ture was distilled at the pressure of the water aspirator with occasional warming on the steam-bath. Acetic acid and unreacted bromine were thus removed and a pale yellow crystalline solid remained. This product was digested with boiling ether for a few minutes and filtered to give almost colorless crystals (1.96 g., 41%), m.p. 180–185° dec. Recrystallization from ethyl acetate–cyclohexane afforded colorless needles, m.p. 195–200° dec. (cap. tube inserted at 180°), undepressed on admixture with the product obtained by bromination in chloroform; the infrared spectra of the two products were identical; ultraviolet maxima (CH_3CN)[25]: 246 mμ (log ε 3.72), 323 mμ (log ε 2.26).

Anal. Calcd. for $C_{15}H_{15}O_4Br_3$: C, 36.10; H, 3.03; Br, 48.05. Found: C, 35.96; H, 2.99; Br, 48.12.

Hydrolysis of Tribromosantonin.—Tribromosantonin (0.2 g.) and an aqueous solution (25 ml.) of sodium bicarbonate (1.1 g.) were boiled under reflux. The tribromosantonin dissolved slowly and after about 15 minutes a brown amorphous solid began to separate from the solution. After 30 minutes the reaction mixture was cooled and filtered. The clear, red-brown filtrate was washed with ether and acidified. The turbid liquid was extracted with ether and the extracts were washed with water and dried over anhydrous magnesium sulfate. Removal of solvent at room temperature *in vacuo* left an acidic brown gum (*ca.* 0.050 g.) from which no crystalline product could be obtained.

Reduction of Tribromosantonin with Chromous Chloride. Formation of II and IIIb.—A three-necked round-bottomed flask was fitted with a gas inlet tube, a wide rubber sleeve connected to an erlenmeyer flask, and an outlet tube fitted with a sintered glass filter end leading *via* a stopcock to the reaction vessel; the outlet tube was initially raised above the contents of the flask. A solution of chromic chloride (20 g.) in 95% ethanol (140 ml.) and concentrated hydrochloric acid (30 ml.) was placed in the round-bottomed flask and granulated zinc (16 g.) was placed in the erlenmeyer flask. The apparatus was thoroughly purged of air with nitrogen and the zinc was added to the solution in portions over a period of 10 minutes. After 4 hours the reduction of chromic chloride to chromous chloride was complete as evidenced by the change in the color of the solution from deep green to pale blue. The outlet tube was then lowered into the solution so that the sintered glass filter end dipped well beneath the surface. Chromous chloride solution (0.75 *M*) was then forced out by means of nitrogen pressure into the reaction vessel.

Tribromosantonin (0.90 g., 0.0018 mole) dissolved in acetone (40 ml.) was treated with chromous chloride solution (12 ml.) prepared in this manner. After standing for 2 hours at room temperature, the reaction mixture was brought to *p*H 6 with sodium bicarbonate and most of the acetone and ethanol were removed by distillation at room temperature *in vacuo*. The residue was diluted with water and extracted with ether. The ethereal solution was extracted with saturated aqueous sodium bicarbonate and the aqueous layer was acidified and extracted with ether. This ethereal extract was dried over anhydrous magnesium sulfate and was freed of solvent. The residue was a viscous oil (0.25 g.) which could not be induced to crystallize; the oil had infrared bands ($CHCl_3$) at 2.9–3.5, 5.79 and 6.00 μ, and an ultraviolet maximum (95% EtOH) at 238 mμ.

(25) The spectrum of tribromosantonin in ethanolic solution had similar maxima; measured values of extinction coefficient were unreliable in this case, however, because of rapid decomposition, as witnessed by a brown coloration of the solution.

The oily reduction product (0.10 g.) was heated in a sublimation apparatus at 140° and 0.3 mm. pressure; a colorless oil distilled which solidified completely on scratching. The white solid (0.015 g., 8% based on tribromosantonin) had m.p. 160–165° and gave a negative test for halogen. Recrystallization of the product from ethyl acetate–cyclohexane afforded white needles, m.p. 168–172°; infrared bands ($CHCl_3$): 5.45, 5.65, 5.98 μ; ultraviolet maxima (95% EtOH): 237 mμ (log ε 4.00), 323 mμ (log ε 1.61).

Anal. Calcd. for $C_{15}H_{18}O_4$: C, 68.68; H, 6.92. Found: C, 68.20; H, 6.99.

Reduction of Tribromosantonin with Zinc and Acetic Anhydride. Formation of IIIb.—Acetic anhydride (200 ml.) was added to a solution of tribromosantonin (15 g., 0.030 mole) in ethyl acetate (400 ml.). The solution was boiled under reflux with mechanical stirring and to it was added zinc dust (75 g.) over a period of 1 hour. The hot mixture was filtered and the filtrate was concentrated to small volume *in vacuo;* the concentrate was taken up in ether (100 ml.). The ethereal solution was washed with water containing a few ml. of dilute hydrochloric acid, dried over anhydrous magnesium sulfate, and freed of solvent. The crude product (3.1 g., 40%) remained as a yellow crystalline solid, m.p. 155–165°. Two recrystallizations from ethyl acetate–cyclohexane afforded white needles (1.2 g.), m.p. 168–172°, undepressed on admixture with the product, m.p. 168–172°, obtained *via* chromous chloride reduction; the infrared spectra of the two products were identical.

Anal. Calcd. for $C_{15}H_{18}O_4$: C, 68.68; H, 6.92. Found: C, 68.66; H, 6.93.

Hydrolysis of this product with warm aqueous sodium bicarbonate gave an acid, m.p. 162.5–164.5°, whose infrared spectrum was very similar to that of the acidic gum obtained by reduction of tribromosantonin with chromous chloride.

Hydrogenation of the zinc reduction product in ethyl acetate solution over 10% palladium–charcoal proceeded with the uptake of one molar equivalent of hydrogen and the formation of a white crystalline product, m.p. 110–112°; infrared spectrum ($CHCl_3$): 5.45, 5.64, 5.83 μ; no high intensity ultraviolet absorption.

Ozonolysis of IIIb. Formation of α-Santoric Acid (IV).—A solution of IIIb (0.40 g., 0.0015 mole) in ethyl acetate (20 ml.), cooled in an acetone–Dry Ice mixture, was treated with a stream of ozonized oxygen until the effluent gas gave a positive test for ozone in a potassium iodide trap. Solvent was then removed from the reaction mixture below −5° at 0.01 mm. pressure. The residual oil was treated with boiling water (5 ml.) containing 30% hydrogen peroxide (0.5 ml.) for 10 minutes. Platinum oxide (0.01 g.) was added to the cooled aqueous solution to decompose any remaining hydrogen peroxide and the solution was subjected to continuous extraction with ether overnight. The ethereal extract was dried over anhydrous magnesium sulfate and evaporated at room temperature yielding a pale yellow, viscous oil (0.200 g.). This residue was dissolved in acetonitrile (1 ml.) and the solution was kept at 5° for 3 days. The solid which separated was filtered and recrystallized once from acetonitrile and twice from ether–chloroform, affording white crystals (0.020 g.), m.p. 182–183°. A mixture of this product with α-santoric acid,[11] m.p. 184–185°, had m.p. 183–184.5°. The infrared spectra (Nujol) of the product and of α-santoric acid were identical.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MD.]

Oxidative Cleavage of Tyrosyl–Peptide Bonds. III. Synthesis and Cleavage of Peptides Containing Sulfur Moieties[1]

BY JOHN G. WILSON[2] AND LOUIS A. COHEN

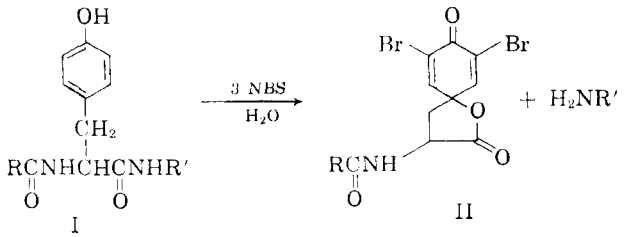
RECEIVED SEPTEMBER 21, 1962

A series of N-acylated tyrosyl-S-alkylcysteine dipeptides was found to undergo facile oxidative cleavage with N-bromosuccinimide. Similar cleavage occurs, without difficulty, in simple tyrosylcystine peptides. Evidence is presented for intramolecularly-catalyzed ester hydrolysis in β-sulfoalanine *t*-butyl ester. The preparation of cystine di-*t*-butyl ester is described.

The oxidative splitting of tyrosyl–peptide bonds (I → II) by the use of N-bromosuccinimide (NBS) has been applied to a variety of tyrosine derivatives and simple peptides.[1] Despite the apparent generality of the reaction, evidence was at hand to indicate that some difficulty might be encountered in the cleavage of tyrosyl–cysteine bonds. Thus, it had been observed by du Vigneaud and his associates that tyrosylcysteic acid failed to undergo oxidative cleavage with bromine

(1) For paper II, *cf.* G. L. Schmir and L. A. Cohen, *J. Am. Chem. Soc.*, **83**, 723 (1961).

(2) Division of Plant Industry, C.S.I.R.O., Canberra, Australia.



although the tyrosyl–isoleucine bond was readily split under the same conditions.[3] Furthermore, early studies with ribonuclease (*cf.* following paper) suggested that the tyrosyl–cystine bond in the enzyme also resists cleavage.

Suspecting that such refractory behavior might be associated with the generation or presence of a highly acidic sulfonic acid residue,[1] we studied the cleavage of tyrosine peptides in which oxidation of sulfur could proceed only as far as the sulfone. As was to be expected, substrates in which the sulfhydryl had been converted into S-methyl, S-benzyl and S-carboxymethylthioethers were cleaved smoothly and in moderate yield; nor was any difficulty encountered with the tyrosyl–methionine bond. The results are summarized in Table I.

TABLE I

NBS CLEAVAGE OF COMPOUNDS CONTAINING THIOETHER RESIDUES

Compound	Solvent[d]	Equiv. of NBS	Cleavage, % Ninhydrin[a]	Cleavage, % Dienone[b]
Z-Tyrosyl-S-Bz-cysteine[c]	A	5	18	
Z-Tyrosyl-S-Bz-cysteine	A	6	36	
Tos-Tyrosyl-S-Bz-cysteine	A	6	47	
Z-Tyrosyl-S-Bz-cysteine·OEt	B	5		55[e]
Tos-Tyrosyl-S-Bz-cysteine·OMe	B	5		57[f]
Tos-Tyrosyl-S-CM-cysteine	B	5		55
Tos-Tyrosyl-S-CM-cysteine	A	6	67	
Tos-Tyrosyl-S-Me-cysteine	A	5	50	64
Tos-Tyrosyl-methionine·OEt	B	5		61
Tos-Tyrosyl-methionine	A	6	45	

[a] The appropriate amino acids or their derivatives were used as standards. [b] Yield based on crystalline material isolated and compared with an authentic sample. [c] Z = carbobenzyloxy, Bz = benzyl, CM = carboxymethyl, Me = methyl. [d] A = 50% aq. acetic acid, B = 50% acetonitrile–0.01 *N* sulfuric acid. [e] Ref. 1. [f] *Cf.* Experimental.

Quite surprisingly, cleavage of the tyrosyl–cystine bond was found to occur in excellent yield (Table II), indicating that, at least in simple compounds, sulfonic acid formation does not interfere with oxidative cleavage.[4]

TABLE II

NBS CLEAVAGE OF COMPOUNDS CONTAINING DISULFIDE RESIDUES

Compound	Solvent[a]	Equiv. of NBS	Cleavage, % ninhydrin
Bis-(Z-tyrosyl)-cystine·OBu-*t*	50% acetic acid	11	79
	80% acetic acid	11	50
	90% acetic acid	11	58
	B	11	48[b]
	C	12	25
Bis-(Z-tyrosyl)-cystine	50% acetic acid	11	40
	50% acetic acid	12	90

[a] B = 50% acetonitrile–0.01 *N* sulfuric acid, C = 50% acetonitrile–acetate buffer (*p*H 4). [b] Dienone isolated in 34% yield.

(3) C. Ressler, S. Trippett and V. du Vigneaud, *J. Biol. Chem.*, **204**, 861 (1953); C. W. Roberts and V. du Vigneaud, *ibid.*, **204**, 871 (1953).

(4) During the course of this work, oxidative cleavage of the tyrosyl–cysteic acid bond in oxidized insulin was reported by E. O. P. Thompson, *Australian J. Biol. Sci.*, **13**, 106 (1960).

The cleavage of bis-(carbobenzyloxytyrosyl)-cystine di-*t*-butyl ester was examined in detail, the best cleavage yields being obtained in 50% aq. acetic acid, using a slight excess of oxidant. From the data of Table III it appears probable that cleavage of the disulfide bond competes with peptide bond cleavage.

TABLE III

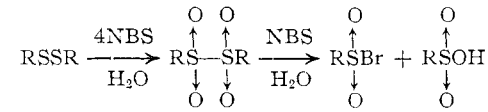
STOICHIOMETRY IN NBS CLEAVAGE OF BIS-(Z-TYROSYL)-CYSTINE-OBu-*t*

Equiv. of NBS	Cleavage, % by ninhydrin[a]
9	29
10	59
11[b]	79
12	90
13	69

[a] All runs in 50% aq. acetic acid. [b] 11 equivalents = theoretical amount.

N-Acylated amino acids were prepared by standard procedures and coupled with cysteine or methionine derivatives using the carbodiimide method.[5] Despite numerous attempts, bis-(carbobenzyloxytyrosyl)-cystine could not be induced to crystallize.[6] In order to avoid alkaline saponification of the methyl ester precursor, a synthesis was undertaken using the *t*-butyl ester.[7] L-Cystine was esterified with isobutylene and concentrated sulfuric acid in tetrahydrofuran and the ester isolated as the hydrochloride in 42% yield. The acid obtained by cleavage of bis-(carbobenzyloxytyrosyl)-cystine di-*t*-butyl ester with hydrogen chloride in dioxane once again failed to crystallize although its analysis was satisfactory. Crystalline products were more readily obtained by using *p*-tolylsulfonyl as the blocking group.[8]

Since the oxidative cleavage of disulfide bonds with NBS may involve the formation of a sulfonyl bromide as an intermediate, precautions are necessary to pre-



vent undesired sulfonylation reactions. Thus, oxidation of cystine di-*t*-butyl ester (III) with NBS at *p*H 4–5 results in the formation of a neutral product, most likely the β-sultam IV.[9] In acidic media, where the amino group remains fully protonated, such cyclization does not occur and the sulfonic acid V is formed. Analogous difficulties might be expected in a complex peptide where a γ-sultam VII can be formed with the peptide bond following cystine. Formation of such γ-sultams was observed following the oxidation of disulfide bonds in wool with chlorine or performic acid.[10] The surprising cyclization with the latter reagent suggests the intermediate formation of a formic–sulfonic anhydride (VI).

Oxidation of cystine di-*t*-butyl ester with bromine, NBS in acid or with performic acid leads to cysteic acid *t*-butyl ester (V), accompanied by varying amounts

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

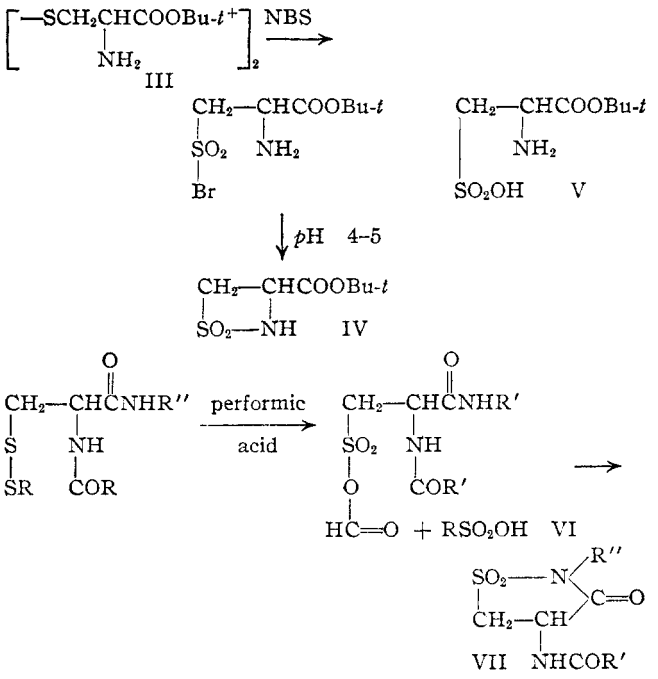
(6) C. R. Harington and R. V. Pitt Rivers (*Biochem. J.*, **38**, 417 (1944)) prepared this compound by carbobenzylation of tyrosylcystine, obtaining a crystalline product, m.p. 150° dec. In the present work, N-carbobenzyloxytyrosine was coupled with cystine dimethyl ester *via* the carbodiimide method and the oily ester saponified.

(7) For earlier use of the *t*-butoxy protective group in peptide synthesis, *cf.* R. W. Roeske, *Chem. Ind.* (London), 1121 (1959); G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960); H. C. Beyerman and J. S. Bontekoe, *Proc. Chem. Soc.*, 249 (1961).

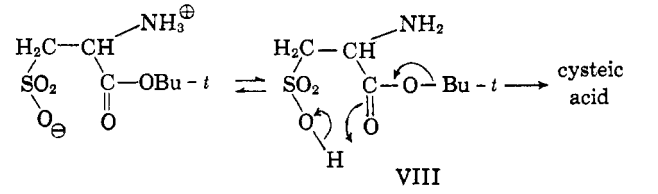
(8) C. Berse, T. Massiah and L. Piche, *J. Org. Chem.*, **26**, 4514 (1961).

(9) *Cf.* H. Baganz and G. Dransch, *Chem. Ber.*, **93**, 784 (1960), for the preparation of the corresponding ethyl ester.

(10) *Cf.* P. Alexander, M. Fox and R. F. Hudson, *Biochem. J.*, **49**, 129 (1951).



of cysteic acid. Preparative electrophoresis at *p*H 1.9 was necessary to obtain the ester in purified form. When aqueous solutions or paper electropherograms containing the ester were heated, decomposition occurred with formation of cysteic acid. Addition of formic acid, performic acid, hydrobromic acid or hydrogen peroxide did not increase the rate of cleavage. Since leucine *t*-butyl ester was completely stable under such conditions, it would appear that ester cleavage may be attributed to internal catalysis by the sulfonic acid residue (I) and may be avoided by cautious work-up of reaction mixtures.



Experimental[11]

N-Carbobenzyloxy-L-tyrosyl-S-benzyl-L-cysteine Ethyl Ester.—To a solution of 2.75 g. (0.01 mole) of S-benzyl-L-cysteine ethyl ester hydrochloride[12] in methylene chloride (40 ml.) containing 1.4 ml. (0.01 mole) of triethylamine was added 3.15 g. (0.01 mole) of N-carbobenzyloxy-L-tyrosine (Mann Research Laboratories). To the clear solution was added 2.06 g. (0.01 mole) of N,N'-dicyclohexylcarbodiimide in 10 ml. of methylene chloride. The mixture was stirred at room temperature for 16 hr. and chilled in ice for 3 hr. The precipitate was separated, 5.5 g., and extracted with 200 ml. of boiling ethyl acetate. After concentration and cooling of the extract the product was collected; 3.4 g., m.p. 167–171°; further concentration yielded an additional 0.48 g., making a total yield of 74%. Recrystallization from ethyl acetate raised the m.p. to 170–171°.[13]

The methyl ester was prepared in an analogous manner from S-benzyl-L-cysteine methyl ester hydrochloride[14] in 64% yield, m.p. 152–154° from ethyl acetate.

Anal. Calcd. for $C_{28}H_{30}O_6N_2S$: C, 64.35; H, 5.79; N, 5.36. Found: C, 64.11; H, 5.95; N, 5.85.

N-Carbobenzyloxy-L-tyrosyl-S-benzyl-L-cysteine was prepared by alkaline saponification of the ethyl ester[15] and crystallized from aqueous methanol; m.p. 132–133°, 165–166°.

N-*p*-Tolylsulfonyl-L-tyrosyl-S-benzyl-L-cysteine Methyl Ester.—To a solution of 2.62 g. (0.01 mole) of S-benzyl-L-cysteine methyl ester hydrochloride in 30 ml. of methylene chloride containing 1.4 ml. of triethylamine was added 3.35 g. (0.01 mole) of N-*p*-tolylsulfonyl-L-tyrosine[16] followed by 2.4 g. (0.01 mole) of CME.DCC[17] in a small volume of the same solvent. The mixture was stirred at room temperature for 24 hr., concentrated to dryness and the residue triturated with water. The water-insoluble solid was dissolved in ethyl acetate, the solution washed with *N* hydrochloric acid and 5% sodium bicarbonate, dried and evaporated to a gum (3.0 g., 56%) which gradually solidified. Recrystallization from aqueous ethanol, ethyl acetate–petroleum ether (60–70°) and finally from aqueous methanol gave the product as prisms (2.0 g.), m.p. 155–156.5°.

Anal. Calcd. for $C_{27}H_{30}O_6N_2S_2$: C, 59.76; H, 5.57; N, 5.16. Found: C, 59.48; H, 5.55; N, 5.22.

N-*p*-Tolylsulfonyl-L-tyrosyl-S-benzyl-L-cysteine was prepared by saponification of the ester according to a published procedure.[8] The acid was recrystallized from ethyl acetate–ligroin (60–70°); m.p. 90–92°. As had been previously observed, the compound separates as a hemihydrate. For analysis, a sample was dried overnight *in vacuo* at 75°; wt. loss 1.29% (calcd. for hemihydrate, 1.67%).

Anal. Calcd. for $C_{26}H_{28}O_6N_2S_2$: C, 59.07; H, 5.34; N, 5.30. Found: C, 58.86; H, 5.41; N, 5.42.

S-Methyl-L-cysteine Ethyl Ester Hydrochloride.—A suspension of 9 g. of S-methyl-L-cysteine in 60 ml. of ethanol was saturated with hydrogen chloride, the mixture refluxed for 2 hr. and the resulting solution concentrated to a solid residue. Two recrystallizations from ethanol–ether afforded 7.2 g. of ester hydrochloride, m.p. 153°.

Anal. Calcd. for $C_6H_{14}O_2NSCl$: C, 36.08; H, 7.06; N, 7.02; Cl, 17.76. Found: C, 36.43; H, 7.24; N, 6.96; Cl, 17.59.

N-*p*-Tolylsulfonyl-L-tyrosyl-S-methyl-L-cysteine Ethyl Ester.—To a stirred solution of S-methyl-L-cysteine ethyl ester hydrochloride (2.0 g.) and triethylamine (1.4 ml.) in methylene chloride (30 ml.) was added N-*p*-tolylsulfonyl-L-tyrosine (3.35) followed by CME.DCC metho-*p*-toluenesulfonate (4.26 g.). The mixture was stirred for 24 hr. at room temperature and, after removal of the solvent *in vacuo*, the residue triturated with water. The water-insoluble material was dissolved in ethyl acetate, the solution washed with *N* hydrochloric acid, 5% sodium bicarbonate, dried and evaporated. Trituration of the residual oil with ligroin (60–70°) effected solidification. Two recrystallizations of the product (3.35 g., 70%) from aqueous acetic acid gave needles (1.8 g.), m.p. 125.5–127.5°.

Anal. Calcd. for $C_{22}H_{28}O_6N_2S_2$: C, 54.98; H, 5.87; N, 5.83. Found: C, 55.27; H, 6.19; N, 5.75.

N-*p*-Tolylsulfonyl-L-tyrosyl-S-methyl-L-cysteine.—To an ice-cooled, stirred solution of the ester (1.6 g.) in dioxane (8 ml.) was added *N* sodium hydroxide (7 ml.) over 10 min. Stirring was continued at room temperature for 45 min. and, after dilution with water (40 ml.) and filtration, the mixture was acidified to *p*H 2 with 6 *N* hydrochloric acid. The oily mixture was extracted with ethyl acetate and the organic layer, after washing with water, was extracted with 5% sodium bicarbonate. Acidification of the bicarbonate extracts liberated an oil which rapidly solidified on scratching. Recrystallization from aqueous ethanol afforded needles (0.7 g.), m.p. 187.5° with some presintering. For analysis, the solvated material was dried overnight *in vacuo* at 100°.

Anal. Calcd. for $C_{20}H_{24}O_6N_2S_2$: C, 53.08; H, 5.34; N, 6.19. Found: C, 53.48; H, 5.34; N, 6.29.

S-Carboxymethyl-L-cysteine Diethyl Ester Hydrochloride.—A suspension of S-carboxymethyl-L-cysteine[18] (15.0 g.) in ethanol (100 ml.) was saturated with hydrogen chloride. After 72 hr. at room temperature, followed by heating at reflux for 2 hr., the solvent was removed *in vacuo*. Following two further evaporations with ethanol, the residue was covered with dry ether and triturated, crystallization being complete after several days. The product was collected, washed well with ether and dried *in vacuo* over phosphorus pentoxide (very hygroscopic) (13.3 g.); m.p. 79–80°. A sample was recrystallized from ethanol–ether as needles, m.p. 80–81°.

Anal. Calcd. for $C_9H_{18}O_4NSCl$: C, 39.78; H, 6.68; N, 5.15. Found: C, 40.21; H, 6.90; N, 5.34.

(11) Melting points are uncorrected. Ultraviolet spectra were run on a Cary recording spectrophotometer, model 14. The authors thank Mr. H. G. McCann and his associates of this Institute for performing the microanalyses.

(12) J. A. MacLaren, W. E. Savige and J. M. Swan, *Austral. J. Chem.*, **11**, 345 (1958).

(13) C. W. Roberts and V. du Vigneaud (*J. Biol. Chem.*, **204**, 871 (1953)) prepared this compound by the azide procedure and report a m.p. of 168.5–169°.

(14) R. A. Boissonnas, St. Guttmann, P. A. Jaquenoud and J. P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).

(15) C. R. Harington and R. V. Pitt Rivers, *Biochem. J.*, **38**, 417 (1944).

(16) E. Fischer and W. Lipschitz, *Ber.*, **48**, 374 (1915).

(17) CME.DCC = 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide (Aldrich Chem. Co.).

(18) L. Goodman, L. O. Ross and B. R. Baker, *J. Org. Chem.*, **23**, 1251 (1958).

N-*p*-Tolysulfonyl-L-tyrosyl-S-carboxymethyl-L-cysteine Diethyl Ester.—The S-carboxymethylcysteine peptide was prepared by coupling as described above for the S-methylcysteine peptide. After thorough trituration of the residual oil with ligroin (60–70°) it solidified; m.p. 87–90° (78%). For analysis a sample was recrystallized from ethyl acetate–ligroin (60–70°) and then from aq. methanol as needles, m.p. 89.5–92.5°.

Anal. Calcd. for $C_{25}H_{32}O_8N_2S_2$: C, 54.33; H, 5.84; N, 5.07. Found: C, 54.38; H, 5.96; N, 5.15.

N-*p*-Tolylsulfonyl-L-tyrosyl-S-carboxymethyl-L-cysteine.—To a stirred solution of the diester (0.55 g.) in dioxane (3 ml.) was added *N* sodium hydroxide (3 ml.) over 30 min. with ice cooling. After 30 min. at room temperature the reaction mixture was diluted with water (10 ml.) and acidified to *p*H 2. The oily mixture was extracted with ethyl acetate and the organic layer washed with water and extracted with 5% sodium bicarbonate. Acidification of the bicarbonate extracts gave an oil which crystallized after several days at 0°. Recrystallization from water afforded needles (0.38 g., 76%), m.p. 130–132° with presintering. For analysis a sample was recrystallized from water; m.p. 132–134° with slight presintering.

Anal. Calcd. for $C_{21}H_{24}O_8N_2S_2$: C, 50.79; H, 4.87; N, 5.64. Found: C, 50.58; H, 4.91; N, 5.50.

N-*p*-Tolylsulfonyl-L-tyrosyl-L-methionine Ethyl Ester.—The solution obtained by adding N-*p*-tolylsulfonyl-L-tyrosine (1.7 g.) to L-methionine ethyl ester hydrochloride[19] (1.05 g.) in methylene chloride (15 ml.) containing triethylamine (0.7 ml.) was treated with CME. DCC metho-*p*-toluenesulfonate (2.13 g.) and stirred for 30 hr. at room temperature. After removal of the solvent, the residue was extracted with water and the water-insolouble material taken into ethyl acetate. The organic layer was washed with *N* hydrochloric acid, 5% sodium bicarbonate, dried ($MgSO_4$) and concentrated to a gum which crystallized readily from aqueous ethanol as needles (1.15 g., 45%), m.p. 136–140°. For analysis a sample was recrystallized from aqueous ethanol; m.p. 140–142°.

Anal. Calcd. for $C_{23}H_{30}O_6N_2S_2$: C, 55.85; H, 6.11; N, 5.66. Found: C, 56.13; H, 6.45; N, 5.56.

N-*p*-Tolylsulfonyl-L-tyrosyl-L-methionine.—To a stirred, ice-cooled solution of the ester (0.8 g.) in dioxane (4 ml.) was added *N* sodium hydroxide (3.5 ml.) over 10 min. After 40 min. at room temperature the mixture was diluted with water (30 ml.), filtered and acidified to *p*H 2. The mixture was extracted with ethyl acetate and the extract washed with 5% sodium bicarbonate. Acidification of the bicarbonate extracts gave an oil which solidified on chilling (0.5 g.); m.p. 182–183°. Recrystallization from water afforded needles (0.43 g.), m.p. 184–185°.

Anal. Calcd. for $C_{21}H_{26}O_6N_2S_2$: C, 54.06; H, 5.62; N, 6.00. Found: C, 54.21; H, 5.75; N, 5.96.

L-Cystine Di-*t*-butyl Ester Dihydrochloride.—Concentrated sulfuric acid (30 ml.) was added in portions to well cooled, freshly distilled tetrahydrofuran (150 ml.) to give a violet solution. L-Cystine (18 g., 0.075 mole) was gradually added and dissolved to form a greenish solution which was equally divided among four pressure bottles and, with thorough cooling (Dry Ice–acetone), liquid isobutylene (40 ml.) was added to each bottle. The securely stoppered bottles were shaken overnight at room temperature. After thorough cooling the contents of the bottles were poured into a large volume of water (600 ml.) and the solution extracted with ether. The aqueous layer was adjusted to *p*H 12 with ice–salt cooling and again extracted with ether several times after saturation with sodium chloride. The combined dried ($MgSO_4$) extracts were evaporated to a volume of *ca.* 120 ml. and a stream of dry hydrogen chloride passed into the cooled ether solution until saturated. The crystalline hydrochloride which separated was collected and washed with dry ether to give 13.2 g. (42%), m.p. 190–192° dec. Recrystallization from ethanol–ether gave needles, m.p. 193–194° dec. with slight presintering.

Anal. Calcd. for $C_{14}H_{30}O_4N_2S_2Cl_2$: C, 39.52; H, 7.11; N, 6.59. Found: C, 39.20; H, 7.15; N, 6.61.

Bis-(N-carbobenzyloxy-L-tyrosyl)-L-cystine Di-*t*-butyl Ester.—Triethylamine (5.6 ml., 0.04 mole) was added to a suspension of L-cystine di-*t*-butyl ester dihydrochloride (8.5 g., 0.02 mole) in methylene chloride (150 ml.) and the mixture stirred until clear (*ca.* 20 min.). N-Carbobenzyloxy-L-tyrosine (12.6 g., 0.04 mole) was added followed by N,N′-dicyclohexylcarbodiimide (8.2 g., 0.04 mole) in 15 ml. of methylene chloride. The reaction mixture was stored for 16 hr. at room temperature and 3 hr. at 0° and filtered from a precipitate of dicyclohexylurea. The filtrate was washed with water, *N* hydrochloric acid, 5% sodium bicarbonate, dried and allowed to evaporate slowly. Two crops of crystals were obtained (14.9 g., 79%), m.p. 166–170°.

Anal. Calcd. for $C_{48}H_{58}O_{12}N_4S_2$: C, 60.88; H, 6.17; N, 5.90. Found: C, 60.89; H, 6.21; N, 6.03.

Bis-(N-carbobenzyloxy-L-tyrosyl)-L-cystine.—A solution of the *t*-butyl ester (8.8 g.) in dioxane (120 ml.) was saturated with hydrogen chloride. After 14 hr. at room temperature the solvent was removed *in vacuo*, the residue dissolved in ethyl acetate and the acid extracted with 5% sodium bicarbonate. Acidification of the alkaline extract gave an oily precipitate which readily solidified on cooling; 1.23 g., m.p. 110–125°. Unhydrolyzed ester from several runs could be recovered and retreated with hydrogen chloride–dioxane. The product could not be obtained crystalline but reprecipitation from bicarbonate solution afforded amorphous material which gave a satisfactory analysis.

Anal. Calcd. for $C_{40}H_{42}O_{12}N_4S_2$: C, 57.54; H, 5.07; N, 6.71. Found: C, 57.82; H, 5.25; N, 6.72.

Harington and Pitt Rivers[6] reported the crystallization of this compound from aqueous ethanol as prisms, m.p. 150° dec.

3-(*p*-Tolylsulfonamido)-7,9-dibromo-1-oxaspiro-[4,5]-deca-6,9-dien-2,8-dione.—N-*p*-Tolylsulfonyl-L-tyrosine (0.34 g.) in acetonitrile (5 ml.) was treated with a solution of N-bromosuccinimide (0.55 g., 3 equiv.) in 5 ml. of acetonitrile. After 1 hour the mixture was diluted with water, chilled and the precipitate collected. Two recrystallizations of the product from aqueous acetonitrile gave needles (0.17 g.), m.p. 223–224° dec. with some preliminary darkening; ultraviolet spectrum (ethanol): λ_{max} 260 mμ, ϵ_{max} 11,300.

Anal. Calcd. for $C_{16}H_{13}O_5NSBr_2$: C, 39.12; H, 2.67; N, 2.85. Found: C, 39.22; H, 2.91; N, 2.97.

Oxidation of L-Cystine Di-*t*-butyl Ester Dihydrochloride. A. In Acetate Buffer.—To a stirred solution of 2.12 g. (0.005 mole) of L-cystine di-*t*-butyl ester dihydrochloride in 50 ml. of acetate buffer (*p*H 5.0) was added dropwise a solution of 4.45 g. (0.025 mole) of NBS in 25 ml. of acetonitrile. A gummy precipitate began to separate almost immediately. When addition of NBS was complete (30 min.) the mixture was stirred an additional 30 min., concentrated to remove acetonitrile and the gum taken up in ethyl acetate. The extract was washed with *N* hydrochloric acid, 5% sodium bicarbonate, dried and concentrated. Again a gum was obtained (1.0 g.) which resisted all attempts at crystallization, even after chromatography on alumina. The material was insoluble in water but soluble in the common organic solvents including ligroin, and was ninhydrin negative. The infrared spectrum (chloroform) showed only a weak NH band and an ester carbonyl. After acid hydrolysis, cysteic acid was identified by paper electrophoresis as the only ninhydrin-positive material. From the preceding evidence, the β-sultam structure IV is postulated.

Anal. Calcd. for $C_7H_{13}O_4NS$: C, 40.58; H, 6.32; N, 6.76. Found: C, 39.60; H, 6.85; N, 6.25.

B. Oxidation in 50% Acetic Acid.—The oxidation was performed as above in 50 ml. of 50% acetic acid as solvent. No precipitation occurred in this case, before or after removal of acetonitrile and dilution with water. Direct examination of the reaction mixture by paper electrophoresis at *p*H 1.9 indicated two products, one migrating toward the anode and corresponding to cysteic acid, the other remaining at the origin. The solution was concentrated *in vacuo* to a sirup at a bath temperature not exceeding 35°. Paper electrophoresis now indicated a considerable increase in the proportion of cysteic acid, hydrolysis of the ester evidently having occurred during the concentration process. Similar results were obtained by oxidation with bromine.

C. Oxidation with Performic Acid.—A mixture of 98% formic acid (20 ml.), 30% hydrogen peroxide (2.25 ml.) and water (1 ml.) was allowed to stand at room temperature for 30 min. It was then cooled to −10° and the ester (0.38 g., 0.009 mole) in 5 ml. of formic acid added. The mixture was lyophilized after 40 min. and the residue, after solution in 5 ml. of water, again lyophilized. A white, crystalline product was obtained which was again found to be a mixture of both substances.

The oxidation was repeated, as above, but in place of lyophilization, 100 ml. of ether was added to the reaction mixture. There was immediate precipitation of crystalline material, consisting primarily of cysteic acid, which was removed by filtration. When the filtrate was stored overnight at 0°, a crystalline product separated which was only slightly contaminated with cysteic acid. A solution of 50 mg. of the material in 0.4 ml. of water was applied along two lines on a large sheet of SS 598 paper and subjected to electrophoresis[20] at *p*H 1.9 (950 v., 35 ma.) for 1.5 hr. The paper was dried *at room temperature* and the appropriate portions (determined from guide strips) cut out and eluted with several portions of methanol at 25°. The methanol extract was filtered and concentrated to a crystalline product (*ca.* 30 mg.) which showed a negligible cysteic acid content by electrophoresis. An analytical sample was

(19) D. Fleš and A. Markovac-Prpić, *Croat. Chem. Acta*, **29**, 79 (1957).

(20) Pherograph Apparatus, manufactured by L. Hormuth, Inc.: W. E. Vetter, Wissenschaftliche Apparate, Heidelberg-Wiesloch, Germany.

dried at 25° *in vacuo* for 24 hr. The compound foams at 160° with gas evolution and the residue is cysteic acid.

Anal. Calcd. for $C_7H_{15}O_5NS$: C, 37.33; H, 6.71; N, 6.22. Found: C, 37.52; H, 6.65; N, 6.42.

Solutions of the ester were subjected to a variety of conditions for 1 hr. followed by electrophoretic examination: (a) 98% formic acid; (b) 30% hydrogen peroxide; (c) performic acid, as prepared above; (d) 2 *N* hydrobromic acid; (e) water. In none of these mixtures was any significant amount of cysteic acid found. When solutions d and e were heated on steam for 0.5 hr. significant but approximately equal conversion to cysteic acid was observed. When a wet paper electropherogram containing the ester was dried at 80°, the material eluted and reanalyzed by electrophoresis, a significant conversion to cysteic acid was evident. Leucine *t*-butyl ester[8] showed no evidence of cleavage under any of these conditions.

Cleavage of Tyrosine Peptides with NBS. A. Isolation of the Dienone-Lactone.—To a solution of N-carbobenzyloxy-L-tyrosyl-S-benzyl-L-cysteine ethyl ester (0.268 g., 0.005 mole) in 25 ml. of acetonitrile and 25 ml. of 0.01 *N* sulfuric acid was added a solution of NBS (0.45 g., 5 equiv.) in acetonitrile (10 ml.) and acid (10 ml.) dropwise over 20 min. Crystals of the dienone-lactone began to separate in *ca.* 40 min. and after further dilution of the mixture with water and cooling, the product was collected (0.125 g., 55%). It melted at 217–219°. When mixed with an authentic sample,[1] the m.p. was 218–220°. Comparison was also made by ultraviolet and infrared spectra. No attempt was made to recover additional material from mother liquors, although its presence was evident. Other peptides were oxidized in a similar manner, using the modifications listed in Tables I–III.

B. Ninhydrin Assay.—The acylpeptide (10^{-4} mole) was dissolved in acetic acid (5 ml.) and the solution diluted with water (5 ml.); NBS (6 moles, 108 mg.) was added to the stirred solution and stirring continued for 2 hr. Solutions were centrifuged and 1 ml. of the supernatant diluted with 50% acetic acid to a concentration appropriate for colorimetry (25–50 ml.). To 1 ml. of the diluted mixture was added *ca.* 0.3 g. of sodium bicarbonate to raise the *p*H to 3–4 and ninhydrin assay performed by standard procedures.[21] Cleavage mixtures were examined by paper chromatography to verify the amino acid resulting from splitting of the peptide bond. The oxidation products of thioethers were invariably found to be sulfones, with negligible amounts of sulfoxides appearing on paper chromatograms. Ninhydrin yields were not increased by storage of reaction mixtures for 24 hr. or by heating on steam following the 2-hr. reaction time.

Standards for Chromatography and Ninhydrin Assay.—S-Benzyl-L-cysteine sulfoxide was prepared by oxidation of S-benzyl-L-cysteine with 30% hydrogen peroxide.[22] S-Methyl-L-cysteine sulfoxide,[23] S-carboxymethyl-L-cysteine sulfoxide,[18] S-methyl-L-cysteine sulfone[24] and S-carboxymethyl-L-cysteine sulfone[18,25] were prepared according to published procedures.

S-Benzyl-L-Cysteine Sulfone.—To a solution of N-carbobenzyloxy-S-benzyl-L-cysteine sulfone[1] (0.5 g.) in 3 ml. of glacial acetic acid in a dry 250-ml. flask was added 1 ml. of 32% hydrogen bromide in acetic acid. The mixture was left for 1 hr. with occasional shaking. Dry ether (100 ml.) was added and after several hours the precipitated solid (0.40 g.) was collected and washed with ether. The sulfone was recrystallized from hot water; m.p. 175° dec.

Anal. Calcd. for $C_{10}H_{13}O_4NS$: C, 49.38; H, 5.39; N, 5.76. Found: C, 49.60; H, 5.41; N, 5.76.

Direct oxidation of S-benzyl-L-cysteine, either with bromine or with hydrogen peroxide, invariably led to difficultly separable mixtures of sulfoxide and sulfone.

(21) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

(22) A. Stoll and E. Seebeck, *Helv. Chim. Acta*, **32**, 866 (1949).

(23) S. Yurugi, *J. Pharm. Soc. Japan*, **74**, 519 (1954); *C. A.*, **49**, 8301 (1955); C. J. Morris and J. F. Thompson, *J. Am. Chem. Soc.*, **78**, 1605 (1956).

(24) H. Rinderknecht, D. Thomas and S. Aslin, *Helv. Chim. Acta*, **41**, (1958).

(25) B. J. Finkle and E. L. Smith, *J. Biol. Chem.*, **230**, 679 (1958).

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MD.]

Oxidative Cleavage of Tyrosyl–Peptide Bonds. IV. The Oxidative Degradation of Ribonuclease and of S-Carboxymethylribonuclease[1]

BY JOHN G. WILSON[2] AND LOUIS A. COHEN

RECEIVED SEPTEMBER 21, 1962

Oxidative cleavage of ribonuclease with N-bromosuccinimide results in the splitting of five of the six tyrosyl–peptide bonds present, the tyrosyl–cysteic acid bond (from disulfide oxidation) failing to cleave. However, all six amide bonds are split in S-carboxymethylribonuclease, in which oxidation of sulfur proceeds only to the sulfone stage. It is suggested that the highly acidic sulfonic acid residue, when constrained in a complex matrix, may inhibit cleavage by protonation of an amide nitrogen. All amino terminals liberated agree with the published sequence of the enzyme. A limited correlation between tertiary structure and rate of cleavage or order of release of amino acids is indicated.

The application of oxidative cleavage with N-bromosuccinimide (NBS) to simple tyrosine peptides has been described in earlier communications in this series.[1,3] The ultimate utility of such a technique lies in its applicability to complex polypeptides and proteins. A number of factors exist, however, which may limit the ability of a protein to exhibit the straightforward behavior found among tyrosyl peptides: for example, limited penetration into a three-dimensional matrix; geometrical and conformational rigidity of peptide bonds, limiting free rotation; competition by suitably placed but extraneous nucleophiles; and side-reactions limiting the release of new amino terminals.[3a]

For an initial investigation, bovine pancreatic ribonuclease was chosen as the test protein, since it is available in a state of high purity, contains no tryptophan[4] and has a well-established sequence.[5] Although it was of interest to confirm the assignment of the six tyrosyl–amino acid bonds derived from enzymatic degradation studies,[5] our main concern was to examine the extent to which the factors enumerated above may complicate NBS degradation of a protein.[6]

Exploratory studies were performed by measuring the increase in absorption at 260 mμ following addition of successive amounts of NBS to aqueous solutions of ribonuclease. Figure 1, curve A, and Fig. 2 show the results of one such experiment. Curve C of Fig. 1 represents the expected optical density increases for the hypothetical case, in which all NBS added is directed toward tyrosine bromination and oxidation. The induction period appearing at the base of both curves represents a repression of dienone formation in 0.1 *N* sulfuric acid; in acidic media, bromination of the phenolic ring is essentially complete before dienone

(1) For paper III, *cf.* J. G. Wilson and L. A. Cohen, *J. Am. Chem. Soc.*, **85**, 560 (1963).

(2) Division of Plant Industry, C.S.I.R.O., Canberra, Australia.

(3) (a) G. L. Schmir and L. A. Cohen, *J. Am. Chem. Soc.*, **83**, 723 (1961); (b) G. L. Schmir, L. A. Cohen and B. Witkop, *ibid.*, **81**, 2228 (1959).

(4) A. Patchornik, W. B. Lawson, E. Gross and B. Witkop, *ibid.*, **82**, 5923 (1960).

(5) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **235**, 633 (1960); D. G. Smyth, W. H. Stein and S. Moore, *ibid.*, **237**, 1845 (1962); J. T. Potts, A. Berger, J. Cooke and C. B. Anfinsen, *ibid.*, **237**, 1851 (1962); E. Gross and B. Witkop, *ibid.*, **237**, 1856 (1962).

(6) No attempt was made, within the scope of this investigation, to follow loss of enzyme activity, or to isolate peptides as fragmentation products.