Amino Acids (1999) 16: 403–413

Amino
Acids

Spectral analysis of a series of partially protected and deprotected tetrapeptides, analogues of AS-I phytotoxin

Review Article

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Accepted October 10, 1998

Summary. A series of six tetrapeptides, analogues of AS-I phytotoxin, pathogenic to sunflower, have been synthesized either in solution and/or by solid phase methods and have been tested for phytotoxic activity in various plants and cytotoxic activity in three cancer cell lines. These peptides were identified as model compounds by fast atom bombardment (FAB), plasma desorption (PD), electrospray ionization (ESI) mass spectrometry and by ^{1}H, ^{1}H-^{1}H, ^{13}C and ^{1}H-^{13}C NMR. The data presented show that in protected tetrapeptides the molecular ion was easily identified whereas some difficulties appeared with the fully deprotected peptides. NMR spectra are given.

Keywords: Amino acids – Phytotoxin AS-I analogues – FAB – PD – ESI mass spectrometry – ^{1}H, ^{13}C and 2D NMR

Introduction

Structural elucidation and characterization of peptides requires a combination of a variety of chromatographic and spectroscopic techniques. An impressive array of modern analytical instruments and techniques is being used with increasing efficiency to identify and characterize complex biomolecules such as peptides and proteins. FAB, PD and ESI mass spectrometry can serve for analytical measurements and can give detailed structural information.

Structural elucidation and sequencing of peptides by Mass Spectrometry is considered to be the most popular technique today. In addition ^{1}H, ^{1}H-^{1}H, ^{13}C and ^{1}H-^{13}C NMR also serve as valuable tool for structural elucidation of peptides.

A series of six tetrapeptides, analogues of AS-I phytotoxin, pathogenic to sunflower were identified by the combination of the above mentioned

spectroscopic techniques. FAB and ESI mass spectra were obtained from partially protected and deprotected tetrapeptides to obtain the molecular ion peak in relative high intensities. The NMR data of the compounds confirmed their structure. Furthermore PD mass spectra were recorded from fully deprotected peptides.

Materials and methods

The tetrapeptides, reported here, were synthesized either in solution or/and by SPPS as reported (Liakopoulou-Kyriakides et al., 1997, 1998).

All reagents and solvents used were of analytical grade.

Mass spectra of the tetrapeptides

PD mass spectra of the fully deprotected tetrapeptides were obtained as reported (Liakopoulou-Kyriakides et al., 1997).

FAB mass spectra were recorded on a VG ZAB2F mass spectrometer equipped with FAB ion source. Matrix: glycerol or glycerol/H^+.

Electrospray ionization mass spectra were reported on a TSQ 7000 Finnigan MAT spectrometer. Conditions used: infusion 3μL/min using a Harrord syringe, Capillary temp. 200°C, Spray voltage +4.5 KV, sheath gas 35 units.

Sample preparation

A standard solution of each peptide 1mg/μL was prepared. The solvent for tetrapeptides II, III, IV and VI was MeOH/H_2O 50:50 containing 0.5% CH_3COOH, whereas for tetrapeptides I and V methanol containing 0.5% CH_3COOH was used.

HPLC separation of peptides

Partially protected peptides I–VI were purified by HPLC using Waters 991 Multi Chromatogram on Nucleosil 58 column. Gradient: 10% Acetonitrile → 90% Acetonitrile.

NMR measurements

Compounds I–VI were dissolved in dimethyl sulfoxide (DMSO-d_6) in 5 mm o.d. sample tubes. Tetramethylsilane (TMS) was used as an internal standard. The 400.13 and 100.62 MHz ^1H and ^{13}C NMR spectra, respectively, were obtained at 298 K on a Bruker AM 400 WB spectrometer. All the 90 transmitter and decoupler pulses were carefully calibrated (7.6-13.9μs).

The ^1H NMR spectra were obtained with presaturation of the solvent signal. The homonuclear Overhauser enhancements were determined by subtracting the unperturbed FID with off-resonance irradiation from the perturbed FID with on-resonance irradiation followed by Fourier transformation and phasing. The broad-band decoupled ^{13}C NMR spectra were acquired using the standard one-pulse, DEPT and spin-echo experiments.

^2D ^1H NMR chemical shift correlations were established using the standard techniques, COSY, TOCSY and ROESY, while for ^1H-^{13}C the HXCO shift correlation pulse sequence was used. The heteronuclear 2D, spin-echo and DEPT experiments were optimized for one-bond couplings of 140 Hz.

Results and discussion

All peptides reported here were identified previously by classical chemical techniques e.g. amino acid hydrolysis and determination of the products by HPLC, amino acid sequencing by Edman degradation. Here we report their spectroscopic analysis by mass spectra and ^{1}H, ^{1}H-^{1}H, and ^{1}H-^{13}C NMR. PD mass spectra were recorded on fully deprotected peptides. Table 1 gives the fragments of the positive ion mass spectra of three tetrapeptides. The nomenclature used for designating these sequence ions is that reported by Roepstorff and Fohlman, 1984; Biemann, 1988. As it can be concluded from this table the fragments A_1, A_3, I_2, I_3, I_4, Y''_1, Y''_2 and B_2 appeared for the three peptides studied whereas A_2, B_1 and Y''_3 appeared in some of them. From this fragmentation pattern their structure can be deduced. Table 2 gives the protonated molecular ions of the partially protected tetrapeptides as well as their peak intensity as they were estimated by ESI mass spectra. Similar results were obtained with FAB mass of the same peptides (data not shown). Purification of the partially protected peptides included in Table 2 was accomplished by HPLC as reported in Materials and Methods.

Analysis by electrospray requires the prior formation of analyte-related ions in solutions. For compounds that are not ionic, analyte-related ions are

Table 1. Positive ion PD mass spectra of the three tetrapeptides

Peptide	MH^+	A_1	A_2	A_3	I_2	I_3	I_4	Y''_1	Y''_2	Y''_3	B_1	B_2
Val-Ser-Gly-Glu	391	72	–	216	30	60	102	148	235	292	–	163
Ser-Val-Gly-Glu	391	60	158	216	72	30	57	148	205	–	88	187
Ser-Val-Glu-Gly	391	60	158	288	72	57	30	76	205	–	88	187

$A_1 = I_1$; $I_1 = H_2N - \overset{+}{C}H - R$; $Y_1 = \overset{+}{H_3}N - CH(R) - COOH$

Table 2. Protonated molecular ions of the six partially protected tetrapeptides obtained by electrospray ionization(ESI) mass spectroscopy

Peptide	$[M + H]^+$	Peak intensity
I Val-Ser(Bu^t)-Gly-Glu(Bu^t)-OH	503.1	very high
II Tyr(Bu^t)-Val-Gly-Glu(Bu^t)-OH	579.71	high
III Cys(Trt)-Val-Gly-Glu(Bu^t)-OH	705.88	Very small
IV His(Trt)-Val-Gly-Glu(Bu^t)-OH	739.88	relatively high
V Hse(Trt)-Val-Gly-Glu-(Bu^t)-OH	702.85	very small
VI Thr(Bu^t)-Val-Gly-Glu(Bu^t)-OH	517.1	very high

usually prepared by solution chemistry methods. For example, peptides and proteins can be analyzed in the positive ion mode after being dissolved in equal proportions of methanol and water together with acetic acid to effect protonation of basic amino acids. As it has been pointed out (Hakansson et al., 1989) the presence of certain impurities in peptides can lead to complex spectra. The presence of cations such as sodium or potassium can lead to the formation of adduct ions such as $[M + Na]^+$ and $[M + K]^+$. The electrospray method is, however unsatisfactory for highly aqueous solvents (Roepstorff,

Table 3. ^1H and ^{13}C NMR data for the peptides **I**, **II** and **III**

AA	Proton	δ-value (coupling)	Carbon	δ-value
		H_2N-Val-Ser(Bu^t)-Gly-Glu(Bu^t)-OH (**I**)		
Val	H_α*	4.09 (unresolved m)	C_α	59.57
	H_β	2.08 (m)	C_β	30.84
	H_γ	0.97 (d, J 6.8 Hz)	C_γ	19.12
	$H_{\gamma'}$	0.89 (d, J 6.8 Hz)	$C_{\gamma'}$	16.86
			C=O	171.55
Ser	H_α	4.29 (unresolved m)	C_α	53.42
	H_β	3.68 (dd, J 4.8 and 5.3 Hz)	C_β	61.56
	$H_{\beta'}$	3.60 (dd, J 6.1 and 5.8 Hz)	C=O	172.54
	NH	8.42 (br. s)		
Gly	H_α	3.86 (d, J 6.2 Hz)	C_α	42.22
	$H_{\alpha'}$	3.82 (d, J 6.0 Hz)	C=0	168.21
	NH	8.32 (unresolved t)		
Glu	H_α*	4.09 (unresolved m)	C_α	51.57
	H_β	1.97 (m)	C_β	26.96
	$H_{\beta'}$	1.81 (m)	C_γ	31.30
	$H_\gamma/H_{\gamma'}$	2.226 (br. m)	C_δ	173.39
	NH	7.73 (d, J 7.1 Hz)	C=0	169.90
Others	Bu^t(Ser)	1.17 (s)	Bu^t(Ser)	72.85
				27.19
	Bu^t(Glu)	1.44 (s)	Bu^t(Glu)	79.57
				27.78
		H_2N-Tyr(Bu^t)-Val-Gly-Glu(Bu^t)-OH (**II**)		
Tyr	H_α	3.57 (unresolved m)	C_α	55.72
	H_β	2.97 (dd, J 8.7 and 9.6 Hz)	C_β	35.97
	$H_{\beta'}$	2.67 (dd, J 8.2 and 9.1 Hz)	C_{ar}	153.83
				132.68
				129.77
				123.13
	H_m	7.13 (d, J 8.3 Hz)	C=O	172.68
	H_o	6.86 (d, J 8.3 Hz)		
Val	H_α	4.13 (unresolved dd)	C_α	57.66
	H_β	1.97 (m)	C_β	30.43
	$H_\gamma/H_{\gamma'}$	0.82 (dd, J 7.0 and 7.0 Hz)	C_γ	19.09
	NH	8.17 (br. s)	$C_{\gamma'}$	17.97
			C=O	171.53

1990). The spectrum quality is much poorer in the presence of alkali metal salts (Roepstorff, 1989; Roepstorff et al., 1990).

A much more satisfactory procedure for peptides and proteins is to adsorb the sample onto a nitrocellulose layer that has previously been deposited on the sample foil. The main advantage of nitrocellulose is the ability to remove metal ions, by rinsing with deionized water or mild acid solutions, while adsorbed peptides remain bound to the surface.

PDMS developed (Macfarlane and Torgerson, 1976) somewhat earlier than FAB has always been able to analyze higher molecular weight samples than FAB, principally because of the very much more energetic primary

Table 3. *Continued*

AA	Proton	δ-value (coupling)	Carbon	δ-value
Gly	$H_\alpha/H_{\alpha'}$	3.72 (d, J 5.7 Hz)	C_α	41.93
	NH	8.35 (unresolved t)	C=O	168.93
Glu	H_α	4.09 (unresolved m)	C_α	51.48
	H_β	1.91(m)	C_β	26.90
	$H_{\beta'}$	1.76 (m)	C_γ	31.25
	$H_\gamma/H_{\gamma'}$	2.19 (m)	C_δ	173.26
	NH	7.82 (d, J 6.9 Hz)	C=O	170.96
Others	Bu^t(Tyr)	1.26 (s)	Bu^t(Tyr)	77.54
				27.76
	Bu^t(Glu)	1.37 (s)	Bu^t(Glu)	79.61
				28.61
		H_2N-Cys(Trt)-Val-Gly-Glu(Bu^t)-OH (**III**)		
Cys	H_α	3.26 (m)	C_α	54.01
	H_β	2.37 (dd, J 5.5 and 5.1 Hz)	C_β	31.53
	$H_{\beta'}$	2.30 (dd, J 7.2 and 7.7 Hz)	C=O	172.65
Val	H_α	4.17 (unresolved dd)	C_α	57.79
	H_β	1.96 (m)	C_β	31.24
	$H_\gamma/H_{\gamma'}$	0.82 (dd, J 6.7 and 6.7 Hz)	C_γ	19.61
	NH	8.00 (unresolved d)	$C_{\gamma'}$	18.35
			C=O	172.10
Gly	H_α	3.88 (dd, J 3.7 and 6.1 Hz)	C_α	42.05
	$H_{\alpha'}$	3.70 (dd, J 5.8 and 5.4 Hz)	C=O	168.91
	NH	8.29 (t, J 2.6 and 3.8 Hz)		
Glu	H_α	4.13 (m)	C_α	51.72
	H_β	1.92 (m)	C_β	27.15
	$H_{\beta'}$	1.74 (m)	C_γ	36.97
	$H_\gamma/H_{\gamma'}$	2.19 (br. m)	C_δ	173.44
	NH	7.96 (d, J 8.0 Hz)	C=O	171.33
Others	Trt	7.31–7.11 (m)	Trt	144.83
				129.53
				128.45
				127.15
	Bu^t	1.38 (s)	Bu^t	80.13
				28.17

*The Val-H_α proton and the Glu-H_α resonate in the same δ region.

beam particles. Thus, until the introduction of the electrospray ionization, PDMS was the method of choice for the analysis of high molecular weight samples.

The principle of the FAB method is known (Barber et al., 1982) and more details can be found in relevant textbooks. According to this method, a beam of energetic particles, directed to strike a sample film carried on a clean metal support, produces an intense thermal spike whose energy is then dissipated through the outer layer of the sample surface.

Table 4. ^{1}H and ^{13}C NMR data for the peptides **IV**, **V** and **VI**

AA	Proton	δ-value (coupling)	Carbon	δ-value
		H_2N-His(Trt)-Val-Gly-Glu(Bu^t)-OH (**IV**)		
His	H_α*	3.98 (unresolved m)	C_α	54.11
	H_β	2.89 (dd, J 3.8 and 4.6 Hz)	C_β	28.74
	$H_{\beta'}$	2.72 (dd, J 4.2 and 5.1 Hz)	C_2	138.14
	H_2	8.54 (br. s)	C_4	119.16
	H_4	6.76 (s)	C_5	136.94
			C=O	172.75
Val	H_α	4.09 (br. m)	C_α	57.83
	H_β	2.00 (m)	C_β	30.24
	$H_\gamma/H_{\gamma'}$	0.82 (dd, J 6.8 and 6.8 Hz)	C_γ	19.26
	NH	7.63 (br. s)	$C_{\gamma'}$	18.03
			C=O	171.88
Gly	H_α	3.69 (br. dd, J 4.1 and 5.3 Hz)	C_α	42.13
	$H_{\alpha'}$	3.65 (br. dd, J 4.8 and 5.6 Hz)	C=O	168.19
	NH	8.54 (unresolved t)		
Glu	H_α*	3.98 (unresolved m)	C_α	52.30
	H_β	1.87 (m)	C_β	27.32
	$H_{\beta'}$	1.72 (m)	C_γ	31.23
	H_γ	2.15 (m)	C_δ	173.10
	$H_{\gamma'}$	2.08 (m)	C=O	171.05
	NH	7.33 (unresolved d)		
Others	Trt	7.40–7.35 (m) and 7.08 (m)	Trt	142.17
				129.25
				128.20
				128.05
	Bu^t	1.35 (s)	Bu^t	79.46
				27.77
		H_2N-Hse(Trt)-Val-Gly-Glu(Bu^t)-OH (**V**)		
Hse	H_α	3.47 (m)	C_α	51.74
	H_β	2.02 (m)	C_β	33.75
	$H_{\beta'}$	1.78 (m)	C_γ	60.44
	$H_\gamma/H_{\gamma'}$	3.13 (m)	C=O	172.51
Val	H_α	4.13 (br. t)	C_α	57.49
	H_β	1.94 (m)	C_β	30.48
	$H_\gamma/H_{\gamma'}$	0.80 (dd, J 6.9 and 6.9 Hz)	C_γ	19.12
	NH	8.22 (br. s)	$C_{\gamma'}$	18.00
			C=O	171.61

Barber's original work used fast neutral atoms e.g. Ar^0, or Xe^0, whereas later workers (Aberth et al., 1982) introduced the use Cs^+. With a dry deposited sample there is a rapid decay of the secondary ion yield due to surface damage by the intense incident beam. This can be circumvented by preparing the sample in a liquid matrix such as glycerol (Barber et al., 1981; Barber et al., 1982). This liquid medium is then able to provide continuous surface renewal so that intense primary beam may be used (Magee, 1983).

By means of FAB-MS analysis of peptides containing side protection we have observed that the predominant signal often is not the protonated mo-

Table 4. *Continued*

AA	Proton	δ-value (coupling)	Carbon	δ-value
Gly	$H_\alpha/H_{\alpha'}$	3.69 (d, J 5.6 Hz)	C_α	41.72
	NH	8.37 (t, J 5.0 and 5.8 Hz)	C=O	168.23
Glu	H_α	4.09 (unresolved m)	C_α	51.56
	H_β	1.90 (m)	C_β	26.87
	$H_{\beta'}$	1.73 (m)	C_γ	31.03
	$H_\gamma/H_{\gamma'}$	2.19 (m)	C=O	170.90
	NH	7.84 (d, J 7.5 Hz)	C_δ	172.83
Others	Trt	7.37–7.23 (m)	Trt	143.84
				128.09
				126.83
	Bu^t	1.36 (s)	Bu^t	79.47
				27.63
		H_2N-Thr(Bu^t)-Val-Gly-Glu(Bu^t)-OH (**VI**)		
Thr	H_α	3.94 (m)	C_α	57.64
	H_β	3.23 (d, J 3.7 Hz)	C_β	67.36
	H_γ	1.09 (s)	C_γ	19.81
			C=O	171.12
Val	H_α	4.15 (br.t)	C_α	58.89
	H_β	1.97 (m)	C_β	31.04
	$H_\gamma/H_{\gamma'}$	0.88 (dd, J 4.7 and 4.7 Hz)	C_γ	19.08
	NH	8.31 (br. s)	$C_{\gamma'}$	18.01
			C=O	171.68
Gly	$H_\alpha/H_{\alpha'}$	3.71 (d, J 5.9 Hz)	C_α	44.64
	NH	8.42 (t, J 4.8 and 5.7 Hz)	C=O	168.12
Glu	H_α	4.07 (m)	C_α	54.35
	H_β	1.92 (m)	C_β	27.00
	$H_{\beta'}$	1.74 (m)	C_γ	31.16
	$H_\gamma/H_{\gamma'}$	2.19 (m)	C_δ	172.90
	NH	7.80 (d, J 7.5 Hz)	C=O	170.90
Others	Bu^t(Thr)	1.10 (s)	Bu^t(Thr)	73.39
				27.63
	Bu^t(Glu)	1.38 (s)	Bu^t(Glu)	81.47
				28.19

* The His-H_α proton and the Glu-H_α resonate in the same δ region.

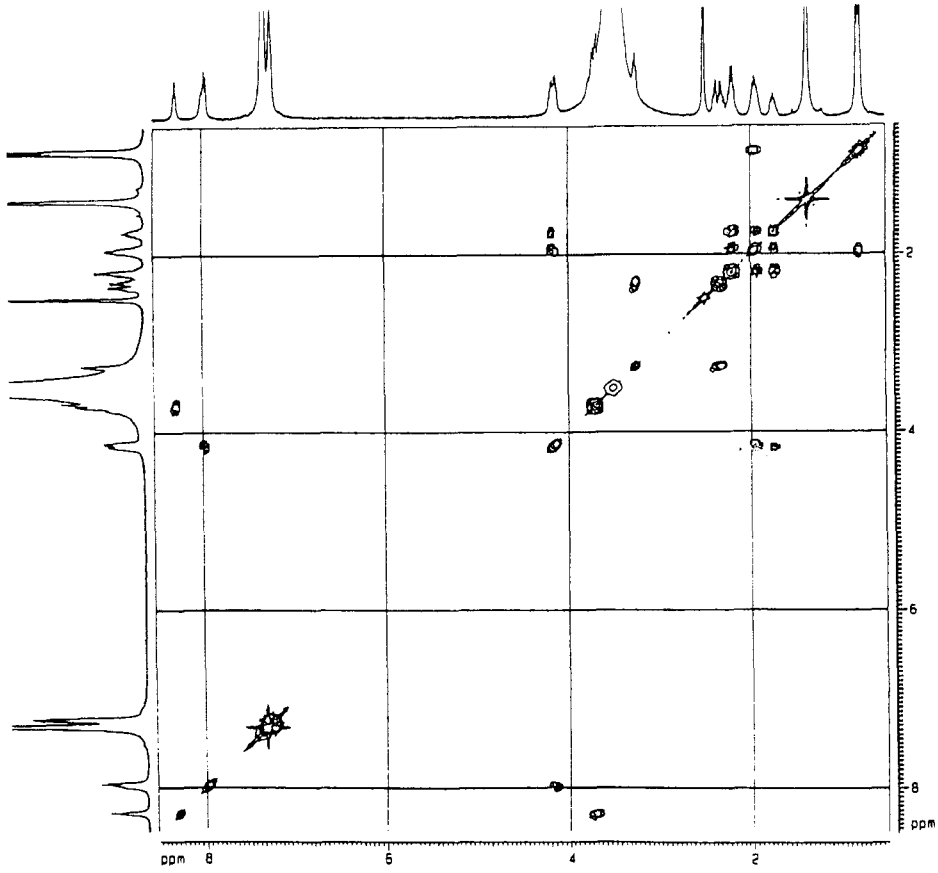

Fig. 1. COSY spectrum of peptide Cys(Trt)-Val-Gly-Glu(Bu^t) -OH (III) in dimethyl sulfoxide (DMSO-d_6)

lecular ion $[M + H]^+$, but instead corresponds to ions that have eliminated multiple oxygen atoms $[M - O + H]^+$.

Tables 3 and 4 give the NMR data of the same six peptides. Since the NMR spectra of a polypeptide chain depend primarily on the amino acid composition and can also be affected by the amino acid sequence, NMR can be employed as a complementary technique for the chemical investigation of the covalent structures of polypeptide chains.

Peak assignments in the ^1H NMR spectra of the tetrapeptides were made on the basis of two dimensional COSY and TOCSY spectral analysis. ^{13}C NMR assignments were made by using spin-echo and DEPT experiments and

Fig. 2. HXCO spectrum of peptide Cys(Trt)-Val-Gly-Glu(Bu^t) -OH (III) in dimethyl sulfoxide (DMSO-d_6)

Fig. 3. TOCSY spectrum of peptide Cys(Trt)-Val-Gly-Glu(Bu^t) -OH (III) in dimethyl sulfoxide (DMSO-d_6)

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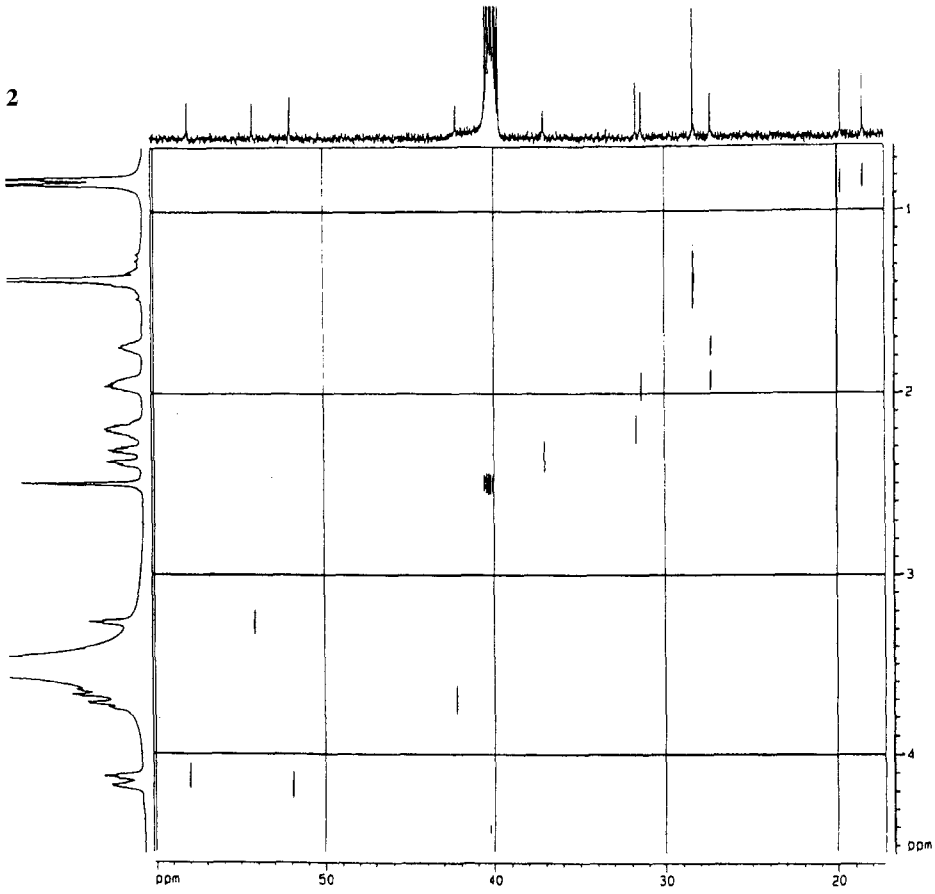

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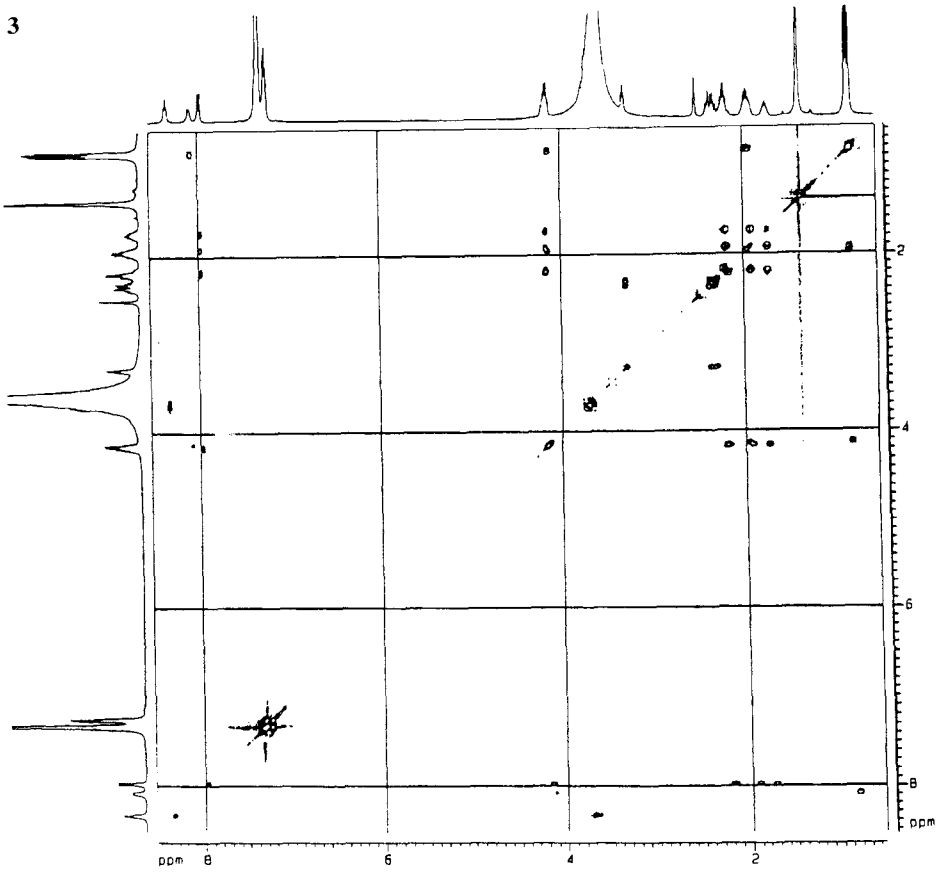

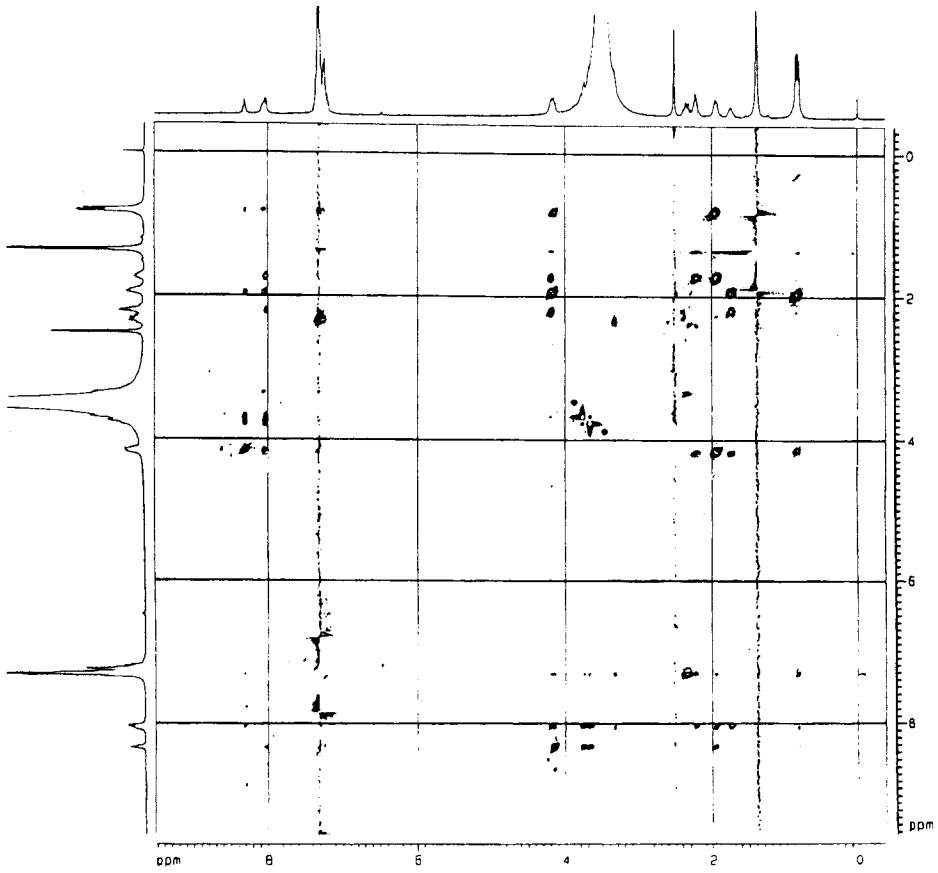

Fig. 4. ROESY spectrum of peptide Cys(Trt)-Val-Gly-Glu(Bu^t) -OH (III) in dimethyl sulfoxide (DMSO-d_6)

two dimensional ^{1}H-^{13}C correlation spectroscopy with detection at the ^{1}H frequency (inverse experiment). This experiment involves ^{1}H-detected heteronuclear multiple quantum coherence (HXCO) providing information about C-H carbon atoms via $^1J_{CH}$ scaler coupling. ROESY experiments, applicable in peptides of low molecular weight, provide information for intramolecular constrains (Kessler et al., 1988; Magafa et al., 1998). Tables 3 and 4 give the ^{1}H and ^{13}C NMR data of the peptides I–VI. As it is shown in these tables similar results are obtained for all of them. The H^α protons of all amino acids incorporated into the tetrapeptides absorb between 3.26–4.17 ppm, H^β protons from 1.72–3.23 ppm and H^γ from 0.80–2.19 ppm (δ-value). The GluNH proton resonates at higher field than the other NH protons. On the other hand the NH proton of the second amino acid in the chain (Val or Ser) gives a broad single peak. Experiments with D_2O showed that all NH protons are exchanged very slowly. The ROESY spectra of all peptides showed a series of crosspeaks, other than the sequential ones, like the following: GluNH-GlyNH, GluNH-GlyH_α, GlyNH-GluH_α, GlyNH-ValH_β and GluH_α-ValH_γ. These crosspeaks provide evidence that the corresponding

protons are close to each other in space in a turn-type structure adopted by the peptides. Figures 1–4 show the characteristic NMR spectra obtained with all the above mentioned techniques for peptide III.

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Received February 23, 1998