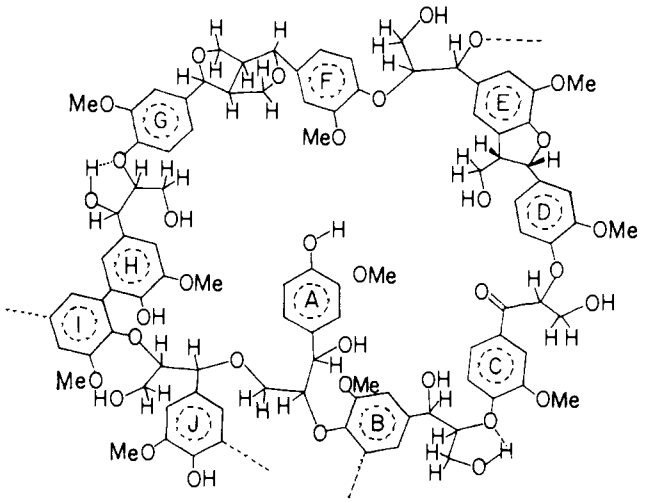


Fig. 12.—Speculative structure of segment of gymnosperm lignin molecule.

attached to side chain carbon atoms per phenylpropane unit; and (d) about eight aliphatic hydroxyl groups, of which about three are benzylic, are present per ten phenylpropane units. The presence of the large scale cross-linking or ring systems indicated in Fig. 12 as being present in lignin molecules is not proved, but the existence of a somewhat rigid structure is suggested by the broadness of the signals observed in the n.m.r. spectra. Evidence for hydrogen bondings of the types shown in BC and GH has been found with model compounds in chloroform solution, and such bonding may also occur in lignins in other solvents and in the dry state.

Experimental

The n.m.r. spectra were taken using the instrumentation and procedures previously described.[1] Solutions of 7 to 15% by weight of the acetylated lignins in $CDCl_3$ containing hexamethyldisiloxane internal reference were used in standard degassed and sealed 5-mm. tubes.

The integrations of n.m.r. spectra were done using an integrator described by Varian Associates.[12] Prior to integration of the n.m.r. spectrum of each lignin preparation a sample of ethanol was integrated to determine that the integrator was adjusted correctly. The integrator was considered to be properly adjusted only when the ratio of methylene protons to methyl protons in the ethanol was found to be 2.00 to 3.00 within confidence limits of 0.01. The stability of the integrator was further checked by allowing the sweep to cover several parts per million at the beginning and end of each spectrum to be sure there was no appreciable drift. Lignin solutions of 10% or greater concentration were found to give more reliable integration results than weaker solutions.

A correction for the small amount of signal from chloroform protons in the deuteriochloroform solvent was made in each case as follows. By integrating an n.m.r. spectrum of our solvent deuteriochloroform containing a known weight of hexamethyldisiloxane it was found to contain about 0.0042% protons by weight. Using this figure the percentage of the total integrated signal coming from chloroform protons, A, was found for each sample by applying the following equation in which x = the weight per cent of lignin preparation in the sample, $100 - x$ = the weight per cent of deuteriochloroform in the sample, and H = fraction of hydrogen in lignin. (The small amount of hexamethyldisiloxane was neglected.)

$$A = \frac{(0.0042)(100 - x)(100\%)}{(Hx) + (0.0042)(100 - x)}$$

Acknowledgment.—The authors are grateful to Professor Eric Adler and Dr. F. E. Brauns for their generous gifts of Bjorkman and Brauns lignins used in these studies and to Professor K. V. Sarkanen for his many helpful suggestions in the preparation of this manuscript.

(12) The NMR-EPR Staff of Varian Associates, "NMR and EPR Spectroscopy," Pergamon Press, New York, N. Y., 1960, Chapter 15.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, THE WEIZMANN INSTITUTE OF SCIENCE, REHOVOTH, ISRAEL]

On the Synthesis of Cysteine Peptides[1]

BY MORDECHAI SOKOLOVSKY, MEIR WILCHEK, AND ABRAHAM PATCHORNIK

RECEIVED AUGUST 14, 1963

The preparation of various peptides of cysteine is described. The basis of the method used is the selective removal of the carbobenzoxy group from either the amino or thiol group without causing racemization. The action of hydrogen bromide in acetic acid at room temperature removes the carbobenzoxy moiety from the amino group almost quantitatively, without affecting the S-carbobenzoxy group. On the other hand, the action of excess sodium methoxide in anhydrous solvent causes rapid alcoholysis of the S-carbobenzoxy group with a quantitative liberation of the free thiol group as determined iodometrically. The method described was successfully applied to the total synthesis of glutathione in 25% over-all yield.

In the course of recent studies on the development of a method for the nonenzymatic cleavage of peptide at cysteinyl residues[2] it was found necessary to prepare cysteine-containing peptides as model compounds.

Methods for the preparation of peptides containing S-protected cysteinyl residues have been known for a long time.[3] However, the methods used so far for the selective removal of the S-protecting group suffer from a number of disadvantages: *e.g.*, low yields, racemization, and side reactions such as splitting of peptide bonds.[4] The problems arising during the synthesis of such peptides have been reviewed recently by Young.[5]

In the present paper, the preparation of various S- and N-carbobenzoxy derivatives of cysteine and their application to the synthesis of cysteine peptides is described. The basis of this method is the selective removal of the carbobenzoxy group from either the amino or thiol group in high yields without causing racemization. The action of hydrogen bromide in acetic acid for 15 min. at room temperature removes the carbobenzoxy moiety from the amino group almost quantitatively without affecting the S-carbobenzoxy group. On the other hand, the action of excess sodium methoxide (5 equiv.) for 5–10 min. at room temperature, under nitrogen, causes rapid alcoholysis of the S-carbobenzoxy group with almost quantitative liberation of the free thiol group, as determined iodometrically.

Such selective removal of the protecting group may be conveniently used in the synthesis of long peptide

(1) A recent report by L. Zervas, I. Photaki, and N. Ghelis (*J. Am. Chem. Soc.*, **85**, 1337 (1963)), which reached us after this work had been completed, contains results similar to some of those described in this paper.

(2) A. Patchornik and M. Sokolovsky, "Vth European Peptide Symposium, Oxford, 1962," Pergamon Press, 1963, p. 253, and in the following papers.

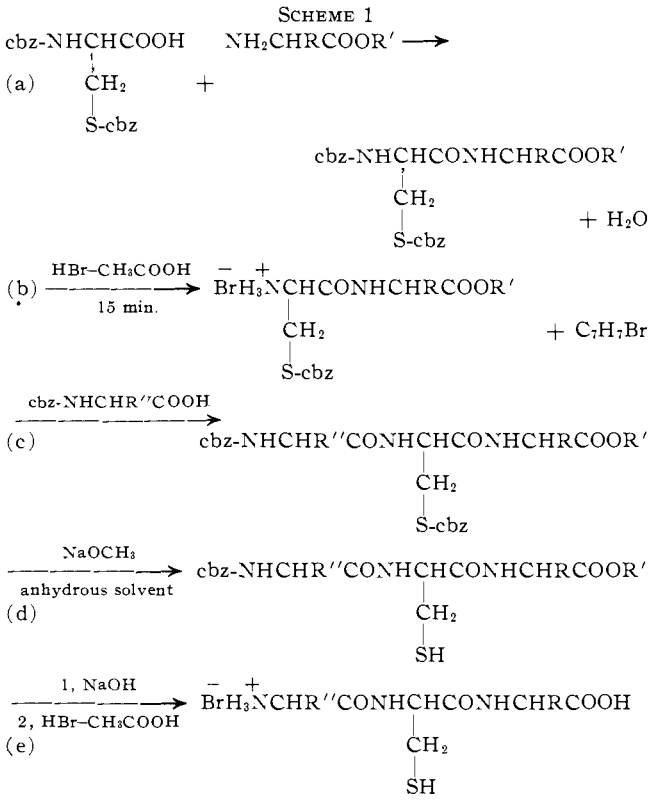
(3) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961.

(4) S. Sarid and A. Patchornik, *Israel J. Chem.*, **1**, 63 (1963).

(5) G. T. Young, *Collection Czech. Chem. Commun.*, **24**, 114 (1959).

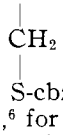
chains containing cysteine residues as exemplified by Scheme 1.

SCHEME 1



abbreviations: cbz = carbobenzoxy group, R, R'' = amino acid residue, R' = carboxyl protecting group

In step a, N,S-dicarbobenzoxy-L-cysteine is coupled with an amino acid ester by known methods of coupling.[3] In a similar fashion, S-carbobenzoxy-L-cysteine ester can be coupled with carbobenzoxy amino acids yielding peptides of the type cbz-NHCHRCONHCHCOOR'.



By the use of the carbodiimide method,[6] for example, high yields (85–90%) of pure protected peptides can be obtained. Treatment of these peptides with 3 equiv. of hydrogen bromide in glacial acetic acid yields the corresponding hydrobromides (step b). This reaction goes to completion within 15 min. at room temperature. For example, N,S-dicarbobenzoxy-L-cysteinylglycine benzyl ester (I) gives the corresponding hydrobromide in 75% yield. Under these conditions, the S-carbobenzoxy moiety was not removed as could be shown titrimetrically.[7] It should be mentioned that even under more drastic conditions (100° for 30 min.) only 30% removal of the S-carbobenzoxy group occurred.

Step c is carried out similarly to step a. By this procedure, various protected peptides were synthesized in 80–90% yields: *e.g.*, N,S-dicarbobenzoxy-L-cysteinylglycine benzylester (I), N,S-dicarbobenzoxy-L-cysteinylglycine ethyl ester (II), carbobenzoxy-L-phenylalanyl-S-carbobenzoxy-L-cysteinylglycine ethyl ester (III), N,S-dicarbobenzoxyglutathione dibenzyl ester (IV), N-carbobenzoxy-L-alanyl-S-carbobenzoxy-L-cysteine benzyl ester (V), N,S-dicarbobenzoxy-L-cysteinyl-L-leucine methyl ester (VI), and N,S-dicarbobenzoxy-L-cysteinyl-L-phenylalanine benzyl ester (VII). Furthermore, the procedure was applied to the synthesis of a tetrapeptide corresponding to the sequence C-terminal of oxytocin: N,S-dicarbobenzoxy-L-cysteinyl-L-prolyl-L-leucylglycine amide (VIII) in 86% yield.

Upon addition of 5 equiv. of sodium methoxide per mole of peptide for 10 min. (step d) all these derivatives resulted in the formation of the corresponding peptides containing a free thiol group. Thus, compounds II, III, VII, and poly-S-carbobenzoxy-L-cysteine[8] yielded N-carbobenzoxy-L-cysteinylglycine ethyl ester (IX), N-carbobenzoxy-L-phenylalanine-L-cysteinylglycine ethyl ester (X), N-carbobenzoxy-L-cysteinyl-L-phenylalanine methyl ester (XI), and poly-L-cysteine, respectively, in high yields (80–90%). The action of alkali on cysteine derivatives is known to cause a β-elimination reaction.[9] However, under the conditions used in the procedure described it could be shown, by determination of the pyruvic acid known to be formed on acid hydrolysis of dehydroalanine containing peptides,[10] that the β-elimination reaction, in which S-protected cysteine peptides are converted to the corresponding dehydroalanyl derivatives,[9,11] occurred to the extent of less than 3–4%.

It is essential to carry out the reaction under anhydrous conditions so as to avoid hydrolysis of the ester bond. In order to avoid transesterification the base used must be derived from the same alcohol as the ester used for esterification of the carboxyl groups. Thus, when compound VII was treated with sodium methoxide in methanol, N-carbobenzoxy-L-cysteinyl-L-phenylalanine methyl ester (XI) and not the benzyl ester was obtained.

In order to estimate whether racemization occurred by this procedure the following determinations were carried out: (a) N,S-dicarbobenzoxyglutathione (XII) was treated with 5 equiv. of sodium methoxide in absolute methanol for 10 min.; the pH was lowered to 8, and recarbobenzoxylation was effected by addition of 5 equiv. of benzyl chloroformate. The isolated compound had a specific rotation identical with that of the original N,S-dicarbobenzoxylglutathione. (Identical results were obtained when the base used was aqueous sodium hydroxide.) (b) Similarly, compound VIII was transformed to the corresponding N-carbobenzoxy-S-benzyl derivative in 90% yield, and this compound had $[\alpha]^{25}D$ $-61°$ (*c* 2, dimethylformamide); the reported values are $[\alpha]^{22}D$ $-60°$,[12] $-62°$.[13] These tests clearly indicates that step d does not cause racemization.

When a peptide contains N-terminal cysteine with a free amino group and a S-carbobenzoxy group, it must be borne in mind that under alkaline conditions (step d), the carbobenzoxy group may migrate from the sulfur to the nitrogen.[14] Indeed, when S-carbobenzoxycysteine was treated with sodium methoxide as described above, and then oxidized with iodine under acidic conditions, dicarbobenzoxycysteine was isolated in 61% yield. Analogously, S-carbobenzoxy-L-cysteinyl-L-phenylalanine benzyl ester hydrobromide (XIII) gave N-carbobenzoxy-L-cysteinyl-L-phenylalanine in 60% yield. This rearrangement may occur as the result of

(6) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

(7) A. Patchornik and S. Ehrlich-Rogozinski, *Anal. Chem.*, **33**, 803 (1961).

(8) A. Berger, J. Noguchi, and E. Katchalski, *J. Am. Chem. Soc.*, **78**, 4483 (1956).

(9) H. T. Clarke and J. M. Inouye, *J. Biol. Chem.*, **94**, 541 (1931); J. M. Swan, *Nature*, **18**, 643 (1957); M. Sokolovsky, M. Wilchek, and A. Patchornik, *Bull. Res. Council Israel*, **11A**, 79 (1962).

(10) A. Patchornik and M. Sokolovsky, *J. Am. Chem. Soc.*, **86**, 1206 (1964).

(11) M. Sokolovsky, T. Sadeh, and A. Patchornik, *ibid.*, **86**, 1212 (1964).

(12) M. Bodansky and V. du Vigneaud, *ibid.*, **81**, 2504 (1959).

(13) H. C. Beyerman, J. S. Bontekoe, and A. C. Koch, *Rec. trav. chim.*, **78**, 935 (1959).

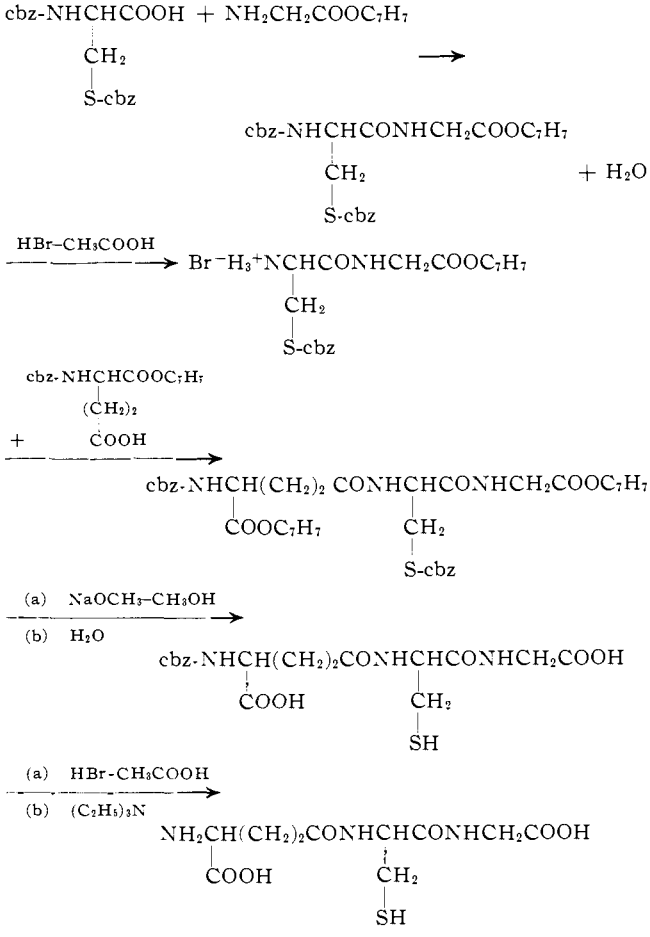
(14) H. P. Burchfield, *Nature*, **181**, 49 (1958); L. A. Cohen and B. Witkop, *Angew. Chem.*, **73**, 253 (1961).

an S → N shift, or may be due to a nucleophilic attack of the α-amine on the S-carbobenzoxy group which may be regarded as an "active ester." The possibility of such a shift can be avoided by removing the S-carbobenzoxy group before removing the N-protecting group.

In order to obtain peptides containing protected amino groups and free thiol and carboxyl groups, it is sufficient to add small amounts of water to the reaction mixture after completion of step d. Under the alkaline conditions prevailing, hydrolysis of the ester is completed within 30 min. at room temperature. Under such conditions, N,S-dicarbobenzoxyglutathione dibenzyl ester (IV) yielded N-carbobenzoxyglutathione in 79% yield.

The methods illustrated in Scheme 1 were successfully applied to the total synthesis of glutathione in 25% over all yield, as shown in Scheme 2.

SCHEME 2



Experimental

All melting points are uncorrected. Prior to analysis, the compounds were dried *in vacuo* over phosphorus pentoxide at 45°. The hydrogen bromide used was free of bromine and the ether used was free from peroxides. The content of the free thiol was determined by titration with 0.1 *N* iodine.

N,S-Dicarbobenzoxy-L-cysteinylglycine Benzyl Ester (I).—To a solution of 35 g. of glycine benzyl ester *p*-toluenesulfonate (0.1 mole) and 14.4 ml. of triethylamine (0.1 mole) in 150 ml. of dichloromethane, 38.8 g. of N,S-dicarbobenzoxy-L-cysteine (0.1 mole)[8] in 150 ml. of dichloromethane and 20.5 g. of dicyclohexylcarbodiimide (0.1 mole) were added at 0°. The solution was then stirred overnight at room temperature. Dicyclohexylurea was removed by filtration and the filtrate was washed with 0.5 *N* hydrochloric acid, water, 5% sodium bicarbonate solution, and water, and finally dried over sodium sulfate. The solvent was evaporated *in vacuo*. Upon adding petroleum ether, the residue crystallized and was recrystallized from ethyl acetate; yield 90%, m.p. 118–119°; after recrystallization from ethanol, m.p. 108–109°, $[\alpha]^{25}D$ −44.5° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{28}H_{28}N_2O_7S$: C, 62.68; H, 5.26; N, 5.22; S, 5.96. Found: C, 62.90; H, 5.32; N, 5.35; S, 5.98.

S-Carbobenzoxy-L-cysteine Benzyl Ester Hydrochloride.—Dry phosgene was passed at room temperature through a suspension of 25.4 g. of S-carbobenzoxy-L-cysteine (0.1 moles) in anhydrous dioxane (300 ml.) until a clear solution was obtained (about 1 hr.). Phosgene was removed by a stream of dry nitrogen and the solvent was distilled off *in vacuo* at 45°. Benzyl alcohol (60 ml.) and dry ether (300 ml.), previously saturated with gaseous HCl (10 g.) at 0°, were added and the solution was left overnight at room temperature. The ester which separated was filtered off and washed with ether. The ester was recrystallized from hot water or methyl alcohol–ether; yield 30 g. (78%), m.p. 106–107°.

Anal. Calcd. for $C_{18}H_{20}NO_4SCl$: C, 56.69; H, 5.24; N, 3.67; S, 8.39. Found: C, 56.75; H, 5.37; N, 3.50; S, 8.51.

L-Phenylalanine benzyl ester hydrochloride was prepared in the same manner as described for S-carbobenzoxy-L-cysteine benzyl ester hydrochloride, and it was recrystallized from water; yield 80%, m.p. 208°, $[\alpha]^{25}D$ −23° (*c* 2, 0.25 *N* hydrochloric acid); reported[15] m.p. 203°, $[\alpha]^{25}D$ −22.5° (*c* 1, 0.25 *N* hydrochloric acid).

Anal. Calcd. for $C_{16}H_{18}NO_2Cl$: N, 4.81; Cl, 12.03. Found: N, 4.84; Cl, 12.12.

The procedure described for the synthesis of compound I was used for the preparation of the following compounds:

N,S-Dicarbobenzoxy-L-cysteinylglycine ethyl ester (II): yield (79%), m.p. 94°; recrystallized from ethyl acetate–petroleum ether, $[\alpha]^{25}D$ −31° (*c* 2, ethanol). *Anal.* Calcd. for $C_{23}H_{26}N_2O_7S$: C, 58.22; H, 5.52; N, 5.90; S, 6.74. Found: C, 58.42; H, 5.48; N, 6.03; S, 6.65.

N-Carbobenzoxy-L-alanyl-S-carbobenzoxy-L-cysteine benzyl ester (V): yield 89%, m.p. 109–110°; after recrystallization from ethanol, $[\alpha]^{25}D$ −40.5° (*c* 2, methanol). *Anal.* Calcd. for $C_{29}H_{30}N_2O_7S$: C, 63.26; H, 5.49; N, 5.09; S, 5.81. Found: C, 62.97; H, 5.35; N, 5.27; S, 5.80.

N,S-Dicarbobenzoxy-L-cysteinyl-L-leucine methyl ester (VI): yield 95%, m.p. 98–100°; after recrystallization from ethyl acetate–petroleum ether, $[\alpha]^{25}D$ −36.5° (*c* 2, methanol). *Anal.* Calcd. for $C_{26}H_{32}N_2O_7S$: C, 60.45; H, 6.24; N, 5.42. Found: C, 60.58; H, 6.20; N, 5.22.

N-Carbobenzoxy-L-phenylalanyl-S-carbobenzoxy-L-cysteinylglycine ethyl ester (III).—N,S-Dicarbobenzoxy-L-cysteinylglycine ethyl ester (5 g.) was treated with 8 ml. of 45% HBr in acetic acid. Carbon dioxide evolution ceased after 15 min. and anhydrous ether was added to precipitate the dipeptide ester hydrobromide, which was triturated with ether until entirely crystalline, then washed with ether and dried *in vacuo;* yield 4 g. This compound was coupled with 3 g. of N-carbobenzoxy-L-phenylalanine by the carbodiimide method as described above. The crude material weighed 5.2 g. (84%). Recrystallization from ethyl acetate yielded 4 g. (64%), m.p. 110°, $[\alpha]^{25}D$ −40.6° (*c* 2, ethanol).

Anal. Calcd. for $C_{32}H_{35}N_3O_8S$: C, 61.83; H, 5.68; N, 6.76; S, 5.15. Found: C, 61.92; H, 5.60; N, 6.82; S, 5.21.

S-Carbobenzoxy-L-cysteinylglycine Benzyl Ester Hydrobromide.—A solution of 10.7 g. of compound I (0.02 mole) in 15 ml. of acetic acid was treated with 16 ml. of 45% HBr in acetic acid, as described above. The oily residue which was precipitated by the addition of ether was dissolved in about 10 ml. of methanol; upon adding ether it crystallized and was recrystallized from methanol–ether or water; yield 7 g. (75%), m.p. 126–128°, $[\alpha]^{25}D$ +12° (*c* 2, methanol).

Anal. Calcd. for $C_{20}H_{23}O_5N_2SBr$: C, 49.69; H, 4.76; N, 5.79; S, 6.63. Found: C, 49.62; H, 4.70; N, 5.91; S, 6.38.

N-Carbobenzoxy-γ-L-glutamyl-(α-benzyl ester)-S-carbobenzoxy-L-cysteinylglycine benzyl ester (N,S-dicarbobenzoxyglutathione dibenzyl ester) (IV) was prepared by coupling of N-carbobenzoxy-α-benzyl ester L-glutamic acid[16] and S-carbobenzoxy-L-cysteinylglycine benzyl ester hydrobromide in the same manner as described for the preparation of I. The peptide was recrystallized from benzene or ethyl acetate; yield 90%, m.p. 158–159°, $[\alpha]^{25}D$ −35.5° (*c* 1, methanol).

Anal. Calcd. for $C_{40}H_{41}N_3O_{10}S$: C, 63.56; H, 5.47; N, 5.56; S, 4.24. Found: C, 63.60; H, 5.55; N, 5.52; S, 4.15.

N,S-Dicarbobenzoxy-L-cysteinyl-L-phenylalanine benzyl ester (VII) was prepared by coupling of N,S-dicarbobenzoxy-L-cysteine with L-phenylalanine benzyl ester in the same manner as described for the preparation of compound I. The crude product was recrystallized from ethyl acetate; yield 82%, m.p. 147–148°, $[\alpha]^{25}D$ −44.8° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{35}H_{34}N_2O_7S$: C, 67.07; H, 5.47; N, 4.47; S, 5.12. Found: C, 66.98; H, 5.56; N, 4.58; S, 5.03.

(15) B. F. Erlanger and R. M. Hall, *J. Am. Chem. Soc.*, **76**, 5781 (1954).

(16) G. Lasse, H. Jeschkeit, and W. Langenbeck, *Chem. Ber.*, **96**, 204 (1963).

N,S-Dicarbobenzoxy-L-cysteinyl-L-prolyl-L-leucylglycine amide (VIII) was prepared by coupling of N,S-dicarbobenzoxy-L-cysteine with L-prolyl-L-leucylglycine amide[17] in dimethylformamide by the use of the carbodiimide method; yield 86%, $[\alpha]^{25}D$ −67° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{32}H_{41}N_5O_8S$: C, 58.61; H, 6.30; N, 10.68; S, 4.88. Found: C, 58.48; H, 6.33; N, 10.88; S, 4.76.

S-Carbobenzoxy-L-cysteinyl-L-phenylalanine Benzyl Ester Hydrobromide (XIII).—To a suspension of 4.5 g. (0.007 mole) of compound VII, 7 ml. of 45% HBr in acetic acid was added. The ester dissolved completely during the first 3 min. Addition of ether after 12 min. caused the above hydrobromide to separate. The product was recrystallized from water; yield 3.7 g. (90%), m.p. 141°, $[\alpha]^{25}D$ −6.5° (*c* 2, methanol).

Anal. Calcd. for $C_{27}H_{29}N_2O_5SBr$: C, 56.56; H, 5.06; N, 4.88; S, 5.58. Found: C, 56.51; H, 5.10; N, 4.99; S, 5.62.

N-Carbobenzoxy-L-phenylalanyl-L-cysteinylglycine Ethyl Ester (X).—To a suspension of 1.24 g. (0.002 mole) of compound VI in 30 ml. of absolute ethanol 6 ml. of ethanolic 2.4 *N* sodium ethoxide was added in an atmosphere of nitrogen. The ester dissolved completely after 2 min. of stirring which was continued for an additional 5 min. On acidification with 1 *N* HCl, a crystalline compound separated out. The precipitate was collected by filtration and was recrystallized from ethyl acetate; yield 93%, m.p. 170°, $[\alpha]^{25}D$ −17.4° (*c* 3, dimethylformamide) (reported[18] m.p. 178–179°, $[\alpha]^{30}D$ −16.8° (*c* 3, dimethylformamide)). The iodine titration revealed 98% free thiol group.

Anal. Calcd. for $C_{24}H_{29}N_3O_6S$: C, 59.13; N, 6.00; N, 8.62; S, 6.57. Found: C, 59.08; H, 5.92; N, 8.81; S, 6.68.

N-Carbobenzoxy-L-cysteinyl-L-phenylalanine Methyl Ester (XI).—To a suspension of 1.25 g. of N,S-dicarbobenzoxy-L-cysteinyl-L-phenylalanine benzyl ester (0.002 mole) in 15 ml. of absolute methanol, 5 ml. of methanolic 2 *N* sodium methoxide was added in an atmosphere of nitrogen. The ester dissolved completely during 4 min. The iodine titration revealed 98% free thiol after 10 min. On acidification with 1 *N* HCl, a crystalline compound separated out, and was recrystallized from ethyl acetate–petroleum ether; yield 0.75 g. (91%), m.p. 98°, $[\alpha]^{25}D$ −11.7° (*c* 1, ethanol).

Anal. Calcd. for $C_{21}H_{24}N_2O_5S$: C, 60.56; H, 5.81; N, 6.73; S, 7.69; $-OCH_3$, 7.45. Found: C, 60.39; H, 5.93; N, 6.58; S, 7.58; $-OCH_3$, 7.21.

N-Carbobenzoxy-L-cysteinylglycine Ethyl Ester (IX).—To a solution of 2.37 g. of compound II (0.005 mole) in 15 ml. of absolute ethanol, 15 ml. of ethanolic 2 *N* sodium ethoxide was added in an atmosphere of nitrogen. After 7 min., the solution was acidified with 1 *N* HCl and water was added. The oil which separated out was dissolved in ethyl acetate. The ethyl acetate solution was dried over sodium sulfate and evaporated to dryness *in vacuo*. The crystalline residue was triturated with ether (free of peroxide) and was filtered off; yield 1.2 g. (70%), m.p. 122–123°, $[\alpha]^{25}D$ −16.9° (*c* 3, ethanol); reported m.p. 118–120°,[19] 123–124°,[18] $[\alpha]^{28}D$ −16.8° (*c* 3, ethanol). Iodine titration revealed 97% of free thiol.

Anal. Calcd. for $C_{15}H_{20}N_2O_5S$: C, 52.92; H, 5.92; N, 8.23; S, 9.42. Found: C, 52.90; H, 5.80; N, 8.40; S, 9.28.

N-Carbobenzoxy-L-cysteinyl-L-phenylalanine.—To a suspension of 2.1 g. of compound VII (0.003 mole) in 15 ml. of absolute methanol 5 ml. of methanolic 3 *N* sodium methoxide was added in an atmosphere of nitrogen. After 10 min., 3 ml. of water was added to the solution which was kept for an additional 30 min. The solution was then acidified by the addition of 0.5 *N* hydrochloric acid. The crystalline product was recrystallized from 80% methanol. Iodine titration revealed 98% of free thiol; yield 1 g. (77%), m.p. 148–150°, $[\alpha]^{25}D$ −7.5° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{20}H_{22}N_2O_5S$: C, 59.69; H, 5.51; N, 6.96; S, 7.96. Found: C, 59.76; H, 5.55; N, 7.05; S, 7.83.

Poly-L-cysteine.—To a solution of 1 g. of poly-S-carbobenzoxy-L-cysteine[8] in 2 ml. of pyridine, 5 ml. of methanolic 2.4 *N* sodium methoxide was added in an atmosphere of nitrogen. After 10 min., 5 ml. of glacial acetic acid and 25 ml. of 3 *N* hydrochloric acid were added. The poly-L-cysteine which separated was collected on a glass filter under nitrogen and washed with air-free water, alcohol, and ether; yield, 300 mg. (65%).

Anal. Calcd. for (C_3H_5NOS): C, 34.95; H, 4.39; N, 13.59; thiol-S, 32.03. Found: C, 35.05; H, 4.71; N, 13.23; thiol-S, 28.60.

The –SH group was estimated by titration with iodine as described by Berger, *et al.*[8] The polymer was identical in its properties with that prepared by alternative procedures.[8]

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N,S-Dicarbobenzoxyglutathione (XII).—A solution of 6.14 g. of reduced glutathione (0.02 mole) was adjusted to pH 9–9.5 with 1 *N* sodium hydroxide and treated with stirring and cooling in an ice bath with benzyl chloroformate (7.5 g., 0.044 mole) over a 30-min. period. During this time the pH was maintained between 8.5–9.5 by addition of 1 *N* sodium hydroxide. After further stirring for 30 min. at room temperature, the reaction mixture was extracted with ether (three 150-ml. portions) and the aqueous layer acidified with 5 *N* HCl. The resulting crystalline product was filtered, washed with water, and recrystallized from methanol–water; yield 10 g. (87%), m.p. 105–107°, $[\alpha]^{25}D$ −32° (*c* 1, methanol).

Anal. Calcd. for $C_{26}H_{29}N_3O_{10}S$: C, 54.25; H, 5.08; N, 7.30; S, 5.57. Found: C, 54.00; H, 5.06; N, 7.40; S, 5.71.

Another preparation of this compound with sodium carbonate in place of sodium hydroxide gave a compound with m.p. 141–143°, $[\alpha]^{25}D$ −32° (*c* 1, methanol).

Anal. Found: C, 53.99; H, 5.09; N, 7.21; S, 5.75.

Attempts to raise the melting point of the product with the low melting point by recrystallization were unsuccessful.

Methanolysis of XII and Recarbobenzoxylation.—To a solution of 1.15 g. of compound XII (0.002 mole) in 20 ml. of anhydrous methanol, 7 ml. of methanolic 1 *N* sodium methoxide was added under nitrogen. After 7 min., when iodine titration of an aliquot revealed 98% methanolysis, the pH was lowered to 8.5 with dilute acetic acid. The solution was diluted with water, and 1.7 g. of benzyl chloroformate (0.01 mole) was added. The reaction mixture was stirred for 0.5 hr. at 0°. During this time the pH was maintained at 8.5–9 by addition of 0.1 *N* sodium hydroxide. After further stirring for 30 min. at room temperature the reaction mixture was extracted twice with ether and once with petroleum ether, and the pH was adjusted to 2 with dilute hydrochloric acid. The resulting crystalline product was recrystallized from methanol–water; yield 1 g. (85%), m.p. 105–106°, $[\alpha]^{25}D$ −31.9° (*c* 1, methanol). The m.p. was not depressed on admixture with a sample of compound XII.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine Amide.—To a solution of 0.655 g. of compound VIII (0.001 mole) in 20 ml. of absolute methanol 2 ml. of methanolic 2.5 *N* sodium methoxide was added under nitrogen. After 10 min. when iodometric titration revealed 99% methanolysis, 0.3 ml. of glacial acetic acid was added. The pH was brought to 8 with triethylamine and 0.25 g. of benzyl chloride (0.002 mole) was added with stirring. After 30 min. water was added and on cooling a crystalline product was produced and was recrystallized from ethyl acetate; yield 0.52 g. (86%), m.p. 104–105°, $[\alpha]^{25}D$ −61° (*c* 1, dimethylformamide); reported m.p. 161–164°,[13] $[\alpha]^{22}D$ −62° (*c* 0.98, dimethylformamide), m.p. 170–171°,[12] $[\alpha]^{22}D$ −60° (*c* 2, dimethylformamide).[12]

Transformation of S-Carbobenzoxy-L-cysteine[8] to N,N′-Bis-carbobenzoxy-L-cysteine.—Methanolysis of 2.54 g. of S-carbobenzoxy-L-cysteine (0.01 mole) was carried out as described above. After acidification to pH 4, the product was oxidized by addition of 10 ml. of a 1 *N* iodine solution. The product was extracted twice with 100 ml. of ethyl acetate, washed with water, and dried over sodium sulfate. The solvent was evaporated *in vacuo*. Upon adding petroleum ether the product crystallized, and was recrystallized from carbon tetrachloride; yield 1.55 g. (61%), m.p. 121–123°, $[\alpha]^{25}D$ −91° (*c* 5, acetic acid); reported[20] m.p. 123°, $[\alpha]^{20}D$ −91.7° (*c* 6.7, acetic acid). The m.p. was not depressed on admixture with an authentic sample.

Transformation of XIII to N-Carbobenzoxy-L-cysteinyl-L-phenylalanine.—To a solution of 1.1 g. of compound XIII (0.002 mole) in 20 ml. of anhydrous methanol, 5 ml. of methanolic 2 *N* sodium methoxide was added under nitrogen. After incubation for 7 min. at room temperature, 1 ml. of water was added and the reaction mixture was kept at room temperature for 30 min. The mixture was concentrated *in vacuo* until most of the methanol had been removed. On addition of water a crystalline product separated out; yield 0.53 g. (66%), m.p. 149–150°. Iodine titration revealed 97% free thiol group. The m.p. was not depressed on admixture with an authentic sample.

Synthesis of Glutathione. (a) N-Carbobenzoxyglutathione.—To a suspension of 1.5 g. of compound IV (0.002 mole) in 20 ml. of anhydrous methanol 5 ml. of methanolic 2 *N* sodium methoxide was added under nitrogen. The compound dissolved within 2–3 min., and after stirring for 7 min. had undergone 98% methanolysis as revealed by iodometric titration. Water, 6 ml., was added and the reaction mixture allowed to stand for a further 30 min. The solution was diluted with water to 120 ml., extracted twice with 100 ml. of ether, and was then acidified and extracted three times with 100 ml. of ethyl acetate. The ethyl acetate was washed with water and dried over sodium sulfate. After removal of the solvent *in vacuo* an oily product was obtained which crystallized on addition of 10 ml. of ethyl acetate at 0°.

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The product was recrystallized from ethyl acetate; yield 0.7 g. (79%), m.p. 143–145°, $[\alpha]^{25}D$ −26.5° (*c* 1, methanol).

Anal. Calcd. for $C_{18}H_{23}N_3O_8S$: C, 48.98; H, 5.25; N, 9.52; neut. equiv., 220.5. Found: C, 49.17; H, 5.41; N, 9.51; neut. equiv., 223.

The neutral equivalent was obtained by titration with 0.1 *N* sodium methoxide using thymol blue in ethanol as indicator.[21] Iodometric titration revealed 97% free thiol groups. The same product was obtained by methanolysis of compound XII.

(b) **Glutathione.**—N-Carbobenzoxyglutathione (0.44 g., 0.001 mole) was suspended in 2 ml. of 45% hydrobromic acid in acetic acid. After 5 min. the compound had dissolved and the solution was kept for an additional 10 min. at room temperature. Anhydrous ether, 100 ml., was added, and the hydrobromide derivative then precipitated. The precipitate was repeatedly washed at the centrifuge with anhydrous ether and then dissolved in 3 ml. of oxygen-free water. The pH was adjusted to 2.9 with dilute triethylamine and was concentrated to 0.2 ml. at 25° *in vacuo*. Upon addition of ethanol the glutathione precipitated. The precipitate was repeatedly washed at the centrifuge with ethanol; yield 0.154 g. (50%), m.p. 189–190°.[22] On oxidation with iodine (80% of the theoretical value), the product behaved chromatographically and electrophoretically like an authentic sample. For further purification the method described by du Vigneaud, *et al.*,[22] was found to be necessary; m.p. 190°, $[\alpha]^{25}D$ −21.8° (reported[22] $[\alpha]^{27}D$ −21.3°).

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Acknowledgment.—This investigation was supported by Grant AM-5098 of the National Institutes of Health, United States Public Health Service.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, THE WEIZMANN INSTITUTE OF SCIENCE, REHOVOTH, ISRAEL]

Nonenzymatic Cleavages of Peptide Chains at the Cysteine and Serine Residues through their Conversion into Dehydroalanine. I. Hydrolytic and Oxidative Cleavage of Dehydroalanine Residues[1,2]

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RECEIVED AUGUST 14, 1963

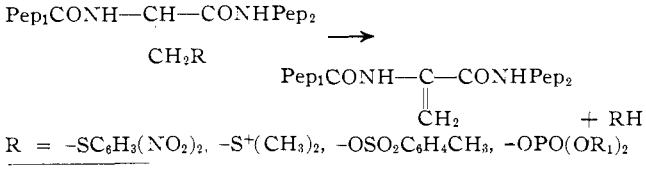
In connection with the specific chemical fragmentation of peptides and proteins, the conditions for the cleavage of dehydroalanine residues, which can be formed from cysteine and serine residues, was investigated. Dehydroalanine residues are cleaved hydrolytically at the N–C_α bond with nearly quantitative yield by heating the compound to 100° at pH 2 for 1 hr. The cleavage can be facilitated by oxidative activation of the molecule. Oxidation by bromine or performic acid leads to a labile intermediate which is easily cleaved at alkaline pH at room temperature. Both methods lead to the formation of two fragments: $Pep_1CONHC(CH_2)CONHPep_2 \rightarrow Pep_1CONH_2 + XCH_2COCONHPep_2$ (X = H without oxidation, X = OH with oxidation). The nitrogen of the dehydroalanine residue appears as a new terminal amide group on one of the fragments and the rest of the molecule as a terminal pyruvoyl group on the other. The pyruvoyl group can be removed by oxidation with alkaline hydrogen peroxide, a step which liberates the new terminal amino group. A method for the quantitative estimation of dehydroalanine residues in peptides is described.

Introduction

A primary step in the elucidation of the chemical structure of proteins is the selective fragmentation of separated peptide chains. Such fragmentation can be achieved by enzymatic hydrolysis, but only few proteolytic enzymes in use are sufficiently selective.[3] Furthermore, the number of amino acids next to which proteolytic enzymes are known to cleave in a selective way is limited and side reactions sometimes complicate interpretation.[4]

Recently a number of selective nonenzymatic methods for the cleavage of peptide bonds involving a particular amino acid were reported[5–11] and reviewed by Witkop.[12] This new approach has been used recently in studies on the structure of several proteins[8,9,12–14] and natural peptides.[1c,7,8,14]

The methods described so far for the specific chemical cleavage of peptide bonds[12] have been based on interaction of the side chain of an amino acid residue (sometimes after specific chemical modification) with the adjacent peptide bond. This bond is labilized by the formation of intermediate cyclic structures which facilitate the hydrolysis of the peptide chain into two fragments.[12] A new approach to the labilization of peptide chains not involving a cyclic intermediate consists in the conversion of cysteine and serine[1c,15] residues into dehydroalanine residues. This can be achieved by the conversion of the cysteine or serine residue into a derivative which possesses a good "leaving group," *e.g.*, a sulfonium or thiodinitrophenyl group in the case of cysteine, and tosyl or phosphoryl group in the case of serine. These derivatives can be converted to the corresponding dehydroalanine derivative by an elimination reaction under basic conditions.



R = $-SC_6H_3(NO_2)_2$, $-S^+(CH_3)_2$, $-OSO_2C_6H_4CH_3$, $-OPO(OR_1)_2$

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