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Oxidative Cleavage of Tyrosyl-Peptide Bonds. II. Effects of Variation in Structure and *p*H

BY GASTON L. SCHMIR[1] AND LOUIS A. COHEN

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In the oxidative cleavage of tyrosyl-peptide bonds by N-bromosuccinimide (NBS), the highest yields are realized in aqueous acetic or dilute mineral acid. Cleavage occurs readily with a variety of N-acylated tyrosyl peptides. Competition studies demonstrated the selective cleavage of a tryptophyl-peptide bond in the presence of a tyrosyl-peptide. Although sulfur and imidazole groups consume NBS rapidly, cleavage of tyrosyl-peptide bonds can be effected in their presence by use of an excess of reagent. Cleavage of the tyrosyl-valyl bond of hypertensin has also been demonstrated.

The oxidative cleavage of tyrosyl-peptide bonds by the action of N-bromosuccinimide (NBS) or of bromine was recently reported from this Laboratory.[2] Certain aspects of the reaction have now been studied in greater detail in order to apply oxidative cleavage to complex polypeptides in which reactive centers other than phenolic hydroxyl groups are unavoidably exposed to the reagent.

Phloretylglycine[2] (I) was oxidized with three equivalents of NBS in various buffer mixtures and the extent of cleavage determined both by ninhydrin assay for glycine and by the intensity of the dienone peak at 260 mμ in the ultraviolet. From the results (Table I) it is evident that cleavage is favored by increasing the acidity of the reaction mixture.

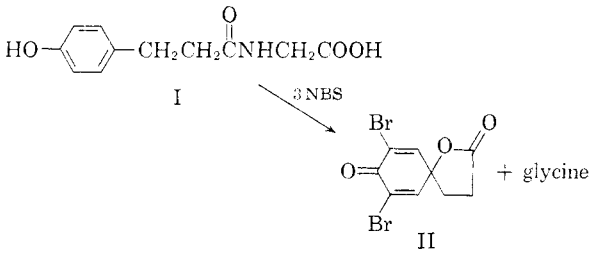


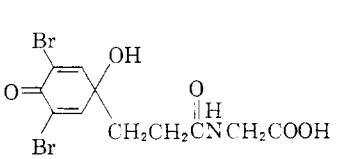
TABLE I

*p*H DEPENDENCE OF THE CLEAVAGE OF PHLORETYLGLYCINE[a]

*p*H	Glycine, %	Dienone, %
1.9	73	83
2.3	59	80
3.5	53	77
4.7	56	75
5.2	42	73
5.9	35	71
6.8	36	59
8.6	16	No peak

[a] For conditions, see Experimental.

It is further apparent that increasing alkalinity affects release of glycine and formation of dienone to different extents and possibly in different ways. Although II is unstable in alkaline media, Fig. 1 reveals that the destruction of preformed dienone-lactone by this pathway can be no more than 5% under the reaction conditions employed. Compounds which show dienone absorption but fail to release glycine, such as III[3] and IV,[4] may also be formed at higher *p*H values, although their lability is probably no greater than that of II.[5] In addition, the extensive degradation of amino acids by positive halogen reagents[6] must be considered not only as a means of destroying glycine but also of consuming a significant portion of the limited amount of oxidant added. By working in media of relatively high acidity, the oxidation of a simple amino acid by NBS can be successfully inhibited, as shown in Fig. 2. Further, it is evident from the data in Table II that consistently high cleavage yields may be obtained in dilute aqueous acids, including acetic, as reaction media. Whereas ortho bromination and dienone formation are competitive reactions at *p*H 4.6, acidic media tend to retard the latter reaction without



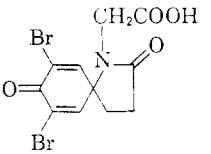


TABLE II

NBS CLEAVAGE OF PHLORETYLGLYCINE IN ACIDIC MEDIA

Solvent	Glycine, %	Dienone, %
0.016 *N* HCl	73	83
1.8 *N* HCl	80	82
0.01 *N* H_2SO_4	76	79
0.08 *N* H_2SO_4	84	86
20% Acetic acid	90	[b]
50% Acetic acid	89[a]	[b]
80% Acetic acid	84	[b]

[a] Average of several runs varying in yield from 82–96%. [b] Spectroscopic assay obscured by high concentrations of acetic acid.

(1) Department of Biochemistry, Yale University School of Medicine, New Haven, Connecticut.

(2) G. L. Schmir, L. A. Cohen and B. Witkop, THIS JOURNAL, **81**, 2228 (1959).

(3) S. Goodwin and B. Witkop, *ibid.*, **79**, 179 (1957).

(4) The possibility of either the oxygen or nitrogen of an amide serving as the nucleophile in participation reactions has been observed in related cases. For example, *cf.* F. L. Scott, R. E. Glick and S. Winstein, *Experientia*, **13**, 183 (1957); H. W. Heine, P. Love and J. L. Bove, THIS JOURNAL, **77**, 5420 (1955); C. J. M. Stirling, *J. Chem. Soc.*, 255 (1960).

(5) Both the formation and degradation of dienone-lactones in alkaline media appear to be of a complex nature and are currently under investigation.

(6) For example, E. W. Chappelle and J. M. Luck, *J. Biol. Chem.*, **229**, 171 (1957); N. Konigsberg, G. Stevenson and J. M. Luck, *ibid.*, **235**, 1341 (1960); A. Schönberg, R. Moubasher and M. Z. Barakat, *J. Chem. Soc.*, 2504 (1951); S. Goldschmidt, *et al.*, *Ann.*, **456**, 1 (1927); K. Langheld, *Ber.*, **42**, 2360 (1904).

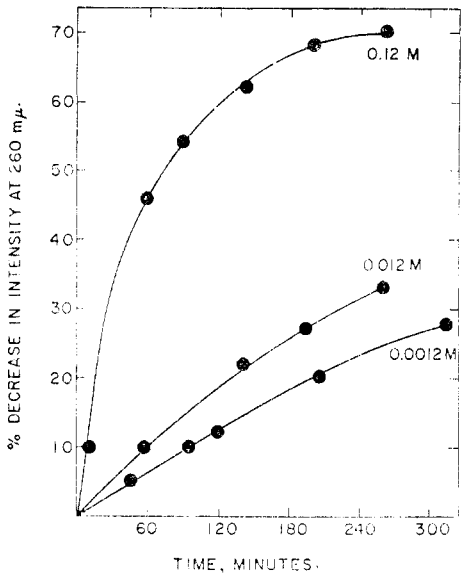


Fig. 1.—Stability of dienone-lactone II in bicarbonate solution.

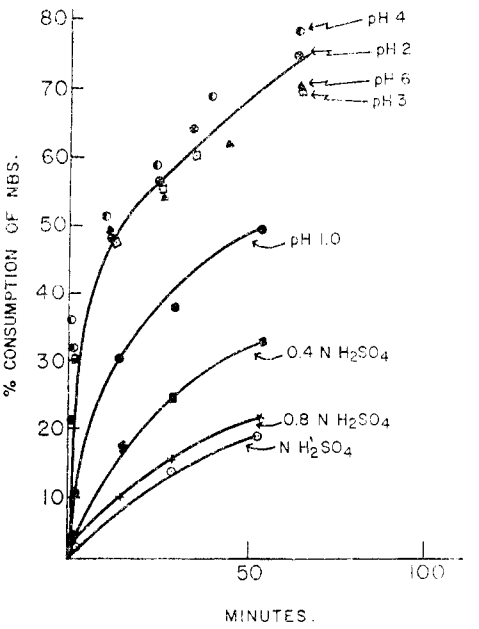


Fig. 2.—Effect of *p*H on rate of consumption of *N*-bromosuccinimide (0.04 *M*) by leucine (0.01 *M*).

affecting ortho bromination. These conclusions are evident from the families of curves shown in Figs. 3a and 3b. In addition, the excellent correspondence between ninhydrin yield and dienone intensity shown in Fig. 4 indicates that, at least in acidic media, spectral intensity may be used as a valid measure of the degree of cleavage.

Spectroscopic assay of reactions run in aqueous halogen acids (0.1–2 *N*) may be temporarily obscured by strongly absorbing polyhalide ions. For example, addition of NBS or of bromine to *N* hydrochloric acid produces an intense maximum at 235 mμ, probably due to the $BrCl_2^-$ ion or to $HBrCl_2$. The absorption is rapidly discharged by a reducing agent such as phenol or formic acid or by passing nitrogen through the solution. In the presence of phloretylglycine the polyhalide peak decreases until the 260 mμ peak for dienone emerges (Fig. 5). Spectral data for several polyhalide species are recorded in Table III.

TABLE III

SPECTRAL DATA FOR POLYHALIDES

Compound[a]	Solvent	Species of polyhalide	Max.
NBS or Br_2	0.1 *N* HCl	....	End absorption
NBS or Br_2	1.0 *N* HCl	$HBrCl_2$[c]	233 mμ 22,000
NBS or Br_2	6.0 *N* HCl	$HBrCl_2$	233 mμ 21,000
NBS or Br_2	*N* NaCl	....	End absorption
NBS or Br_2	2.0 *N* H_2SO_4	....	End absorption
NBS or Br_2	2.0 *N* HBr	HBr_3	265 mμ 25,000
NBS or Br_2	*N* NaBr	....	End absorption
NCS[b]	1.0 *N* HCl	HCl_2	End absorption

[a] Concentrations = 3×10^{-5} *M*. [b] NCS = N-chlorosuccinimide. [c] In preference to HBr_2Cl (*cf.* R. L. Scott, THIS JOURNAL, 75, 1550 (1953)).

Experiments with a series of N-acylated tyrosine peptides indicated that cleavage yield was relatively independent of the nature of the N-acyl group preceding or of the amino acid following tyrosine (Table IV).[7]

(7) The cleavage of tyrosyl-cystine bonds will be described in a forthcoming publication.

In order to determine the degree and rate of consumption of NBS by other functional groups in a peptide chain, a number of amino acids and their derivatives were treated with NBS, the disappearance of positive halogen being followed by thio-

TABLE IV

NBS CLEAVAGE OF SUBSTITUTED TYROSYL PEPTIDES

Substrate	Solvent	Cleavage yield Ninhydrin[e]	Cleavage yield Dienone
Phloretylglycine[a]	0.01 *N* H_2SO_4	76	79
N-Benzoyltyrosylglycine	.01 *N* H_2SO_4	73	
N-Acetyltyrosylglycine	.01 *N* H_2SO_4	73	77
N-Acetyltyrosylglycylglycine	.01 *N* H_2SO_4	77	80
Z-Tyrosylhistidine[b]	.01 *N* H_2SO_4	[c]	70
Phloretylglycine	50% acetic acid	82	
Z-Tyrosylphenylalanine·OEt	50% acetic acid	68	
Z-Tyrosylalanine·OEt	50% acetic acid	80	
Z-Tyrosylserine·OMe	50% acetic acid	77	
Z-S-Bz-Cysteinyltyrosylisoleucine[d]	50% acetic acid	79	

[a] Three equivalents of NBS were used in all cases except where noted. [b] Five equivalents of NBS; Z = carbobenzyloxy. [c] Ninhydrin assay obscured by formation of ammonia. [d] Four equivalents of NBS; isoleucine identified by paper chromatography. [e] The appropriate amino acids or their derivatives were used as colorimetric standards.

sulfate titration. As summarized in Table V, there is no strong competition by simple amino acids or by hydroxyamino acids in acidic media. A more rapid reaction is observed with the basic amino acids, phenylalanine and aspartic acid, and a very rapid consumption of NBS by the sulfur amino acids, tryptophan and histidine.

Early experiments with the tripeptide carbobenzyloxy-S-Bz-cysteinyltyrosylisoleucine[8] gave cleavage yields which were disappointingly low until it became evident that the thioether linkage was competing with tyrosine for the available

(8) C. Ressler and V. du Vigneaud, THIS JOURNAL, 79, 4511 (1957).

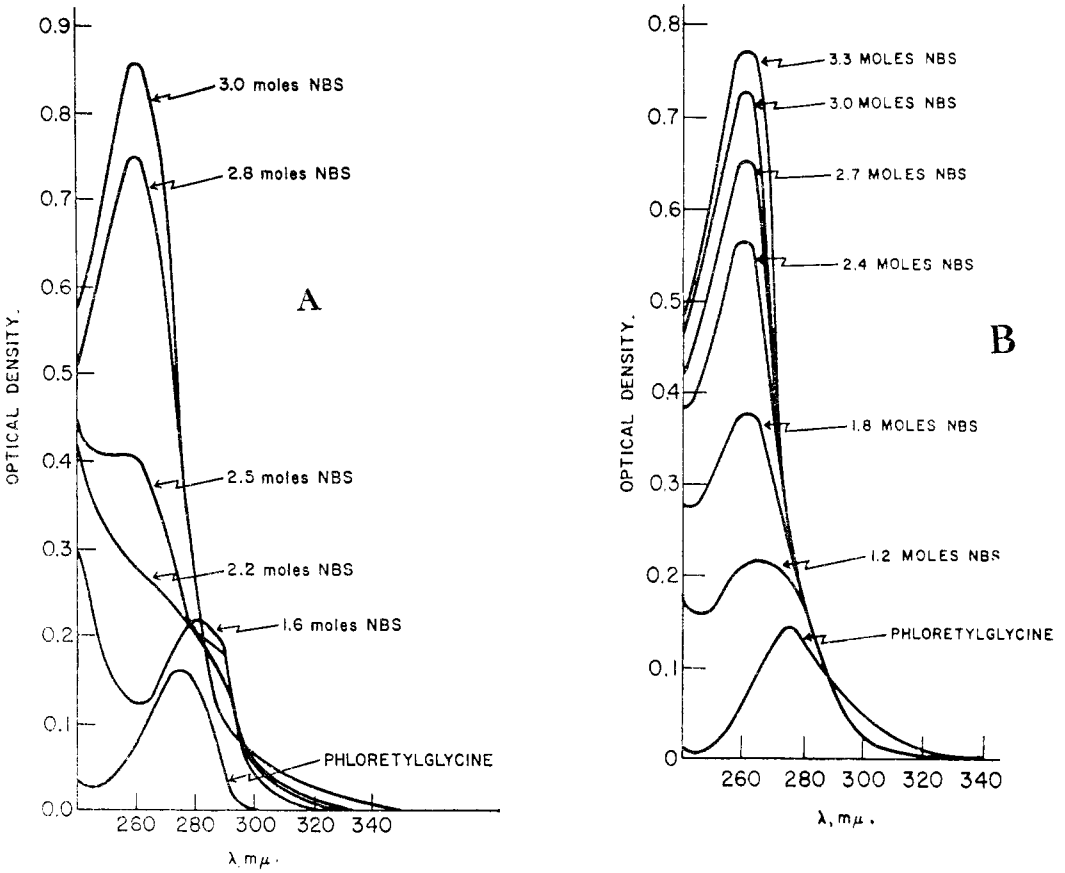


Fig. 3.—(A) Ultraviolet spectra resulting from the cleavage of phloretylglycine (10^{-4} M) with successive increments of N-bromosuccinimide in 0.8 N H_2SO_4. (B) Ultraviolet spectra resulting from the cleavage of phloretylglycine (9.2×10^{-5} M) with successive increments of N bromosuccinimide at pH 4.6.

NBS. From the data of Table VI it is apparent that both methionine and cystine offer significant competition for NBS and reduce cleavage yield. Results with the tripeptide also suggest that tyrosyl–peptide cleavage is slower than sulfoxide formation but faster than that of sulfone. In a preparative experiment, carbobenzyloxy-DL-methionine sulfone was obtained in 61% yield by oxidation of carbobenzyloxy-DL-methionine with two equivalents of NBS; the product was identical

TABLE V

CONSUMPTION OF NBS BY AMINO ACIDS AND DERIVATIVES

Substrate	Per cent. disappearance of NBS in 15 min.	60 min.
Phenylalanine	16	36
Aspartic acid	13	32
Arginine	10	30
Lysine	11	28
Glutamic acid	5	17
Alanine	4	14
Valine	3	12
Serine (threonine)	3	10
Asparagine	2	7
Glycine	1	4
Hydroxyproline	1	2
Proline	0	1
Aceturic acid	0	0
Z-Aspartic acid	1	2
Glycylleucine	1	2
Ditosyltyrosine	2	10
Histidine[b]	100	..

[a] Substrate = 10^{-4} M, NBS = 10^{-4} M, solvent = 0.8 N H_2SO_4 containing 1% acetonitrile (solvent for NBS addition). [b] The following substrates consumed one equivalent of NBS within two minutes: histidine, imidazole, tyrosine, tryptophan, methionine, cystine.

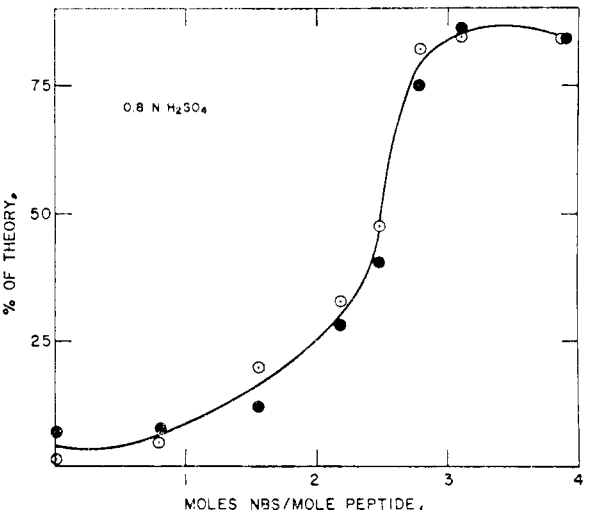


Fig. 4.—Reaction of phloretylglycine with N-bromosuccinimide: ●, ultraviolet absorption at 260 mμ; ⊙, ninhydrin color based on glycine.

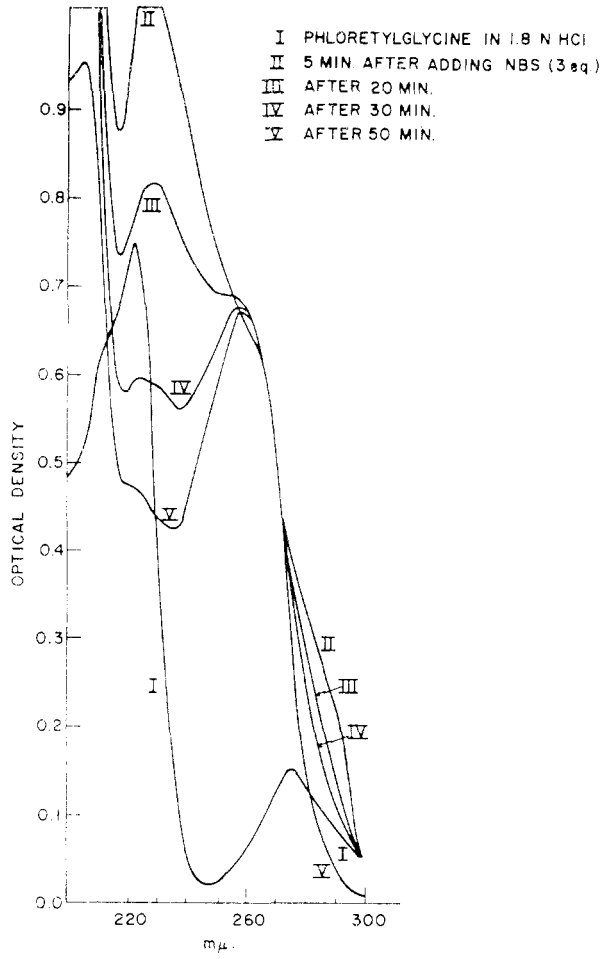


Fig. 5.—Ultraviolet spectra resulting from the reaction of phloretylglycine with NBS in 1.8 *N* HCl.

to that obtained by carbobenzoxylation of DL-methionine sulfone. Similarly, carbobenzyloxy-S-Bz-L-cysteine was converted into its sulfone in 78% yield by reaction with NBS and in 92% yield by reaction with hydrogen peroxide.

TABLE VI

EFFECT OF SULFUR GROUPS ON CLEAVAGE YIELD[a]

Substrate	Addition	Moles NBS/ Mole substrate	Ninhydrin yield (%)
Phloretylglycine	None	1	8
Phloretylglycine	None	2	36
Phloretylglycine	None	3	96
Phloretylglycine	Z-methionine (1 eq.)	1	9
Phloretylglycine	Z-methionine (1 eq.)	2	11
Phloretylglycine	Z-methionine (1 eq.)	3	37
Phloretylglycine	Di-Z-cystine (1 eq.)	2	34
Phloretylglycine	Di-Z-cystine (1 eq.)	3	52
Tripeptide[b]	None	1	1
Tripeptide	None	2	5
Tripeptide	None	3	8
Tripeptide	None	4	79
Tripeptide	None	5	81

[a] In 50% acetic acid, substrate 10^{-4} *M*. [b] Z-S-Bz-cystyrileu.

Since it had already been demonstrated that tryptophyl–peptide bonds can also be cleaved by NBS,[9] the competition between these two amino acids was studied *via* model compounds. When mixtures of equimolar quantities of phloretylglycine and indolepropionyl-DL-phenylalanine[10] in 50% acetic acid were oxidized with increasing proportions of NBS, increasing amounts of phenylalanine were liberated as shown by paper chromatography. Glycine began to appear only after three equivalents of NBS had been added, demonstrating (at least for simple systems) a high degree of selectivity in the fission of these two peptide bonds. A preferential cleavage at tryptophyl-peptide bonds has also been observed in more complex peptides. Thus, glucagon has been split selectively at the tryptophyl bond,[9] and similar results have been obtained with tobacco mosaic virus and with serum albumins.[11]

When phloretylglycine was oxidized with NBS in the presence of imidazolepropionic acid,[12] formation of dienone was not seriously inhibited and apparently proceeds faster than attack on the imidazole ring (Table VII). It is evident that

TABLE VII

EFFECT OF THE IMIDAZOLE RING ON THE CLEAVAGE OF PHLORETYLGLYCINE

Substrate	NBS, eq.	Ninhydrin,[b] %	Dienone, %
PG[a]	1.5	9	10
PG	3.0	59	73
IP[a]	0.75	93	..
IP	1.5	142	..
IP	3.0	136	..
PG + IP	1.5	12	11
PG + IP	2.25	44	39
PG + IP	3.0	90	69
PG + IP	3.75	167	75
PG + IP	4.5	197	73

[a] PG = phloretylglycine, IP = imidazolepropionic acid, both 10^{-4} *M* in 0.01 *N* HCl. [b] Glycine standard.

considerable ninhydrin-positive material, probably ammonia, is released in the reaction of the imidazole ring with NBS.[13] Somewhat greater interference is shown by the imidazole ring in the oxidative cleavage of carbobenzyloxy-tyrosylhistidine. With the addition of three equivalents of NBS, the dienone intensity is only 57% of theory, five equivalents being necessary to reach a value of 70%. In a preparative experiment, the dienone-lactone of carbobenzyloxy-L-tyrosine was isolated in 36% yield after the addition of a total of six equivalents of NBS. In titration experiments no measurable difference in the rate of uptake of NBS by imidazole could be detected in the *p*H range 1.0 to 8.0 and from spectral data it was evident that neither imidazole nor its oxidation products contributed significantly to 260 mμ absorption.

(9) A. Patchornik, W. B. Lawson and B. Witkop, THIS JOURNAL, **80**, 4747, 4748 (1958).

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

(11) L. K. Ramachandran and B. Witkop, *ibid.*, **81**, 4028 (1959).

(12) Prepared by hydrogenolysis of α-chloroimidazolepropionic acid with 5% rhodium on alumina (T. W. Beiler, unpublished experiments).

(13) Further details on the course of this reaction will be published shortly.

In another test of tyrosine-histidine competition, samples of synthetic valyl-hypertensin (V)[14] were

H·Asp(NH_2)–Arg–Val–Tyr–Val–His–Pro–Phe·OH (V)

oxidized with three equivalents of NBS at *p*H values from 1.1 to 5.0. Spectral intensities at 260 mμ reached values of 70–95% of theory within 8 minutes. From cleavage experiments in 50% acetic acid, yields of DNP-valine up to 15% were obtained.[15]

Experimental[16]

Peptide Derivatives.—N-Benzoyltyrosylglycine, N-acetyltyrosylglycine and N-acetyltyrosylglycylglycine were the gift of Dr. Nobuo Izumiya and N-carbobenzyloxy-tyrosyl-histidine was supplied by Dr. Bernard J. Jandorf.

N-Carbobenzyloxy-L-tyrosyl-L-phenylalanine Ethyl Ester.—To a suspension of 760 mg. (0.0033 mole) of L-phenylalanine ethyl ester hydrochloride in 15 ml. of ice-cold methylene chloride was added 0.46 ml. (0.0033 mole) of triethylamine. The mixture was shaken vigorously and added to a suspension of 946 mg. (0.003 mole) of N-carbobenzyloxy-L-tyrosine in 15 ml. of cold methylene chloride. After brief stirring of the reaction mixture a solution of 620 mg. (0.003 mole) of N,N′-dicyclohexylcarbodiimide in 3 ml. of methylene chloride was added. After 26 hr. at room temperature the reaction mixture was chilled for 15 hr. and the crystalline precipitate of N,N′-dicyclohexylurea removed by filtration. The filtrate was washed with 5% sodium bicarbonate, *N* hydrochloric acid and twice with water. The solution was dried over magnesium sulfate and concentrated *in vacuo*. The residual solid was crystallized first from methanol–water and then from ethyl acetate–ligroin, yielding 770 mg. (52%), m.p. 141–143°. For analysis, the product was recrystallized from ethyl acetate–ligroin and dried at 100° overnight over P_2O_5, melting point unchanged.

Anal. Calcd. for $C_{25}H_{30}N_2O_6$: C, 68.56; H, 6.17; N, 5.71. Found: C, 68.59; H, 6.21; N, 5.76.

N-Carbobenzyloxy-L-tyrosyl-L-serine Methyl Ester.—This compound was prepared by the carbodiimide procedure as above. The oily product was crystallized from ethyl acetate–ligroin, yielding hard colorless crystals of m.p. 147–149° in 35% yield. For analysis, the compound was recrystallized from the same solvent, the melting point increasing to 149–150°.[17]

Anal. Calcd. for $C_{21}H_{24}N_2O_7$: C, 60.57; H, 5.81; N, 6.73. Found: C, 60.70; H, 5.73; N, 6.54.

N-Carbobenzyloxy-L-tyrosyl-L-alanine Ethyl Ester.—The above procedure was also employed in the preparation of this compound, which was obtained in 50% yield. The crude product melted at 100–110° and appeared to be solvated. After two recrystallizations from ethyl acetate–ligroin, followed by extensive drying *in vacuo* over P_2O_5 at 65, 80 and 100° successively, the compound melted at 140–142°.

Anal. Calcd. for $C_{22}H_{26}N_2O_6$ C, 63.75; H, 6.32; N, 6.76. Found: C, 63.82; H, 6.38; N, 6.71.

With less rigorous drying, melting generally occurred at 100–120° and occasionally was followed by resolidification and melting at 140°.

N-Carbobenzyloxy-DL-methionine Sulfone. A. From NBS Oxidation.—To a solution of 283 mg. (0.001 mole) of N-carbobenzyloxy-DL-methionine in 25 ml. of acetonitrile and 20 ml. of acetate buffer (0.1 *M*, *p*H 4.7) was added a solution of 356 mg. (0.002 mole) of NBS in 10 ml. of acetonitrile and 10 ml. of acetate buffer. After 30 minutes at room temperature the acetonitrile was removed under reduced pressure. The faintly cloudy solution was acidified to about *p*H 2 with 6 *N* HCl, partially concentrated *in vacuo* and the oily suspension stored in the cold for 5 days while crystallization occurred. After filtration there was obtained 192 mg. (61%) of material melting at 138–139°. Before analysis, the product was recrystallized from methanol–water and dried at 100°, m.p. 139–140°.

Anal. Calcd. for $C_{18}H_{17}NO_6S$: C, 49.51; H, 5.44; S, 10.17. Found: C, 49.43; H, 5.55; S, 10.25.

B. From Methionine Sulfone.—To a solution of 905 mg. (0.005 mole) of DL-methionine sulfone in 15 ml. of water and 1.5 ml. of 4 *N* NaOH was added in 4 equal portions 1.33 ml. of carbobenzyloxy chloride and 1.5 ml. of 4 *N* NaOH, with chilling and vigorous shaking. After 15 minutes the reaction mixture was extracted twice with ether and acidified with 6 *N* HCl. The oily suspension was seeded and the crystalline product collected after 30 minutes to yield 807 mg. (51%), m.p. 138.5–140°. Before analysis, the substance was recrystallized from methanol–water (m.p. 140–141°) and dried *in vacuo* at 100°.

Anal. Calcd. for $C_{18}H_{17}NO_6S$: C, 49.51; H, 5.44; S, 10.17. Found: C, 49.46; H, 5.33; S, 10.38.

The compounds prepared by the two methods showed identical infrared spectra (chloroform) and gave no melting point depression upon admixture.

N-Carbobenzyloxy-S-benzyl-L-cysteine Sulfone. A. By Oxidation with Hydrogen Peroxide.—To a solution of 345 mg. (0.001 mole) of N-carbobenzyloxy-S-benzyl-L-cysteine in 10 ml. of glacial acetic acid was added 0.4 ml. of 30% aqueous hydrogen peroxide. The reaction mixture was warmed on a steam bath for 1 hr., about one-half the solvent removed *in vacuo* and the residual solution poured into ice-water. The crystalline precipitate was filtered, washed with water and dried, yielding 346 mg., 92%, m.p. 158–160°. For analysis, the product was recrystallized from methanol–water and dried *in vacuo* at 100°. The substance melted partially at 120–125°, resolidified and melted again at 159–161°. If the temperature of the melting point bath was allowed to increase slowly, the initial melting was sometimes not observed.

Anal. Calcd. for $C_{18}H_{19}NO_6S$: C, 57.31; H, 5.07; S, 8.49. Found: C, 57.11, H, 5.18; S, 8.58.

B. By Oxidation with NBS.—To a solution of 345 mg. (0.001 mole) of N-carbobenzyloxy-S-benzyl-L-cysteine in 25 ml. of acetonitrile and 25 ml. of acetate buffer (0.2 *M*, *p*H 4.6) was added a solution of 356 mg. (0.002 mole) of NBS in 10 ml. of acetonitrile and 10 ml. of acetate buffer. After 10 minutes at room temperature the acetonitrile was removed *in vacuo*, during which time a crystalline solid separated. The reaction mixture was cooled, acidified with 6 *N* HCl and the product collected, washed with water and dried, 295 mg., 78%. The compound was recrystallized twice from methanol–water, melting at 157–159°. Its behavior during melting was identical to that described above. The infrared spectra were identical and there was no melting point depression upon admixture.

Anal. Calcd. for $C_{18}H_{19}NO_6S$: C, 57.31; H, 5.07; S, 8.49. Found: C, 57.31; H, 5.08; S, 8.52.

Oxidative Cleavage of N-Carbobenzyloxy-L-tyrosyl-L-histidine. Isolation of Spirolactone.—To a solution of 316 mg. (0.0007 mole) of N-carbobenzyloxy-L-tyrosyl-L-histidine in 140 ml. of 20% acetonitrile–0.01 *N* H_2SO_4 was added dropwise and with stirring a solution of 410 mg. (0.0023 mole) of NBS in 40 ml. of acetonitrile–water. Crystals began to separate after 10 minutes and the reaction mixture was cooled for 1 hr. The crystalline product (50 mg., 15%, m.p. 214–216° d.) was collected and to the filtrate was added another 410 mg. (0.0023 mole) of NBS in 40 ml. of 10% acetonitrile–water. Crystalline material began to deposit shortly afterwards. After 11 hr. in the cold, the product was collected (70 mg., 21%, m.p. 210–215° d.). The combined products were recrystallized from acetonitrile–water, yielding 82 mg., of spirodienone-lactone, m.p. 218–220°. The mixed melting point with an authentic sample of m.p. 218–220° (prepared from N-carbobenzyloxy-L-tyrosine[2] was 218–220°. The infrared and ultraviolet spectra were identical.

Effect of *p*H on the Cleavage of Phloretylglycine.—Aliquots of a solution of phloretylglycine in acetonitrile (spectral grade) were diluted with the buffers listed in Table VIII to final concentrations of 10^{-4} *M* containing 1% acetonitrile. To each solution was added 3 equivalents of NBS

(14) We are indebted to Dr. R. Schwyzer of Ciba, Ltd., for a gift of this material.

(15) No correction factor for hydrolytic or chromatographic losses has been applied to these results.

(16) Melting points are uncorrected. Infrared spectra were run on a Perkin–Elmer recording spectrophotometer, model 21. Ultraviolet spectra were run on a Cary recording spectrophotometer, model 14.

(17) R. F. Fischer and R. R. Whetstone (THIS JOURNAL, **76**, 5076 (1954)) prepared this compound by the azide method and reported m.p. 151–152°.

(3×10^{-4} *M*) in aqueous acetonitrile (1%). After 45 minutes the dienone intensity at 260 mμ was measured. Aliquots were removed and assayed with ninhydrin against a glycine standard.[18] The results are recorded in Table I. Control experiments showed that ninhydrin values were unaffected by the presence of dienone-lactone, even after boiling.

TABLE VIII

*p*H	Buffer	Final conc., *M*	*p*H	Buffer	Final conc., *M*
1.9	HCl	0.016	5.2	Acetate	0.08
2.3	Phosphate	.04	5.9	Acetate	.08
3.5	Acetate	.08	6.8	Phosphate	.04
4.7	Acetate	.08	8.6	Bicarbonate	.016

Similar methods were used to obtain the data summarized in Tables II, IV, VI, VII and Figs. 3a, 3b and 4. Where necessary, dilute potassium carbonate was used to neutralize acidic solutions prior to ninhydrin assay.

Dibromophloretylglycine, when reacted with 1 equivalent of NBS or bromine in 4% methanol–water over the *p*H range 2.2–8.4, exhibited a similar range of results.

Stability of Dienone-lactone (II) in Sodium Bicarbonate Solution.—Solutions of II (10^{-4} *M*) in aqueous sodium bicarbonate (1% acetonitrile) were examined by spectrophotometric assay at 260 mμ. The decrease in intensity with time is recorded in Fig. 1 at several concentrations of bicarbonate. No difference in rate of destruction was observed in the presence of glycine (10^{-4} *M*) or succinimide (3×10^{-4} *M*).

Effect of *p*H on NBS Oxidation of Leucine.—To solutions of leucine in sulfuric acid or buffer (Table VII) was added 4 equivalents of NBS in acetonitrile (final concentrations-amino acid 0.01 *M* and NBS 0.04 *M*, 1% acetonitrile) and the disappearance of positive halogen followed by thiosulfate titration. The results are recorded in Fig. 2.

Competition Between Tyrosine and Tryptophan Cleavage.—To reaction mixtures containing phloretylglycine and indolepropionyl-DL-phenylalanine (each at 2.5×10^{-3} *M* in 50% acetic acid–6% acetonitrile–water) was added either 1.5, 2.25 or 3 equivalents of NBS (per mole of either peptide). After 5 minutes at room temperature, the solutions were concentrated to dryness *in vacuo*, the residues dissolved in water and the solutions reconcentrated and the residues finally dissolved in 0.5 ml. of 50% ethanol.

Aliquots were spotted on Whatman #1 paper and the chromatograms run in the descending technique using either methyl ethyl ketone–propionic acid–water (15:5:6) or methanol–benzene–*n*-butanol–water (1:1:1:1) with concentrated ammonia added to 1% of total volume. The chromatograms were dried in air and developed with a spray of 0.25% ninhydrin in acetone containing 5% acetic acid.

By visual estimation increasing amounts of phenylalanine were observed. Glycine appeared only after 3 moles of NBS has been added and only in trace amounts. In a control experiment which omitted the tryptophyl analog a considerable amount of glycine was released.

Cleavage of Valyl-Hypertensin.—Solutions of hypertensin (2×10^{-4} *M*) in 50% acetic acid were treated with 3, 4 and 5 equivalents of NBS and with 4 equivalents in water alone. After 10 minutes, the solutions were concentrated to dryness and treated with fluorodinitrobenzene in bicarbonate solution.[19] The resulting mixtures of DNP-peptides were hydrolyzed at 105–110° for 14 hr. Dinitrophenol was removed by sublimation and the residual materials separated by two-dimensional chromatography with DNP-valine as a standard.[19] Actual recoveries of DNP-valine were determined by elution of the spots and spectrophotometric assay. Results are summarized in Table IX.

TABLE IX

RECOVERIES OF DNP-VALINE FROM HYPERTENSIN CLEAVAGE

NBS, moles	Solvent	DNP-valine, %
3	50% acetic	5
4	50% acetic	7
5	50% acetic	15
4	Water	2

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An Improved Synthesis of Isoleucine[5] Angiotensin Octapeptide[1]

BY KIKUO ARAKAWA[2] AND F. MERLIN BUMPUS

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Both high yield and high purity have been achieved in the synthesis of angiotensin octapeptide. The product was also more active pharmacologically than those previously synthesized. This great increase in biological activity probably is due to an increased optical purity of the peptide. The synthesis was started from the C-terminal end by adding amino acids one at a time except for valyl-tyrosine. An excess of the carboxyl entering amino acids insured high yields. As a C-terminal blocking group, *p*-nitro-benzyl ester proved more stable to hydrogen bromide cleavage than the benzyl ester. Despite this, cleavage occasionally occurs which introduces difficulties if the hydrobromide salt of the resulting peptide is hard to crystallize.

Introduction

Angiotensin I (a decapeptide), the product of the action of renin on renin substrate, has been isolated in highly purified form and its structure postulated in two laboratories.[3,4] This decapeptide is converted to angiotensin II (an octapeptide) by a plasma enzyme by removal of dipeptide L-histidyl-L-leucine from the C-terminus.[5] The octapeptide was first synthesized in our Laboratory.[6] The asparaginyl[1] analog of angiotensin II was simultaneously reported[7] and has similar physiological properties to angiotensin II. Several angiotensin-peptide syntheses have been reported more recently.[8]

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(2) Research Fellow of the Frank E. Bunts Educational Institute.

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