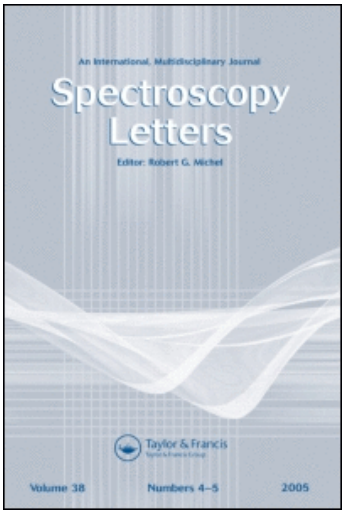

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FAB of peptides study: Deuterated glycerol matrix and selectively deuterated peptides. Part II

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FAB of peptides study : deuterated glycerol matrix and selectively deuterated peptides. Part II*

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ABSTRACT

Two peptides, H-Gly-Lys-Thr-Gly-Pro-Gly-OH (1) and Leu-enkephalin (2) FAB-PI spectra in normal and deuterated glycerol have been studied. Two C-deuterated and D_2O exchanged analogs have been used to compare their fragmentation to unlabelled peptides.

The mass spectrometry of peptides has become a particularly interesting field with the wide spread adoption of the FAB technique. This technique enables one to record the quasi-molecular ion (in both positive and negative modes as M+1 or M-1 respectively) and to extract from the spectrum valuable information on the peptide sequence.

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* Part I, see reference 2

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In the positive mode peptides produce an intense M+1 ion. The origin of the hydrogen added to the molecular mass is not known and may be related to :

i) the matrix e.g. glycerol-OH hydrogen transfer to the peptide ;

ii) autoprotonation under FAB conditions

In 1983, McCloskey [1, 6] recorded a spectrum of tetradecapeptide in deuterated glycerol (Fig. 1). The complex ion cluster in the M^+ region shows an intense $M+2^+$ ion. The use of deuterated glycerol and nondeuterated peptide did not determine the mechanism of formation of the quasimolecular ion. Similarly in 1983, Klöppel [8] recorded the FAB spectra of a simple aminoacid after deuteration in a DCl/D_2O system.

Continuing this study, we have designed a simple two-part experiment. First, we recorded four spectra of the underivatised hexapeptide 1, H-Gly-Lys-Thr-Gly-Pro-Gly-OH as follows :

i) nondeuterated peptide in nondeuterated glycerol ; (Fig. 2)

ii) nondeuterated peptide in deuterated glycerol-d_3 ; (Fig. 3)

iii) peptide deuterated, under D_2O/base exchange, in nondeuterated glycerol ; (Fig. 4)

IV) the peptide obtained in iii) in deuterated glycerol.

The spectra are presented in Figures 2 - 4 and Table 1. The quasimolecular ion area has been referred to the spectrum of nondeuterated peptide in nondeuterated glycerol.

Secondly, we recorded spectra of Leu-Enkephalin, 2, H-Tyr-Gly-Gly Phe-Leu-OH, its two selectively deuterated ^{2}Gly-d_2 and ^{1}Tyr-d_7 analogs and one D_2O deuterated-dn (Figures 5 - 12) in both glycerols in order to study the sequence and fragmentation rules under FAB conditions. This short series enabled us to compare the advantage of specific C-H deuteration versus labelling of all mobile protons.

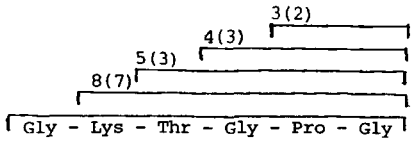
Finally, we investigated the possibility of C-D bond cleavage on the matrix, particularly from the labelled glycine, in order to determine the origin of the hydrogen in the M+1 or P+1 ions (P - any fragment observed).

SPECTRA OF HEXAPEPTIDE 1 IN GLYCEROL AND GLYCEROL-d_3

The hexapeptide 1 represents a system with 10 mobile protons : two NH_2 groups, one carboxylic, one hydroxyl and four amide protons. A deuterated peptide was prepared under basic conditions by two consecutive lyophilisations in D_2O. The peptide exchanges back all mobile deuteriums in glycerol-d_o*; the spectrum was identical to that of the nondeuterated peptide in normal glycerol (Table 1). The nondeuterated peptide (Fig. 3) in glycerol-d_3 shows an exchange of 6 to 7 protons and the deuterated, under similar conditions, of 8 to 9 protons. Assuming that the deuteration of the second hydrogen of the NH_2 group is usually weaker and that the carboxylic proton is involved in the zwitterion interaction, the observed exchange means that all four amide hydrogen atoms, the OH of threonine and one hydrogen from the lysine side-chain amine group have been exchanged. For the deuterated peptide all but one proton have been exchanged. This hypothesis is confirmed by the sequence ions study. All labelled fragments compared to the corresponding unlabelled peptide spectrum in glycerol-d_o confirm this assignment.

Simulation of spectrum of peptide 1 [2] (Table 3) reveals the presence of four intense ions, all corresponding to the route established by Barber [3] for fragmentation from the amine end of the peptide. Four sequence ions are observed at 459 (M+1 - Gly), 331 (-Lys), 230 (-Thr) and 173u (-Gly). In the spectrum of the labelled peptide in glycerol-d_3, these ions are shifted to 467, 336, 234 and to 176u respectively, which implies the exchange of eight* protons on the pentapeptide fragment Lys-Thr-Gly-Pro-Gly, five on the tetrapeptide Thr-Gly-Pro-Gly, four on the tripeptide Gly-Pro-Gly and three on the Pro-Gly fragments.

Since all these ions follow the same fragmentation rule the labelling pattern is as follows :

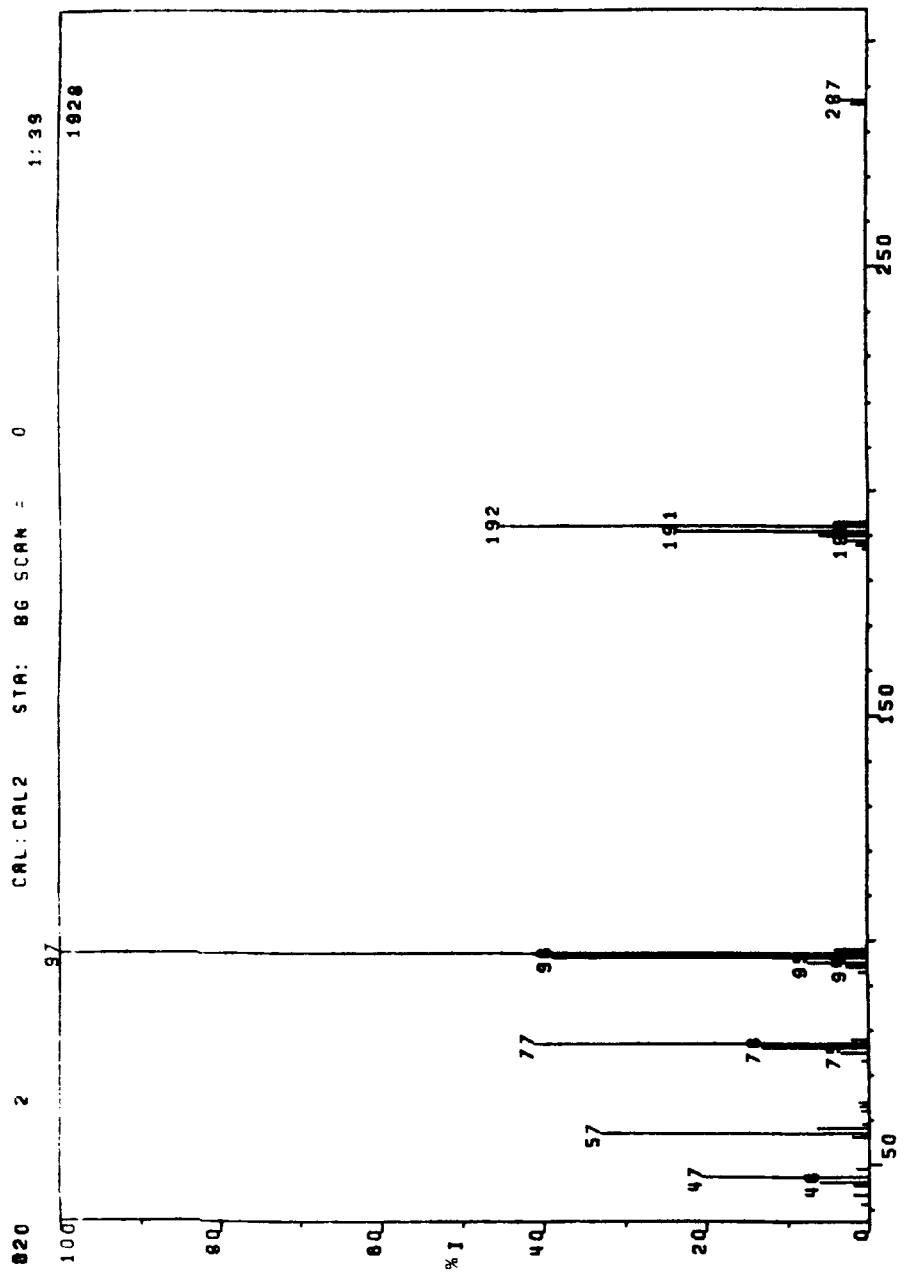


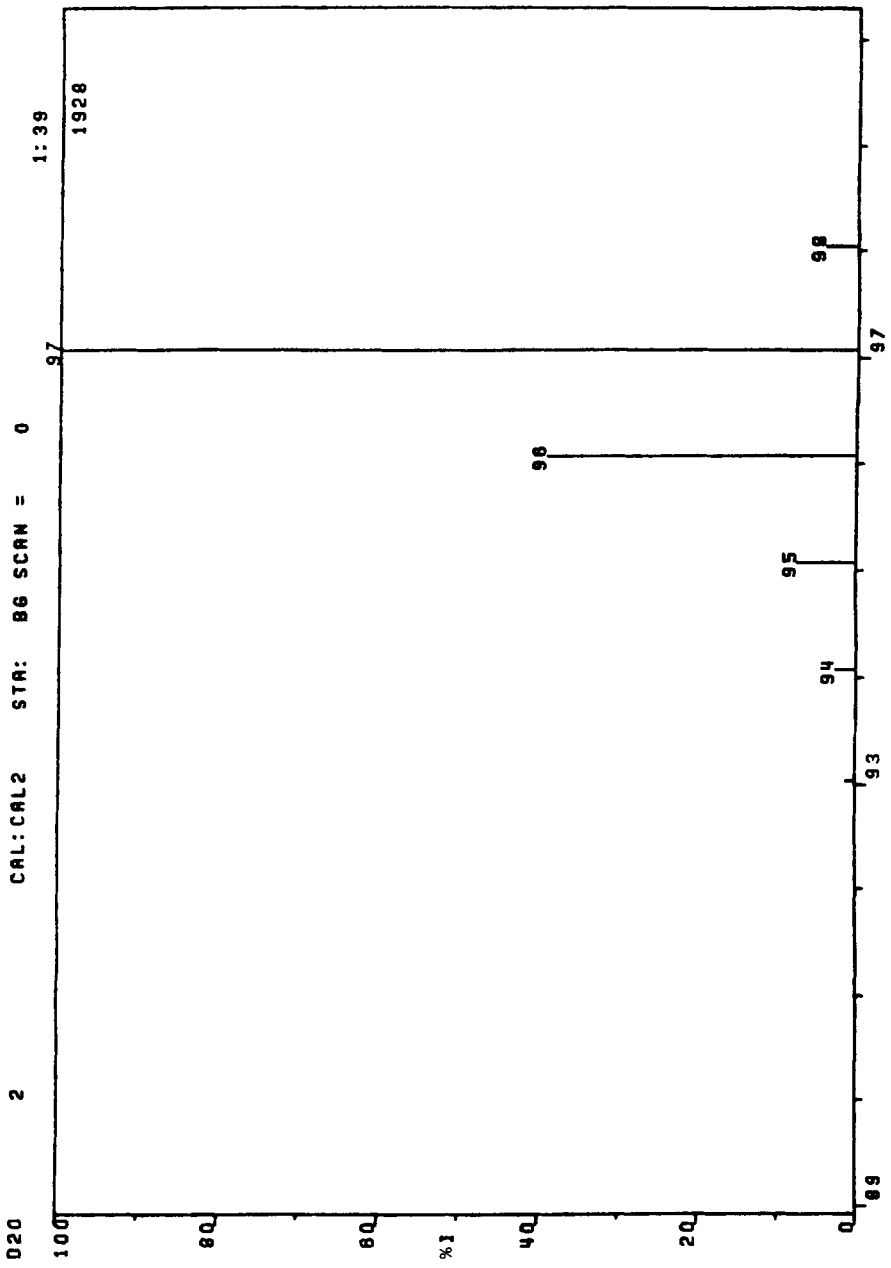
in glycerol-d_3 after deuteration.

w/t deuteration values in ( ).

* Incidentally the Referee of this paper suggests that because of similar kinetics two of the deuteriums must be more difficult to replace. When the deuterated peptide - 1 spectrum is recorded fast, just after mixing it with glycerol-d_o, the M+2 ion intensity is slightly higher.

* For the most intense peaks of the cluster.





. Figure 1 - FAB-PI glycerol-d_3 Spectrum

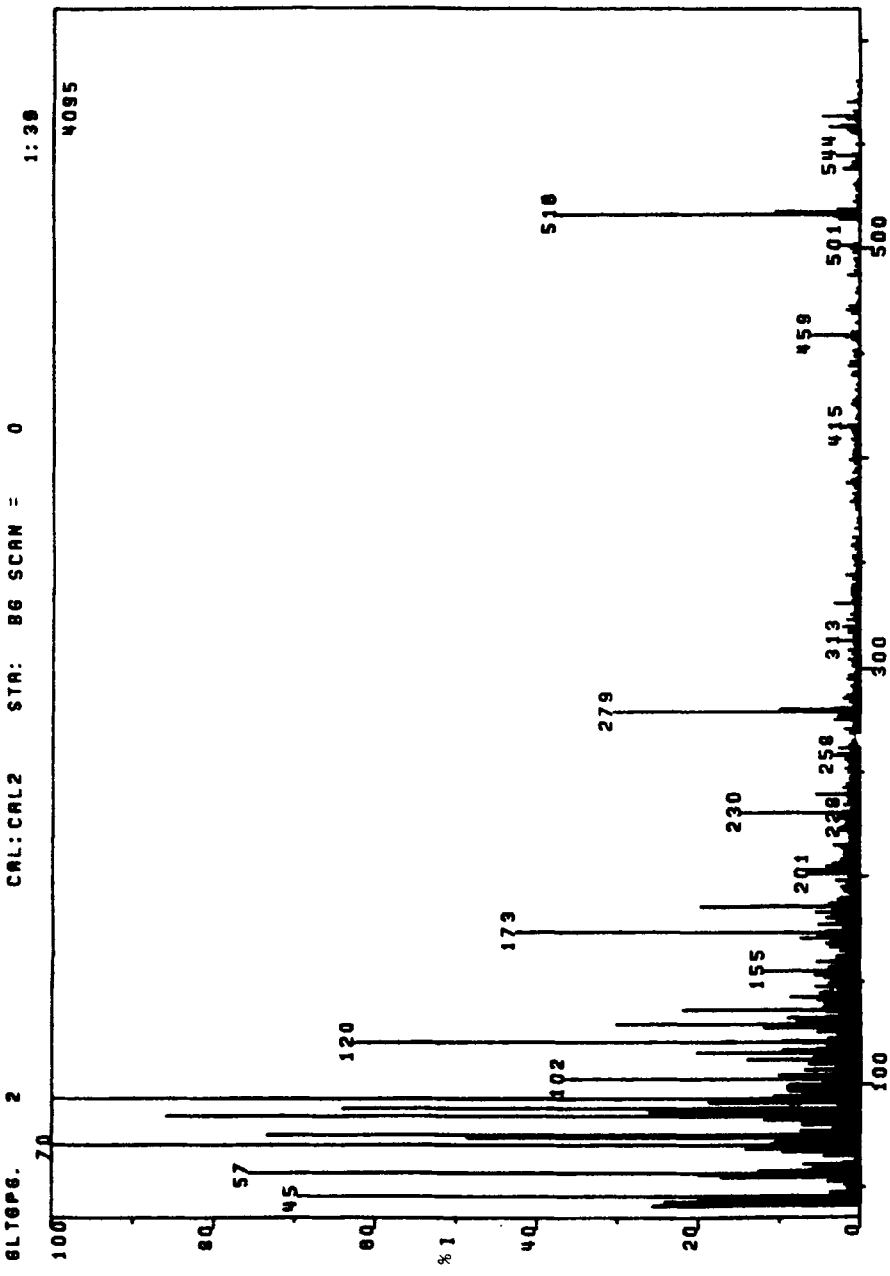
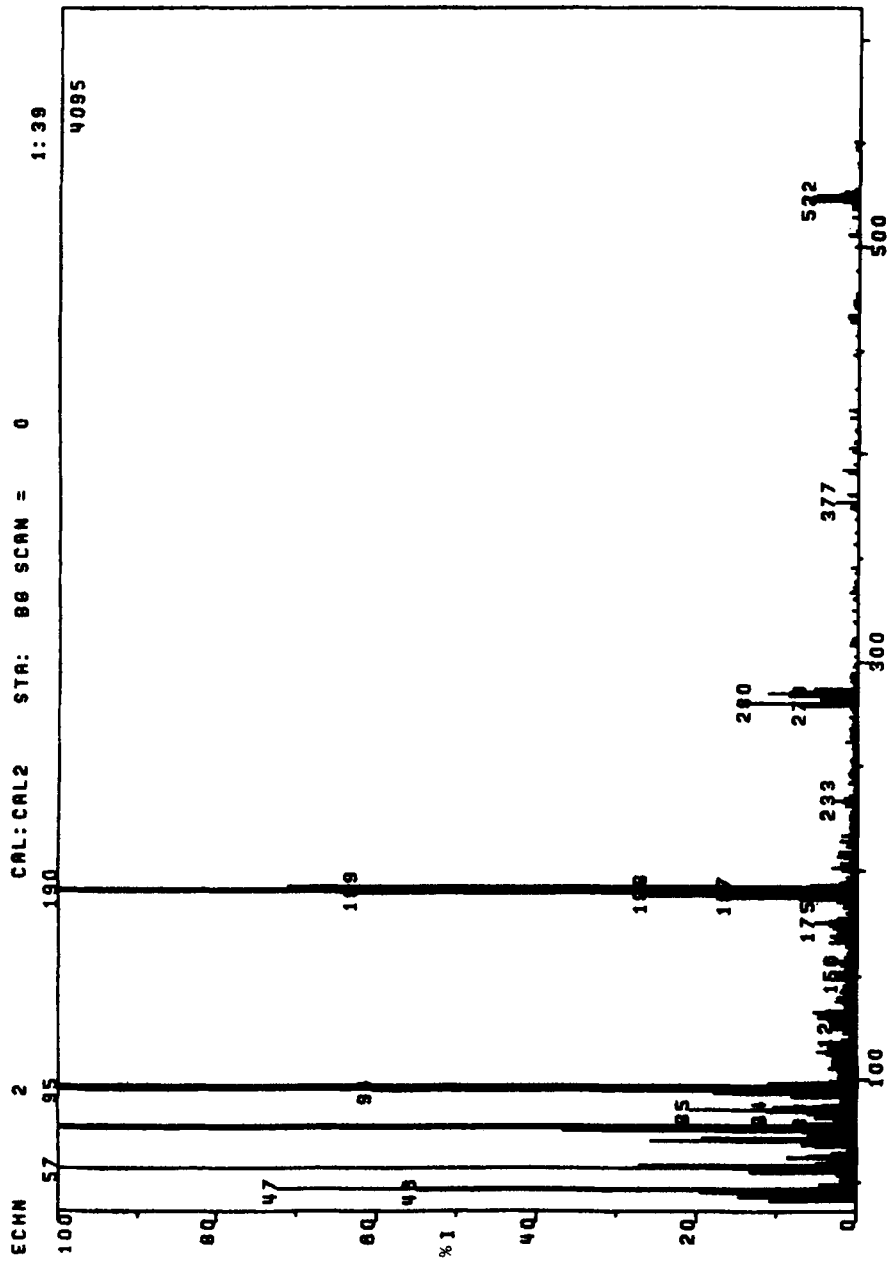
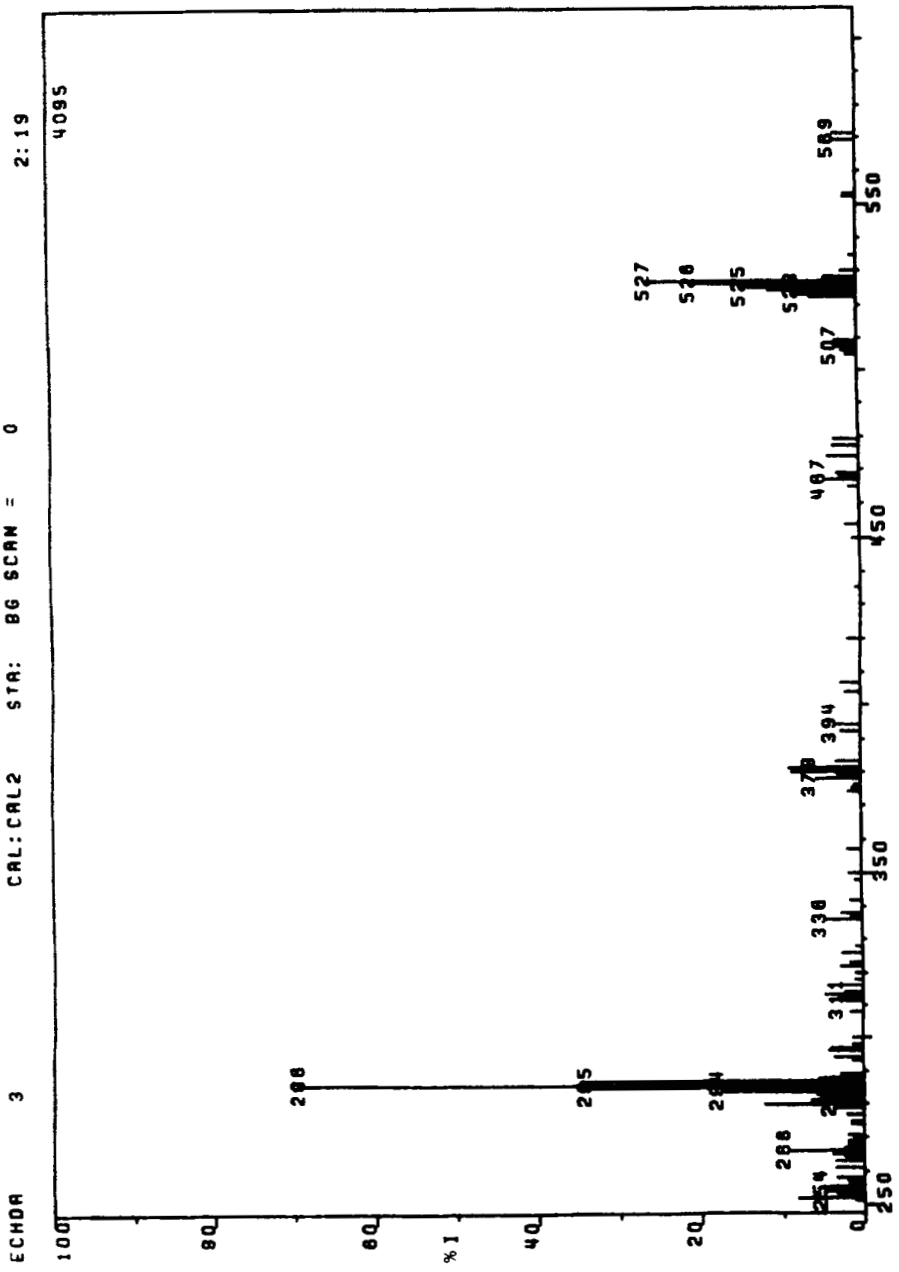
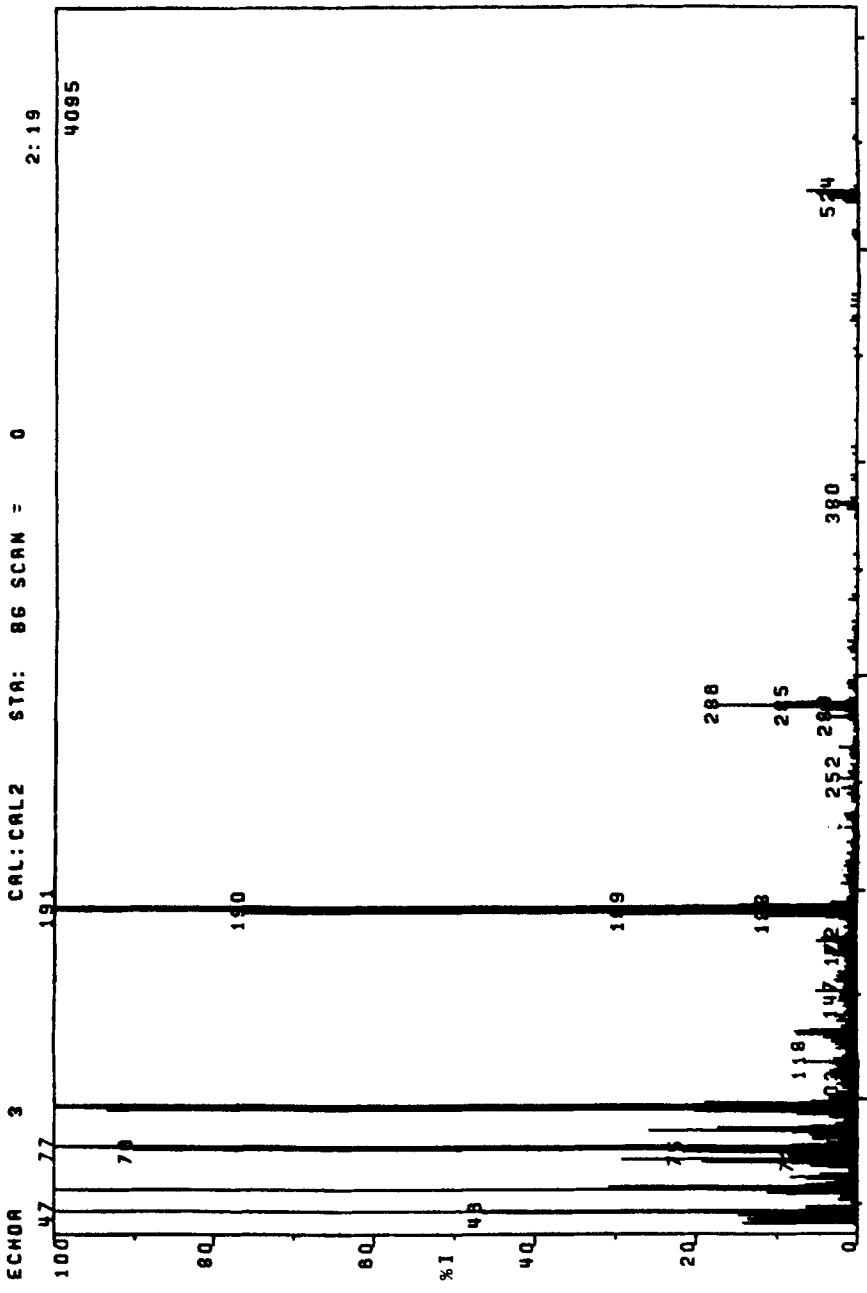


Figure 2 - Peptide 1-d_o spectrum in glycerol-d_o

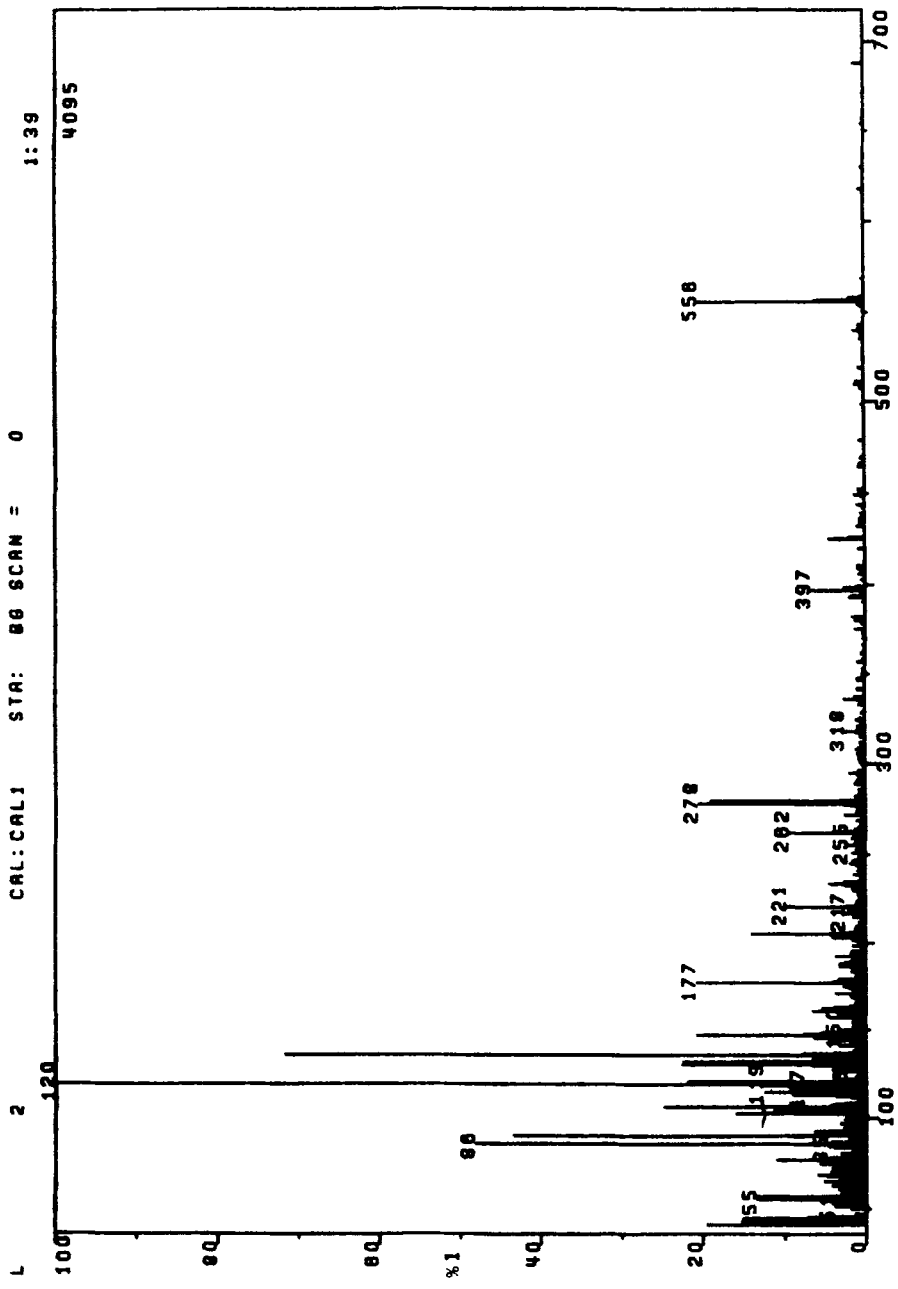


. Figure 3 - Peptide $\underline{1}$-d_0 spectrum in glycerol-d_3





. Figure 4 - Peptide $\underline{1}$-d_n spectrum in glycerol-d_3



. Figure 5 - Peptide 2-d_o spectrum in glycerol-d_o

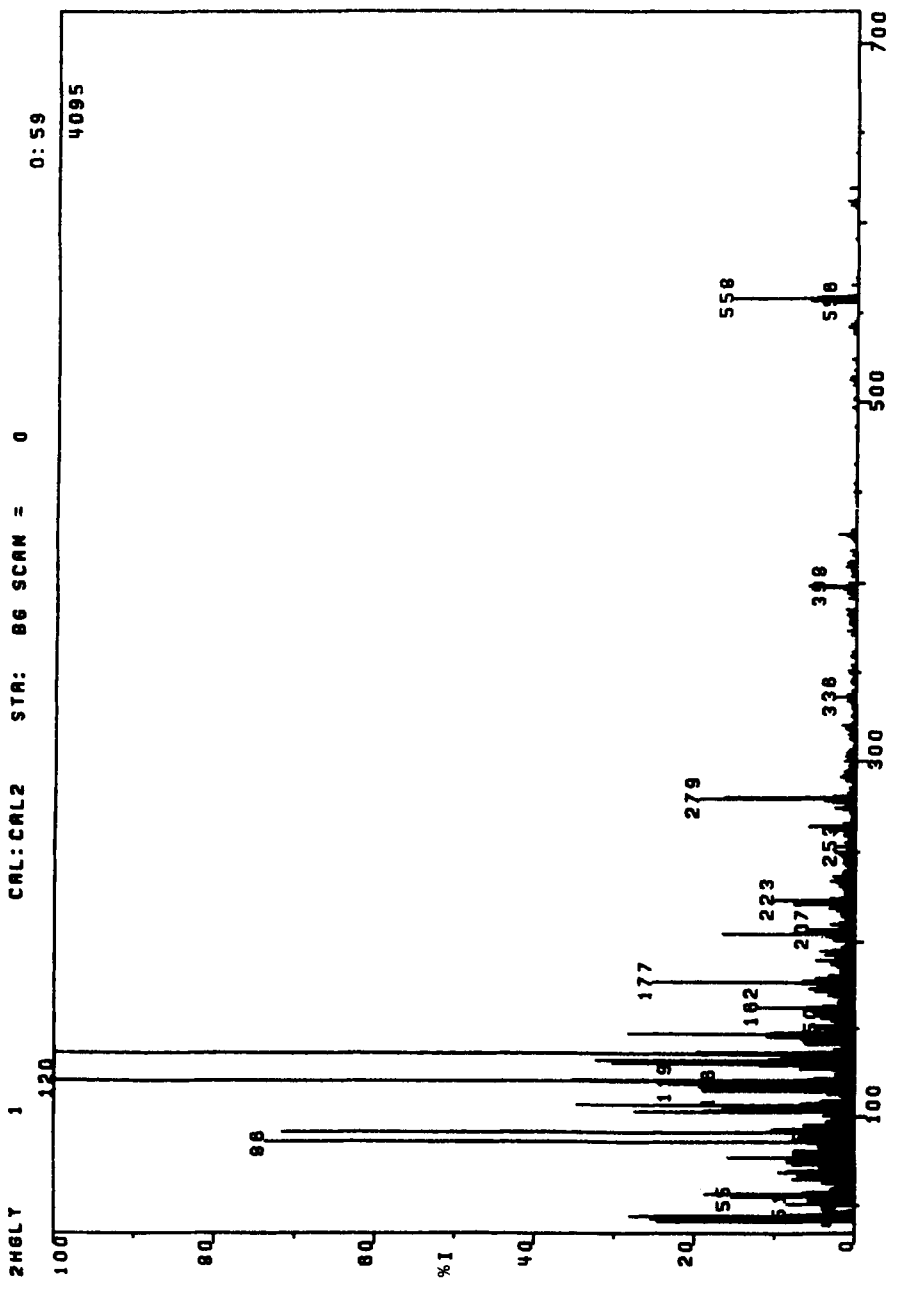
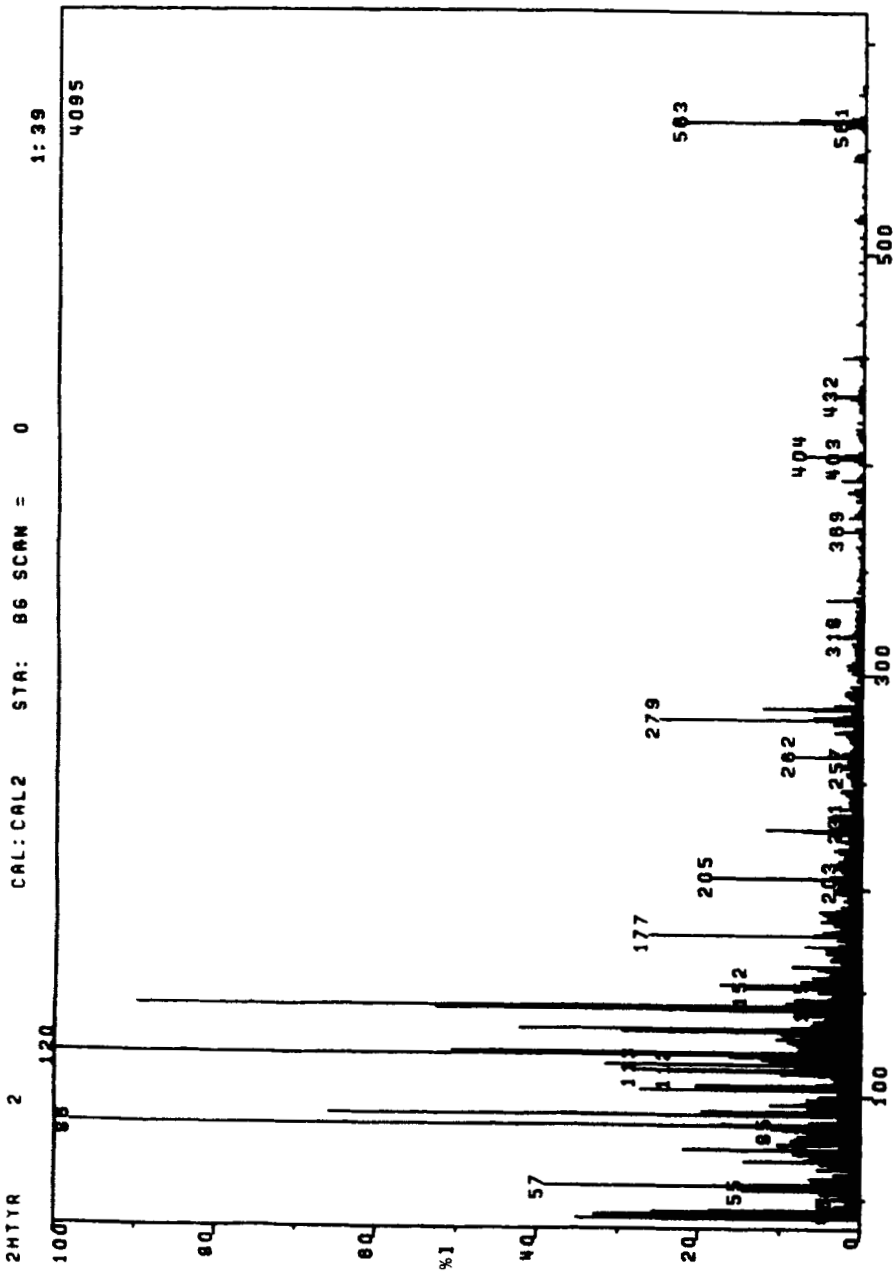
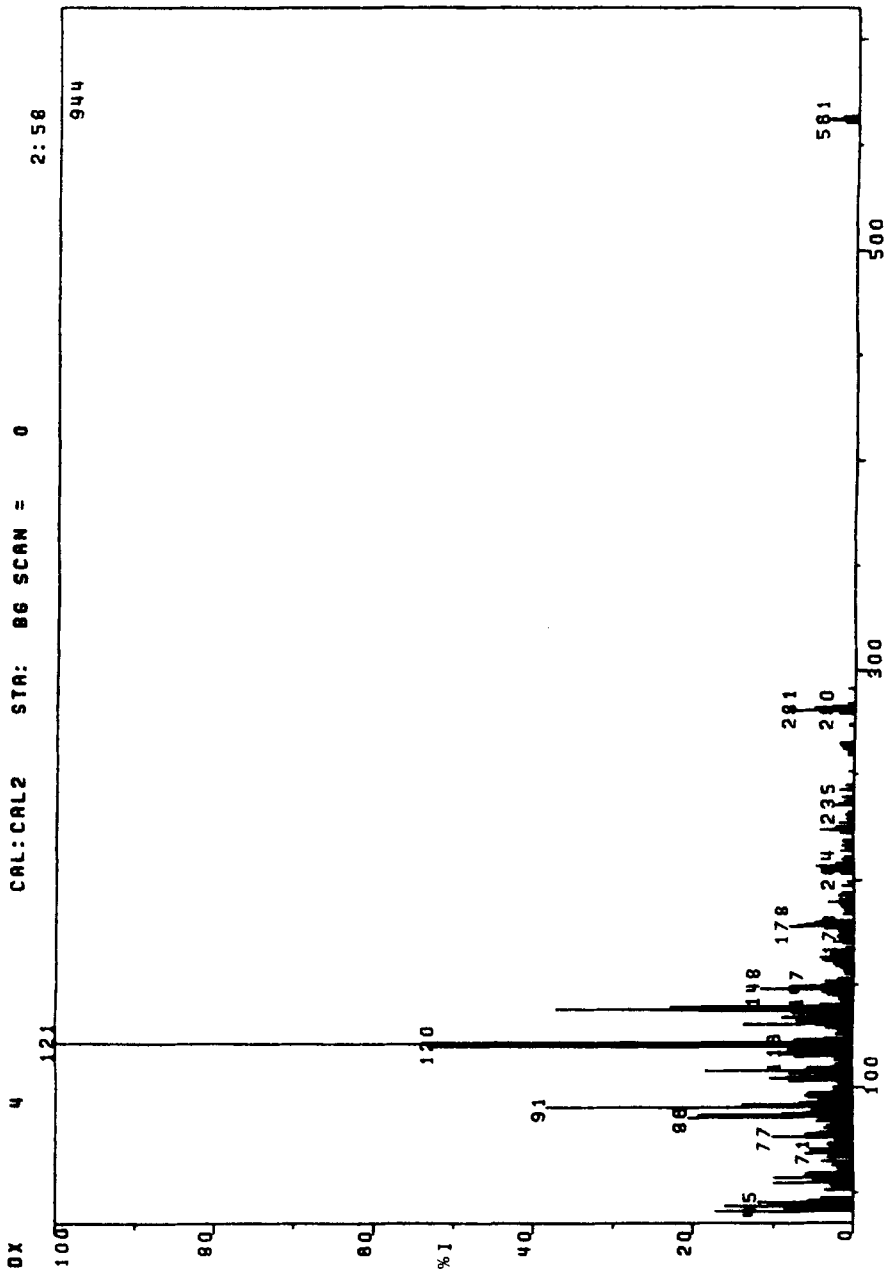


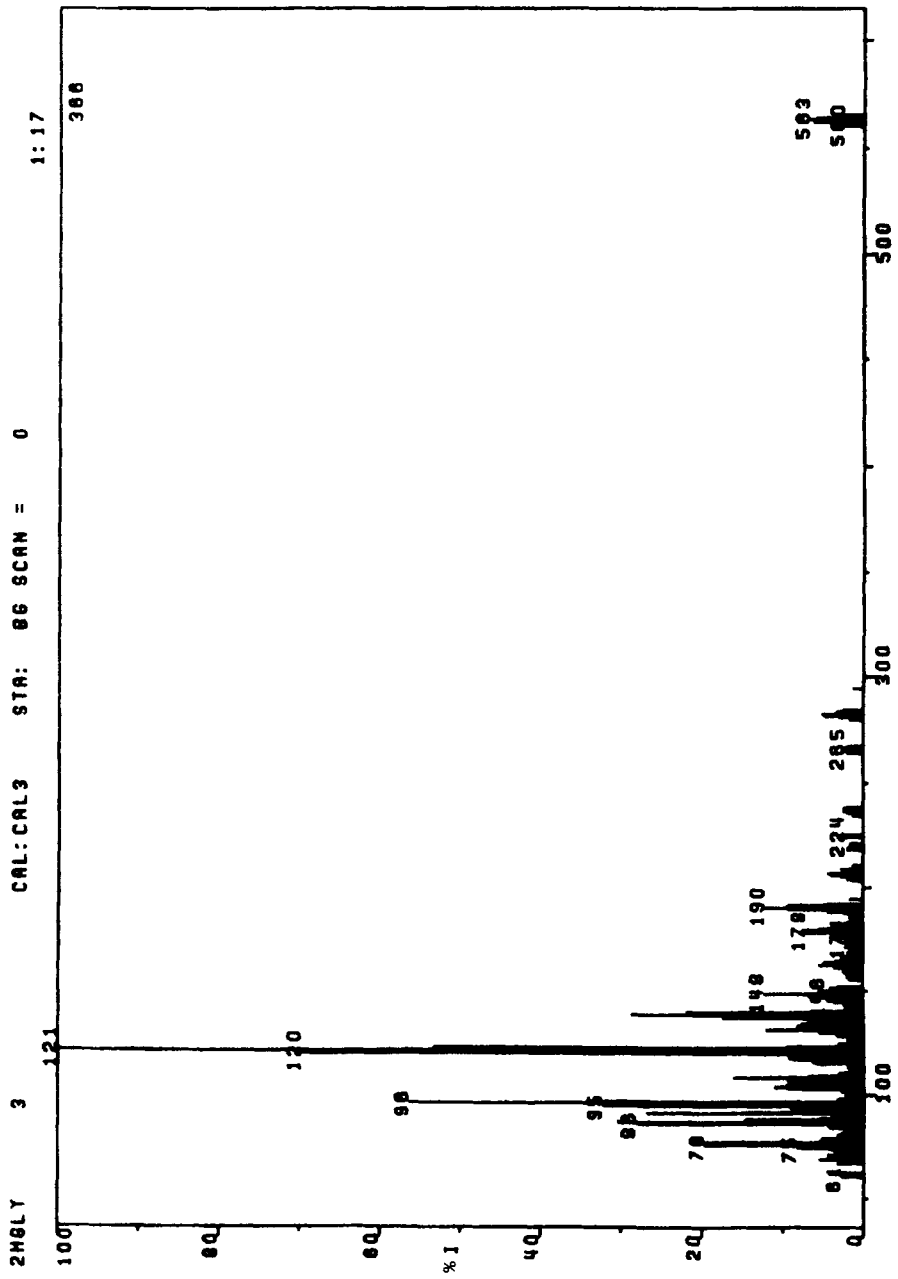
Figure 6 - Peptide Gly-d_2-2 spectrum in glycerol-d_o



. Figure 7 - Peptide Tyr-d_{7-2} spectrum in glycerol-d_o



. Figure 8 - Peptide 2-d_0 spectrum in glycerol-d_3



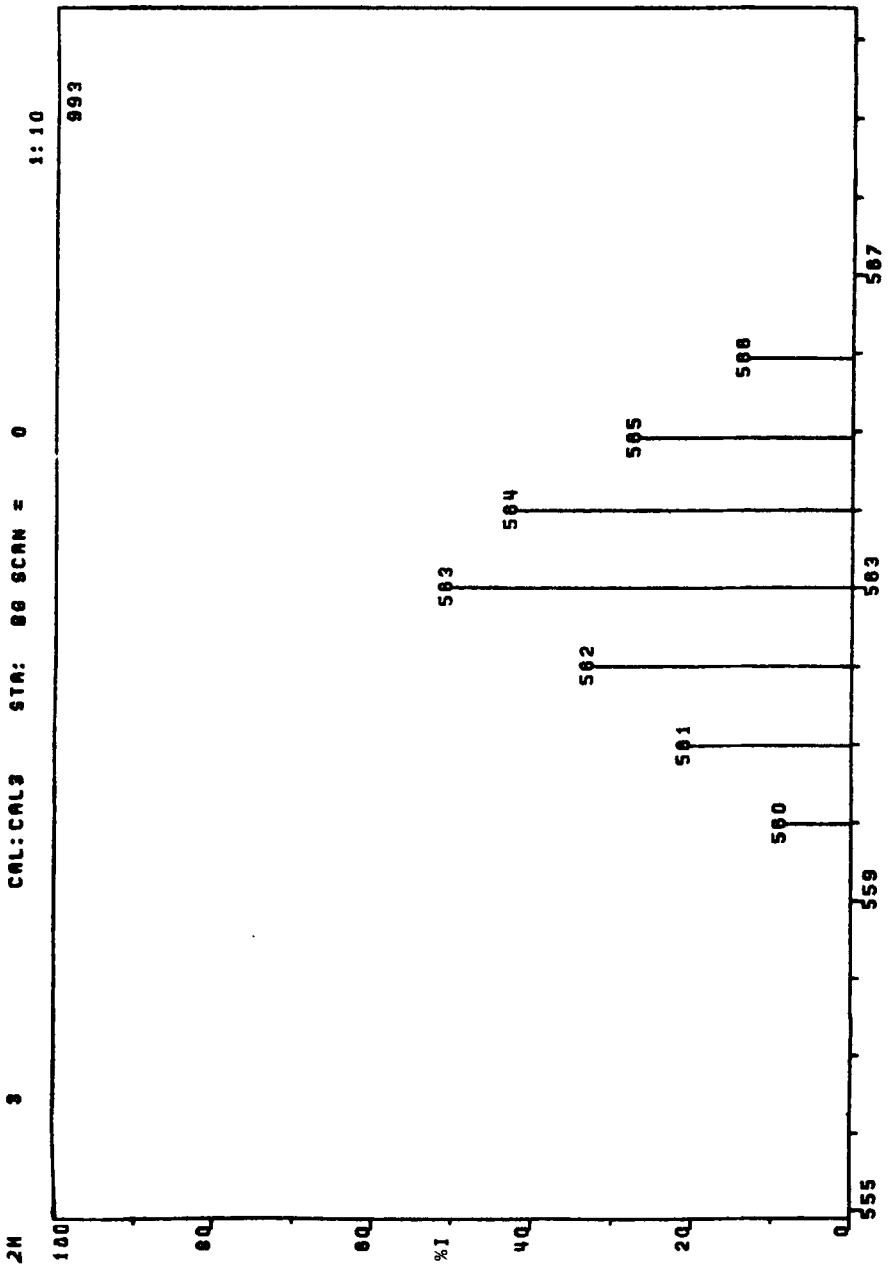
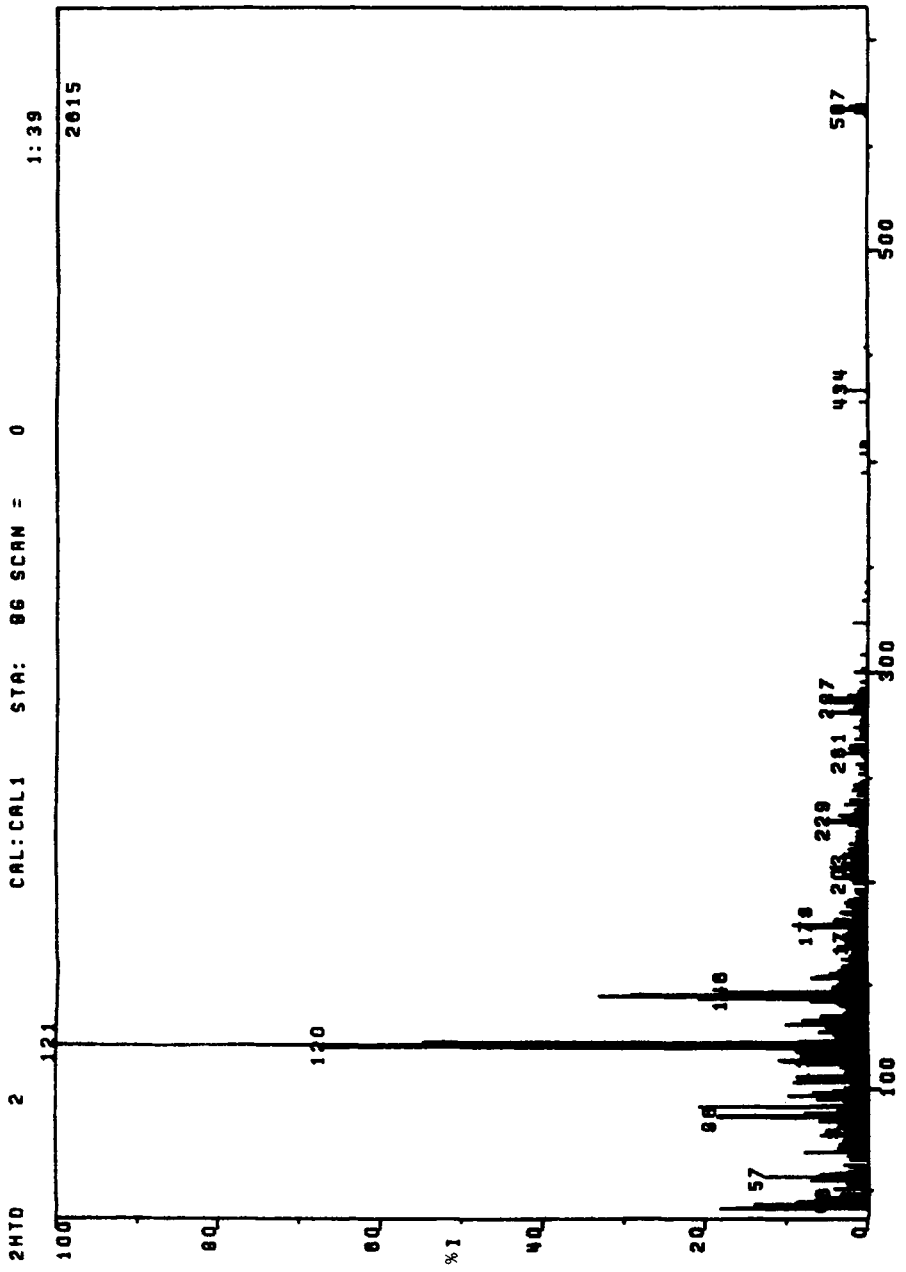
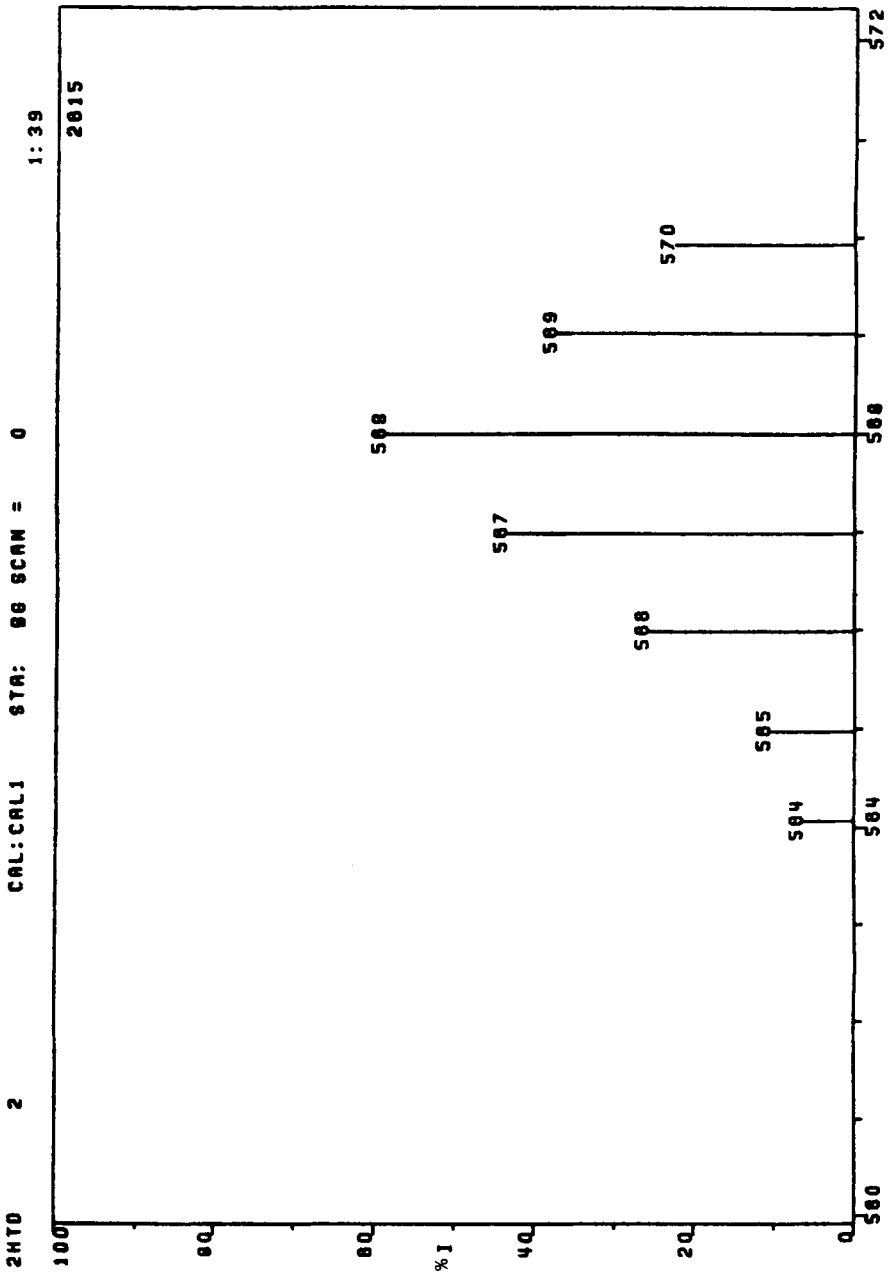
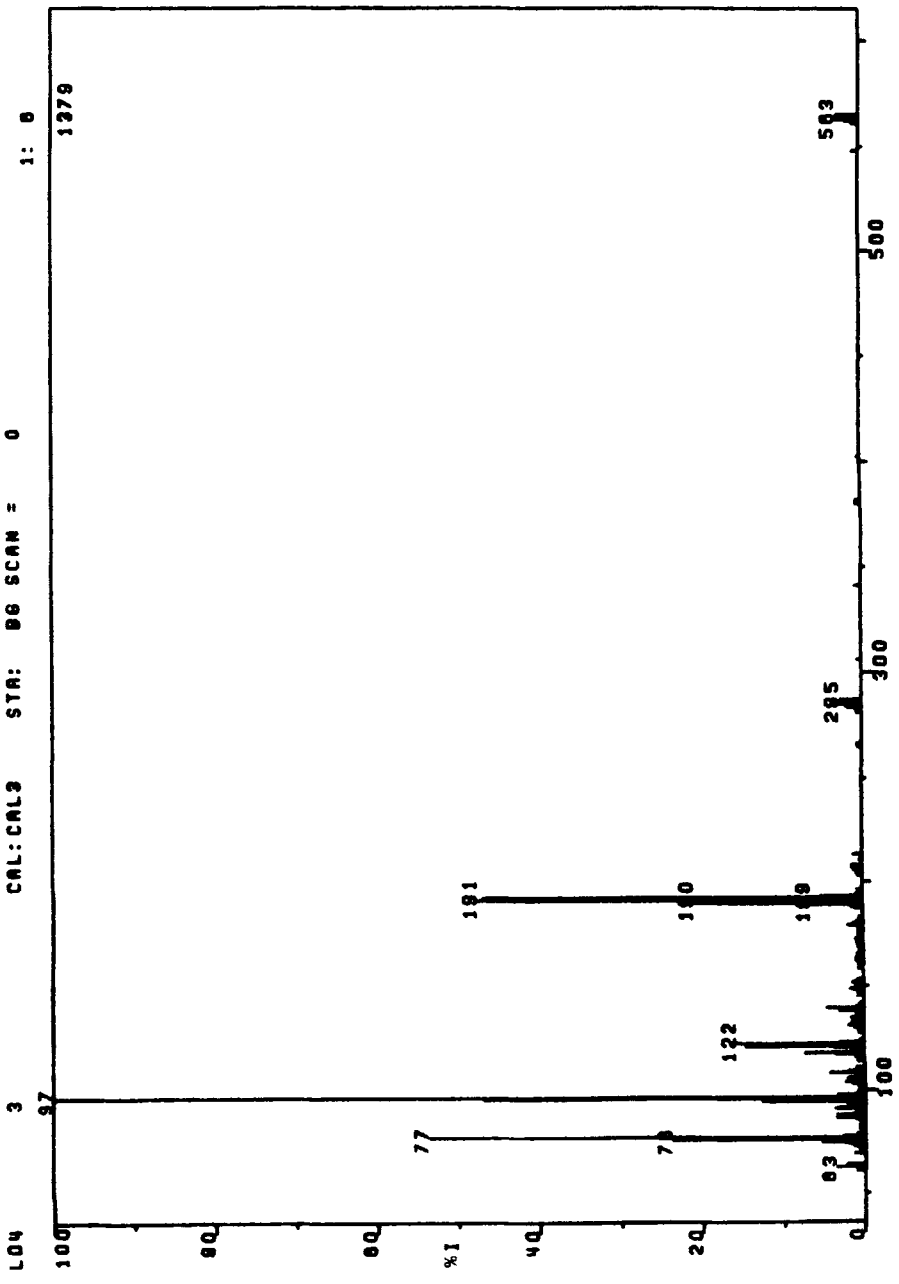


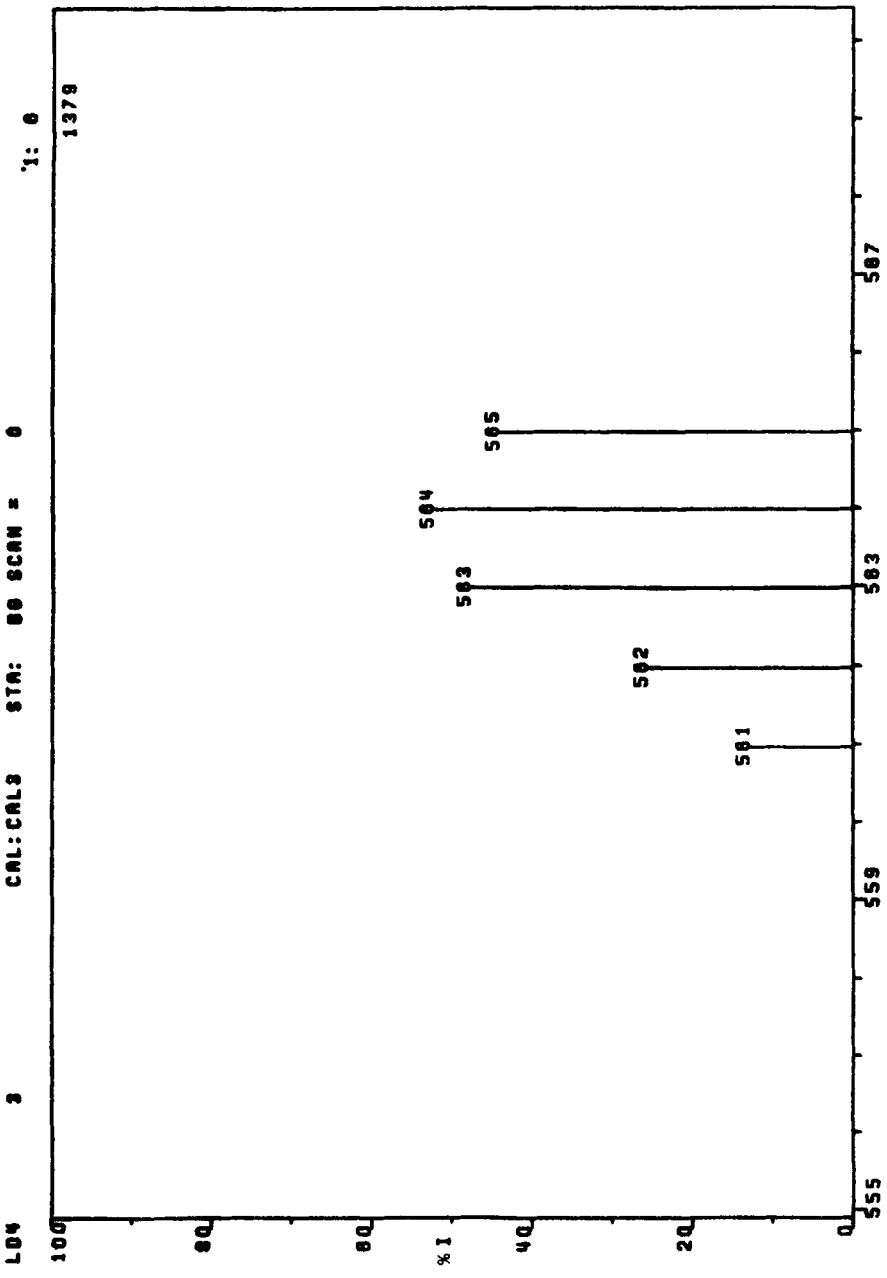
Figure 9 - Peptide Gly-d_2-2 spectrum in glycerol-d_3



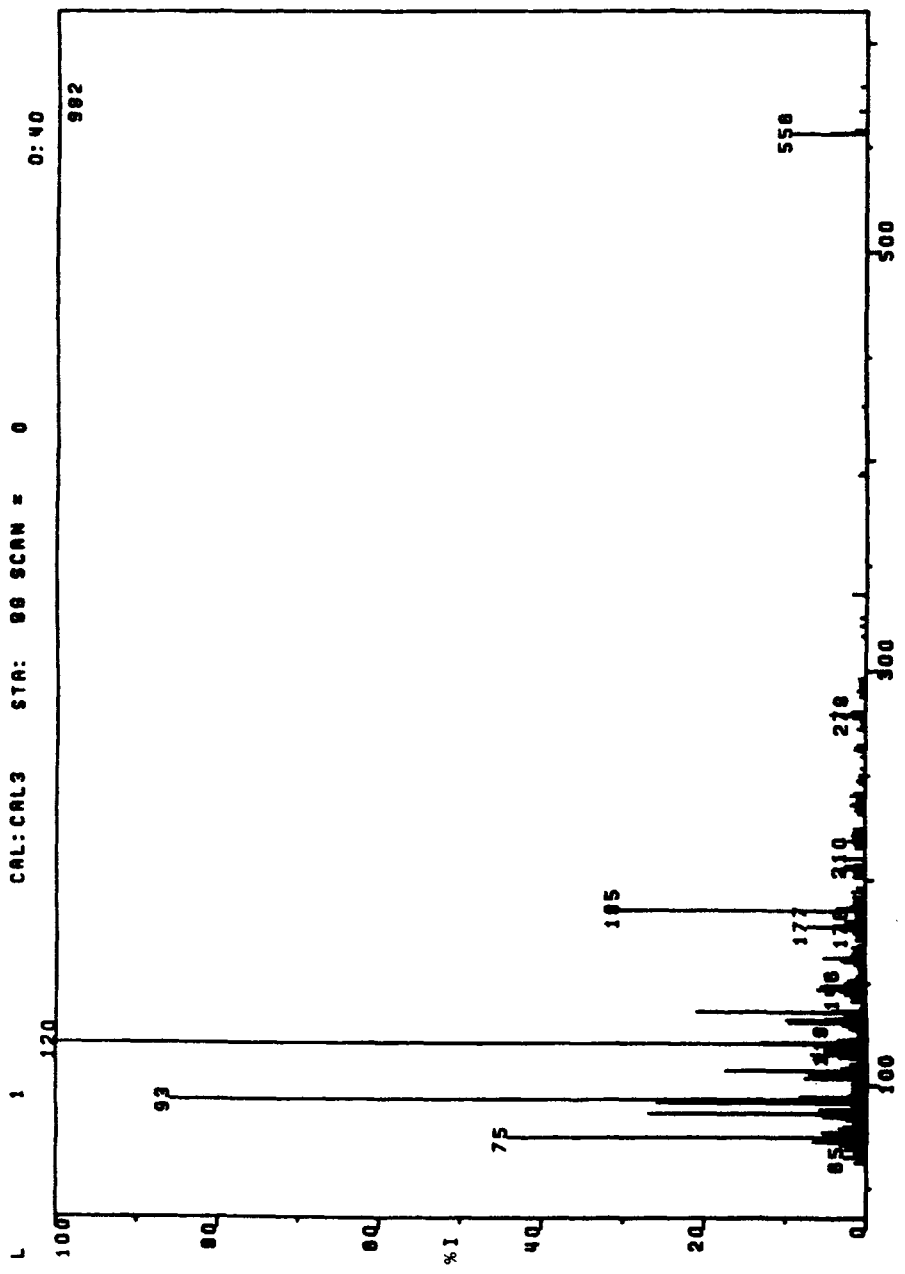


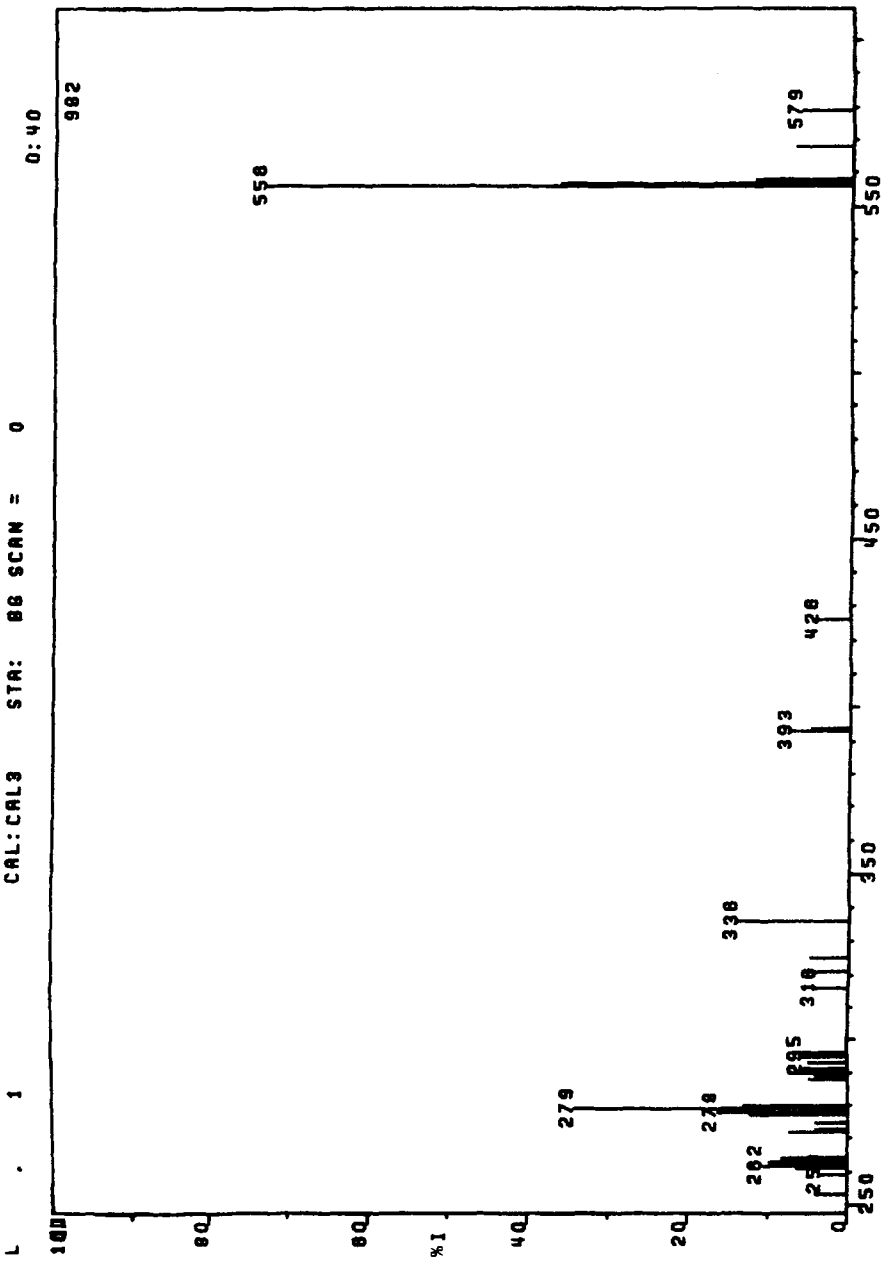
. Figure 10 - Peptide Tyr-d_7-2 spectrum in glycerol-d_3





. Figure 11 - Peptide 2-d_n spectrum in glycerol-d_3





. Figure 12 - Peptide 2-d_n spectrum in glycerol-d_o

Table 1

Molecular ion clusters

Peptide	Matrix	M.W.		515	516	517	518	519	520	521	522	523	524	525	526	527	530		
1	Glycerol	515	d_o	11	100	38	7.5	3											
			dn*	12	100	53	7	3											
	Glycerol-d_8	515	d_o					24	11	63	55	100	75	34	27	9.5			
			dn				11	10	9	28	78	34	83	100	70	36	10		
2	Glycerol	555	d_o	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570
				4	100	30	8.5	2											
		557	^{2}Gly-d_2		37	36	100	23	8										
		562	^{1}Tyr-d_7							8.5	-	100	37	7	2				
			dn	2	100	38	15												
	Glycerol-d_8	555	d_o						41	94	100	53							
		557	^{2}Gly-d_2						16	40	65	100	83	33	24				
		562	^{1}Tyr-d_7										10	17	42	74	100	63	38
			dn							23	47	90	100	75					

* dn - product after liophylisation in D_2O.

For the spectrum of peptide 1-d_n in glycerol-d_3 these results show additional labelling at NH_2 and COOH groups. The difference of two units noted between pairs of spectra in glycerol-d_3 (of d_o and d_n peptides) has been observed for both peptides (1 and 2) (Table 1). This means that slow deuteration with D_2O followed by glycerol-d_3 under FAB conditions exchanges even the second hydrogen of the NH_2 terminal group. However, glycerol-d_3 alone does not exchange both NH_2 hydrogens. The corresponding ions for unlabelled peptide 1 in glycerol-d_3 are 466, 333, 233 and 175u. The corresponding shifts are then seven, three, three and two atomic mass units (compared to the unlabelled spectrum of 1 in glycerol-d_o).

The differences between the two deuterations are situated on the dipeptide Pro-Gly fragment : one more mobile hydrogen on this system means that one hydrogen is shifted from the amine end of the peptide during fragmentation. In fact it is impossible to exchange more than two hydrogens on such a peptide because of the tertiary amide nature of the peptide bond involving proline.

The deuterium in the P + D fragment, where P is a mass of any given sequence fragment, originates from glycerol as previously proposed by McCloskey (1). The systematic difference of +1 between the two series certainly implies deuteration on the COOH group. As for the last fragment, m/z 76, interference by glycerol prevents interpretation of the structural variations. The m/z 75 fragment, usually very strong in the glycerol-d_o spectrum, is replaced for glycerol-d_3, by very strong 76 and 77 fragments (see Experimental).

Comparison of four differently labelled peptides 2 enables us to identify several important ions, to discuss their origin and to relate them to the sequence of this peptide.

SPECTRA OF PEPTIDE 2 IN GLYCEROL-d_o

The peptide 2 spectra have previously been recorded by Katakuse [4] and by Kratos group [5] and reported in the catalog of Jeol spectra [9].

The peptide 2 and its deuterated analogs show intense quasimolecular ions (Table 1). The ^{2}Gly-d_2 and ^{1}Tyr-d_7 peptides both show the molecular ion at the expected masses - two and seven units higher than the unlabelled peptide. The molecular ion clusters of all four patterns are very similar. Starting from these ions several cleavages could be identified. The first cleavage of peptide occurs on the Phe-Leu bond (ion 425) although, the cleavage of the terminal Tyr moiety is practically absent from the spectrum (Table 2). Contrary to the earlier work [4] we find that the area of 556-425u region is

empty.* The corresponding tetrapeptide ions Tyr-Gly-Gly-Phe were recorded for both C-deuterated and 2-d_n peptides and the loss of CO leads to the ion 397u (or its deuterated equivalents). The tripeptide fragment Tyr-Gly-Gly was recorded at 278u for all peptides studied. Another fragment important for sequence determination is the 336 ion corresponding to Gly-Gly bond cleavage which is observed for all three compounds without any labelling. This implies that the H-shifts involve NH protons rather than CH protons.** The sequence determining ions are then easily correlated to the structure and confirmed by labelling.

The analysis of the ion at 318u (320u for the 2-d_2 peptide), seems to show that this ion contains the second glycine residue. However, the ions 136 and 147u contain the tyrosine residue (2-d_7, peptide characteristic labelling). The ion at 262 u which has been identified /4/ as resulting from the McLafferty rearrangement on the Leu-Phe end shows typical glycine-labelling which means that it cannot originate from such a rearrangement***.

Finally the group of ions at 221, 205, 177 and 120u is present independently of deuteration and does not show any systematic mass variations. It seems however that they are not of unique origin. These ions do show some specific labelling on deuteration with glycerol-d_3. As for compound 1, the 2-d_n peptide spectrum in glycerol-d_o shows exchange with the matrix giving a spectrum in all respects identical to 2-d_o. More careful study of this reexchange leads as previously to similar conclusion (M+2 ions) (Table 2). The fast recording of 2-d_n spectrum in glycerol-d_o shows the retention of two more deuteriums which are reexchanged with longer glycerol-d_o exposure time.

SPECTRA OF 2 IN GLYCEROL-d_3

The peptide 2 represents a system of eight mobile protons (three on the NH_2 and COOH terminus, four peptide bonds and one phenolic (Tyr)). The two exchange systems studied are glycerol-d_3 with the unlabelled peptide and with the peptide previously deuterated with D_2O. All four labelled peptides 2 have been examined in glycerols : d_o, ^{2}Gly-d_2, ^{1}Tyr-d_7 and d_n where n denotes an unspecific labelling with D_2O.

First, the molecular ion clusters of the four peptides are presented in Table 1, assuming the in glycerol-d_3 the M+2 (M+D) quasi-molecular ion is

---

* No intense ions observed in that region.

** The single bond enthalpies difference between these two bonds is 22 KJ $mole^{-1}$ (C-H 413 and N-H 391 KJ $mole^{-1}$) (12).

*** It is necessary to remark that other ions at 295, 442, 443, 499 and 511u observed by Katakuse in the spectrum of peptide 2 are extremely weak.

Table 2

Fragmentation of peptide 2 in glycerol-d_o matrix

	M+1									
d_o*	556	425	397	318	278(279)	336	262	147	136	221, 205
^{2}Gly-d_2	558	427	399(398)	320	279(280)	336	264	147	136	177, 120
^{1}Tyr-d_7	563	432	404(403)	318	279	336	262	153	143(142)	
d_n*										

* The spectrum of 2-d_n peptide in glycerol-d_o (fig. 12) is identical to d_o (fig. 5)

( ) denotes : also present.

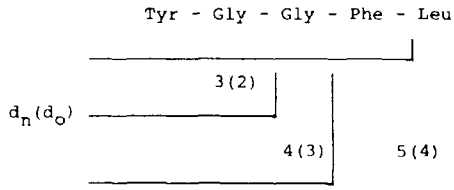
formed. For the unlabelled 2-d_o peptide the exchange of five protons is observed, while for the two C-labelled peptides the exchange of four to five protons is observed (the molecular ion clusters show three intense peaks at M+3, M+4 (100) and M+5 compared to corresponding spectra in normal glycerol). The mass difference between the highest ions in the clusters for both d_2 and d_7 peptides remains 5 units (the experimental conditions have been rigorously the same). The last labelled peptide of the series 2-d_n shows, the exchange of seven protons (clusters M+6, M+7 (100), M+8) • as expected and by analogy to the peptide 1.

The nonspecifically deuterated peptide d_n in glycerol-d_3 shows a difference of two more exchangeable protons compared to d_o (the highest peak in the cluster).

Because of the possibility of an H or D shift during the six fragmentation routes the exact assignment of fragmentation is difficult. For example, in the block of ions which is common for the glycerol-d_o spectrum series several mass shifts have been interpreted. The fragments at 120 and 178 u are shifted to 121 and 178 u (one mobile hydrogen) except for the d_n peptide showing 122 and 179 u (two mobile hydrogens), and the 205 fragment which shows a shift to 204 in the d_o peptide and to 203 u in both C-deuterated peptides.

Generally speaking the intensity of all sequence-important ions are weaker than in normal glycerol spectra. In spite of these restrictions sequence cleavage at ^{4}Phe-^{5}Leu (ions 397 and 425) could be confirmed in the unlabelled spectrum. The ion 278 is shifted to 281 for d_o, 282 in d_2, 287 in d_7 and 285 in d_n spectra : the ion 262 is shifted to 266, 265, 264 and 265 and the ion 147 to 148, 148, 147 and 147 respectively. However, the ion at 336 is observed at 341.

The general picture of the labelling in glycerol-d_3 deduced from these fragments probably follows the scheme :



• Cluster referred to M+D quasi-molecular ion.

However our results imply that great caution must be exercised regarding reported peptide 2 ion structure assignments (4). The fragmentation patterns in the glycerol matrices are more complex than expected because of the H (or D) shifts.

SIMULATION OF SPECTRA FOR PEPTIDES 1 and 2

Using the simulation program previously developed in our laboratory* [2] we have obtained a good simulation of the FAB spectra of both peptides (Table 3). The prediction of sequence-characteristic fragments for peptide 1 in particular shows very good agreement between experimental spectra and simulation according to the rules of Barber [3] and Williams [7, 10]. This agreement is particularly good for the acyl-amine cleavages with retention of charge on the carboxylic end of the peptide (four ions present).

The pentapeptide 2 simulation of sequence-important cleavages is worst. This difference may be attributed to the presence of Tyr and Phe, both amino acids involved in several α-carbon substituent cleavages. However, each peptide bond is characterised by at least one intense ion present in the experimental spectrum.

It is necessary to remark that these two peptides do not contain even one common peptide bond.

Our program permits much easier identification of peptide fragments. Its utility for the labelled peptide 2 is assured by introduction a twenty-second hypothetical amino-acid XXX of variable mass. In the case of 2-d_2 for instance the glycine residue has been replaced by XXX = 59.

The difficulty of dealing with such a simulation program could be shown for the group of 397-425 u ions. The 28 u difference between there two masses support the carboxylic-terminal Leu position - however, this group of ions could also be obtained from other fragmentation routes. The advantage of our program consists of the use of six families of cleavages. If there is no evidence for specific cleavages within the same complete cleavage mode for all peptide bonds of any given peptide, the partial information should be synthesised into complete sequence confirmation. For instance for the peptide 2, Gly-Phe and Gly-Gly bond cleavage is characterised by the first type of fragmentation : Tyr-Gly and Phe-Leu by the second one. Both are additionally confirmed by other ions present.

---

* Copy available on request.

Table 3

Simulation of spectra of peptide 1, 2-d_0, 2-^{2}Gly-d_2 and 2-^{1}Tyr-d_7 in glycerol-d_0

		Fragmentation mode					
		1	2	3	4	5	6
	Mass	H-end	OH-end				
1	515	459	413(141)	457	444	443	441
		331	316	329	316	346	344
		230	259(258)	228	215	289	287
		173	158	171	158	183	186(185)
		76(75)	30	74	61	60	58
2-d_0	555	393	397	391	378	427	425
		336	250	334	321	280	278
		279	193	277	264	223	221
		132	136	130	117	166	164
2 ^{2}Gly-d_2	557	395	399	393	380	429	427
		336	252	334	321(320)	282	280
		279	195	277	264	225	223
		132	136	130	117	166	164
2 ^{1}Tyr-d_7	562	393	404	391	378	434	432
		336	257	334	321	287	285
		279	200	277	264(262)	230	228
		132	143	130	117	173	171

( ) means ion found in the nearest environment of the expected one.

CONCLUDING COMMENTS

We have observed that deuterium exchange of mobile peptide protons using double heavy water exchange with lyophilisation alone is not complete. The spectra of the undeuterated peptides recorded in glycerol-d_3 shows a significant increase in deuteration. The spectra of peptides deuterated by the first method recorded in heavy glycerol show a maximum of deuteration. It has been shown that e.g. carboxylic acid proton primary amine group or amide proton exchange is very dependent on the conditions of deuteration.

The potential of analysis by the recording spectra in glycerol-d_o and d_3, is interesting. The main utility of such a comparison remains the study of fragmentation mechanism and fragment structure and sometimes is a better method for studying the sequence or mechanism. The combined technique of specific deuteration and recording of spectra in heavy glycerol of course gives more precise information about both. Simulation of peptide FAB spectra has shown its value in the two cases studied.

The disadvantage of both deuteration methods is the intensity loss of sequence-characteristic ions (isotopic dilution and incomplete deuteration) as well as the H or D-shifts mentioned.

The fragmentation schemes of both peptides have been discussed. It is interesting to note the high number of ions from fragmentation of the carbosylic acid quasi-molecular ion (with retention of charge on this part of the peptide) as well as the good agreement between fragmentation of the peptide itself and its deuterated analogs (specifically or randomly deuterated).

The origin of the hydrogen added to M in the quasi-molecular ion is certainly the mobile glycerol proton.

Several practical conclusions follow : first, the possible use of perdeuterated glycerol (or even glycerol-d_3) for calibration of medium range FAB spectra (up to 1500 daltons). The effective mass gain (92 to 100) at, for example, the 1200-1300 daltons level means ~ 120 daltons shift to higher masses, which extends the applicability of glycerol or glycerol-alkali halide cluster ions for calibration purposes.

A second, more important, practical conclusion is the fact that the glycerol-d_3 matrix acts as an excellent deuteration agent on-the-tip-probe. The exchange of mobile protons is at the ~ 90 % level (checked by McLafferty's method [6]). However, the α-to carbonyl and other acidic protons (C-H bond) remains unexchanged under FAB conditions.

Finally the FAB probe should be washed with deuterated glycerol prior to experiments. The partial retention of active hydrogens cannot thus disturb the deuteration count and the deuteration level remains high and stable from one determination to the other. For this purpose the use of glycerol with a few drops of heavy water or deuterated alcohol is certainly very useful.

For both peptides it is interesting to note that double deuteration (D_2O and glycerol-d_3) exchanges two more hydrogen atoms (e.g. 523-525 for 1 and 562-564 u for 2), presumably since glycerol is more acid than D_2O. Both the corresponding C-deuterated series for peptide 2 exchange five of eight possible protons with glycerol-d_3 (555-563 and 563-568 u). This observation means that glycerol-d_3 systematically exchanges the same kind of protons. For the D_2O-exchanged product 2 such a measurement is impossible because of the previously mentioned fast back-exchange in normal glycerol.

EXPERIMENTAL

All FAB-PI spectra have been recorded on VG 70-35E mass spectrometer with PDP-8 computer equipped with saddle field gas gun (Ion Tech. Ltd.). The Xe atoms have been produced (~ 8 kV, gun current ~ 0.4 mA) and projected against a stainless steel probe tip which has been covered with suspension of ~ 100 nmols of peptide in glycerol.

The glycerol-d_o (bidistilled) or glycerol-d_3 have been obtained from "Service des Molécules Marquées" of CEN de Saclay.

The glycerol-d_o has been stabilised with either methanol-d_3 or heavy water. In such a manner the normal base catalysed deuteration of the products (normally at ~ 90 % of deuteration level) has been stabilised.

The underivatised peptide 1 and the specifically deuterated peptides 2 have been synthesised by the solid state method described by ourselves [11]* ; the peptide 2 has been purchased from Serva Chem. Co. (Heidelberg, Germany).

The deuteration of all mobile hydrogens has been done by dissolving a small sample of peptide in 1 mL of D_2O and stirring overnight (room temperature), then lyophilising till dryness. The deuteration has been repeated a second time in a similar manner.

The glycerol-d_3 FAB spectrum shows the monomer M+D ion cluster (Fig. 1) with a highest peak at m/z 97 (a cluster m/z (I %) : 93(1), 94(3), 95(7), 96(38, M+H), 97(100, M+D) and 98(4)). The dimer peaks cluster at 192 and 191 have been observed (188(1), 189(2.5), 190(6), 191(23), 192(45) and 193(4)), followed by a weak trimer cluster (at 283(0.5), 284(0.8), 285(1),

*Using uniform labelled aminoacids which are biosynthetically produced in our laboratory [12].

286(2) and 287(3.5)), tetramer (at 379(0.2), 380(0.5), 381(0.5) and 383(0.7)) and traces of cluster at 477.

The fragments of glycerol-d_3 at m/z 75, 76 and 77 have been observed (respectively 20, 82, 100 %, intensity referred to m/z 77), compare to glycerol-d_o cluster in m/z 73-77 area with the intensities of 73(17), 74(25), 75(100), 76(12) and 77(8).

The recording of D or L pentapeptide 2 spectra lead to exactly the same results (fragment presence and intensities, the quasi-molecular ion).

The expended spectra (Fig. 1-12) are available on request from the authors.

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