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SEMISYNTHESIS I - CLEAVAGE OF METHIONYL PEPTIDE BONDS BY SULPHENYL CHLORIDES.

I.J. Galpin* and D.A. Hoyland

The Robert Robinson Laboratories, The University of Liverpool, P.O.Box 147, Liverpool, L69 3BX, England.

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Abstract - Methoxycarbonyl sulphenyl chloride (Scm.Cl) and o-nitrophenyl-sulphenyl chloride (Nps.Cl) have been shown to cleave methionyl peptide bonds in a manner similar to that found with cyanogen bromide. These new methods of cleavage of methionyl peptide bonds were illustrated by digestion of H.Gly.Met.Ala.OH trifluoroacetate, the (1-32) fragment of adrenocorticotropic hormone {(1-32) ACTH} and Hen Egg-White Lysozyme.

Protein semi-synthesis[1] is a technique which may be employed to convert natural proteins into analogues. The technique has evolved in parallel with total peptide synthesis and several intermediate stages are used during the transformation from the native protein to an analogue. Important stages in protein semi-synthesis are protection prior to cleavage of the protein chain, fragmentation of the peptide chain, total synthesis or modification of cleaved fragments, followed by coupling and final deprotection.

One of the most limiting stages of this sequence of events is peptide bond cleavage. Enzymic cleavage has been used on a variety of occasions and trypsin is the most commonly used enzyme[2] allowing cleavage at the peptide bond adjacent to basic residues (arginine and lysine). There are several limitations to enzymic cleavage as frequently secondary factors may influence the cleavage of the peptide bond.[3,4] In order to circumvent these problems endolytic chemical cleavage is an attractive proposition. However, relatively few methods exist and by far the most common is the use of cyanogen bromide[5,6] for cleavage at the carboxyl side of methionine. This mode of cleavage is well established and relies upon attack by the sulphur atom in the thio-ether linkage of methionin on cyanogen bromide. This reaction leads to the formation of homoserine iminolactone with the expulsion of methylthiocyanate. Hydrolysis of the iminolactone under acidic conditions (either 0.1M HCl[5,6] or 70% aqueous formic acid[7]) then yields C-terminal homoserine lactone in place of the original methionyl peptide bond. As well as being used in semisynthesis[1], cleavage by cyanogen bromide has also recently been used in genetic engineering to excise synthetic proteins after ribosomal synthesis.[8]

Very few reliable methods of endolytic chemical cleavage exist, although the use of 2-(2-nitrophenylsulphenyl)-3-methyl-3-bromoindolenine (BPNS)-skatol[9,10] and DMSO/Conc. aqueous HBr[11] allow cleavage at a tryptophan residue, although this cleavage does not appear to be as satisfactory as that employing cyanogen bromide to cleave methionyl peptide bonds. A number of other chemical methods have been developed and have been reviewed by Spande *et al.*,[12] but few of these have been used to any great extent.

In recent work on the semisynthesis of hen egg white lysozyme analogues, we envisaged the use of the methoxycarbonyl sulphenyl protecting group[13] for the protection of the thiol functions of reduced hen egg white lysozyme. In the proposed scheme the thiols of reduced lysozyme would be

† The nomenclature and abbreviations used conform to the 1983 recommendations of IUPAC/IUB Joint Commission on Biochemical Nomenclature (JCBN).

protected with this group and then digested at the two methionine residues at positions 12 and 105, with cyanogen bromide according to the standard method.[6,7] During preliminary studies it was found that treatment of hen egg white lysozyme which had been reduced with dithiothreitol with methoxycarbonyl sulphenyl chloride[13] not only gave protection of the sulphydryl groups in the expected manner, but also gave chain cleavage at one or both of the methionine residues. This prompted us to investigate cleavage of methionyl peptide bonds with methoxycarbonyl sulphenyl chloride (Scm chloride) and o-nitrophenylsulphenyl chloride (Nps chloride). Both these reagents are used for other applications in peptide synthesis, the Scm chloride being used as mentioned above for protection of sulphydryl functions[13] and Nps chloride being used for the masking of amino groups.[14]

The tripeptide H.Gly.Met.Ala.OH trifluoroacetate was prepared as a model peptide which could be used to study the effect of Scm and Nps chlorides on methionyl peptides. The peptide was prepared by conventional solution methods,[15] Boc-methionine was coupled to the p-toluene sulphonate of alanine phenyl ester[16] using the DCCI/HOBt procedure.[17] The Boc group was removed by treatment with 5M HCl in ethyl acetate to give the corresponding dipeptide hydrochloride which was then coupled with Boc glycine again by the DCCI/HOBt method. The protected tripeptide was then subjected to phenyl ester cleavage,[16] being treated with sodium hydroxide at pH 10.5 in the presence of hydrogen peroxide. In this case dimethyl sulphide was used as a scavenger to prevent oxidation of methionine. The Boc group was then removed from the N-protected tripeptide by acidolysis with 90% trifluoroacetic acid in the presence of 2-mercaptoethanol and dimethyl sulphide.

Samples of the tripeptide were then treated with cyanogen bromide, methoxycarbonyl sulphenyl chloride[13] and o-nitrophenylsulphenyl chloride. In each case the tripeptide was dissolved in anhydrous formic acid and the digestion carried out for three hours. The reaction mixture was then diluted with water and stirred for a further 15 hours. The crude product was applied to Sephadex G10 eluting with 0.1M acetic acid. The product from the cyanogen bromide digestion showed two major peaks, both of which were positive to fluorescamine[18] indicating the presence of a free amino group in each component. The products contained in the two peaks from the gel filtration were then subjected to amino acid analysis. The first peak did not give an analysis for a single amino acid, but after conventional acid hydrolysis it was shown to contain glycine and homoserine (normally co-eluting with glutamic acid), the second peak was identified as alanine. These results confirmed that the anticipated cleavage of the tripeptide by cyanogen bromide had occurred, and that glycyl homoserine lactone and alanine had been produced. The digests with Scm chloride and Nps chloride were then examined in the same way and virtually identical results were obtained. In the case of Scm chloride some minor impurities were also present in the first fraction, but comparison by t.l.c. indicated that glycyl homoserine lactone and alanine were produced in all three cases. These results confirm that the two sulphenyl chlorides are able to cleave a peptide chain at methionine in the same way as cyanogen bromide.

In the second study digestion of the 1-32 sequence of human adrenocorticatropic hormone (ACTH) was examined. The amino terminal tetrapeptide sequence of this hormone is Ser.Tyr.Ser.Met, there being no other methionine residue present in the remainder of the sequence; therefore cleavage at methionine would result in the liberation of the tetrapeptide lactone Ser.Tyr.Ser.Hse and the remaining 35 residue fragment.

The 1-32 ACTH was then subjected to standard CNBr digestion, and the lyophilised product subjected to gel filtration on Sephadex G25. Two well resolved peaks were obtained and amino acid analysis indicated that the cleavage at methionine-4 had proceeded as anticipated.

Amino acid analysis of the tetrapeptide (see Figure 1) confirmed its identity as only serine, tyrosine and homoserine were present. As can be seen from Figure 1, the three methods of digestion all gave the tetrapeptide, but the amino acid ratios are rather variable due to the fact that serine, homoserine and to some extent tyrosine are all degraded during amino acid analysis. Analysis of the 5-32 fragment showed a trace of methionine after digestion with Scm chloride and Nps chloride, and thus it was assumed that the digest was not quite complete. These results, again demonstrate the fact that cleavage of the methionyl peptide bond can be achieved with the two sulphenyl chlorides.

Cleavage Agent	Ser	Tyr	Hse
CN.Br	1.86	1.25	0.75
Scm.Cl	1.98	1.08	0.92
Nps.Cl	2.13	1.00	1.34

FIGURE 1 Amino-acid analysis of (1-4) ACTH produced by cleavage at Met-4 in (1-32) ACTH.

Digestion of reduced hen egg white lysozyme was then studied, using the digestion of octasulphityl reduced hen egg white lysozyme[19] with cyanogen bromide as a control. In the control experiment the three fragments 1-12, 15-105 and 106-129 were produced, gel filtration allowed separation, and the identity of the fragments was confirmed by amino acid analysis. The amino acid ratios for the three fragments are shown in Figure 2. Treatment of reduced hen egg white

Fragment	1-12			13-105			106-129		
	A	B	C	A	B	C	A	B	C
Tyr		Nd	Nd	3	Nd	Nd	3	Nd	Nd
Lys	1	1.11	1.05	4	3.81	3.56	1	1.12	1.28
His				1	0.87	1.01			
Arg	1	1.00	0.93	6	5.73	6.42	4	3.59	3.64
Asp				18	16.55	14.98	3	2.91	2.95
Thr				6	5.18	5.87	1	0.95	0.80
Ser				10	7.07	7.82			
Pro				2	2.18	2.32			
Glu(Hse*)	1	1.23	1.16	3	3.78	3.60	1	1.13	1.09
Gly	1	1.12	1.08	9	8.70	8.35	2	1.99	2.04
Ala	3	2.92	2.98	6	6.92	6.77	3	3.08	2.92
½Cys	1	Nd	Nd	5	Nd	Nd	2	Nd	Nd
Val	1	0.98	0.93	3	3.11	3.16	2	1.94	1.97
Met	1	0.09	0.00	1	0.08	0.26			
Ile				5	4.45	4.66	1	0.89	1.17
Leu	1	0.99	1.01	6	5.82	6.06	1	1.12	0.91
Tyr				3	3.17	2.77			
Phe	1	0.94	0.81	2	2.01	1.89			

FIGURE 2 Amino-acid analyses of fragments after digestion of Hen Egg-White Lysozyme derivatives. A-Theoretical composition of fragment; B - CNBr digestion of octasulphityl lysozyme; C - Nps.Cl digestion of reduced lysozyme; Nd indicates residue not determined; Hse* - homoserine and glutamic acid co-elute.

lysozyme[20] with Nps chloride resulted in a similar trace on gel filtration using Sephadex G50, eluting with 70% aqueous formic acid, and once again three fragments were obtained. The amino acid ratios being shown for comparison against those obtained with cyanogen bromide in Figure 2.

Treatment of reduced hen egg white lysozyme[20] with Scm chloride resulted in a more complex situation and as will be shown in the following paper[21] only two fragments were obtained. The fragments being a bridged 1-12/106-129 fragment and the central 13-105 portion.

These results again confirm that Nps chloride and Scm chloride are able to cleave peptide chains containing methionine in the same way as cyanogen bromide.

Such a cleavage can be rationalised in a manner similar to that which is accepted for cleavage by cyanogen bromide. Thus, cleavage according to the Scheme shown in Figure 3 is initiated by attack of the sulphur in the thioether linkage of the methionine side chain on the electrophilic sulphenyl chloride. This gives a sulphonium salt which then cyclises to give the iminolactone with expulsion of a disulphide. Hydrolysis of the iminolactone in the second stage of the reaction then gives the homoserine lactone.

This new method of cleaving methionine containing peptides could presumably be extended by using other sulphenyl chlorides, although we have not investigaged this possibility. Caution

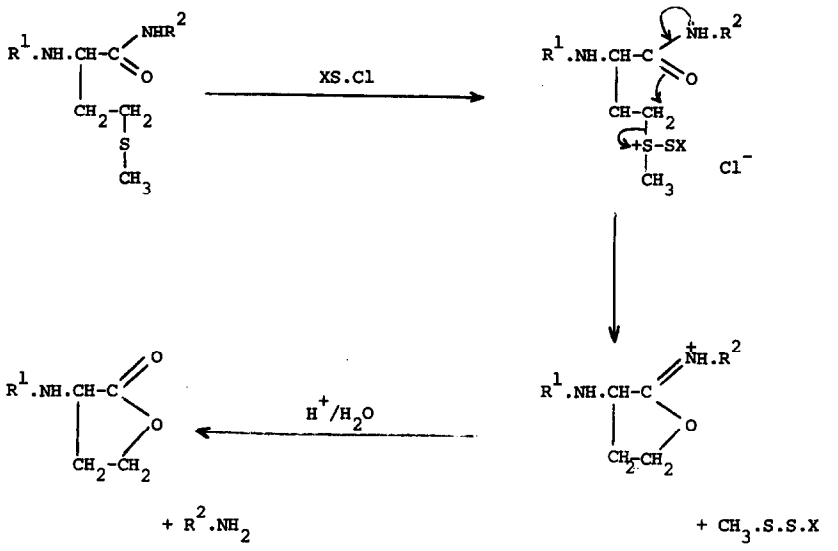

FIGURE 3. Mechanism of cleavage of methionyl peptide bonds; where X = methoxycarbonyl or o-nitrophenyl.

should however be exercised when the peptide contains tryptophan as there is the possibility of electrophilic attack on the indole ring system. In our studies UV evidence suggested that the aliphatic methoxycarbonyl sulphenyl chloride did not react with tryptophan[21] and other workers have found that tryptophan was undamaged by treatment with cyanogen bromide.[22] However, it is well established that the aromatic o-nitrophenylsulphenyl chloride can react with tryptophan,[23] and indeed, this reaction forms the basis of one of the methods of estimating the tryptophan content of peptides.

EXPERIMENTAL

Materials and general techniques

Hen egg-white lysozyme (crystallised, salt free), cyanogen bromide and sodium sulphite were obtained from Fluka AG, o-nitrophenylsulphenyl chloride and methoxycarbonylsulphenyl chloride was prepared[13] from trichloromethanesulphenyl chloride obtained from Aldrich chemicals, dithiothreitol from BDH chemicals, and 1-32 ACTH was a gift of Dr. D. Medzihradszky.

Melting points (uncorrected) were determined using a Gallenkamp melting point apparatus; optical rotations were measured on a Thorn-NPL automatic polarimeter type 243 and amino acid analyses were determined on a Jeol JLC 6AH amino-acid analyser. Samples for amino-acid analysis were dissolved in twice distilled, constant boiling (6M) hydrochloric acid (3 ml) and placed in an evacuated sealed tube at 110° for 18 - 24 hours. The tube was then opened and the acid evaporated *in vacuo*, the residue was then dissolved in pH 2.2 citrate buffer for amino-acid analysis. Ultraviolet spectra were determined on a Pye Unicam SP8-100, infrared spectra on a Unicam SP1000 and ^1H n.m.r. spectra on either a Perkin-Elmer R12 (60 MHz) or R34 (220 MHz). Thin layer chromatograms were run on Alugram SIL/UV254 plates and visualised by UV fluorescense (254 nm), fluorescence at long wavelength after spraying with fluorescamine or spraying with starch/KI after exposure to chlorine gas. Eluants were A - ^{n}BuOH/AcOH/H_2O, 3/1/1; B - $CHCl_3$/MeOH, 9/1.

Preparation of $CF_3.CO_2^-\ H_2^+$.Gly.Met.Ala.OH.

Boc.Met.Ala.OPh. Boc.Met.OH (3.9g, 16mM), $TosO^-\ H_2^+$.Ala.OPh (5.0g, 15 mM) and NMM (1.56 ml, 15 mM) were dissolved in DMF (50 ml) and the solution was cooled to 0°. DCCI (3.7g, 18 mM) and N-hydroxybenzotriazole (4.0g, 30 mM) were added and the reaction mixture stirred at room temperature overnight. A few drops of glacial acetic acid were added and the reaction mixture cooled to 0° for 2 hours. The resulting dicyclohexyl urea was filtered and the filtrate evaporated to give a residue which was dissolved in EtOAc. This solution was washed with 10% citric acid solution and saturated sodium bicarbonate, then dried over $MgSO_4$. Evaporation of the

solvent gave a residue which was crystallised from EtOAc giving the protected dipeptide 4.8g (81%), m.p. 85 - 86, R_f(A) 0.9, $[\alpha]_D^{20}$ - 66.7 (c = 1, MeOH), $Met_{0.98}$ $Ala_{1.00}$, (Found: C, 57.49; H, 7.19; N, 7.10; $C_{19}H_{28}N_2O_5S$ requires: C, 57.56; H, 7.12; N, 7.07%).

Boc.Gly.Met.Ala.OPh Boc.Met.Ala.OPh (4.3g, 11 mM), 2-mercaptoethanol (37.3 ml, 0.55 M) and dimethylsulphide were dissolved in a solution of HCl (5M) in EtOAc (6 ml) and the solution stirred for 1 hour. Excess Et_2O was then added to precipitate the dipeptide ester hydrochloride, filtration gave the crude product (2.5g, 78%) which was dissolved in dry DMF (50 ml). After cooling to 0° Boc.Gly.OH (1.56g, 8.9 mM), NMM (1.15 ml, 8.5 mM), hydroxybenzotriazole (3.0g, 18 mM) and DCCI (2.2g, 11 mM) were added and the reaction mixtured stirred for 15 hours. The reaction mixture was then cooled to 0° for 2h, and the precipitated dicyclohexyl urea removed by filtration. The filtrate was evaporated in vacuo and the residue dissolved in EtOAc; this solution was washed with 10% citric acid, saturated $NaHCO_3$ solution and water, then dried over $MgSO_4$. The solvent was evaporated and the residue crystallised from EtOAc/petroleum ether giving the required protected tripeptide, (2.6g, 67%), m.p. 130 - 131, R_f(B) 0.6, $[\alpha]_D^{20}$ - 56.3 (c = 1, MeOH), $Gly_{1.00}Met_{0.96}Ala_{1.00}$, (Found: C, 55.80; H, 7.11; N, 9.05; $C_{21}H_{31}N_3O_6S$ requires: C, 55.61; H, 6.84; N, 9.27%).

$CF_3.CO_2^-$ H_2^+.Gly.Met.Ala.OH The above protected tripeptide (0.4g, 0.8 mM) was dissolved in DMF/H_2O (3:2) (80 ml); dimethylsulphide (0.3 ml, 40 mM) and 100 ml hydrogen peroxide (0.8 ml) added and the pH of the solution adjusted to 10.5 by the addition of 1M NaOH. The pH was maintained at this value for 30 min., then brought to 4.0 with 2M HCl and the solution volume reduced to ca. 15 ml. A saturated solution of NaCl was added to precipitate the product and the resulting solid washed with water and dried in vacuo.

This material was dissolved in 90% trifluoroacetic acid (80 ml) in the presence of 2-mercaptoethanol (4.3 ml, 50 mM) and dimethylsulphide (3.7 ml, 50 mM). After 2 hours excess Et_2O was added and the resulting precipitate filtered and washed with further portions of Et_2O. The solid was dissolved in distilled water and freeze dried giving the required tripeptide hydrochloride (0.3g, 70%), m.p. 82.5 - 83.5, R_f(A) 0.7, $[\alpha]_D^{20}$ - 25.0 (c = 1, MeOH), $Gly_{1.00}Met_{0.94}Ala_{1.00}$.

Digestion of $CF_3.CO_2^-$ H_2^+.Gly.Met.Ala.OH.

The tripeptide hydrochloride (20 mg, 50 μM) was dissolved in 70% formic acid (20 ml) and cyanogen bromide (1.06g, 10 mM), MeO.CO.S.Cl (0.9 ml, 10 mM) or Nps.Cl (1.9g, 10 mM) added. The solution was stirred for 3 hours then distilled water (6 ml) was added and the solution stirred for 15 hours. The solvent was evaporated in vacuo and the residue dissolved in 70% formic acid (2 ml) and diluted with water (20 ml) prior to freeze drying. The products were then chromatographed on Sephadex G10 eluting with 0.1M acetic acid.

Digestion of (1-32) ACTH

(a) CNBr digest. (1-32) ACTH (0.02g, 5.5 μM) was dissolved in 70% formic acid (20 ml) and cyanogen bromide (117 mg, 1.1 mM) added. The solution was stirred for 3 hours then for a further 15 hours, after the addition of distilled water (6 ml). The same procedure as in the digestion of the above tripeptide hydrochloride was then adopted, followed by gel filtration on Sephadex G25 eluting with 0.1M acetic acid.

(b) Sulphenyl chloride digests. A similar procedure to that described in (a) was used except that the solvent was anhydrous formic acid (14 ml); MeO.CO.S.Cl (140 mg, 1.1 mM) or Nps.Cl (209 mg, 1.1 mM) were used in these digestions.

Cyanogen bromide digestion of octasulphityl hen egg-white lysozyme

Octasulphityl hen egg-white lysozyme[19] (100 mg, 6.99 μM) was dissolved in 70% aqueous formic acid (10 ml) and cyanogen bromide (37 mg, 349 μM) added. The reaction was stirred for 15 hours then diluted by the addition of distilled water (100 ml). This solution was freeze dried and the resulting solid subjected to gel filtration on Sephadex G50 eluting with 70% aqueous formic acid.

o-Nitrophenylsulphenyl chloride (Nps.Cl) digestion of reduced hen egg-white lysozyme.

Reduced Hen egg-white Lysozyme[20] (100 mg, 6.95 μM) was dissolved in anhydrous formic acid (20 ml). Nps.Cl (530 mg, 2.8 mM) was added and the solution stirred for 3 hours. Distilled water (8.5 ml) was added and the reaction mixture stirred for a further 15 hours then this solution was frozen and freeze dried. The resulting solid was subjected to gel filtration on Sephadex G50 eluting with 70% aqueous formic acid.

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