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CHLORINATION STUDIES*

II. THE REACTION OF AQUEOUS HYPOCHLOROUS ACID WITH α -AMINO ACIDS AND DIPEPTIDES

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SUMMARY

HOCl by oxidative decarboxylation converts several α -amino acids into a mixture of the corresponding nitriles (major) and aldehydes (minor product). In addition, chlorination of the ring of tyrosine was observed. Cysteine when reacted with HOCl yielded cystine and cysteic acid, while with cystine, cysteic acid was the only product identified. The amide nitrogen bond of several dipeptides was found to be resistant to aqueous HOCl at room temperature. Chlorination of these compounds gave the corresponding *N,N*-dichlorodipeptide.

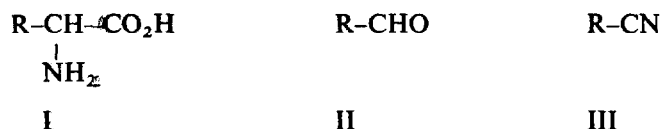
INTRODUCTION

Although Cl₂ has been used to disinfect public water supplies for over 70 years², very little is known at the molecular level of the mechanism by which Cl₂ kills bacteria³. One possible mode of attack could involve modification and inactivation of enzymes critical to the survival of the bacterium^{4,5}. We wish to describe the chemical action of aqueous HOCl⁶ on L-phenylalanine, L-tyrosine, L-glutamic acid, L-cystine and L-cysteine and compare these results with this reagent's action on four typical dipeptides, Gly-L-Ala, Gly-DL-Phe, DL-Ala-DL-Leu and L-Val-L-Val.

The reaction of α -amino acids (I) with NaOCl was investigated by Langheld⁷ who observed the production of an aldehyde (II), CO and NH₃. Subsequently, Dakin^{8,9} demonstrated that one equivalent of the *N*-chloro compound, chloramine T [(*N*-chloro-*p*-toluenesulfonamido) sodium] converted α -amino acids to their corresponding aldehydes (II) while if two equivalents of reagent were used¹⁰ the corresponding nitriles (III) resulted. Wright¹¹ found that aldehydes were formed from several α -amino acids by reaction with NaOCl while nitriles predominated at lower pH values. Van Tamelen and co-workers¹² studied in detail the NaOCl-induced oxidative decarboxylation of several amino acids as models for *in vivo* alkaloid transfor-

* Part I: Patton, W., Bacon, V., Duffield, A. M., Halpern, B., Hoyano, Y., Pereira, W. and Lederberg, J. (1972) *Biochem. Biophys. Res. Commun.* 48, 880-884

mations. More recently a report has appeared¹³ describing the paper chromatographic separation of the products from the action of NaOCl on several α -amino acids.



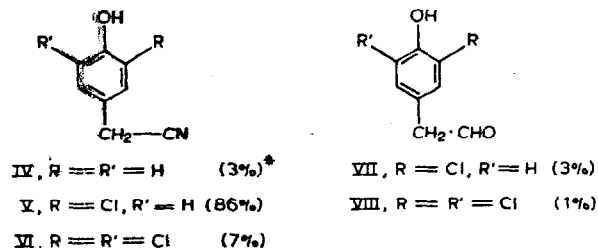
In contrast to the prolific amount of research devoted to the study of the action of NaOCl with α -amino acids, comparatively little attention has been directed to the action of this reagent at the molecular level with peptides and proteins. Briggs¹⁴, and later Baker¹⁵, examined the action of NaOCl with protein molecules in order to investigate this reagent's application as an antiseptic in surgical procedures. Wright¹⁶ also examined the gross physicochemical changes occurring when NaOCl was added to aqueous solutions of several proteins. Rydon and Smith¹⁷ demonstrated that peptides, proteins, diketopiperazines and acylated amino acids, can be visually detected on chromatograms by chlorination with gaseous Cl_2 and subsequent spraying with starch-KI solution. The visual color was suggested to result from the liberation of I_2 by the formed *N*-chloropeptide bond. A subsequent modification¹⁸ utilized aqueous NaOCl solution instead of gaseous Cl_2 .

None of the previous workers characterized the products from the action of NaOCl on proteins as being *N*-chloro compounds. In order to investigate the stability of the peptide bond to aqueous HOCl⁶, we commenced a study using four representative dipeptides as substrates. Before commencing this investigation however, it was felt necessary to determine the effect of aqueous HOCl on several α -amino acids, since previous investigators⁷ had used solutions of NaOCl at a pH > 7.

RESULTS AND DISCUSSION

Reaction of *L*-tyrosine with HOCl

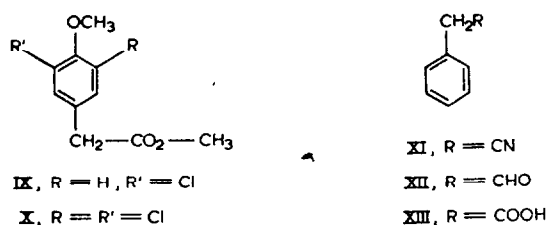
Gas chromatography indicated that five components were present in this reaction product. Examination of the mass spectra of each of the five constituents enabled the Structures IV-VIII to be assigned to these products (see Experimental section for details of their mass spectra). It is interesting that we observed ring chlorination of tyrosine since it was not previously detected⁷, while the suggestion¹³ that 3'-chloro- and 3',5'-dichlorotyrosine was formed from tyrosine at pH 2 could not be substantiated by our results.



* Approximate percentage of the product mixture as determined from gas chromatography.

The ratios of the products obtained from the reaction of L-tyrosine with HOCl remained approximately the same regardless of whether 1 or 2 equiv of HOCl were used.

Hydrolysis of the crude reaction mixture with 75 % H₂SO₄ containing NaCl under reflux, and methylation of the crude product yielded two chlorinated *p*-methoxyphenylacetic acid methyl esters (IX and X). These compounds were identified from their mass spectral fragmentation patterns (see Experimental) obtained from combined gas chromatography-mass spectrometry.

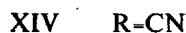


Reaction of L-phenylalanine with HOCl

Two products were obtained from the reaction of HOCl with L-phenylalanine. They were identified by gas chromatography-mass spectrometry as phenylacetonitrile (XI, 95 % of the reaction mixture) and phenylacetaldehyde (XII, 5 %). Acid hydrolysis of the crude reaction mixture yielded phenylacetic acid (XIII) which was identified from its m.p., mixed m.p. with an authentic sample and mass spectrum.

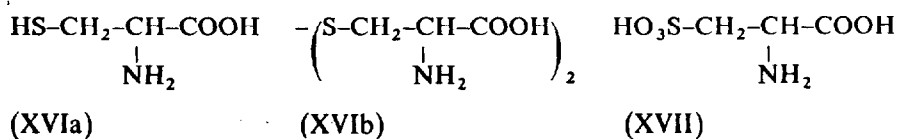
Reaction of L-glutamic acid with HOCl

The product from this reaction was an oil. One portion was methylated (diazomethane in methanol) and gas chromatography-mass spectrometry confirmed the presence of the methyl ester of β -cyanopropionic acid (XIV). From the other unmethylated portion, β -aldehydopropionic acid (XV, R=CHO) was isolated as its 2,4-dinitrophenylhydrazone and identified by mass spectrometry. Both the free acid corresponding to XIV and XV had previously been identified from the action of NaOBr with L-glutamic acid¹⁹.



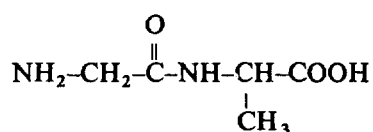
Reaction of cysteine with HOCl

Two products, plus recovered starting material, were isolated from the action of either 1 or 2 g equiv of HOCl at room temperature with L-cysteine (XVIa). The products were identified as cysteic acid (XVII) and cystine (XVIb).

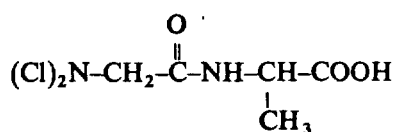


Reaction of cystine with HOCl

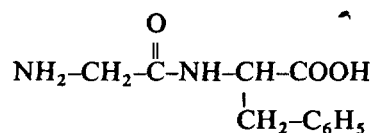
Cystine (XVIb) when reacted with HOCl (1 or 2 g equiv) at room temperature yielded cysteic acid (XVII) as the only identifiable product thus confirming a recent observation¹³.

Reaction of dipeptides with HOCl

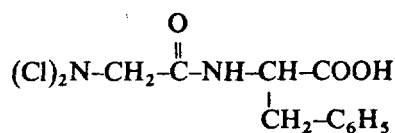
XVIII



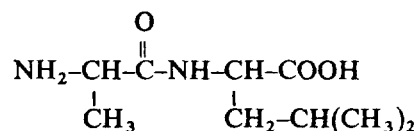
XXII



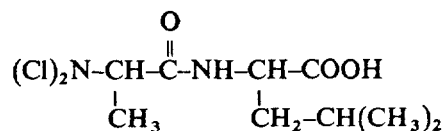
XIX



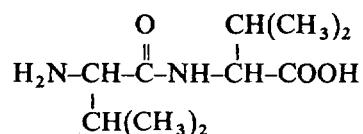
XXIII



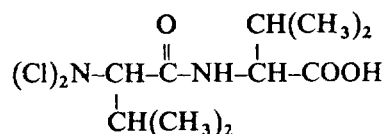
XX



XXIV



XXI



XXV

Four dipeptides, Gly-L-Ala (XVIII), Gly-DL-Phe (XIX), DL-Ala-DL-Leu (XX) and L-Val-L-Val (XXI) were each reacted with 2 g equiv of aqueous HOCl⁶ at room temperature. In each case the product from these reactions was shown to be the corresponding *N,N*-dichlorodipeptides (XXII-XXV). Although crystalline (except XXIV), these compounds, on standing at room temperature overnight, decomposed with the evolution of HCl. The structures of the *N,N*-dichlorodipeptides followed from their mass spectra (see for instance Figs 1 and 2 for the mass spectra of XXIII and XXIV). The origins of the diagnostic ions in Figs 1 and 2, critical to structural assignment, are rationalized in Schemes 1 and 2. High resolution mass measurements show that these fragmentation patterns (Figs 1 and 2) require the assignment of both chlorine atoms to the N-terminal nitrogen atom of the dipeptides.

Ethanollic NaOH at room temperature, smoothly dehydrochlorinated XXIV and XXV to the corresponding *N*-chloroimines XXVI and XXVII. The assignment of these structures follow from their mass spectra (Figs 3 and 4) in which diagnostic ions are rationalized in terms of Schemes 3 and 4. Although the imine XXVI failed to

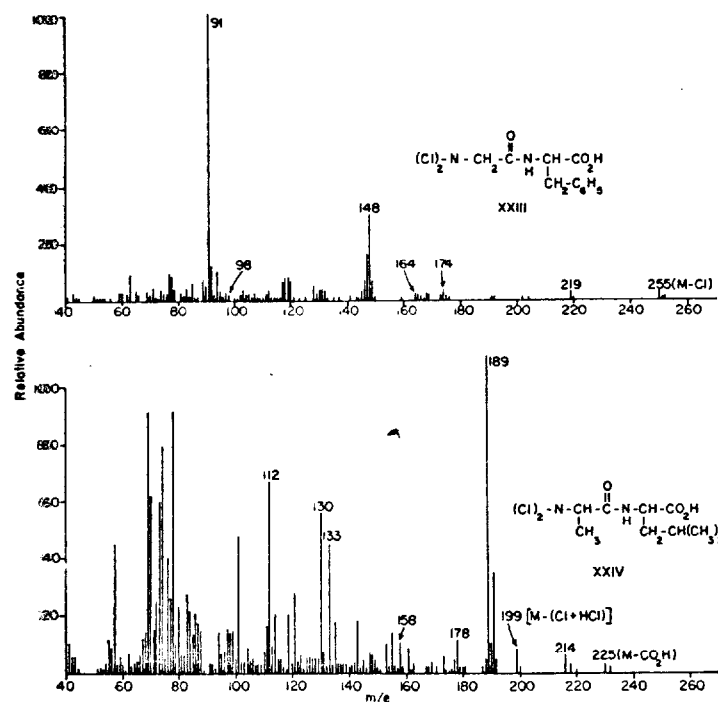
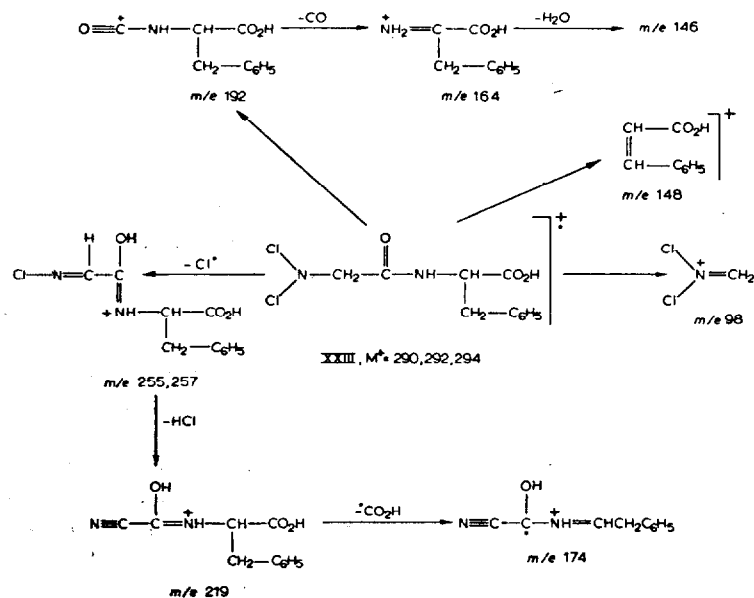
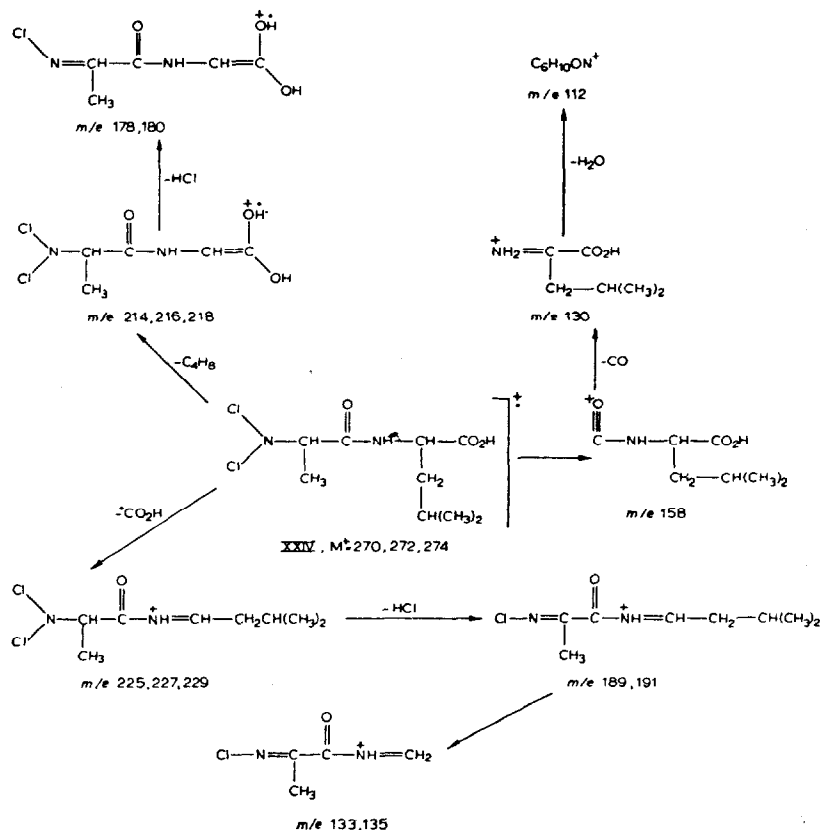


Fig. 1. Mass spectrum (70 eV) of XXIII. Fig. 2. Mass spectrum (70 eV) of XXIV.

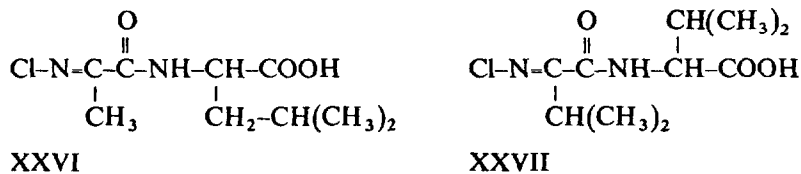


Scheme 1. Rationalizations of the origins of some significant ions in the mass spectrum (Fig. 1) of XXIII. High resolution mass spectrometry established the fragment ions to have the indicated molecular compositions.



Scheme 2. Rationalizations of the origins of some significant ions in the mass spectrum (Fig. 2) of XXIV. High resolution mass spectrometry established all the fragment ions to have the indicated molecular compositions.

show a detectable molecular ion in its mass spectrum (Fig. 3), its fragmentation pattern is consistent with its assigned structure.



Our results establish that the amide nitrogen atom of dipeptides is not attacked by aqueous HOCl at room temperature contrary to a previous suggestion¹⁸. This was supported by the observation that *N*-acetyl-L-alanine (XXVIII) failed to form an *N*-chloro compound with an excess of aqueous HOCl at room temperature over a period of several days. This result should be contrasted to the recent report²⁰ that *tert*-butylhypochlorite readily chlorinates the amide nitrogen atom of several polypeptides and proteins.

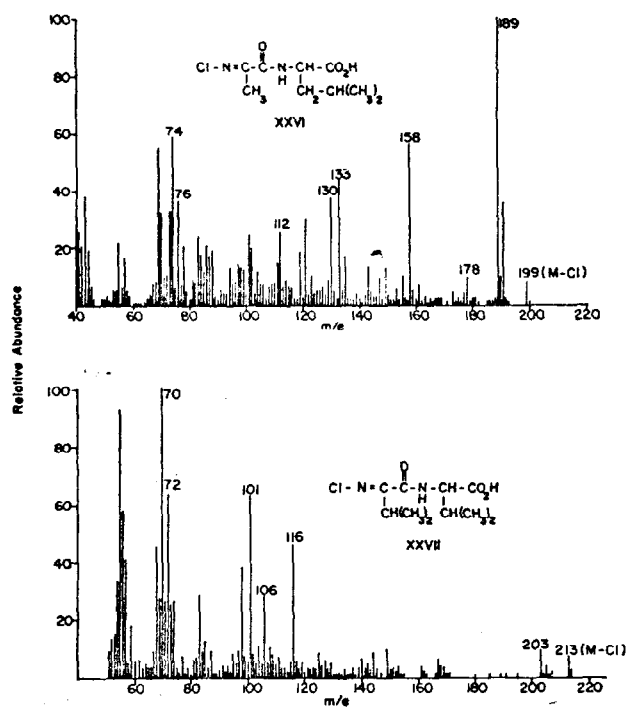
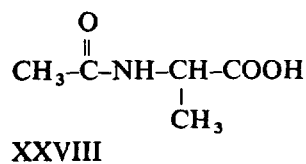
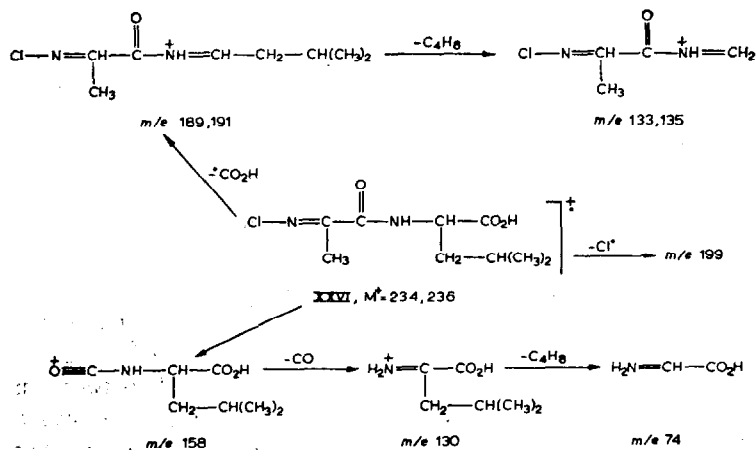
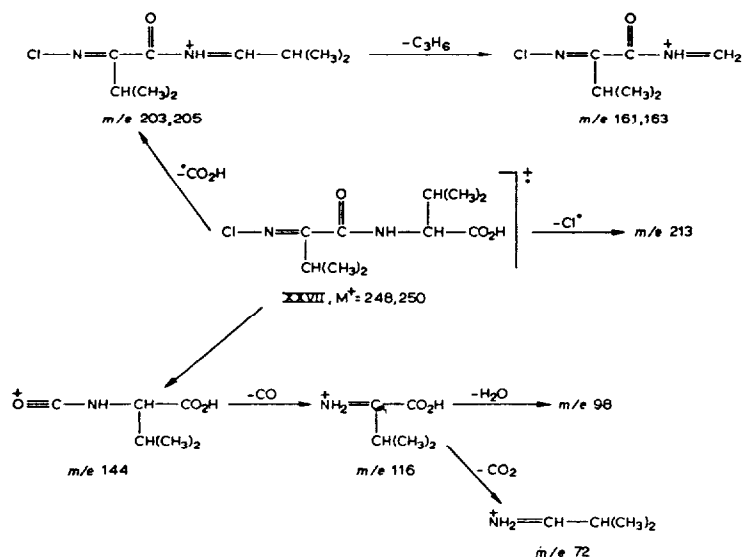


Fig. 3. Mass spectrum (70 eV) of XXVI. Fig. 4. Mass spectrum (70 eV) of XXVII.



Scheme 3. Rationalization of the origin of significant ions in the mass spectrum (Fig. 3) of XXVI. High resolution mass spectrometry established the fragment ions to have the indicated molecular compositions.



Scheme 4. Rationalization of the origin of significant ions in the mass spectrum (Fig. 4) of XXVII. High resolution mass spectrometry established the fragment ions to have the indicated compositions.

The overall effect of aqueous HOCl on dipeptides parallels the behavior observed by Goldschmidt and Strauss²¹ from the reaction of alkaline NaOBr on dipeptides where terminal *N,N*-dibromodipeptides were formed.

EXPERIMENTAL

Gas chromatography-mass spectrometry data were obtained with a Varian Aerograph Model 1200 gas chromatograph coupled by a membrane separator²² to a Finnigan 1015 Quadrupole mass spectrometer. High resolution mass measurements were obtained with an A.E.I. MS-9 mass spectrometer interfaced with the ACME computer system of the Stanford University Medical School. Gas chromatographic separations were carried out using a 6-ft glass coiled column (internal diameter 1/8 inch) packed with 3% OV-61 coated on CHROM G (80-100 mesh).

Reagents

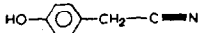
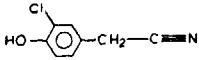
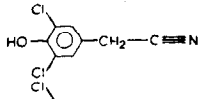
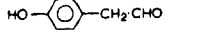
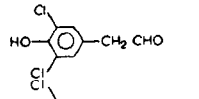
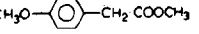
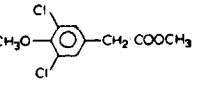
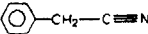
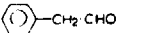
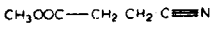
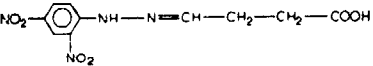
HOCl was prepared from commercial Chlorox (5.25% NaOCl) according to the procedure of Higuchi and Hasegawa²³. The molarity of the aqueous HOCl was determined by titration with Na₂S₂O₃ of the I₂ liberated from an acidified solution of KI using starch as an indicator. The pH of the standardized solution was in the range 3.5-3.0.

General reaction of amino acids with HOCl

HOCl (4 mmoles) was added dropwise during 2 h to a stirred solution of the amino acid (2 mmoles) in water (20 ml). After 4 h the solution was saturated with

TABLE I

PRINCIPAL PEAKS IN THE MASS SPECTRA OF THE PRODUCTS (AND THEIR DERIVATIVES) OBTAINED FROM THE ACTION OF HOCl ON AMINO ACIDS

Structure		<i>m/e</i> (% relative abundance)	<i>m/e</i> (% relative abundance)	<i>m/e</i> (% relative abundance)
	(IV)	133* (100)	132 (66)	
	(V)	167*, 169* (32, 11)	132 (100)	
	(VI)	201*, 203*, 205* (35, 23, 3)	166, 168 (100, 36)	
	(VII)	170*, 172* (20, 7)	141, 143 (100, 39)	
	(VIII)	204*, 206*, 208* (23, 16, 3)	175, 177, 179 (100, 70, 11)	
	(IX)	214*, 216* (21, 7)	155, 157 (100, 37)	
	(X)	248*, 250*, 252* (23, 16, 1)	189, 191, 193 (100, 70, 11)	
	(XI)	117* (100)	116 (48)	
	(XII)	120* (13)	91 (100)	
	(XIV)	113* (1)	82 (100)	54 (87)
		282* (90)	247 (54)	85 (100)

* Molecular ion.

NaCl and extracted with ether. Removal of the ether, after drying over Na_2SO_4 , yielded a residue which was dissolved in methanol and analyzed by gas chromatography-mass spectrometry. This served for the identification of the reaction products which were further characterized, if present in sufficient quantity, by derivative formation.

Reaction of cysteine and cystine with HOCl

Cysteine and cystine were reacted under the same conditions as above and the products examined by thin-layer chromatography on silica gel using methanol-water (7:3, v/v) as solvent.

The cysteine reaction mixture showed three main components identified by their R_F values as cystine (R_F 0.44), cysteic acid (R_F 0.80) and unreacted cysteine (R_F 0.68). These compounds were separated by column chromatography on Bio-Rad AGI-X8 anion exchange resin and their identification confirmed by comparison with authentic samples.

The cystine reaction yielded a single product, cysteic acid, identified by comparison with authentic material.

General reaction of dipeptides with HOCl

The dipeptide (0.5–2 mmoles) was dissolved in water (5 ml) at room temperature and HOCl (2 g equiv) was added dropwise with stirring. After 30 min the solvent was removed *in vacuo* and the product dried in a desiccator over P_2O_5 . Thin-layer chromatography of each product was carried out on silica gel, using ethylacetate-methanol (10:6, v/v) as mobile phase. Mass spectra (low and high resolution) were in agreement with structural assignment. The following yields and physical constants were recorded.

XXII. The compound had m.p. 99–101 °C. Yield 80%. Single spot ($R_F = 0.42$) on thin-layer chromatography.

XXIII. The compound had m.p. 113–115 °C. Yield 85%. Thin-layer chromatography showed that the product was contaminated with a small amount of starting material. Purification was achieved by chromatography on a 19 cm × 1 cm carboxymethyl-cellulose column using ethylacetate-methanol (10:1, v/v) to elute the product. Single spot ($R_F = 0.46$) on thin-layer chromatography. Found: C, 45.31; H, 4.15; N, 9.75; Cl, 24.10. $\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_3\text{Cl}_2$ requires C, 45.38; H, 4.15; N, 9.62; Cl, 24.36%.

XXIV. The compound was an oil. Yield 85%. Single spot ($R_F = 0.64$) on thin-layer chromatography.

XXV. The compound had m.p. 104–106 °C. Yield 98%. This compound precipitated from the reaction medium and was isolated by ether extraction. Single spot ($R_F = 0.62$) on thin-layer chromatography. Found: C, 42.21; H, 6.30; N, 9.80; Cl, 24.97. $\text{C}_{10}\text{H}_{18}\text{N}_2\text{O}_3\text{Cl}_2$ requires C, 42.11; H, 6.36; N, 9.82; Cl, 24.86%.

Dehydrochlorination of XXIV and XXV

N,N-Dichloropeptides (XXIV and XXV, 0.25 mmole) were separately added to a cold solution (0 °C) of NaOH (0.3 mmole) in ethanol (1.5 ml). The solution was kept at 0 °C for 30 min and then diluted with 5 ml of water. The solution was chromatographed on a cation-exchange resin (Bio-Rex 70 in the H^+ form, 200–400 mesh, carboxyl type) and the column eluted with 50 ml of a 70% aqueous ethanol

solution. The eluate was evaporated to dryness at room temperature and the crystalline residue dissolved in ether, dried (Na_2SO_4), filtered and the ether removed *in vacuo*.

XXVI. The compound was an oil. Yield 60%. Single spot ($R_F = 0.58$) on thin-layer chromatography.

XXVII. The compound had m.p. 105–107 °C. Yield 98%. Single spot ($R_F = 0.60$) on thin-layer chromatography.

Reaction of N-acetyl-L-alanine (XXVIII) with HOCl

N-Acetyl-L-alanine (131 mg) was dissolved in HOCl (4 g equiv), and kept at room temperature for 20 h. An aliquot was evaporated to dryness at room temperature, and mass spectrometry of the product showed it to be starting material. After a further 48 h, only starting material was identified.

ACKNOWLEDGMENT

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