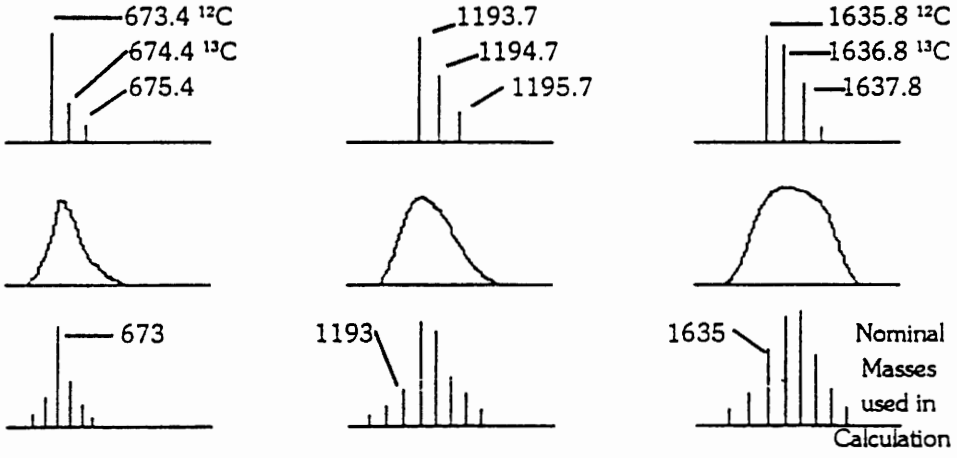
Strategy for the Interpretation of Peptide CID Spectra

The following general strategy will be illustrated with CID spectra (#2A and 2B) of the model peptide, GXDXQK (X refers to either Leu or Ile). Nominal masses (monoisotopic, ^{12}C, masses rounded to the nearest whole number) for the predicted fragment ions of type b and y from this peptide (free acid and methyl ester) are shown above and below the structure, repectively.

58	171	300	413	541	701	Type b (COOMe)
58	171	286	399	527	673	Type b (COOH)
Gly	Lxx	Asp	Lxx	Gln	Lys	
673	616	503	388	275	147	Type y (COOH)
701	644	531	402	289	161	Type y (COOMe)

Note that all spectra have been generated on a triple quadrupole instrument operating at less than unit resolution. Accordingly the observed signals are often as many as 6-8 mass units wide. Below are shown the predicted isotope patterns for three peptides containing 6, 10, and 14 amino acids, respectively. The actual peak shape recorded on the triple quadrupole instrument is shown on line 2 of the diagram. The third line below shows the experimentally observed pattern after the computer has summed the total signal intensity observed in each 1 mass window within the unresolved multiplet.



Note that at m/z greater than 800 the signal due to the monoisotopic mass of the ion is the most abundant in the cluster and has a value that is essentially identical to that of the rounded nominal mass. At m/z 1100 or so, the monoistopic mass remains the most abundant but is now about one mass unit higher than the rounded nominal mass. Near mass 1600, signal for the ^{13}C isotope becomes approximately equal to that of the ^{12}C isotope and the nominal mass occurs at a value 1 mass unit below the first of the two large peaks. For the purpose of calculating mass values for b and y type ions we will almost always choose the signal on the low mass side of the most abundant signals in a particular cluster. For ions below mass 7-800, we will select the most abundant ion as being that characteristic of the rounded nominal mass value.

1) Measure the mass of the $(M+H)^+$ ions at unit resolution of both the peptide and the corresponding methyl ester. The mass shift observed for $(M+H)^+$ ion on conversion of the peptide to the corresponding methyl ester should be a multiple (n) of 14 Da, where (n-1) is the number of acidic residues (Asp, Glu, and Cmc) in the molecule (assuming that the C-terminus of the peptide is not blocked).

   In the above example, the $(M+H)^+$ ion shifts from 673 to 701 (28 mass units) n-1 = 1, therefore, there is a single acidic residue in the molecule.

   Caution! Under the conditions employed for converting peptides to the corresponding methyl esters, Gln and Asn residues can also esterify. Here the shift is 15 mass units, -CONH2 ——> -COOCH3.

2) Acetylate the peptide or its methyl ester on the solids probe and remeasure the mass of the $(M+H)^+$ ion. The mass shift observed for the $(M+H)^+$ ion upon acetylation should be a multiple (n) of 42 Da, where (n-1) = the number of Lys residues in the peptide (assuming that the amino terminus of the peptide is not blocked). If no mass shift is observed, the amino terminus of the peptide must be blocked. Lys and Gln, two residues having the same mass, are differentiated by the above procedure. Only Lys suffers acetylation.

   In the above example, the $(M+H)^+$ ion shifts from 673 to 757, (84 mass units). n -1 = 1, therefore, there is a single Lys residue in the molecule.

   Caution! In the absence of base catalysis, acetylation usually occurs only on the N-terminus of the peptide and on the side chain of Lys. Cys will also be acetylated if it has not already been carboxymethylated. If the acetylation reaction is catalyzed by base, phenolic residues (Tyr) and the alcohol side chains (Thr and Ser) can also be acetylated. Partial formation of methyl esters can also result if the peptide is treated with methanolic/acetic anhydride for a prolonged period of time (15-60 min).

3) If the peptides being analyzed are generated in a tryptic digest, look at the low mass end of the spectrum for the ion of type y_1 that is characterisic of the expected C-terminal residues, Lys or Arg. Lys affords a y ion at m/z 147 in the spectrum of the free acid (COOH) and at m/z 161 in the spectrum of the methyl ester (COOMe). The corresponding y_1 ions for arginine occur at m/z 175 (COOH) and 189 (COOMe), respectively. These ions lose ammonia readily from the side chain to produce fragments at m/z 158 (COOH) and 172 (COOMe), respectively. In most cases, the m/e 175 ion will be more abundant in the COOH spectrum and the 172 ion will be more abundant in the COOMe spectrum.

Spectra 2A and 2B contain ions at m/z 147 and 161, respectively. Accordingly, Lys is assigned as the C-terminal residue and the following partial structure is generated.

	701	Type b (COOMe)
	673	Type b (COOH)
	Lys	
673	147	Type y (COOH)
701	161	Type y (COOMe)

4) Examine the low mass end of the spectrum for fragment ions having the formula, $+NH_2=CHR$. Ions of this type are characteristic of the amino acid composition of the peptide but are not observed for all amino acids. The following fragment ion masses in the spectrum of either the free acid or the methyl ester are particularly diagnostic:

70 = Pro (Arg, Asn)	120 = Phe
72 = Ala	134 = Cmc
86 = Lxx	136 = Tyr
104 = Met	159 = Trp
110 = His	

In the present example only m/z 86 characteristic of Lxx is observed.

5) Label signals in the methyl ester spectrum with whole numbers (+0, +1, +2, etc.) to indicate how many methyl groups were incorporated into each fragment as a result of the esterification process. Ions of type y contain the C-terminus of the peptide and thus should all shift to higher mass by at least 14 mass units (more if the fragment contains an acidic residue such as Asp, Glu or CmCys). Ions of type b contain the N-terminus of the peptide and should not shift to higher mass in the ethyl ester spectrum, unless the fragment also contains an acidic residue such as Asp, Glu, or CmCys.

Spectrum 2B is labeled for the present example.

6) Proceed to the high mass end of the spectrum and look for the fragment ion of type b, formed by loss of the C-terminal residue. If the C-terminal residue is Lys, then the highest ion of type b will be observed at $(M+H)^+$ - (H2O) - 128 (the residue mass for Lys, see Table 1). In the methyl ester spectrum, the b ion will appear at $(M+H)^+$ - 32 (MeOH) - 128.

If the C-terminal residue is Arg, the corresponding b ions will be observed at $(M-H)^+$ - 18 -156 and $(M+H)^+$ - 32 -156.

If the C-terminal residue is not known, the highest mass ion of type b will be found by using the formula $(M+H)^+$ - 18(H_2O) - X, where X = each of the twenty residue masses in Table 1. In the methyl ester spectrum this ion appears at m/z = $(M+H)^+$ - 32 - X. Note that highest mass ion of type b contains at least one less COOH group than the (M+H) ion (two less COOH groups

if the C-terminal residue has an acidic side chain, Asp, Glu, Cmc). According to the labeling scheme discussed in step #5 above, the highest mass ion of type b will be labeled with a whole number that is at least one less than the label on the $(M+H)^+$ ion.

In the present example the C-terminal residue is Lys, and the high mass b ion is calculated to occur at 673 - 18 - 128 = 527 (COOH) and 701 - 32 - 128 = 541. The following partial structure results.

	541	701	Type b (COOMe)
	527	673	Type b (COOH)
		Lys	
673		147	Type y (COO)
701		161	Type y (COOMe)

Note !!! (important). Once you know the mass of any type b ion, the mass of the corresponding type y ion (formed by cleavage of the same amide bond) is obtained by subtracting the mass of the b ion from the $(M+H)^+$ ion and adding 1 ($y = m/z$ $(M+H)^+ - b + 1$). Look to see if the spectrum contains a signal for the predicted type y ion each time you locate a type b ion.

Note that loss of CO from ions of type-b is quite common. The resulting species is referred to as a type-a ion. Look for this ion 28 mass units lower than the corresponding b-ion and label it as an ion of type-a. If you find two ions separated in mass by 28 units in a spectrum, they usually belong to an a,b pair.

$$\underset{b}{NH_2CH(R)CO^+} \longrightarrow \underset{a}{^+NH_2{=}CH(R)} + CO$$

Type a and type b ions are often accompanied by other fragments that result from the loss of water and/or ammonia. Look to see if these other fragments exist and label them with an o (water) and * (ammonia). Loss of water occurs in fragments that contain the amino acids Ser, Thr and Glu (only if the latter residue is at the N-terminus of the fragment). Loss of ammonia occurs from fragments that contain the amino acids Arg, Lys, Gln, and Asn.

7) Examine the high mass end of the spectrum and locate the signal corresponding to the fragment of type y formed by elimination of the N-terminal amino acid. The m/z value of this ion will be equal to that of the $(M+H)^+$ ion minus one of 21 possible residue masses in Table 1. Since the smallest residue mass is 57 units (Gly) and the largest is 186 (Trp), the ion in question will be found in a window 57-186 mass units below the $(M+H)^+$ ion. Note that in the methyl ester spectrum this ion and the $(M+H)^+$ ion should be shifted to higher mass by the same increment and thus be labeled with the same whole number, unless the N-terminal amino acid is either Asp, Glu, or Cmc.

Note !!! (important). Once you know the mass of any type y ion, the mass of the corresponding type b ion (formed by cleavage of the same amide bond) is obtained by subtracting the mass of the y ion from the $(M+H)^+$ ion and adding 1 ($b = m/z\ (MH)^+ - y + 1$). Look to see if the spectrum contains a signal for the predicted type b ion each time you locate a type y ion.

Type y ions are often accompanied by other fragments that result from the loss of water or ammonia. Look to see if these other fragments exist and label them with an ° (water) and * (ammonia). Loss of water from type y ions occurs in fragments that contain the amino acids Ser, The and Glu (only if the latter is at the N-terminus of the fragent). Loss of ammonia occurs from fragments that contain the amino acids Arg. Lys, Gln and Asn.

Caution! Note that all peptide CID spectra contain a series of abundant fragment ions within 60 amu of the $(M+H)^+$ ion. These appear to result from the loss of multiple units of water, ammonia, plus 45-46 and 59-60 mass units (HCOOH and CH_3COOH, ?). Signals due to the loss of the smallest amino acid, Gly (57 units) fall within this 60 mass unit window. Accordingly one must always check to see if there is a signal 57 units below the $(M+H)^+$ ion. Note also that several combinations of two residue masses add up to the same mass as a single larger residue . Gly-Gly, for example, has the same residue mass (114) as Asn. Be sure to select the signal closest to the $(M+H)^+$ ion that fits for the loss of a residue mass.

Gly-Gly	114 = Asn	AcGly	99 = Val
Gly-Ala	128 = Gln or Lys	AcAla	118 = Leu/Ile
Val-Gly	156 = Arg	AcSer	129 = Glu
Gly-Glu	186 = Trp	AcAsn	156 = Arg
Ala-Asp	186 = Trp		
Ser-Val	186 = Trp		

In the present example the signals at m/z 616 (COOH) and 644 (COOMe) fit for the loss of Gly. The following partial structure is generated.

58		541	701	Type b (COOMe)
58		527	673	Type b (COOH)
Gly			Lys	
673	616		147	Type y (COOH)
701	644		161	Type y (COOMe)

8) If the low mass end of the spectrum contains ions characteristic of Pro (m/z 70) or His (m/z 110), examine the spectrum for fragments that result from internal cleavage at these residues. Often the most abundant signal in the spectrum will correspond to the ion of type y that contains elther of these two residues at

the N-terminus. Since the structure of this ion is the same as that for the $(M+H)^+$ ion for a shortened peptide containing either Pro or His at the N-terminus, the observed type y ion can undergo further fragmentation to produce a series of type b ions characteristic of this shortened peptide. A search for these ions is often the quickest way to solve the structure of an unknown peptide.

In renin tetradecapeptide, DRVYIHPFHLLVYS, the most abundant ions in the CID spectrum correspond to ions of type b derived from the y ion of sequence PFHLLVYS. PFVH, PFHLL, PFHLLV, PFHLLVY, and PFHLLVYS are all observed.

None of these types of ions are found in the present example.

9) To continue the sequence analysis, search the spectrum for additional ions of type b or type y. Start at the high mass end of the spectrum and work backwards since the number of signals in that region is generally smaller than that found at low mass. Additional ions of type b or y are found by subtracting each of the twenty one residue masses in Table 1 from the m/z value of an existing b or y ion until a new signal is encountered. Note that the signal corresponding to the next lower member of a given series will be labeled with the same whole number from step #5 above, unless the residue lost contains a carboxylic acid (Asp, Glu, Cmc).

The following points should be noted:

a) A unique b_1 ion will not be observed. The only way to determine the order of the first two amino acids in the chain is to find the appropriate ion of type y, acetylate the amino terminus and look for the b_1 ion that has increased in mass by 42 units, or perform manual Edman degradation on the sample and measure the mass of the $(M+H)^+$ that results from the peptide shortened by one residue.

b) The signal intensity for ions of type b drops dramatically when the next amino acid in the chain is either Pro, Gly or His. Lys and Arg also cause this phenomenon.

c) When a type y or b ion is formed by cleaving an amide bond before or after the residue, Arg, it is not uncommon for the y^* and b^* ions to be much more abundant than the b or y ions themselves.

d) Quite often one will observe the situation where a particular series or b type ions disappears and the corresponding ions of type y become much more abundant. The opposite situation occurs just as frequently. This is usually the case when one encounters either Pro, or a basic residue such as His, Lys or Arg in the middle of the peptide chain.

To determne the identity of the second amino acid in the chain, we search for the second highest mass ion of type y. This can be found either by subtracting all of the residue mass values in Table 1 from the previously identified y ion at m/z 616, or by simply looking for another signal at lower mass that contains the same number of methyl groups as m/z 616. The signal at m/z 503 indicates that a mass of 113 has been lost. Accordingly the Lxx is placed in the structure at position two. The mass of the b_2 ion can now be calculated as 58 + 113 = 171. The existence of this ion in the spectrum provides additional support for the structure.

```
58  171                 541  701  Type b (COOMe)
58  171                 527  673  Type b (COOH)

Gly  Lxx                     Lys

673  616  503                147  Type y (COOH)
701  644  531                161  Type y (COOMe)
```

Since none of the remaining ions in the spectrum have the same number of methyl groups as that of the $(M+H)^+$ ion, we conclude that either the next lower ion of type y is missing or it is formed by loss of a residue containing a carboxylic acid group, Asp, Glu or Cmc. When we subtract the residue mass for these three amino acids from m/z 503, we find that m/z 388 fits for the loss of Asp. This becomes the third residue in the chain and the corresponding ion of type b is calculated, 171 + 115 = 286. This ion appears in the spectrum.

```
58  171  300          541  701  Type b (COOMe)
58  171  286          527  673  Type b (COOH)

Gly  Lxx  Asp               Lys

673  616  503  388          147  Type y (COOH)
701  644  531  402          161  Type y (COOMe)
```

The fourth residue in the chain is deduced from the mass separation beteen the signals at m/z 388 and 275, 113 = Lxx.

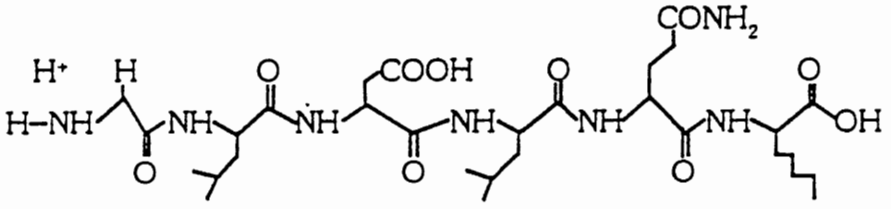
```
58   171  300  413  541  701  Type b (COOMe)
58   171  286  399  527  673  Type b (COOH)

Gly  Lxx  Asp  Lxx       Lys

673  616  503  388  275  147  Type y (COOH)
701  644  531  402  289  161  Type y (COOMe)
```

The mass separation between 275 and 147 indicates that the missing amino acid has a residue mass of 128. The molecule is acetylated twice, there can only be one Lys in the molecule and it has already been located. Residue 5 is thus assigned as Gln.

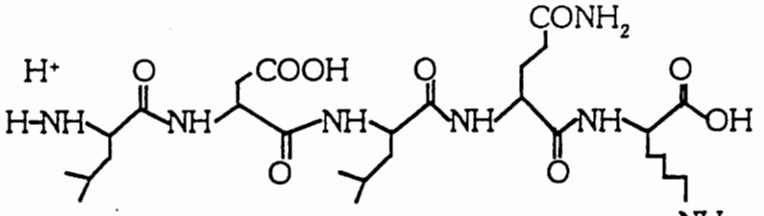
58	171	300	413	541	701	Type b (COOMe)
58	171	286	399	527	673	Type b (COOH)
Gly	Lxx	Asp	Lxx	Gln	Lys	
673	616	503	388	275	147	Type y (COOH)
701	644	531	402	289	161	Type y (COOMe)

Fragments of Type y

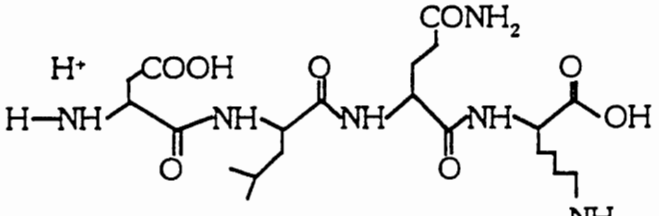
Gly	Leu	Asp	Leu	Gln	Lys
673	616	503	388	275	147
673.5	616.3	503.2	388.2	275.2	147.1



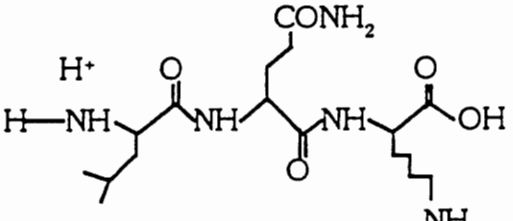
$(M+H)^+ = 673$



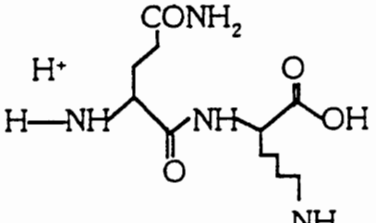
$y_5 = 616$



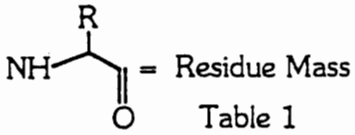
$y_4 = 503$



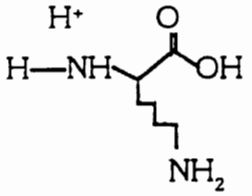
$y_3 = 388$



$y_2 = 275$



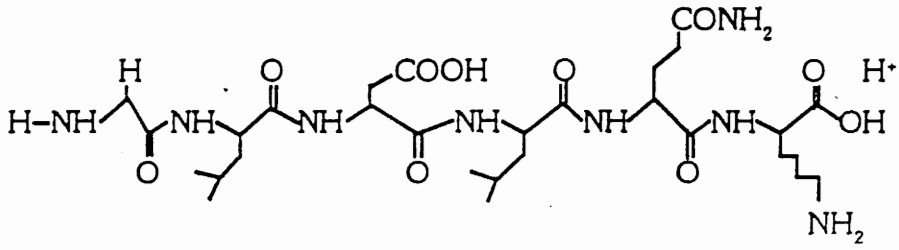
= Residue Mass Table 1



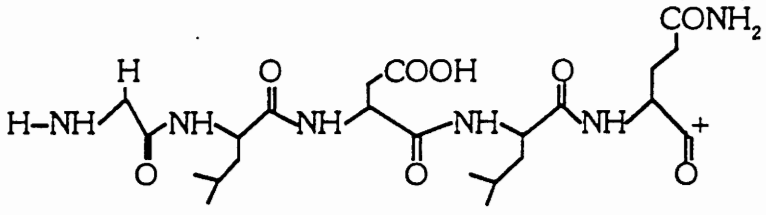
$y_1 = 147$

Fragments of Type b

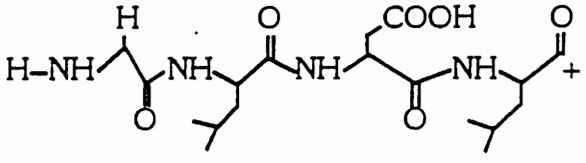
58.0	171.1	286.1	399.2	527.3	673.4
58	171	286	399	527	673
Gly	Leu	Asp	Leu	Gln	Lys



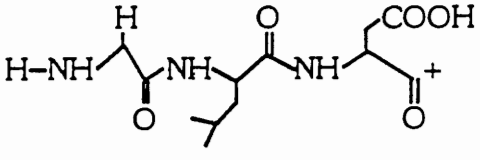
$(M+H)^+ = 673$



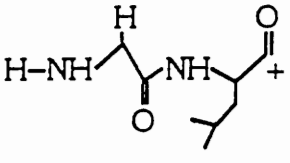
$b_5 = 527$



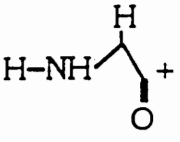
$b_4 = 399$

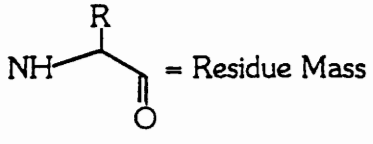


$b_3 = 286$



$b_2 = 171$





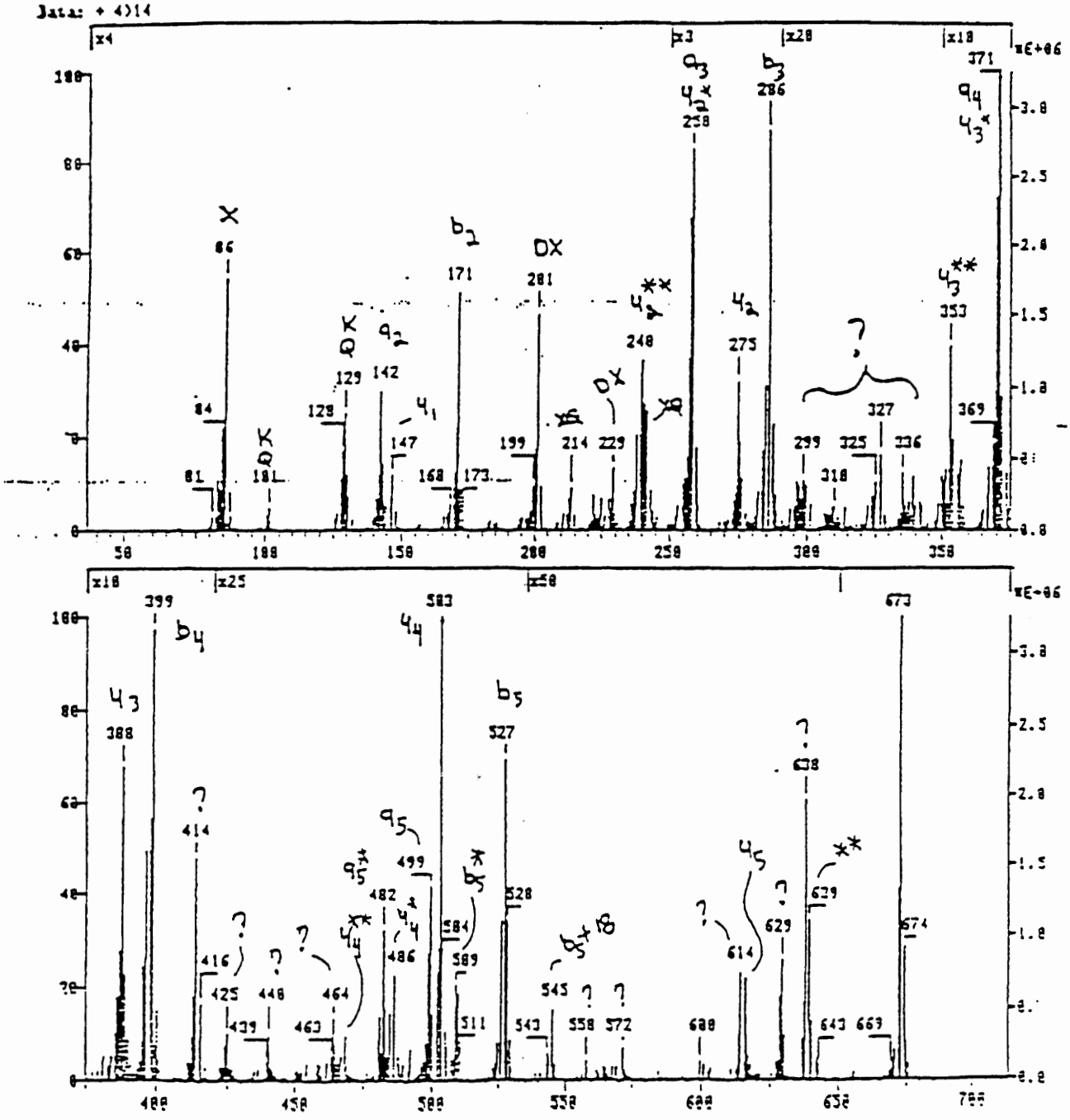
= Residue Mass

Table 1

$b_1 = 58$

Tryptic Peptide
CAD 673.4 ⇌ 3A
Acetylation ⇌
673.4 → 757.5

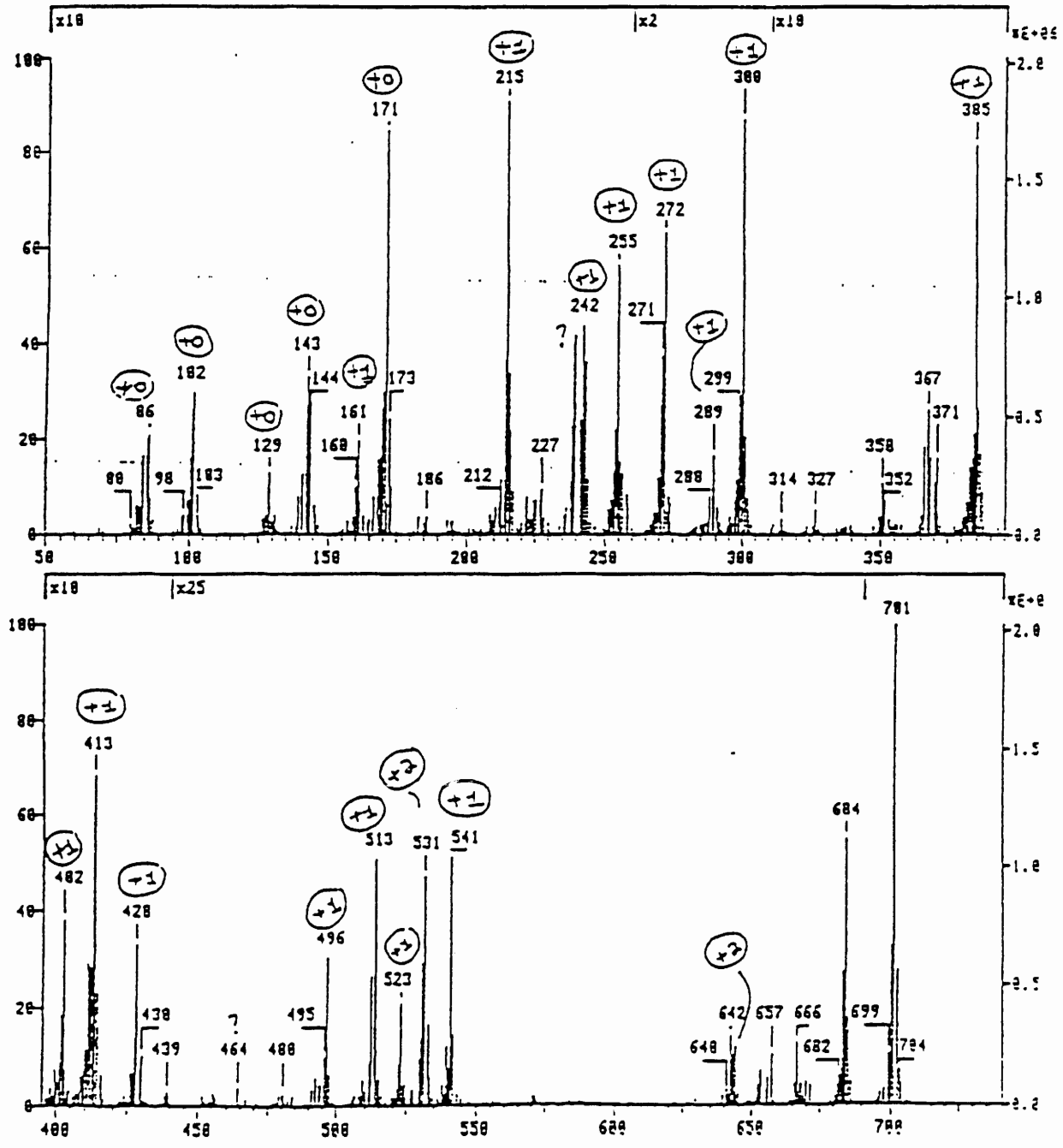
SPEC: DFH58 ver 12 on UIC 4 1 16-NOV-89 DERIVED SPECTRUM 9
Samp: BLG, TRYPTIC DIGEST Start : 16:43:48 16
Cmnt: 03 CAD 673
EI +DAUGHTERS OF 673.8 @ -15eV HMR GAS UP LR
Oper: JS Inlet :
Base: 673.1 Inten : 3244798 Masses: 50) 700
Norm: 673.1 RIC : 26884634 0 peaks: 702
Peak: 1888.88 amu
Data: + 4)14



Spectrum 2A - Tryptic Peptide CID 673.4 Acetylation 673.4 —> 757.5

Methyl Ester
MH+ = 701.4
673.4 → 701.4
#2B

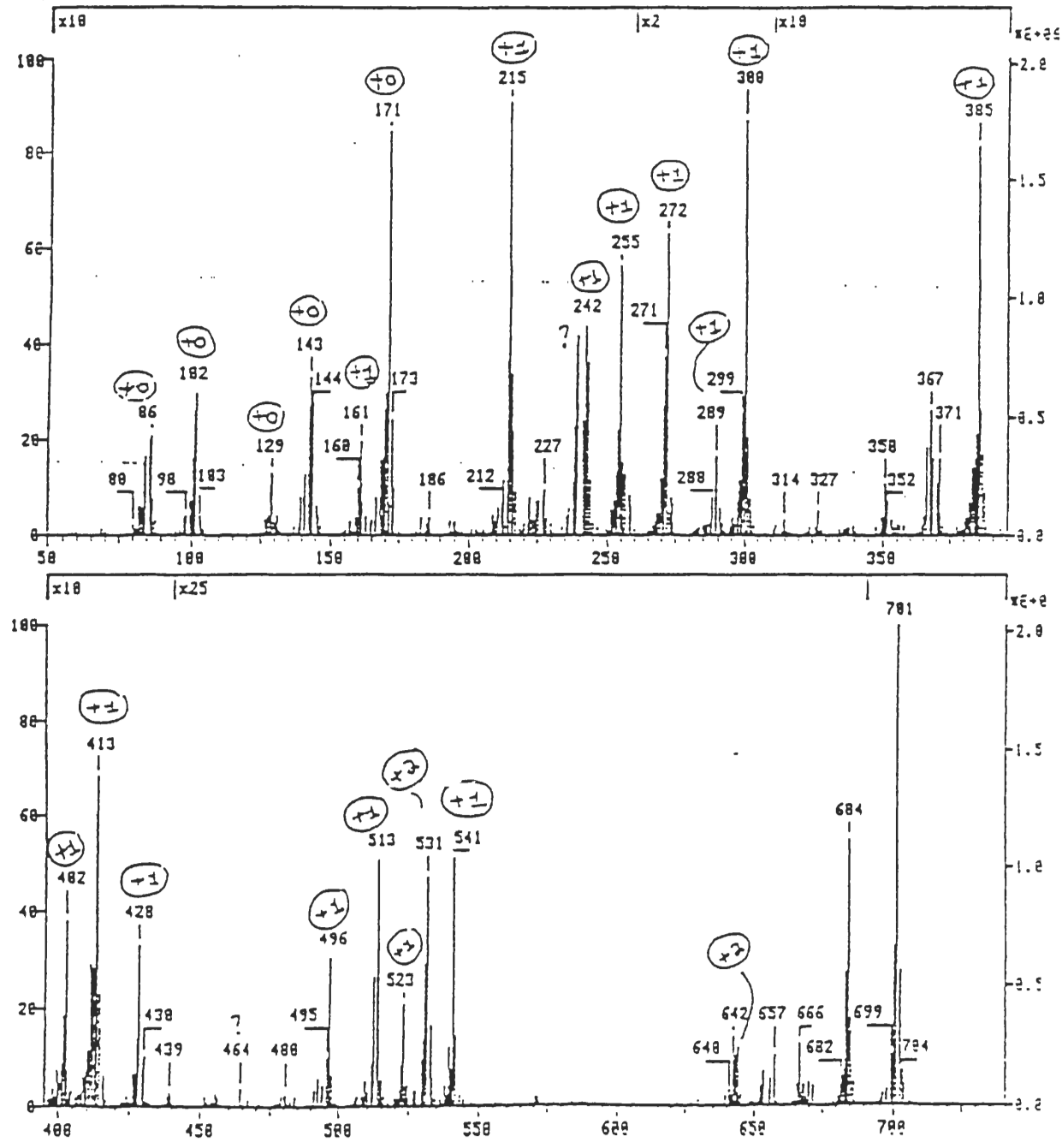
SPEC: DFHS2 ver 1 on UIC 4 1 20-NOV-89 DERIVED SPECTRUM 9
Samp: BLG-TRYPTIC DIGEST-OME Start : 01:38:27 15
Comm: 83 CAD 701
Mode: EI +DAUGHTERS OF 701.0 @ -15eV HMR GAS UP LR
Oper: JS Inlet :
Base: 700.9 Inten : 2828832 Masses: 50 > 750
Norm: 700.9 RIC : 14196592 0 peaks: 672
Peak: 1000.00 amu
Data: + S>15



Spectrum 2B - Methyl Ester MH^+ = 701.4 673.4 —> 701.4

Methyl Ester
MH+ = 701.4
673.4 → 701.4
#2B

```
SPEC: DFHS2 ver 1 on UIC 4 1          20-NOV-89 DERIVED SPECTRUM     9
Samp: DLG-TRYPTIC DIGEST-OME                    Start : 01:38:27    15
Comm: #3 CAD 701
Mode: EI +DAUGHTERS OF 701.0 @ -15eV HMR GAS UP LR
Oper: JS                                        Inlet :
Base: 700.9              Inten : 2828832        Masses: 50 ) 750
Norm: 700.9              RIC   : 14196592       # peaks: 672
Peak: 1000.00 amu
Data: + 5)15
```



Spectrum 2B - Methyl Ester MH^+ = 701.4 673.4 —> 701.4

Chemical and Enzymatic Methods for Peptide and Protein Sequence Analysis

Formation of Peptide Methyl Esters

A standard solution of 2 N HCl in methanol is prepared by adding 800 ul of acetyl chloride dropwise with stirring to 5 ml of methanol. After the solution has stood at room temperature for 5 min, 100 uL aliquots of the reagent are added to lyophilized HPLC fractions. Esterification is allowed to proceed for 2 hr at room temperature, and the solvent is then removed by lyophilization of the solution.

N-Acetylation of Peptides

Acetylation of peptides directly on the instrument probe is performed by exposing the sample in 0.5 - 1.0 ul of monothioglycerol to 0.5 ul of 3:1 methanol:acetic anhydride for a period of 1-3 minutes. Complete acetylation of Lys residues often requires addition of either 0.5 uL of 50 mmol ammonium bicarbonate or 0.5 ul of pyridine to the acetylation mixture. Prolonged acetylation of samples under basic conditions will often acetylate Tyr, Thr and Ser residues. Prolonged acetylation under acidic conditions results in partial conversion of the peptide to the corresponding methyl ester. Detection limits for peptides lacking an Arg residue will decrease by a factor of 5-10 following acetylation. Acetic anhydride-d_6 is frequently used when the peptide is suspected to contain an acetyl blocking group at the N-terminus.

Manual Edman Degradation

Peptides are placed in polypropylene tubes and dissolved in 15 ul of 50% aqueous pyridine. To this solution is added 15 ul of a freshly prepared 5% solution of phenylisothiocyanate in pyridine. The resulting solution is allowed to stand at 37 C for 5 min and then extracted twice with 100 ul portions of 2:1 heptane:ethyl acetate. Following centrifugation of the mixture, the organic layer was removed, and the aqueous layer was lyophilized. The resulting residue was taken up in 25 ul of anhydrous TFA and heated at 37 C for 5 min. TFA was removed by lyophilization and the shortened peptide is then dissolved in 25 uL of distilled water. The resulting solution is extracted twice with 100 ul of n-butyl acetate and the aqueous layer is then lyophilized. The resultant peptide is redissolved in 0.1% TFA for analysis by mass spectrometry. Note that reaction of the E-amino group of lysine and the -SH group of Cys with phenylisothiocyanate will shift the $(M+H)^+$ ion to higher mass by 135 Da.

Performic Acid Oxidation of Met and Cys

Standard reagent is prepared by mixing 85 ul of 100% formic acid with 15 ul of 30% hydrogen peroxide. The mixture is allowed to stand at room temperature for 15 min. On-probe oxidation of Met and Cys residues is conducted by adding 1 ul of the above solution to peptide in 0.5 uL of thioglycerol and allowing the mixture to stand for 30 sec before inserting the probe into the mass spectrometer. The above procedure converts Met to methionine sulfoxide and sulfone. Disulfide bonds are converted to cysteic acid -SO_3H. Trp residues also suffer partial oxidation under the above conditions

Reductive Carboxymethylation and Pyridylethylation

Protein is dissolved in degassed 100 mM Tris-HCl, pH 8.5, to a final concentration of 10 mg/ml. Dissolved oxygen is removed from the Tris buffer by vacuum filtration through a 4.5 uM aqueous filter and by stirring the water vigorously under vacuum for at least 10 min. Removal of dissolved oxygen is necessary to prevent oxidation of sulfur. Disulfide bonds are cleaved by the addition of dithiothreitol (100 nmol/ul), a 10 fold molar excess over disulfide bonds, to the protein. This mixture is allowed to stand at 37 deg C under N_2 for 1 hour. The reduced protein is S-carboxymethylated with iodoacetic acid (500 nmol/ul) or converted to the S-ethylpyridine derivative with 4 vinylpyridine. This is accomplished by the addition of alkylating agent at a 50 molar excess over disulfide bonds. Both reaction mixtures are allowed to stand under nitrogen in the dark at 37 C for l h and then lyophilized.

Proteolytic and Chemical Cleavage Reactions

All cleavage reactions are terminated by lyophilization of the reaction mixtures. Proteolysis with either trypsin and endoprotease Lys-C is carried out by treating the protein (1-2 nmol) in 100 ul of l00 mmol Tris-Cl, pH 8.5, with protease (a 1-2% w/w enzyme/protein ratio) for 4-8 hours at 37 deg C. Cleavage with endoproteinase Asp-N is accomplished by treating 1-2 nmol of protein dissolved in 100 ul of sodium phosphate buffer, 25 uM, pH 8.0, ith 0.2 ug of enzyme for 6-8 hours at 37 deg C. Cleavage with chymotrypsin and pancreatic elastase (@ 1 ug) is carried out in ammonium bicarbonate buffer, 50 mM pH 8.6, for 4-6 h at 37deg C.

Chemical cleavage with cyanogen bromide is performed by the addition of 300 ug of CNBr to 150 uL of 70% TFA (1-2 nmol of protein) that is 100 mM in L-tryptophan. The reaction is allowed to proceed in the dark at room temperature for 12 hour under nitrogen.

Location of Disulfide Bonds

A typical experiment involves digestion of the native protein with trypsin for 24 hours in 100 mM Tris-HCl at pH 7.5 in the presence of 5 mM iodoacetic acid. Addition of the 5 mM iodoacetic acid scavenges trace quantities of free -SH groups and thus prevents scrambling of intact disulfide bonds. The resultant oligopeptide mixture is then fractionated by HPLC. Samples at the 5-20 pmol level are then subjected to molecular weight analysis on the mass spectrometer. After each mass spectrum is recorded, the sample is withdrawn from the instrument and 0.5 ul of ammonium hydroxide is added directly to the probe. In the presence of excess base and thioglycerol, reduction of all disulfide linkages occurs in a few seconds. Following acidification, the sample is then inserted back into the mass spectrometer and additional spectra are recorded. Newly produced $(M+H)^+$ ions, those containing the reduced form of Cys, whose masses sum to a value, 3 Da higher than that of an $(M+H)^+$ ion in the first mass spectrum, are identified as being part of a disulfide linkage in the native protein.