derived from the carboxyl end of cysteine, the secondary amino group in the thiazolidine system which still can be acylated has to be protected. To achieve this, a formyl group is introduced. The facile removal of the formyl group by mild acid solvolysis is discussed in the preceding communication.[8] After condensation through the carboxyl function, the formyl group and the isopropylidine groups are easily removable by mild acid solvolysis.



To extend the peptide chain on the amino function of cysteine, a procedure similar to the preparation of N-caproylpenicillamine[9] was adopted. 3-Aminoacyl-4-carboxyl-2,2-dimethylthiazolidine (IV) can be cleaved by a heavy metallic salt, such as mercuric chloride, to give the mercuric mercaptide of the corresponding aminoacylcysteine. Decomposition of the mercuric mercaptide by hydrogen sulfide then gives rise to the hydrochloride of the aminoacylcysteine. The sequence of reactions may be illustrated as



As a model study, the synthesis of cysteine anilide was investigated. L-4-Carboxy-3-formyl-2,2-dimethylthiazolidine (III) was prepared by treatment of L-cysteine hydrochloride in boiling acetone followed by formylation in a mixture of formic acid, sodium formate and acetic anhydride. The condensation of III with aniline through a mixed carbonic anhydride[10,11] gave L-4-carboxyanilido-3-formyl-2,2-dimethylthiazolidine (V) in 86% yield. Acid hydrolysis of V in dilute methanolic hydrochloric acid yielded cysteine anilide hydrochloride which was characterized by the formation of a S,N-diacetyl derivative. Under the hydrolytic conditions used both the formyl group and the isopropylidine group were removed in one operation.



The method was next applied to the synthesis of L-cysteinylglycine. Compound III was condensed with glycine methyl ester by the mixed carbonic anhydride procedure to give unracemized L-N-(3-formyl-2,2-dimethylthiazolidine-4-carboxyl)-glycine methyl ester (VI) in excellent yield (89–90%). The optical integrity of V was established by comparison with the product prepared by the

(7) G. B. Woodward and E. F. Schroeder, THIS JOURNAL, **59**, 1690 (1937).

(8) J. C. Sheehan and D.-D. H. Yang, *ibid.*, **80**, 1154 (1958).

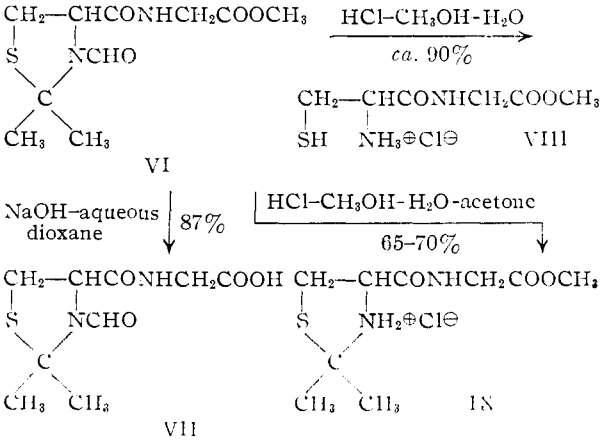
(9) H. T. Clarke, J. R. Johnson and R. Robinson, editors, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 465 and 470.

(10) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

(11) J. R. Vaughan, Jr., THIS JOURNAL, **73**, 3547 (1951).

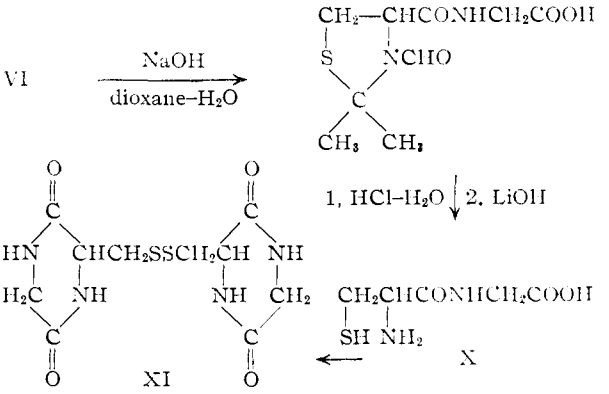
carbodiimide method which is known to avoid racemization in the case of several N-formylamino acids.[8] Previously the use of formylamino acids in peptide synthesis *via* a mixed carbonic anhydride was found to give optically inactive products, which may be attributed to the azlactonization of the mixed anhydride intermediate.[8] Preservation of the active center in this instance is not unexpected since formation of an azlactone-type structure is difficult. The finding is in perfect agreement with the observation that acylprolines are stable under the conditions which generally affect azlactonization and racemization of many acylamino acids.[12]

In order to recover L-cysteinylglycine from VI and to generalize the present synthetic scheme so that the process may be applied repeatedly to the stepwise synthesis of cysteinyl polypeptides, the selective hydrolyses of the ester group, the formyl group and the isopropylidine group in compound VI were carefully studied. Basic hydrolysis in aqueous dioxane removed selectively the ester group, and L-N-(3-formyl-2,2-dimethylthiazolidine-4-carboxyl)-glycine (VII) was obtained in 87% yield. Acid hydrolysis in dilute methanolic hydrochloric acid removed both the formyl group and the isopropylidine group. The product, L-cysteinylglycine methyl ester hydrochloride (VIII) was characterized by the formation of a S,N-diacetyl derivative and oxidative cyclization to anhydro-bis-glycylcystine (XI). The one-step acid hydrolysis of VI in aqueous medium to remove all three of the protective functions was found unsatisfactory. Attempts to hydrolyze the formyl group selectively, including the use of anhydrous methanolic hydrogen chloride and methanolic hydrogen chloride in the presence of acetone, were also unpromising. However, by using concentrated aqueous hydrochloric acid in methanol–acetone, crystalline L-N-(2,2-dimethylthiazolidine-4-carboxyl)-glycine methyl ester hydrochloride (IX) could be isolated. Acetone was added in this case to shift the equilibrium in favor of the thiazolidine. The selective hydrolysis of the formyl group is of particular interest because the introduction of an aminoacyl group at this very position followed by mercuric cleavage would lead eventually to a cysteinyl tripeptide.



(12) V. du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, **99**, 143 (1932).

By a procedure based on the hydrolytic results, L-cysteinylglycine (X) was regenerated from VI by saponification and then by acid hydrolysis to remove the formyl group and the isopropylidine functions. After careful neutralization with lithium hydroxide, crystalline L-cysteinylglycine monohydrate could be isolated directly from the reaction mixture. This is the first successful isolation of crystalline L-cysteinylglycine, although the dipeptide had been isolated from partial hydrolysis of glutathione[13,14] and synthesized[6b] previously. The tendency of L-cysteinylglycine to crystallize as a monohydrate explains the low nitrogen content previously reported[14] which agrees within experimental error with our present data. The dipeptide showed a positive nitroprusside test for sulfhydryl function. Paper chromatography of an acid hydrolysate confirmed the presence of cysteine and glycine. The dipeptide underwent cyclization and oxidation on prolonged standing in aqueous medium to give anhydro-bis-glycylcystine (XI). A similar phenomenon was observed in the isolation of L-cysteinylglycine from partial hydrolysis of glutathione.[15,16]



In the studies of peptide extension utilizing the secondary amino function of the thiazolidine system, we chose to prepare γ-glutamylcysteine, an intermediate of interest in connection with a new synthesis of glutathione. The reaction of L-4-carboxy-2,2-dimethylthiazolidine (I) with phthaloyl-L-glutamic anhydride in glacial acetic acid gave L-4-carboxy-2,2-dimethyl-3-(γ-phthaloyl-L-glutamyl)-thiazolidine (XII), which afforded crystalline L-4-carboxy-3-γ-glutamyl-2,2-dimethylthiazolidine (XIII) (47%) upon hydrazinolysis in aqueous sodium bicarbonate. Compound XIII showed a positive ninhydrin test for α-amino function and a negative nitroprusside test for sulfhydryl function. Cleavage of XIII with mercuric chloride gave a mercuric mercaptide from which crystalline γ-L-glutamyl-L-cysteine hydrochloride (XIV) was generated. Pure γ-glutamylcysteine could be obtained through its cuprous mercaptide, identical in all properties to those reported previously.[5]

It is interesting to note that only L-cysteine hydrochloride could be isolated when the mercuric

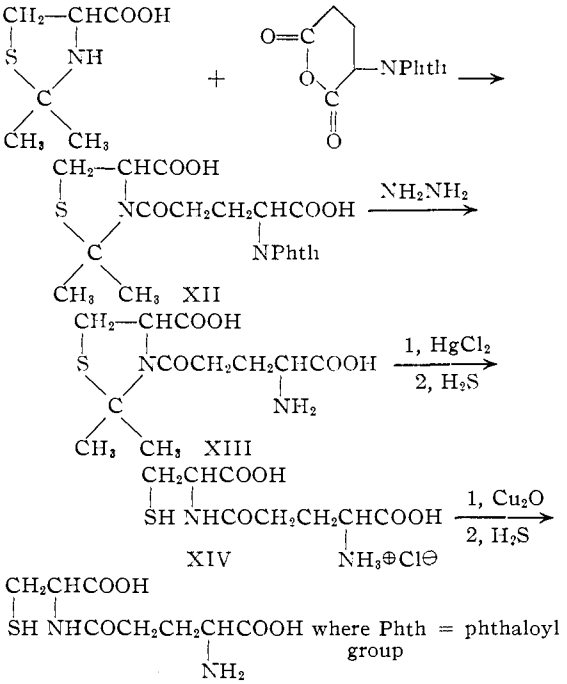
(13) E. C. Kendall, H. L. Mason and B. F. McKenzie, *ibid.*, **88**, 109 (1930).

(14) H. L. Mason, *ibid.*, **90**, 25 (1931).

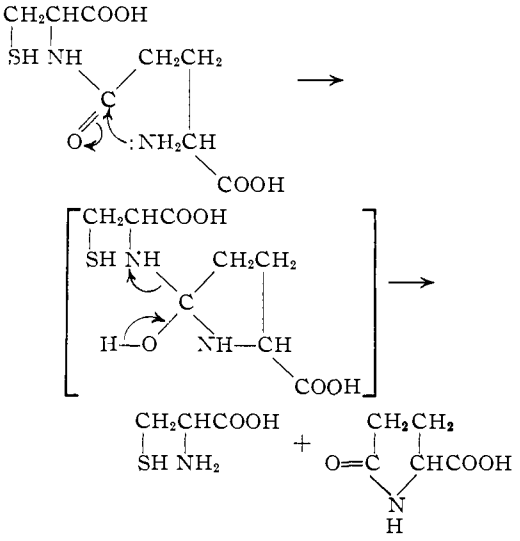
(15) S. Colowick, *et al.*, editors, "Glutathione," Academic Press, Inc., New York, N. Y., 1954, p. 50.

(16) C. K. Olson and F. Binkley, *J. Biol. Chem.*, **186**, 731 (1950).

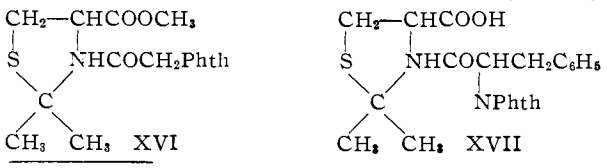
cleavage was conducted at an elevated temperature. Possibly the γ-glutamyl peptide underwent an "autohydrolysis"[16,17] in the following manner to



give 3-pyrolidone-5-carboxylic acid and cysteine. The latter was precipitated as the mercuric mercaptide on cooling.



Other thiazolidine peptides prepared in this series include L-4-carbomethoxy-2,2-dimethyl-3-(phthaloylglycyl)-thiazolidine (XVI) and L-4-carboxy-2,2-dimethyl-3-(phthaloyl-L-phenylalanyl)-thiazolidine (XVII). Both were made from the corresponding



(17) F. Binkley, S. Fujii and J. R. Kimmel, *J. Biol. Chem.*, **186**, 159 (1950).

phthaloylamino acids by the mixed carbonic anhydride method.

It has thus been demonstrated that the thiazolidine protective system is very promising in the synthesis of cysteinyl peptides. One of the advantages in the present approach may be the easy removal of the protective groups by mild acid solvolysis which offers an alternative to the conventional method which requires a reductive step. Another advantage is the direct isolation of the cysteinyl peptide during the synthesis as its mercuric mercaptide by mercuric cleavage of the isopropylidine group, whereas in the other methods the purification of a cysteinyl peptide after removal of the protective groups generally requires a separate similar treatment with mercuric reagent.

Acknowledgment.—D.-D. H. Y. wishes to thank Dr. N. C. Yang for his valuable suggestions during the course of this investigation.

Experimental

L-4-Carboxy-2,2-dimethylthiazolidine Hydrochloride (II).[1,18]—A suspension of 20.0 g. of finely ground L-cysteine hydrochloride monohydrate in 4.5 l. of anhydrous acetone was heated under reflux for six hours. As the powdered cysteine hydrochloride dissolved, crystalline flakes separated slowly. The reaction mixture was concentrated by distillation to a volume of 200–250 ml., and the residual slurry was cooled overnight at 0–5°. The crystalline condensation product, 20.5 g. (82%), was collected by filtration, m.p. 163–165° (reported[19] m.p. 165–168°).

L-4-Carboxy-3-formyl-2,2-dimethylthiazolidine (III).[1,18]—Acetic anhydride (56 ml.) was added dropwise over a period of one hour to a stirring solution of 168 ml. of 98% formic acid containing 20.0 g. (0.010 mole) of L-4-carboxy-2,2-dimethylthiazolidine hydrochloride (II) and 7.8 g. (0.10 mole) of sodium formate at a temperature between 0–5°. The mixture was stirred at room temperature for six hours; at the end of this period, 56 ml. of ice water was introduced. The crystalline precipitate, 17.1 g., m.p. 219–220°, was collected by filtration and the filtrate was concentrated at reduced pressure to a slurry from which a second crop of 1.02 g., m.p. 211–215°, could be isolated after dilution with water. The combined product was recrystallized from methanol–water (3:1) yielding 17.4 g. (92%) of colorless prisms, m.p. 221–222.5°, $[\alpha]^{28}D$ −166.6° (43.2 mg. in 2.0 ml. of 98% formic acid).

Anal. Calcd. for $C_7H_{11}NO_2S$: C, 44.44; H, 5.68; N, 7.40. Found: C, 44.47; H, 5.74; N, 7.35.

L-4-Carboxyanilido-3-formyl-2,2-dimethylthiazolidine (V).—A solution of 0.95 g. (5.0 millimoles) of 4-carboxy-3-formyl-2,2-dimethylthiazolidine (III) and 0.695 ml. (5.0 millimoles) of triethylamine in 10 ml. of methylene chloride was cooled to −5° and a solution of 0.54 g. (5.0 millimoles) of ethyl chloroformate in 5 ml. of methylene chloride was introduced. After 15 minutes of stirring at −5°, a second pre-cooled solution of 0.47 g. (5 millimoles) of aniline in 5 ml. of methylene chloride was added. Stirring was continued for 25 min. at −5° and two hours at room temperature. The reaction mixture was diluted with 80 ml. of methylene chloride and washed thoroughly with 10-ml. portions of 5% hydrochloric acid, 5% sodium bicarbonate and water. Concentration of the dry methylene chloride layer under diminished pressure gave a crystalline residue. One recrystallization from methanol–water (2:1) gave 1.19 g. (90%) of long needles, m.p. 191.5–192.5°, $[\alpha]^{28}D$ −205° (16.5 mg. in 1.5 ml. of glacial acetic acid).

Anal. Calcd. for $C_{13}H_{16}N_2O_2S$: C, 59.06; H, 6.10; N, 10.60. Found: C, 58.76; H, 5.90; N, 10.53.

S,N-Diacetyl-L-cysteine Anilide.—L-4-Carboxyanilido-3-formyl-2,2-dimethylthiazolidine (V) (0.529 g., 2.0 milli-

(18) The procedure for preparing compounds II and III was worked out in this Laboratory by Dr. William R. Armstrong.

(19) Compound II was first prepared by F. Micheel and H. Emde, *Ber.*, **72B**, 1724 (1939), who obtained a 40% yield by treatment of a suspension of cysteine in acetone with hydrogen chloride at room temperature.

moles) was dissolved in a mixture of 2.4 ml. (2.5 meq.) of 5% methanolic hydrochloric acid (11.0 ml. of 38% aqueous hydrochloric acid dissolved in 120 ml. of absolute methanol) and 2 ml. of methanol by gentle warming. The solution was allowed to cool to room temperature and was stored at this temperature for three days. Removal of solvent at reduced pressure gave a gummy residue which was flushed twice with a small amount of chloroform. The crude yield was quantitative; weight 0.50 g.

A sample of 0.21 g. (0.90 millimole) of the crude hydrochloride was dissolved in 10 ml. of water containing 0.076 g. (0.90 millimole) of sodium bicarbonate. The solution was cooled in an ice-bath and two 1-ml. portions of 96% acetic anhydride added with swirling in a 5-minute interval. A colorless precipitate separated immediately. When most of the acetic anhydride had disappeared, the precipitate was dissolved by heating. Fine needles separated from the solution on cooling, weight 0.17 g. (68%), m.p. 173–175°. One recrystallization from water yielded an analytical sample of m.p. 174–175°.

Anal. Calcd. for $C_{13}H_{16}N_2O_3S$: C, 55.69; H, 5.75; N, 10.00. Found: C, 55.38; H, 5.75; N, 9.91.

L-N-(3-Formyl-2,2-dimethylthiazolidine-4-carboxy)-glycine Methyl Ester (VI).[1] A. By the Mixed Carbonic Anhydride Method.—A solution of 0.945 g. (5.0 millimoles) of L-4-carboxy-3-formyl-2,2-dimethylthiazolidine (III) and 6.95 ml. (5.0 millimoles) of triethylamine in 10 ml. of purified methylene chloride was treated with 0.54 g. (5.0 millimoles) of freshly distilled ethyl chloroformate at −8°. The mixture was stirred at this temperature for 18 minutes during which time a large amount of triethylamine hydrochloride separated. To the reaction mixture, a pre-cooled solution of 0.627 g. (5.0 millimoles) of glycine methyl ester hydrochloride and 0.70 ml. (5.0 millimoles) of triethylamine in 40 ml. of methylene chloride was then introduced. After 20 minutes of stirring at −5° and then two hours of stirring at room temperature, the mixture was diluted with 100 ml. of methylene chloride and washed thoroughly with 10-ml. portions of 5% hydrochloric acid, 5% sodium bicarbonate and water. The methylene chloride layer was dried and evaporated under diminished pressure. The oily residue solidified readily to a hard cake on scratching or seeding, yielding 1.18 g. (92%) of a slightly yellow solid, m.p. 105–107.5°. Recrystallization from carbon tetrachloride–petroleum ether gave 1.16 g. (89%) of colorless prisms which had a tendency to adhere in lumps; m.p. 108–109.5°, $[\alpha]^{28}D$ −155.0° (50.0 mg. in 1.4 ml. of methanol). In many runs with quantities ranging from 1 to 20 g., the recrystallized yields were consistently better than 86%.

Anal. Calcd. for $C_{10}H_{16}N_2O_4S$: C, 46.14; H, 6.20; N, 10.86. Found: C, 45.95; H, 6.21; N, 10.61.

B. By the Carbodiimide Method.—A solution of 80 ml. of methylene chloride containing 0.427 g. (3.4 millimoles) of glycine methyl ester hydrochloride, 1.00 g. (4.2 millimoles) of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide metho-*p*-toluenesulfonate[20] and 0.64 g. (3.4 millimoles) of L-4-carboxy-3-formyl-2,2-dimethylthiazolidine (added in the order given) was stirred overnight at room temperature. When the reaction was complete, the mixture was washed four times with 10-ml. portions of 5% hydrochloric acid, once with 5% sodium bicarbonate and once with water. Concentration of the dry methylene chloride solution under reduced pressure gave an oily residue (0.65 g.) which solidified slowly on scratching. The solid was recrystallized from carbon tetrachloride–petroleum ether to give 0.59 g. (63%) of prisms, m.p. 108–109°, $[\alpha]^{28}D$ −155.1° (29.8 mg. in 1.4 ml. of methanol).

L-Cysteinylglycine Methyl Ester Hydrochloride (VIII).—A solution of 0.50 g. (1.92 millimoles) of L-N-(3-formyl-2,2-dimethylthiazolidine-4-carboxyl)-glycine methyl ester (VI) in a mixture of 2 ml. of 1 *N* methanolic hydrochloric acid (prepared by dilution of 2 ml. of 38% aqueous hydrochloric acid to 24 ml. with absolute methanol) and 2 ml. of methanol was stored at room temperature for 60 hours. Removal of solvents at reduced pressure gave a gummy residue. Precipitation of the crude hydrochloride from a methanolic solution by anhydrous ether yielded 0.42 g. (98%) of an amorphous solid which resisted crystallization. A sample was purified by repeated precipitations from the same solvents.

Anal. Calcd. for $C_6H_{12}N_2O_3S$·HCl: C, 31.51; H, 5.73; N, 12.25. Found: C, 31.18; H, 5.99; N, 11.17.

The crude hydrochloride (0.40 g., 1.81 millimoles) was acetylated in a sodium bicarbonate solution (0.153 g., 1.81 millimoles of sodium bicarbonate in 15 ml. of water) at 0° by addition of two 1-ml. portions of acetic anhydride in a 5-minute interval under an atmosphere of nitrogen. The mixture was stirred continuously until all of the acetic anhydride had dissolved. Concentration of the aqueous solution under reduced pressure gave a colorless solid which was taken up in 20 ml. of methylene chloride. The insoluble residue was removed by filtration and washed thoroughly with more methylene chloride. The filtrate, combined with the washings, was dried and concentrated at reduced pressure. Crystallization of the residue from benzene–carbon tetrachloride mixture yielded 0.36 g. (72%) of fine needles, m.p. 142–143°. A sample was recrystallized from the same solvents (m.p. 142–143.5°). The analysis corresponded to S,N-diacetylcysteinylglycine methyl ester, $[\alpha]^{28}D$ 42.6° (27.2 mg. in 1.5 ml. of water).

Anal. Calcd. for $C_{10}H_{16}N_2O_5S$: C, 43.47; H, 5.84; N, 10.14; S, 11.60. Found: C, 43.52; H, 5.85; N, 10.09; S, 11.59.

L-N-(3-Formyl-2,2-dimethylthiazolidine-4-carboxyl)-glycine (VII).—A solution of 2.08 g. (8.0 millimoles) of N-(3-formyl-2,2-dimethylthiazolidine-4-carboxyl)-glycine methyl ester (VI) in 21 ml. of dioxane was treated with 8.0 ml. of 1.0 *N* sodium hydroxide. The mixture was set aside at room temperature. After 50 minutes an additional quantity of 0.6 ml. of 1.0 *N* sodium hydroxide was added and 15 min. later the solution was acidified with an equivalent amount of 1 *N* hydrochloric acid. Removal of solvents under reduced pressure gave a colorless residue. The residue was triturated twice with 10-ml. portions of ethyl acetate and twice with 3-ml. portions of cold water and dried; weight 1.76 g. (90%), m.p. 151–152.5°. Recrystallization from methanol–carbon tetrachloride mixture gave 1.68 g. (87%) of small prisms, m.p. 152–153.5°, $[\alpha]^{26}D$ −169° (28.3 mg. in 1.4 ml. of water).

Anal. Calcd. for $C_9H_{14}N_2O_4S$: C, 43.89; H, 5.73; N, 11.38. Found: C, 43.89; H, 5.73; N, 11.19.

Anhydro-bis-glycylcystine (XI). A. From Cyclization of L-Cysteinylglycine Methyl Ester.—A solution of 1.56 g. (60 millimoles) of N-(3-formyl-2,2-dimethylthiazolidine-4-carboxyl)-glycine methyl ester (VI) in 8.8 ml. of 0.73 *N* methanolic hydrochloric acid and 3.2 ml. of methanol was refluxed for two hours. Removal of solvents at reduced pressure gave a residue which was dissolved in 10 ml. of water and neutralized with aqueous ammonium hydroxide. The mixture was aerated and stored at room temperature for precipitation. At the end of three days, the crystalline precipitate was collected and recrystallized from water, yielding 0.74 g. (78%) of colorless needles, m.p. 266–267° dec., reported[21] m.p. 260°.

Anal. Calcd. for $C_{10}H_{14}N_4O_4S$: C, 37.72; H, 4.43. Found: C, 37.52; H, 4.43.

B. From Cyclization of L-Cysteinylglycine.—A solution of 1.17 g. (4.5 millimoles) of L-N-(3-formyl-2,2-dimethylthiazolidine-4-carboxyl)-glycine (VII) in a mixture of 4.8 ml. of 1.0 *N* hydrochloric acid and 10 ml. of dioxane was refluxed for 110 minutes under an atmosphere of nitrogen. After neutralization with an equivalent amount of 1 *N* sodium hydroxide, the mixture was lyophilized to a powder which was taken up in 10 ml. of water. The solution was stored at room temperature for three days with occasional shaking. During this period, small needles, insoluble in acid or base, separated slowly. They were collected by filtration and recrystallized from water; weight 0.23 g. (32%), m.p. 267–268° dec.

Anal. Calcd. for $C_{10}H_{14}N_4O_4S$: C, 37.72; H, 4.43; N, 17.58. Found: C, 38.08; H, 4.61; N, 17.45.

In a separate run, L-N-(3-formyl-2,2-dimethylthiazolidine-4-carboxyl)-glycine (1.17 g., 4.5 millimoles) was hydrolyzed by the same procedure. The gummy residual hydrochloride was dissolved in 8 ml. of water and chromatographed through a 35 × 1.7 cm. column packed with Amberlite IR-A-400 anion exchange resin. From the chloride-free effluent, needles separated within 15 min.; weight 0.13 g. (17%), m.p. 267–268° dec. A mixed melting point with

(20) J. C. Sheehan and J. J. Hlavka, *J. Org. Chem.*, **21**, 439 (1956).

(21) F. G. Hopkins, *J. Biol. Chem.*, **84**, 269 (1929).

an authentic sample of anhydro-bis-glycylcystine gave no depression.

L-N-(2,2-Dimethylthiazolidine-4-carboxyl)-glycine Methyl Ester Hydrochloride (IX).—A solution of 4.68 g. (0.018 mole) of L-N-(3-formyl-2,2-dimethylthiazolidine-4-carboxyl)-glycine methyl ester (VI) in a mixture of 30 ml. of acetone and 17.1 ml. (0.020 equivalent) of 1.2 *N* methanolic hydrochloric acid (5 ml. of aqueous 38% hydrochloric acid diluted with absolute methanol to 1–50 was refluxed for two hours). Removal of solvents under reduced pressure gave an oily residue which was dissolved in 400 ml. of anhydrous acetone. After storage at room temperature for 14 hours, the acetone solution was refluxed for two hours and concentrated at reduced pressure. A solution of the foamy residue in 8 ml. of methanol was diluted with 32 ml. of anhydrous ether. By scratching gently on the side of the vessel, long needles separated rapidly; weight 3.5 g. (70%), m.p. 113–116° dec. An analytical sample was prepared by two recrystallizations from methanol–ether (1:4); m.p. 118–119° dec., $[\alpha]^{26}D$ −95.3° (33.0 mg. in 1.5 ml. of chloroform).

Anal. Calcd. for $C_9H_{16}N_2O_3S\cdot HCl$: C, 40.22; H, 6.38; N, 10.43. Found: C, 40.02; H, 6.44; N, 10.61.

L-Cysteinylglycine Monohydrate (X).—A solution of 3.20 g. (0.013 mole) of L-N-(3-formyl-2,2-dimethylthiazolidine-4-carboxyl)-glycine (VII) in a mixture of 14.0 ml. of 1 *N* hydrochloric acid and 50 ml. of 1:1 dioxane–water was refluxed under nitrogen for two hours. The mixture was cooled and neutralized with an equivalent amount of 1 *N* lithium hydroxide under an atmosphere of nitrogen. The mixture was immediately lyophilized and the residue was washed thoroughly with ethyl acetate then with ethanol, and dried under vacuum; weight 2.50 g., m.p. 177–180°. Recrystallization under nitrogen from 1:1 methanol–water (oxygen-free),[22] yielded 1.4 g. (55%) of rectangular prisms, m.p. 184–185° dec. From the mother liquor, anhydro-bis-glycylcystine was isolated on long standing. A sample was recrystallized in the same manner and analyzed; m.p. 184–185°, $[\alpha]^{28}D$ +46.1° (38.2 mg. in 1.3 ml. of water).

Anal. Calcd. for $C_5H_{10}N_2O_3S\cdot H_2O$: C, 30.61; H, 6.16; N, 14.28. Found: C, 30.67; H, 5.91; N, 13.87.

This compound gave a positive nitroprusside test and a negative ninhydrin test. Hydrolysis of a small sample in refluxing 6 *N* hydrochloric acid for 12 hours, then paper chromatography showed the presence of glycine and cystine.

L-4-Carboxy-2,2-dimethyl-thiazolidine (I).—The compound was prepared following the procedure of Woodward and Schroeder.[7] From 1.2 g. of L-cysteine and 3 l. of boiling acetone, 14.0 g. (88%) of crystalline product was obtained, m.p. 130–132°. Recrystallization from acetone gave 10.0 g. of rectangular prisms, m.p. 134.5–135°.

L-4-Carboxy-2,2-dimethyl-3-(γ-phthaloyl-L-glutamyl)-thiazolidine (XII).—Finely ground phthaloyl-L-glutamic anhydride[23] (4.15 g., 0.016 mole) was dissolved in 35 ml. of glacial acetic acid containing 2.58 g. (0.016 mole) of L-4-carboxy-2,2-dimethylthiazolidine (I) by gentle warming. After storage at room temperature for four hours, the mixture was lyophilized. A solution of the residue in 200 ml. of ethyl acetate was washed thoroughly with 5% hydrochloric acid until the washings did not give a ninhydrin test. The acid fraction was then extracted from the solution using 5% sodium bicarbonate. The combined sodium bicarbonate extracts were washed with more ethyl carbonate, acidified to *p*H 3 and re-extracted with ethyl acetate. Concentration of the ethyl acetate extracts at reduced pressure gave a foamy residue which was lyophilized to a colorless powder from a dioxane solution; weight 6.5 g. (crude yield 95%).

The powder gave a negative nitroprusside test and no ninhydrin test. It is readily soluble in hot water, from which solution an oil separated on cooling. Many attempts to crystallize the product failed and it was subjected to hydrazinolysis without further purification.

L-4-Carboxy-3-γ-L-glutamyl-2,2-dimethylthiazolidine Monohydrate (XIII).—A solution of 6.22 g. (0.015 mole) of the crude 4-carboxy-2,2-dimethyl-3-(δ-phthaloyl-L-glutamyl)-thiazolidine (XII) and 2.49 g. (0.03 mole) of sodium bicarbonate in 22 ml. of water was treated with 0.80 ml. (0.0165 mole) of 100% hydrazine hydrate. The mixture was set aside at room temperature for two days. At the end of this period, a large amount of crystalline phthaloyl hydrazide separated. The mixture was lyophilized and the residual powder was treated with 52 ml. of *N* hydrochloric acid. After storage at 0–5° for 6 hours, the acid insoluble residue was removed by filtration and the filtrate was lyophilized again to a powder. After trituration of the powder with small portions of absolute methanol, the alcohol-insoluble inorganic salt was removed by filtration. Concentration of the filtrate at reduced pressure yielded 5.8 g. of a crude hydrochloride.

The crude hydrochloride was dissolved in 10 ml. of water, and 0.90 ml. of pyridine was added to adjust the *p*H of the solution to 3.1. At this *p*H, prisms arranged in rosettes began to separate. The mixture was stored at 0–5° and the crystalline product was collected two hours later; weight 2.0 g., m.p. 151.5–153°. From the mother liquor, a second crop of 0.2 g. (m.p. 150–152.15°) could be obtained after dilution with 15 ml. of ethanol and storage overnight at 0–5°. The combined yield was 2.20 g. (45%).

The product gave a positive ninhydrin test and a negative nitroprusside test. One recrystallization from ethanol–water afforded an analytical sample, m.p. 151–153°, $[\alpha]^{30}D$ −57.6° (34.5 mg. in 1.60 ml. of water).

Anal. Calcd. for $C_{11}H_{18}N_2O_6S\cdot H_2O$: C, 42.85; H, 6.54; N, 9.09. Found: C, 42.59; H, 6.60; N, 9.13.

γ-L-Glutamyl-L-cysteine Hydrochloride (XIV).—A solution of 0.80 g. (2.36 millimoles) of L-4-carboxy-3-γ-L-glutamyl-2,2-dimethylthiazolidine monohydrate (XIII) in 30 ml. of water was treated with 1.40 g. (5.2 millimoles) of mercuric chloride in 20 ml. of water in two equal portions. Colorless precipitate deposited immediately and the mixture was swirled occasionally. After storage at room temperature for two days the mercuric mercaptide was collected and washed thoroughly with small portions of oxygen-free water at the centrifuge.

The mercuric mercaptide was suspended in 20 ml. of oxygen-free water and hydrogen sulfide was introduced under slight pressure for 20 minutes, with constant shaking of the reaction mixture. The mercuric sulfide was separated and washed four times with oxygen-free water by centrifugation. The supernatant solution and the washings were combined, acidified with 0.5 ml. of *N* hydrochloric acid and filtered. Excess hydrogen sulfide was removed by passing a stream of nitrogen through the solution until a negative test was obtained with lead acetate test paper. The solution was lyophilized immediately. The powdery residue was washed thoroughly with ethyl acetate and dried under vacuum; weight 0.20 g. (30%), m.p. 163–165°. Crystalline hydrochloride was obtained by slow addition of ether to a methanolic solution of the crude hydrochloride, followed by seeding or scratching. One recrystallization from glacial acetic acid and another from methanol–ether yielded 70 mg. of fine needles, m.p. 173–174°, $[\alpha]^{28}D$ +2.5°, +2.9° (2.5% solution in water). The hydrochloride gave a positive ninhydrin test and a positive nitroprusside test.

Anal. Calcd. for $C_8H_{15}N_2O_5SCl$: N, 9.70. Found: N, 9.50.

A solution of 0.41 g. (1.44 millimoles) of the crude L-γ-glutamyl-L-cysteine hydrochloride in 15 ml. of 0.5 *N* sulfuric acid was treated with a suspension of freshly prepared cuprous oxide[24] at 45°. As the reddish cuprous oxide dissolved, a colorless precipitate possessing a characteristic silky sheen separated. Addition of cuprous oxide was continued until the precipitation appeared to be complete. After separation of the precipitate by centrifugation, the mother liquor was treated with more cuprous oxide whereby an additional quantity of cuprous mercaptide could be obtained. The combined precipitate was washed repeatedly with small portions of oxygen-free water at the centrifuge until the washings were free of sulfate ions. A suspension of the cuprous mercaptide in 15 ml. of oxygen-free water was treated with hydrogen sulfide by the method described above for the decomposition of the mercuric mercaptide. The cuprous sulfide was separated and washed four times

(22) Oxygen-free water was obtained by cooling boiling water under a stream of nitrogen.

(23) Unracemized phthaloyl-L-glutamic anhydride can be prepared successfully by direct fusion of phthalic anhydride and purified L-glutamic acid at 133–138°, followed by treatment with N,N'-dicyclohexylcarbodiimide, a modification of the procedure of J. C. Sheehan and W. A. Bolhofer, THIS JOURNAL, **72**, 2469 (1950).

(24) E. G. Bail, Editor, "Biochemical Preparations," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1952, p. 89.

with oxygen-free water at the centrifuge. The supernatant solution, combined with the washings, was lyophilized immediately to a colorless residue which was taken up in a small amount of 95% alcohol, and stored under nitrogen at 0–5° for precipitation. An aqueous solution of the solid was concentrated in a desiccator over phosphoric oxide to low bulk, left overnight in a desiccator containing sodium hydroxide and a dish of alcohol and evacuated to 300 mm. pressure. The moist crystalline prisms were collected two days later with the aid of a little alcohol, weight 72 mg., m.p. 164–166°, $[\alpha]^{28}D$ +12.4° (20.2 mg. in 1.2 ml. of water). The reported values for γ-L-glutamyl-L-cysteine are m.p. 167°, $[\alpha]D$ +13.6° (110 mg. in 10 ml. of water).[5]

Treatment of XIII with Mercuric Chloride at Elevated Temperature.—A solution of 1.20 g. (3.78 millimoles) of L-4-carboxy-3-γ-L-glutamyl-2,2-dimethylthiazolidine monohydrate (XIII) in 30 ml. of water was heated on a steam-bath and 2.5 g. (9.3 millimoles) of mercuric chloride dissolved in 20 ml. of hot water was added. The mixture was maintained at 60–70° for 15 min. During this time, the mercuric mercaptide that had precipitated at the beginning dissolved slowly, and solid mercaptide separated on cooling. After storage at room temperature for 14 hours, the heating process was repeated in order to effect decomposition and the mixture was allowed to cool gradually. The mercuric mercaptide was collected, washed thoroughly with small amount of water and treated with hydrogen sulfide by the regular procedure. The hydrochloride thus obtained was found to be cysteine hydrochloride, weight 0.24 g. (40%). A mixed melting point with an authentic sample of cysteine hydrochloride gave no depression (m.p. 178–179.5°).

Anal. Calcd. for $C_3H_8NO_2SCl$: N, 8.90. Found: N, 9.10.

L-4-Carbomethoxy-2,2-dimethylthiazolidine Hydrochloride (XV).—The ester was prepared from the corresponding thiazolidinecarboxylic acid following the procedure for the esterification of 4-carboxy-2,2,5,5-tetramethylthiazolidine.[25] A mixture of 15.0 g. (0.069 mole) of 4-carboxy-2,2-dimethylthiazolidine hydrochloride in 300 ml. of absolute methanol, cooled in a water-bath, was saturated with anhydrous hydrogen chloride. The solution was stored at room temperature for 8 hours and at 0–5° for 14 hours. Removal of solvent under reduced pressure gave a crystalline residue which was digested twice with 100-ml. portions of anhydrous acetone for 20 min. The acetone mixture was concentrated at reduced pressure and the residue was recrystallized from 25 ml. of methanol and 40 ml. of acetone, yielding 8.5 g. (60%) of fine needles, m.p. 159–159.5°. A mixed melting point with the starting material (m.p. 161–164°) gave a depression of 20°.

Anal. Calcd. for $C_7H_{13}NO_2S\cdot HCl$: C, 39.72; H, 6.67; N, 6.62. Found: C, 40.01; H, 6.67; N, 6.64.

(25) H. T. Clarke, J. R. Johnson and R. Robinson, Editors, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 960.

L-4-Carbomethoxy-2,2-dimethyl-3-(phthaloylglycyl)-thiazolidine (XVI).—A solution of 2.48 g. (0.012 mole) of phthaloylglycine and 1.68 ml. (0.012 mole) of triethylamine in 10 ml. of methylene chloride was cooled to −8° and treated with 1.30 g. (0.012 mole) of ethyl chloroformate. The mixture was stirred at −8° and a pre-cooled solution of 2.54 g. (0.012 mole) of L-3-carbomethoxy-2,2-dimethylthiazolidine hydrochloride (XV) and 1.68 ml. (0.012 mole) of triethylamine in 50 ml. of methylene chloride was added. After 20 min. of stirring at −8° and 4 hours at room temperature, the solution was diluted with 100 ml. of chloroform. The reaction mixture was washed thoroughly with 5% hydrochloric acid, 5% sodium bicarbonate and water, dried, and concentrated at reduced pressure. The residue (3.8 g., 86%, m.p. 220–223°) was crystallized from chloroform–methanol yielding 1.80 g. (41%) of small needles, m.p. 225–226° dec. An analytical sample was prepared by two recrystallizations from chloroform–methanol; m.p. 227–228° dec., $[\alpha]^{30}D$ +11.4° (7.9 mg. in 1.8 ml. of 1:1 methanol–chloroform).

Anal. Calcd. for $C_{17}H_{18}N_2O_5S$: C, 56.34; H, 5.01; N, 7.73. Found: C, 56.28; H, 4.84; N, 7.80.

L-4-Carboxy-2,2-dimethyl-3-(phthaloyl-L-phenylalanyl)-thiazolidine (XVII).—A solution of 1.95 g. (6.7 millimoles) of phthaloyl-L-phenylalanine and 0.927 ml. (6.7 millimoles) of triethylamine in 18 ml. of 1:1 methylene chloride–dioxane mixture was cooled to −8° and 0.725 g. (6.7 millimoles) of ethyl chloroformate was added. The mixture was stirred at −8° for 12 min. during which time a large amount of triethylamine hydrochloride precipitated. A second pre-cooled solution of 1.07 g. (6.7 millimoles) of 4-carboxy-2,2-dimethylthiazolidine in 50 ml. of dioxane was then added. Stirring was continued at −8° for 20 min. and at room temperature for 6 hours.

The reaction mixture was concentrated at reduced pressure and the residue was dissolved in 150 ml. of chloroform. The chloroform solution was washed thoroughly with 5% hydrochloric acid and water and the acid fraction was extracted from this solution using 5% sodium bicarbonate. The bicarbonate extracts were combined, washed well with ethyl acetate, acidified to *p*H 3 and re-extracted with ethyl acetate. The ethyl acetate extracts, after thorough washing with water, were dried over magnesium sulfate and concentrated at reduced pressure to a viscous oil which solidified readily on scratching. Recrystallization from aqueous ethanol gave 0.20 g. of small needles, m.p. 193–195°. A second crop of 0.48 g. (m.p. 190–193°) could be obtained by concentration of the mother liquor to 10 ml. and inoculation of the cold solution with a seed crystal. The combined yield was 0.68 g. (24%). A sample was recrystallized from aqueous ethanol for analysis; m.p. 196–197°, $[\alpha]^{30}D$ −280° (26.0 mg. in 1.3 ml. of methanol).

Anal. Calcd. for $C_{23}H_{22}N_2O_5$: C, 63.00; H, 5.06; N, 6.39. Found: C, 63.16; H, 5.15; N, 6.35.

CAMBRIDGE 39, MASS.

[CONTRIBUTION FROM THE PASADENA FOUNDATION FOR MEDICAL RESEARCH]

Synthesis of the Optically Active Tripeptides of Valine

BY S. SHANKMAN AND Y. SCHVO

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With the use of N,N′-dicyclohexylcarbodiimide as a condensing agent, all eight optically pure tripeptides of D-valine and L-valine have been prepared. A high degree of optical purity was demonstrated by microbiological assay.

The presence of D-amino acid residues in antibiotics such as the gramicidins,[1,2] tyrocidine,[3] the penicillins,[4] bacitracin[5] and aerosporin[6] has aroused interest in the inhibitory potentialities of peptides

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