Cyanogen Bromide Treatment of Methionine-Containing Compounds†

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ABSTRACT: The preparation of a series of X-Met-Gly-OEt and X-Met-Phe-OMe and their treatment with CNBr in either 70% or 97–100% formic acid at 25 °C are described where X is methanesulfonyl (mesyl), *p*-nitrobenzyloxycarbonyl, phthaloyl, trifluoroacetyl, acetyl, formyl, or *tert*-butyloxycarbonyl. Total cleavage of the peptide esters was found with mesyl-, *p*-nitrobenzyloxycarbonyl-, phthaloyl-, and trifluoroacetylmethionyl derivatives which indicated the suitability of these derivatives as amino protecting groups in peptide synthesis. Treatment of the acetylmethionyl peptide esters with CNBr in 70 and 97–100% formic acid resulted in 92 and 98% cleavage, respectively. With formylmethionyl peptide esters, about 85–95% cleavage was estimated when either 70 or 97–100% formic acid was used as the solvent. With the *tert*-butyloxycarbonylmethionyl derivatives, CNBr treatment in 70% formic acid resulted in about 93% cleavage of peptides, while treatment in 97–100% formic acid led to only 30–33% release of C-terminal amino acid esters. Quantitative cleavage of the carbonylbis(methionyl peptide esters) was observed. The reaction of CNBr with N-terminal methionyl derivatives containing free α-amino groups revealed that free methionine was quantitatively converted to homoserine lactone, whereas methionine ethyl ester and methionyl peptides (Met-Gly and Met-Phe) disappeared from the reaction mixture in 70% formic acid with only partial splitting of the ester (16%) or peptide bond (45%).

Gross (1964) proposed the use of *N*-acetylmethionine as an amino protecting group in a procedure for peptide synthesis in which the acetylmethionyl moiety was removed by CNBr cleavage. Since bovine insulin contains no methionine, methionyl derivatives present attractive possibilities for use in the selective modification of insulin (Carpenter and Shiigi, 1974; Busse and Carpenter, 1974, 1976; Busse et al., 1974). Investigation of the CNBr cleavage of acetylmethionyl peptides as well as tris(acetylmethionyl)insulin revealed a side reaction in which methionine was converted to homoserine without cleavage of the peptide bond (Carpenter and Shiigi, 1973, 1974). This side reaction, which involved participation of the carbonyl of the acetyl moiety, reduced the cleavage yield by about 10%. Although this side reaction did not preclude the use of acetylmethionine as an amino protecting group, it stimulated the investigation of other methionyl derivatives. Carbobenzoxymethionyl peptides were quantitatively cleaved in the CNBr reaction (Shiigi, 1972; Carpenter and Shiigi, 1973, 1974). The methylthiobutyryl residue, which lacks the α-amino function of methionine, was cleaved to an extent of 85% (Shiigi, 1972). A more recent study (Gross and Matsuura, 1975) reported the successful application of methylthiobutyryl group to protect the ϵ-amino group of lysine residues in peptide synthesis. However, no data were presented as to the extent of cleavage of this particular protecting group with CNBr. In the present study, a systematic investigation was conducted on the CNBr cleavage of a number of methionyl derivatives to determine their appropriateness as amino protecting groups in peptide synthesis.

Experimental Section

Materials

Amino acids were purchased from Schwarz/Mann. Phe-OMe·HCl[1] was prepared according to procedures described by Boissonnas et al. (1956). Gly-OEt·HCl was obtained from Eastman Chemicals and was recrystallized from absolute ethanol before use. Other amino acid esters were prepared by esterification of the free amino acids with methanol or ethanol in HCl (Fischer, 1901). Boc-Met was purchased from Beckman. Ac-DL-Met-ONp was provided by Dr. S. M. Shiigi (Carpenter and Shiigi, 1974). Met-Gly and Met-Phe were purchased from Cyclo Chemical. Methanesulfonyl chloride, *p*-nitrobenzyl chloroformate, *p*-nitrophenol, phthalic anhydride, ethyl thiotrifluoroacetate, triethylamine, and CNBr were all obtained from Eastman Chemicals. Dicyclohexylamine and DCC were purchased from Aldrich Chemicals. Acetic anhydride and 88% formic acid were obtained from Mallinckrodt. The latter was diluted with distilled water to give 70% formic acid. Formic acid (97–100%) was purchased from Aldrich Chemicals. Trifluoroacetic acid was from Matheson Coleman and Bell. All other chemicals were of analytical grade and obtained from Aldrich, Fischer, Mallinckrodt, or Sigma.

Methods

I. Synthesis of X-Methionines. Mes-Met was prepared by a procedure modified from that described by Helferich and Mittag (1938) for the preparation of Mes-DL-Ala. The compound was obtained as an oil which was dried under high vacuum to give a yield of 50%. Since attempts to crystallize the oil were unsuccessful, the product was crystallized from ethyl acetate as its dicyclohexylamine salt. The Mes-DL-Met dicy-

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received October 7, 1976.* This study was supported in part by Grants AM00608 and EY00813 from the National Institutes of Health. Abstracted from a dissertation presented to the University of California, Berkeley, 1976, as partial fulfillment of the requirements for the Ph.D. degree. A preliminary account of this work was presented previously (Yeung and Carpenter, 1975).

‡ Recipient of a Travel Award from the Deutsche Forschungsgemeinschaft.

[1] Abbreviations for amino acid derivatives and peptides are those proposed by the IUPAC-IUB Commission on Biochemical Nomenclature (1972); CNBr, cyanogen bromide; DCC, *N,N'*-dicyclohexylcarbodiimide; Mes or mesyl, methanesulfonyl.

TABLE I. Synthesis and Characterization of X-Met-Y.

X	Y	Synthetic Procedure	Solvents for Recrystallization	Yield (%)	Melting Points[a]	$[\alpha]_D^{27}$[b]	Empirical Formulas	Elemental Analyses[c] C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found	S Calcd.	S Found
Mes-	-OH[d]	I	Absolute ethanol	30[d]	200-202°	-5.0[e]	$C_{18}H_{36}N_2O_4S_2$	52.9	52.9	8.8	8.7	6.9	6.8	15.7	15.9
Mes-	-OH[d,f]	I	Absolute ethanol	30	192-194°	-	$C_{18}H_{36}N_2O_4S_2$	52.9	52.9	8.8	8.7	6.9	7.0	15.7	15.7
Mes-	-Gly-OEt	II-A	Ethyl acetate-ethyl ether	43	90-92°	-11.0	$C_{10}H_{20}N_2O_5S_2$	38.5	38.6	6.4	6.3	9.0	9.0	20.5	20.4
Mes-	-Phe-OMe	II-A	2-Propanol	54	135-137°	-21.8	$C_{12}H_{24}N_2O_5S_2$	49.4	49.3	6.2	6.3	7.2	7.2	16.5	16.3
$Z(NO_2)$-	-OH[d]	I	95% Ethanol	50	178-179°	+0.05[g]	$C_{25}H_{39}N_3O_6S$	58.9	58.9	7.7	7.5	8.2	8.3	6.3	6.3
$Z(NO_2)$-	-Gly-OEt	II-A	2-Propanol	56	120-122°	-0.5[h]	$C_{17}H_{23}N_3O_5S_2$	49.4	49.1	5.6	5.9	10.2	10.3	7.8	7.7
$Z(NO_2)$-	-Phe-OMe	II-A	2-Propanol	36	114-115°	-8.00[e]	$C_{23}H_{27}N_3O_7S$	56.5	56.4	5.5	5.5	8.6	8.3	6.5	6.3
Pht<	-OH[f]	I	Water	60	107-109°	-	$C_{13}H_{13}NO_4S$	55.9	55.7	4.7	4.7	5.0	5.0	11.5	11.6
Pht<	-Phe-OMe[f]	II-B	2-Propanol-ethyl ether	22	117-119°	-12.0[h]	$C_{23}H_{24}N_2O_5S$	62.7	62.8	5.5	5.4	6.4	7.0	7.3	7.4
CF_3CO-	-OH	I	10% Acetic acid	63	64-66°[i]	-20.96[e,i]	$C_7H_{10}F_3NO_3S$	34.3	32.6	4.1	4.4	5.7	5.2	13.1	12.2
CF_3CO-	-Phe-OMe	II-B	Ethanol-water	39	119-121°	-12.0	$C_{17}H_{21}F_3N_2O_4S$	50.2	51.1	5.2	5.4	6.9	7.6	7.9	7.3
Ac-	-Gly-OEt[f]	II-D	Abs. ethanol-ethyl ether	35	108-109°[j]	-	$C_{11}H_{20}N_2O_4S$	47.8	47.7	7.3	7.2	10.1	10.2	11.6	11.4
Ac-	-Phe-OMe[f]	II-D	Abs. ethanol-ethyl ether	35	104-106°[k]	-5.5	$C_{17}H_{25}N_2O_4S$	57.9	57.8	6.9	6.8	8.0	7.8	9.1	8.9
HCO-	-OH	I	Ethyl acetate	80	82-84°[l]	+10.99[e,l]	$C_6H_{11}NO_3S$	40.7	40.6	6.3	6.7	7.9	8.1	18.1	18.3
HCO-	-Gly-OEt	II-B	Ethyl acetate	53	97-99°	-14.5	$C_{10}H_{18}N_2O_4S$	45.8	46.7	6.9	7.0	10.7	10.4	12.2	11.2
HCO-	-Phe-OMe	II-B	Ethyl acetate	90	112-114°	-12.98	$C_{16}H_{22}N_2O_4S$	56.8	56.9	6.6	6.8	8.3	8.4	9.5	9.3
Boc-	-Gly-OEt	II-C	Pet. ether (30-60°)-ethyl ether	10	55-57°[m]	-12.0[n]	$C_{14}H_{26}N_2O_5S$	50.3	50.4	7.8	7.9	8.4	8.5	9.6	9.8
Boc-	-Phe-OMe	II-C	Pet. ether (30-60°)-ethyl ether	79	82-84°	-12.17	$C_{20}H_{30}N_2O_5S$	58.5	57.9	7.4	7.2	6.8	7.8	7.8	7.7

[a] The melting points were determined in an unsealed capillary tube, using a Thomas-Hoover capillary melting point apparatus. The values were uncorrected. [b] The optical activity was measured by determining the optical rotation at 5893 nm (corresponding to sodium D light) with a Cary 60 spectrophotometer in absolute ethanol at a concentration of 1 g/100 mL unless otherwise specified. [c] All elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. [d] Dicyclohexylamine salt. [e] Absolute methanol. [f] DL-Methionine was used. [g] c 0.5, absolute methanol. [h] Dimethyl sulfoxide. [i] Lit. mp 69–71 °C; $[\alpha]^{25}_D$ −22.7 °c 2, water; Fones and Lee (1954). [j] Lit. mp 114 °C; Lawson et al. (1962). [k] Lit. mp 113–118 °C; Carpenter and Shiigi (1974). [l] Lit. mp 99–100 °C; $[\alpha]^{25}_D$ −10.00°, c 0.2 g made up to 25 mL of water; Windus and Marvel (1931). [m] Lit. mp 51 °C; $[\alpha]^{27}_D$ −12.6°, c 1, dimethylformamide; De Castiglione (1969). [n] c 0.5.

clohexylamine salt was prepared according to the same procedure.

Z(NO_2)-Met was synthesized following the procedure described by Gish and Carpenter (1953) for the preparation of Z(NO_2)-DL-Met. The product was isolated as an oil which was converted to the crystalline dicyclohexylamine salt by the addition of dicyclohexylamine to a solution of the compound in ethyl acetate.

Pht<DL-Met was prepared by fusing 74 g (0.5 mol) of phthalic anhydride with 75 g (0.5 mol) of DL-methionine in a round-bottomed flask at 180–190 °C in an oil bath for 30 min. The resulting oil was crystallized from warm 2-propanol–water.

CF_3CO-Met was synthesized according to the procedure described by Schallenberg and Calvin (1955) for the preparation of trifluoroacetyl-amino acids.

HCO-Met was prepared by the procedure described by du Vigneaud et al. (1932) for the formylation of DL-cystine.

II. Synthesis of X-Methionyl Peptide Esters. Procedure A. Equimolar quantities of X-L-methionine dicyclohexylamine salt and Gly-OEt·HCl (or Phe-OMe·HCl) were dissolved in 120 mL of chloroform. The mixture was shaken for 4 h at room temperature. DCC (same molar quantities as Gly-OEt·HCl) was added and the resulting solution was shaken for another 4 h at room temperature. After letting the reaction mixture stand at 4 °C overnight, the white precipitate consisting of dicyclohexylamine hydrochloride and dicyclohexylurea was filtered. The filtrate was washed sequentially three times with 50 mL each of water, 1 N HCl, 1 N $NaHCO_3$, and water. The organic phase was dried over anhydrous $MgSO_4$ and filtered and the solvent was evaporated to dryness. The products were crystallized from the solvents noted in Table I.

Procedure B. Gly-OEt·HCl (or Phe-OMe·HCl) (0.01 mol) was dissolved in 10 mL of water. The pH of the solution was adjusted to 9.0 with 0.5 M Na_2CO_3. The ester was extracted into ethyl ether and the resulting ether was removed. The oil was suspended in the appropriate solvent (e.g., ethyl acetate). X-L-Methionine (0.01 mol) was dissolved in the above solution and the reaction mixture was cooled in an ice bath. DCC (0.01 mol) was added and the resulting solution was stirred for 1 h in an ice bath and then left at −10 °C overnight. The dicyclohexylurea was filtered and the solvent was removed. The products were crystallized from the solvents noted in Table I.

Procedure C. X-L-Methionine (0.01 mol) was dissolved in 10 mL of ethyl acetate. This solution was added to 0.01 mol of Gly-OEt·HCl (or Phe-OMe·HCl) in 30 mL of ethyl acetate along with 0.01 mol of triethylamine. The resulting solution was stirred in an ice bath. DCC (0.01 mol) was added to this solution and the final mixture was left at −10 °C overnight.

The dicyclohexylurea was filtered and the solvent was removed. The products were crystallized from the solvents noted in Table I.

Procedure D. Gly-OEt·HCl (or Phe-OMe·HCl), 0.01 mol, was dissolved in 30 mL of ethyl acetate. Triethylamine (0.01 mol) was added and the solution was stirred at 4 °C. X-L-Methionine *p*-nitrophenyl ester (0.012 mol) was added and the reaction mixture was stirred for 23 h at 4 °C. The triethylamine hydrochloride was filtered and the solvent was removed. The residue was suspended in 50 mL of ethyl acetate. The organic phase was washed three times with 20 mL each of 5% HCl, 5% $NaHCO_3$, and water. After drying the organic layer with anhydrous $MgSO_4$ for several hours, the mixture was filtered and the solvent was removed. The products were crystallized from the solvents noted in Table I.

Carbonylbis(L-methionylglycine ethyl ester). Carbonylbis(L-methionine *p*-nitrophenyl ester) (Busse and Carpenter, 1976) (2.26 g, 4 mmol) and Gly-OEt·HCl (1.26 g, 8 mmol) were dissolved in 100 mL of dimethylformamide. Triethylamine (1.1 mL, 8 mmol) was added to the stirred solution at 0 °C. The reaction mixture was kept at 2–4 °C for 18 h. The product admixed with triethylamine hydrochloride was precipitated by the addition of 700 mL of ethyl ether. The product was purified by two recrystallizations from 600 mL of 2-propanol to yield 1.75 g (97%), mp 232 °C, $[\alpha]^{27}_D$ +22.5 (*c* 1, dimethyl sulfoxide).

Anal. Calcd for $C_{19}H_{34}N_4O_7S_2$: C, 46.2; H, 6.9; N, 11.3; S, 13.0. Found: C, 46.2; H, 6.6; N, 11.2; S, 12.8.

Carbonylbis(L-methionyl-L-phenylalanine methyl ester). This derivative was prepared according to the procedure described above for the carbonylbis(L-methionylglycine ethyl ester): yield 76%; mp 208 °C; $[\alpha]^{27}_D$ −2.5 (*c* 1, dimethylformamide).

Anal. Calcd for $C_{31}H_{44}N_4O_7S_2$: C, 57.5; H, 7.0; N, 8.6; S, 9.9. Found: C, 57.0; H, 6.4; N, 8.7; S, 10.0.

Isolation of Carbonylbis(homoserine lactone). CO(Met-Gly-OEt)$_2$ (1 g, 2 mmol) was reacted with CNBr (5 g, 47 mmol) in 50 mL of 70% formic acid for 4 h at room temperature. The solvent and CNBr were removed under reduced pressure and the remaining oil was redissolved in 5 mL of 90% 2-propanol. Tetrahydrofuran (150 mL) was added and Gly-OEt·HBr crystallized in long needles to yield 575 mg (78%), mp 176–177 °C (lit. 177–178 °C, Pettit and Kadunce (1963)); the infrared spectrum was identical with that of Gly-OEt·HCl. The filtrate was taken to dryness under reduced pressure and the oily residue crystallized from 95% 2-propanol, yielding needle shaped crystals, 112 mg (67.5%) of carbonylbis(homoserine lactone), mp 199–200 °C, infrared absorption bands at 1570 (s), 1645 (s), 1778 (s), 3370 (s).

Anal. Calcd for $C_9H_{12}N_2O_5$: C, 47.5; H, 5.3; N, 12.3. Found: C, 47.4; H, 5.5; N, 12.3.

CNBr Treatment of Model Compounds. In general, 14.45 μmol of the methionine derivatives was dissolved in 1.0 mL of either 70 or 97–100% formic acid in a water-jacketed reaction vessel equilibrated at 25 °C. After the removal of a zero-time aliquot (25 μL), 1 mL of a CNBr solution (100 mg/mL) in either 70 or 97–100% formic acid was added. At various times after the addition of the CNBr solution, 50 μL of the reaction mixture was removed and added to 2.0 mL of amino acid sample diluting buffer (0.2 M sodium citrate at pH 2.20). Two hundred and fifty microliters of these samples was applied to the amino acid analyzer to quantitate the amount of C-terminal amino acid ester released.

With the methionyl derivatives and peptides (including Met-OEt, Met-Gly, and Met-Phe), two aliquots of the reaction mixture were taken out at the designated time. One of the aliquots was analyzed directly for the amount of C-terminal amino acid (Gly or Phe) and/or the starting material (Met-OEt, Met-Gly, or Met-Phe). The other aliquot was diluted with 2.0 mL of 6 N HCl and the tube (16 × 125 mm) was sealed under vacuum and heated at 120 °C for 6 h. The acid hydrolysate was dried on the rotary evaporator. To ensure quantitative conversion of homoserine lactone to homoserine, a modified procedure of the one described by Ambler (1965) was used. One milliliter of 0.2 M pyridine–acetate at pH 7.0 was added to the dried acid hydrolysate. The tube was sealed under vacuum and was heated at 120 °C for 1 h. The tube was opened immediately before analysis. In the case of CNBr-treated Met-OEt, any starting material remaining in the reaction mixture is hydrolyzed to Met by the treatment of pyridine–acetate buffer. The Met thus formed as well as the Hse were analyzed. With CNBr treatment of Met, only one aliquot of the reaction mixture was removed and treated with pyridine–acetate buffer as described above, and the sample was analyzed for the amount of Hse and Met on the amino acid analyzer.

Conditions for the Quantitative Determination of the Products. Gly, Met, and Phe were analyzed on the long column (0.9 × 56 cm) of the amino acid analyzer according to the procedure of Spackman et al. (1958). A flow rate of 70 mL/h was used for all these analyses and for those described below. The analyzer was a Beckman Model 120B equipped with a manual sample injector and an Autolab System AA computing integrator for the amino acid analyzer.

Commercial DL-Hse was treated with 0.2 M pyridine–acetate, pH 7.0 at 120 °C for 1 h before applying to the amino acid analyzer. To elute the Hse from the long column (0.9 × 56 cm), 0.2 M sodium citrate buffer, pH 3.26, was used. The retention time was found to be 61 min.

The conditions for analyzing the amino acid esters and methionyl peptides on the amino acid analyzer are shown in Table II.

Results

Extent and Rate of Cleavage of N-Protected Methionyl Peptide Esters. A reaction mechanism for the cleavage of methionyl peptides with CNBr has been proposed by Gross and Witkop (1962). Their mechanism of CNBr cleavage as applied to N-protected methionyl peptide esters is shown in Figure 1. X represents a series of *N*-acyl amino protecting groups that were tested. The N-protected methionyl peptide esters react with CNBr to form the cyanosulfonium bromide. The nucleophilic attack by the cabonyl oxygen of the methionyl portion on the carbon of the cyanosulfonium salt leads to the formation of iminolactone bromide, an intermediate that has been isolated by Inglis and Edman (1970). Upon hydrolysis, the iminolactone bromide breaks down to give the *N*-acylhomoserine lactone and the C-terminal amino acid esters. The course of the reaction was followed by determining the release of the amino acid esters which were quantitated on the amino acid analyzer.

N-Mesylmethionyl Peptide Esters. The kinetics of CNBr cleavage of *N*-mesylmethionyl peptide esters is shown in Figure 2. When 70% formic acid was used as the solvent in the CNBr treatment of these peptides, only 75% of the C-terminal amino acid esters was released. However, 100% cleavage was found when 97–100% formic acid was used as a solvent. Under these conditions, total cleavage of the peptides was attained within an hour. The side chain of the C-terminal amino acid ester did not have any effect on the extent as well as on the rate of cleavage.

TABLE II: Conditions for Analyzing Amino Acid Esters and Methionyl Peptides on the Amino Acid Analyzer.

Compounds	Conditions: Column Size (cm)	Buffer	Temp (°C)	Elution Time[a] (min)
Gly-OEt	0.9 × 7.0[b]	0.35 M Na citrate, pH 5.20	50	88
Phe-OMe	0.9 × 7.0[b]	0.35 M Na citrate-2.0 M NaCl, pH 7.50	58	45
Met-OEt	0.9 × 7.0[b]	0.35 M Na citrmte-2.0 M NaCl, pH 7.50	58	25
Met-Gly	0.9 × 56[c]	0.20 M Na citrate, pH 4.25	50	55
Met-Phe	0.9 × 56[c]	0.20 M Na citrate, pH 5.20	50	95

[a] Flow rate was 70 mL/h. [b] Beckman PA-35. [c] Beckman AA-15.

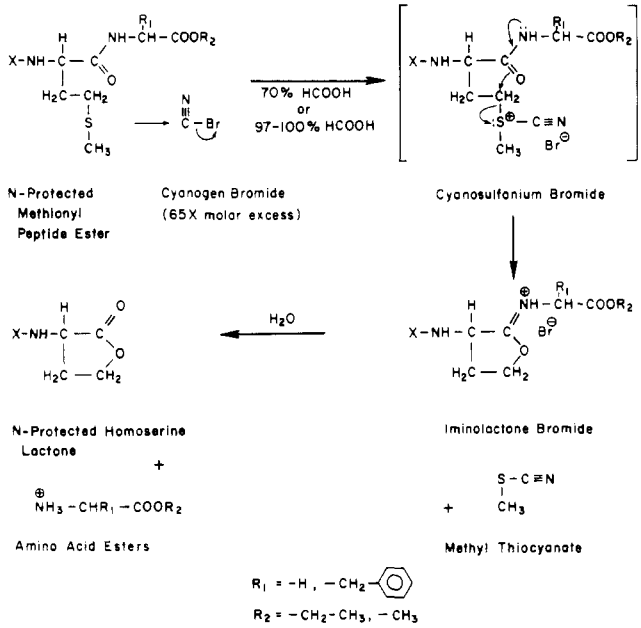

FIGURE 1: Mechanism of CNBr cleavage of N-protected methionyl peptide esters, modified from Gross and Witkop (1962).

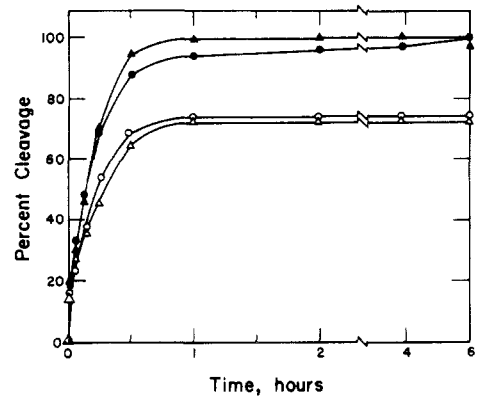

FIGURE 2: Kinetics of CNBr cleavage at 25 °C of Mes-Met-Gly-OEt (○) in 70% formic acid and (●) in 97-100% formic acid and of Mes-Met-Phe-OMe (△) in 70% formic acid and (▲) in 97-100% formic acid.

TABLE III: CNBr Cleavage of N-Protected-Methionyl Peptide Esters.

N-Protecting Group	Extent of Cleavage in % at the End of 6 Hours: 70% HCOOH Gly-OEt	70% HCOOH Phe-OMe	97-100% HCOOH Gly-OEt	97-100% HCOOH Phe-OMe
Mes-Met-	75	75	100	100
$Z(NO_2)$-Met-	100	100		
Pht<Met-		100		
CF_3CO-Met-		100		
Ac-Met-	92	93	96	98
HCO-Met-	85	92	86	95
Boc-Met-	93	93	30	33

N-p-Nitrobenzyloxycarbonyl-, N-Phthaloyl-, and N-Trifluoroacetylmethionyl Peptide Esters. The rate and extent of cleavage of these peptide esters were examined in 70% formic acid. Unlike *N*-mesylmethionyl peptide esters, these three N-protected methionyl peptide esters were cleaved completely within 1-2 h when they were treated with CNBr in 70% formic acid (Table III).

N-Acetylmethionyl Peptides and Peptide Esters. CNBr treatment of Ac-Met-Gly and Ac-Met-Phe in 70% formic acid was investigated by Shiigi (1972). The extent of cleavage of these *N*-acetylmethionyl peptide acids was about 90% (Carpenter and Shiigi, 1974). These two peptides in their free acid form were treated with CNBr in 97-100% formic acid to determine if there would be a change in the extent of cleavage with the more concentrated formic acid as the solvent. In all cases, incomplete cleavages, ranging from 85 to 90%, were found regardless of whether the reaction was performed in either 70 or 97-100% formic acid.

The extent of cleavage by CNBr treatment of *N*-acetylmethionyl peptide esters in 70 and 97-100% formic acid varied from 92 to 98%. However, the recovery of C-terminal amino acid esters was relatively higher when 97-100% rather than 70% formic acid was used as the solvent (Table III).

N-Formylmethionyl Peptide Esters. The range of cleavage in the CNBr treatment of *N*-formylmethionyl peptide esters in 70 and 97-100% formic acid was from 85 to 95%. There was very little solvent effect in the CNBr treatment of these peptide esters. However, the recovery of Phe-OMe was somewhat higher than that of Gly-OEt in both of the solvent systems (Table III).

N-tert-Butyloxycarbonylmethionyl Peptide Esters. Cleavage of *N-tert*-butyloxycarbonylmethionyl peptide esters with CNBr shows significant dependence on solvent. The extent of cleavage was much higher (up to 93%) when 70% formic acid was the solvent as compared with the cleavage (30%) in 97-100% formic acid (Table III).

CNBr Treatment of Carbonylbis(methionyl peptide esters). Treatment of $CO(Met\text{-}Gly\text{-}OEt)_2$ results in formation of glycine ethyl ester hydrobromide and carbonylbis(homoserine lactone) (Figure 3), indicating that the mechanism of the

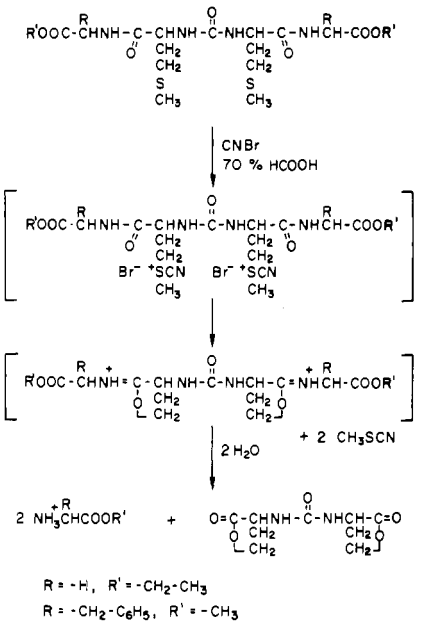

FIGURE 3: Mechanism of the CNBr cleavage of the model compounds CO(Met-Gly-OEt)$_2$ and CO(Met-Phe-OMe)$_2$ which results in formation of 2 mol of the corresponding amino acid esters and carbonylbis(homoserine lactone).

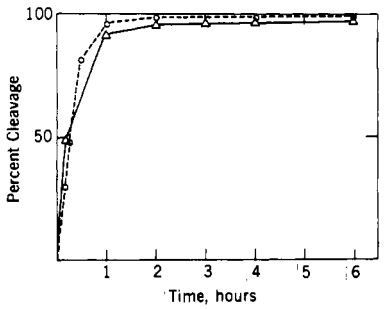

FIGURE 4: Rate of release of Gly-OEt (O–O) and Phe-OMe (Δ–Δ) from the corresponding model compounds, CO(Met-Gly-OEt)$_2$ and CO(Met-Phe-OMe)$_2$, upon CNBr treatment in 70% formic acid at 25 °C.

cleavage reaction is essentially the same as that demonstrated for other methionyl peptides and methionine-containing proteins (Gross and Witkop, 1961, 1962; Gross, 1967).

In quantitative experiments the model peptides CO(Met-Gly-OEt)$_2$ and CO(Met-Phe-OMe)$_2$ were treated with CNBr in 70% formic acid. The time course of the reaction was followed by the determination of the released amino acid esters as shown in Figure 4. The recovery of Gly-OEt as well as Phe-OMe in approximately 98% yield suggests a nearly complete cleavage takes place at both sides of the model compounds.

Extent and Rate of Cleavage of N-Unprotected Methionine Derivatives and Peptides. The mechanism of CNBr cleavage of methionine derivatives in which the amino group is free is not well documented. The results reported here indicate that the mechanism of Gross and Witkop (Figure 1) may not apply in all instances.

Methionine. The rate of reaction of methionine with CNBr was followed by the disappearance of methionine and the appearance of homoserine lactone (Figure 5). The complete conversion of methionine to homoserine lactone occurs in less than 6 h.

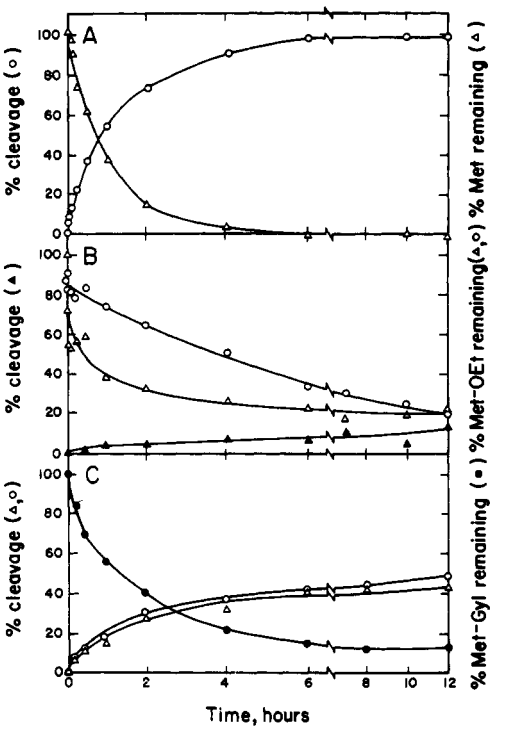

FIGURE 5: (A) Kinetics of CNBr cleavage of methionine at 25 °C in 70% formic acid; (○) formation of homoserine; (△) disappearance of methionine. (B) Kinetics of CNBr cleavage of methionine ethyl ester hydrochloride at 25 °C in 70% formic acid: (▲) formation of homoserine; (△) disappearance of methionine ethyl ester; (○) disappearance of methionine ethyl ester based on methionine detected in the pyridine–acetate hydrolysate. (C) Kinetics of CNBr cleavage of methionylglycine at 25 °C in 70% formic acid; (○) glycine released; (△) homoserine formed; (●) methionylglycine remaining.

Methionine Ethyl Ester Hydrochloride. The disappearance of the starting material was followed by determining directly the amino acid ester remaining in the reaction mixture as well as by quantitating the free methionine after the conversion of the ester to methionine in the pyridine–acetate buffer. The kinetics of disappearance of the starting amino acid ester showed major differences when followed in these two different ways (Figure 5). The direct measurement indicates a much more rapid disappearance of the ester than the indirect. This suggests that an intermediate is formed which can be converted at least in part to methionine upon treatment with the pyridine–acetate buffer. In contrast to the results on the methionine, the methionine ethyl ester gave only a 13% yield of homoserine at a time where 80% of the methionine ethyl ester had disappeared from the reaction mixture.

Methionylglycine. Forty-eight percent of free glycine was detected in the reaction mixture of methionylglycine treated with CNBr at the end of 12 h (Figure 5). The formation of homoserine followed closely the kinetics of release of C-terminal amino acid. However, there was a discrepancy between the disappearance of dipeptide and the appearance of homoserine or C-terminal amino acid. At the end of 12 h, 40% of the dipeptide that had disappeared from the reaction could not be accounted for as homoserine or C-terminal amino acid.

Methionylphenylalanine. The results in the CNBr cleavage of methionylphenylalanine were similar to those found for methionylglycine. After 12 h, about 40% of dipeptide could be accounted for as homoserine and phenylalanine, and another 40% of the dipeptide had disappeared from the reaction mix-

ture and was unaccounted for as known reaction products.

Discussion

The investigation of the CNBr cleavage of free methionyl peptides revealed a discrepancy between the rate of disappearance of the peptide and formation of known cleavage products. In a previous study of CNBr treatment of methionylalanine (Inglis and Edman, 1970), only about 32% of cleavage was observed when the reaction was followed by the release of methyl thiocyanate. The extent of cleavage as determined here either by release of C-terminal amino acids or by formation of homoserine correlates well with that reported by Inglis and Edman (1970) who measured the release of methyl thiocyanate. A survey of the literature shows that partial cleavage at methionine residues when the amino group is free is not uncommon. Link and Stark (1968) reported that the recovery of free homoserine (lactone) never exceeded 33% when ribonuclease A was treated with CNBr. These low yields have been attributed to the poor cleavage of the amino terminal methionyl residue at position 30 by CNBr, based on the assumption that cleavage occurs at methionine-29 first, liberating methionine-30 as the amino terminal. Another observation reported by Black (1967) showed that the amino terminal methionine residue of the lysozyme isolated from phage λ gave rise to only 30% yield of homoserine (lactone) when treated with CNBr in 0.1 N HCl at 30 °C. Recently, in a study to establish the amino acid sequence of flagellin of *Bacillus subtilis* 168 (Chang et al., 1976), the amino terminal methionine residue of this protein was shown to react only partially with CNBr. In fact, close to 0.4 residue of methionine was recovered after treatment with CNBr.

Our investigations showed that, whereas free methionine underwent a complete conversion to homoserine, 40–67% of the methionyl derivatives disappeared from the reaction mixture without cleavage. One of the major differences between methionine and its derivatives is the pK_a of the amino group. Whereas the pK_a of the amino group of methionine is about 9.2, that of the amino group in methionine esters or peptides is about 7.5. Methionine methylsulfonium bromide exhibits a pK_a for the amino group of 7.7 which is considerably lower than the pK_a of the amino group (9.2) of methionine (Lavine et al., 1954). Such a difference in pK_a has been attributed to the effect of the positively charged sulfonium group on the amino moiety. An analogous effect would be expected upon the conversion of methionine ethyl ester and methionyl peptides, which have pK_as of about 7.5, to the cyanosulfonium salts. The amino group of the cyanosulfonium salts of the methionyl derivatives would be expected to have pK_a values of about 5.5. Such a decrease in pK_a of the amino groups of methionyl derivatives of about two units as compared with free methionine would increase the population of the deprotonated form of the amino group of the methionyl derivatives at least 100-fold. At the pH of 70% formic acid, the reaction medium where CNBr cleavage was performed, there will not be a substantial ionization of either type of amino group. However, even at this pH the amount of deprotonated amino groups in methionyl derivatives will be much greater than in free methionine. The uncharged amino group may react with the CNBr in such a fashion as to preclude normal cleavage of the methionyl bond (Yeung, 1976). Although the precise reason for the low cleavage yields with derivatives containing a free amino group in N-terminal methionine is not known, if methionine derivatives are to be used in peptide synthesis, the amino group of the methionine should be covered with a group which is stable to the acidic conditions used in CNBr cleavage.

The acidic media that have been used in the CNBr cleavage of methionyl peptides or methionyl residues in proteins include 0.3 N HCl, 70% formic acid, and 70% trifluoroacetic acid (Spande et al., 1970). All of the amino protecting groups investigated were expected to be stable in these acidic conditions with the exception of the Boc and possibly the formyl group. It has been reported that selective cleavage of Boc group from peptides may be achieved in 85% formic acid (Kinoshita and Kotake, 1974), 98% formic acid (Halpern and Nitecki, 1967), and 50% trifluoroacetic acid (Wang, 1976). The lower cleavage yields found for Boc-peptide in 97–100% formic acid as compared with 70% formic acid as a solvent can be attributed to removal of the Boc group. The less than theoretical cleavage observed for the formyl group may also be due in part to hydrolysis of the formyl residue in the acidic reaction mixture. However, it is also possible that the formyl group participates in a back-sided attack on the intermediate sulfonium bromide in much the same manner as has been demonstrated for the acetyl group (Carpenter and Shiigi, 1974) which results in the conversion of methionine to homoserine without cleavage of the methionyl bond. It was to avoid this side reaction that several other derivatives were investigated. It was known from the work of Carpenter and Shiigi that Z-Met-Gly did not undergo the side reaction and gave a 100% cleavage. This was attributed to a decreased nucleophilicity of carbonyl oxygen of the carbobenzoxy residue as compared with that in the acetyl residue—a property that is attributed to carbobenzoxy residue by Bodanszky and Ondetti (1966) to explain the lack of oxazolone formation by carbobenzoxy residue as compared with acetyl residue. With the mesyl group the nucleophilicity of the sulfonyl oxygen may be decreased as a result of the high electronegativity of the sulfur center in the mesyl moiety. With the phthaloyl group, the nucleophilicity of the carbonyl oxygen may also be decreased as a result of the possible delocalization of the electron density of the carbonyl oxygen over the benzene ring. In addition steric constraints may prevent the phthaloyl group from participating in the back-sided attack noted for the acetyl group. With the trifluoroacetyl group, the electron-withdrawing properties of fluorine may decrease the nucleophilicity of the carbonyl oxygen and hence eliminate the side reaction. In any event, all of these groups under proper conditions allowed for complete cleavage of the methionyl peptide bond in model peptides. However, with the mesyl methionyl derivatives, there was a solvent dependency of cleavage with CNBr in that complete cleavage was noted in 97–100% formic acid but not in 70% formic acid. Sulfanilamide type compounds (Bell and Roblin, 1942; Foernzier and Martin, 1967) are weak acids. Because of high electronegativity, the oxygen atoms of the sulfonyl group are electron withdrawing. The electron-deficient sulfur atom tends to polarize the electron density of the nitrogen and the hydrogen is therefore bound less tightly. The net result is the readiness of the sulfonamide to ionize to liberate a proton in solution. In fact, the pK_a of the sulfanilylglycine is 3.52, and that of *N'*-chloroacetylsulfanilamide is 3.79. The sulfonamide of the mesylmethionyl peptides may be partially deprotonated in 70% formic acid and may react directly with CNBr to form a noncleavable intermediate (Yeung, 1976). With 97–100% formic acid as the solvent, the more highly acidic solvent suppresses the ionization of the amide nitrogen and the consequent side reactions, resulting in complete cleavage.

Since most of the N-protected methionyl derivatives could be removed quantitatively with CNBr under the appropriate conditions, one can conclude that these methionyl derivatives

may be employed as reversible amino protecting groups in peptide synthesis. In the synthesis of peptides or proteins that contain methionine residues, these N-protected methionyl derivatives may still be applicable as amino protecting groups. The approach is to use methionine sulfoxide to substitute for the methionine residues in the peptides or proteins. Upon removal of the N-protected methionyl derivatives with CNBr, the methionine sulfoxide in the peptides and proteins may be reduced with sulfhydryl reagents to regenerate free methionine.

CNBr treatment of the carbonylbis(methionyl peptide esters) yielded a quantitative cleavage. The isolation of the carbonylbis(homoserine lactone) indicates that the reaction follows the mechanism proposed by Gross and Witkop (1962) for methionine residues within a protein. These observations made it possible to develop a bifunctional reagent, $CO(Met\text{-}ONp)_2$, which has been used in a synthetic scheme for the synthesis of insulin (Busse and Carpenter, 1974, 1976; Busse et al., 1974) and which has potential as a reversible cross-linking group for topographical studies on ribosomes and other oligomeric proteins (Slobin, 1972; Sun et al., 1974).

Acknowledgment

The authors are indebted to Robert Ptolemy for aid in the chemical synthesis and to Gene Tisdell for amino acid analyses.

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