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500 MHz NMR characterization of synthetic bombesin and related peptides in DMSO-d_6 by two-dimensional techniques

Carlo Di Bello, Luigia Gozzini*, Mauro Tonellato, Maria Grazia Corradini+, Gabriella D'Auria+, Livio Paolillo+ and Enrico Trivellone°

*Institute of Industrial Chemistry, University of Padua, Padua, *Farmitalia-Carlo Erba spa, Chemical Research and Development, Milan, +Department of Chemistry, University of Naples, Naples* and *°Institute of Chemistry of Molecules of Biological Interest, CNR, Arcofelice, Naples, Italy*

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The proton NMR characterization of bombesin has been carried out at 500 MHz in DMSO-d_6 using two-dimensional homo- and ^1H-^{13}C hetero-correlated techniques. All resonances in the NMR spectra have been assigned and several coupling constants have been measured. The backbone $J_{\alpha CH-NH}$ coupling constants have constant values that vary between 7.8 and 8.2 Hz and indicate an unfolded structure in DMSO-d_6. Discrepancies with data recently obtained at 300 MHz [(1987) Eur. J. Biochem. 168, 193–199] are discussed.

Bombesin; NMR Resonance assignment

1. INTRODUCTION

Bombesin is a tetradecapeptide of formula

pGlu-Glu-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

originally isolated from frog skin by Anastasi et al. [1]. Because of its many biological acitivites [2,3], active research has been performed on bombesin and related peptides. Recently, after our preliminary report [4] on the NMR characterization of bombesin, an NMR work carried out at 300 MHz appeared in the literature [5]. Since several differences in the NH and α-CH assignments were observed, we wish to present our data at 500 MHz and discuss some structural implications.

2. EXPERIMENTAL

2.1. *Synthesis of bombesin*

Bombesin was synthesized using solid-phase methods [6] and purified according to standard procedures [7].

Correspondence address: L. Paolillo, Department of Chemistry, Via Mezzocannone 4, 80134 Naples, Italy

2.2. *NMR measurements*

NMR experiments were carried out with the help of a Bruker WM 500 located at the Institute of Molecules of Biological Interest, CNR, Naples. One- and two-dimensional (2D) spectra were run in DMSO-d_6 (99.99% deuterium purity, Aldrich) at 298 K and at a concentration of ~5 mg/ml. 2D experiments were of the homonuclear-correlated type such as COSY, COSY relayed, NOESY, double quantum and *J*-resolved. ^1H-^{13}C hetero-correlated 2D spectra were also used in order to assign resonances in the highly crowded aliphatic region. Typical parameter values for 2D spectra are reported in the figure legends. TMS was used as internal reference in all spectra.

3. RESULTS

In fig.1a and b the low- and high-field regions of the 500 MHz proton NMR spectrum of bombesin in DMSO-d_6 are shown. Fig.1 demonstrates that even at 500 MHz there are vast signal superimpositions in the NH and α-CH regions. Therefore, an assignment of the resonances can only be achieved with the use of 2D techniques. In fig.2 an expanded region of the COSY spectrum of bombesin shows NH α-CH proton correlations. Despite the good resolution of the cross-peaks in this region, only the following patterns can be clearly identified from the COSY spectrum: three ABMX patterns

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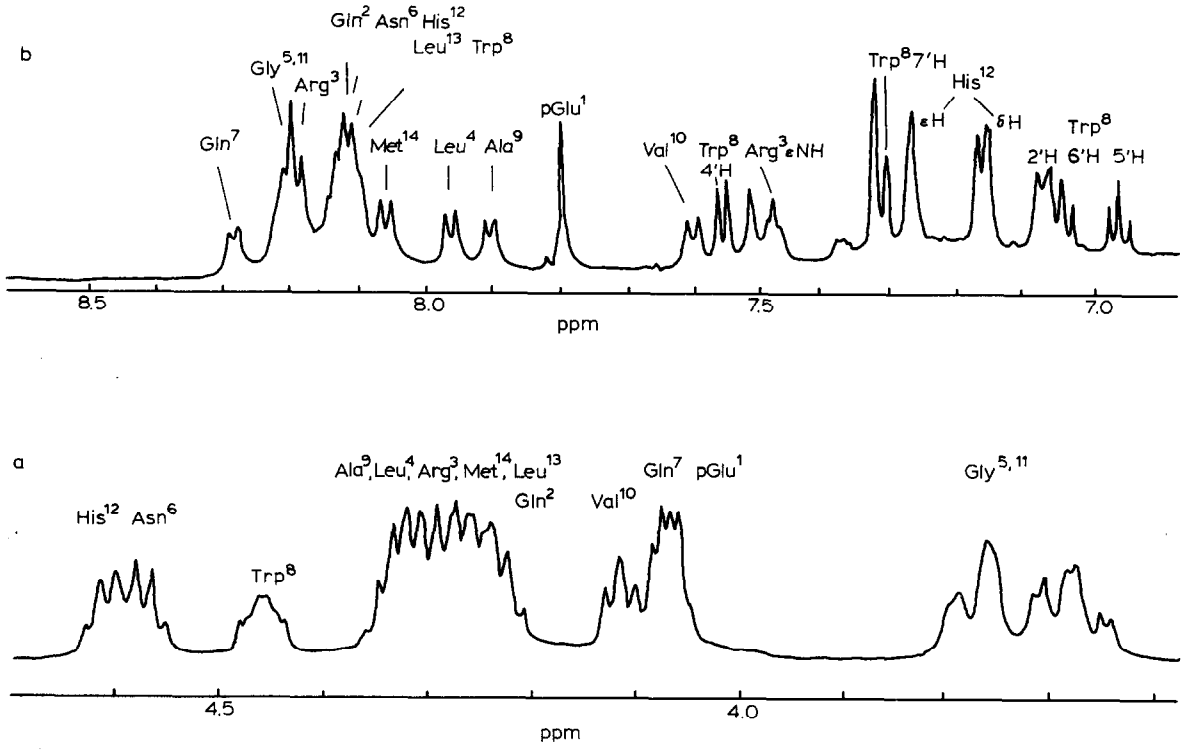

Fig.1. 500 MHz spectrum of bombesin in DMSO-d_6 at a concentration of 5 mg/ml. (a) α-CH region from 3.5 to 4.7 ppm. (b) NH region from 6.8 to 8.6 ppm. Assignments are taken from table I.

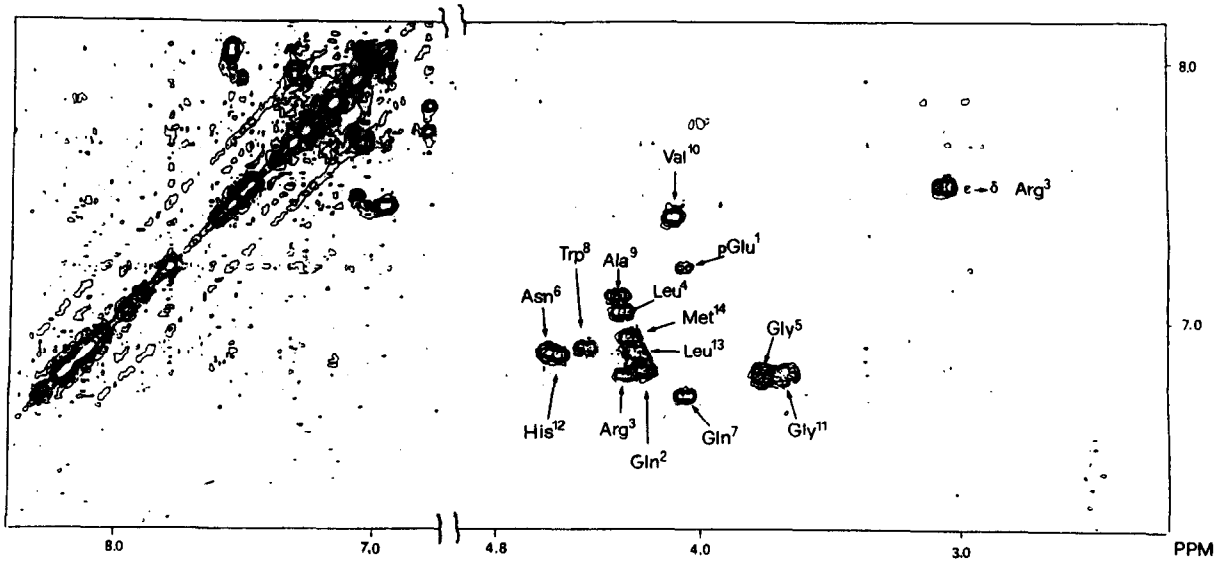

Fig.2. Expansion of the COSY spectrum of bombesin. The cross-peaks correlating NH and α-CH resonances are shown in detail. Spectral parameters: 2K data points in the F_2 dimension, 1K data points in the F_1 dimension, sine bell transformation in both domains.

belonging to Asn-6, Trp-8 and His-12 residues; two ABX patterns attributed to Gly-5 and Gly-11 and one A_3MX pattern due to Ala-9. The Arg-3 system is identified from the guanidino ϵ-NH triplet at 7.48 ppm which is connected to the δ-protons and consequently, although not easily, the β-protons can be located in the very crowded region around 1.5 ppm. The identification of Leu-4, Leu-13 and Val-10 systems is more difficult and exceedingly hard for the pGlu-1, Gln-2, Gln-7 and Met-14 systems that possess rather similar side chains. Some of the correlations found via COSY can be further proved with a COSY relayed coherence transfer experiment as in the case of His-12, Asn-6, Trp-8 and Val-10 systems. Many other correlations belonging to the systems of Gln-7, Gln-2, Arg-3, Met-14, Leu-4, Leu-13 can also be observed. ^1H-^{13}C heteronuclear correlated experiments proved to be crucial for assigning proton resonances in the crowded region (fig.3). Thus, it was possible to obtain chemical shift values for Leu and Val β-proton positions. In addition, double quantum experiments (not shown) demonstrate unequivocally the exact correspondence of correlated α-CH and NH signals. This is particularly important in the areas around 8.26–8.09 and 4.35–4.20 ppm. The experiments outlined thus far, however, provide sufficient information to assign unambiguously only NH, α-CH and side chain resonances of Ala-9, Val-10 and Arg-3 residues. The assignment of all other signals to specific residues along the peptide chain depends heavily on the existence of through-space interactions between backbone protons. In fig.4 a NOESY 2D spectrum is shown in which NOE cross-peaks between NH and α-CH protons due to sequential interactions between neighbouring residues have been identified. Thus, the assigned Ala-9 NH interacts with the α-CH of the preceding Trp-8 residue making Trp proton assignments unambiguous. Trp-8 NH in turn is connected to Gln-7 α-CH etc. Therefore, the NOE contacts can effectively allow the specific

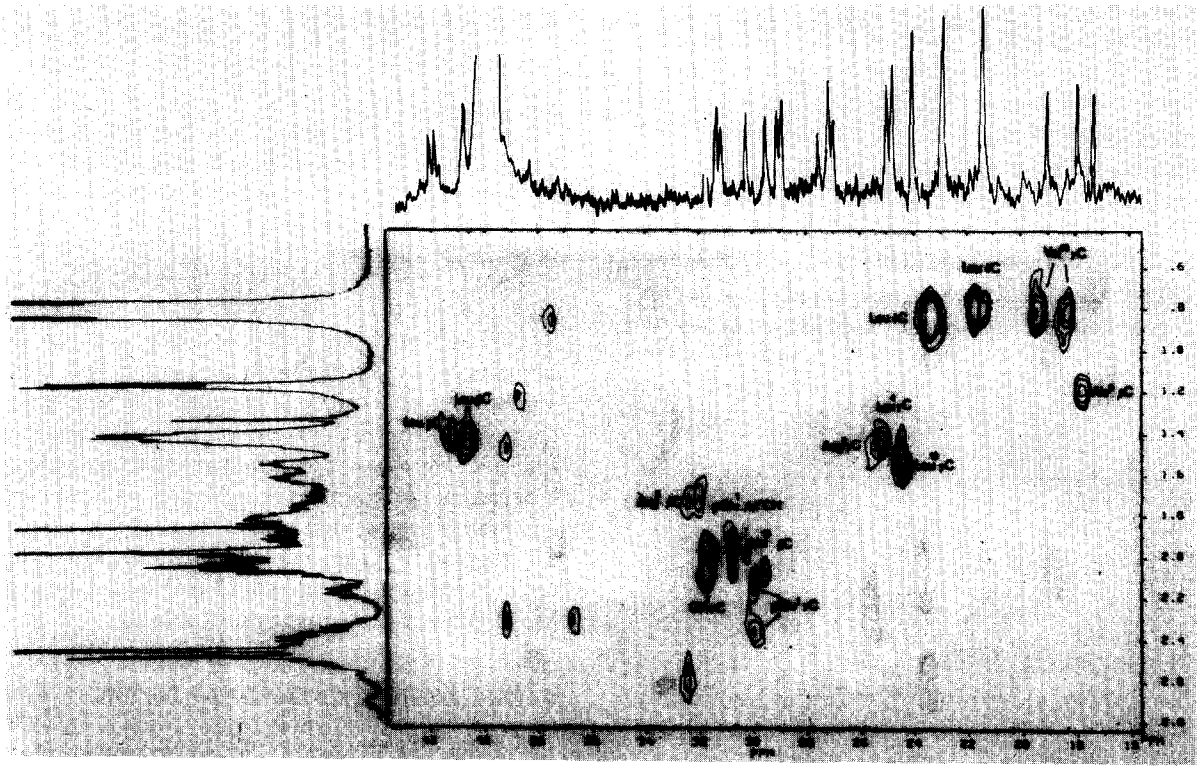

Fig.3. ^1H-^{13}C heterocorrelated 2D NMR spectrum of bombesin in DMSO-d_6. Experimental conditions: 2K data points in F_2, 512 W data points in F_1. Sine bell transformation in both domains.

Table 1

Chemical shifts δ (ppm) and coupling constants J (Hz) of bombesin in DMSO-d_6

Amino acid	NH	α-CH	$^3J_{NH-\alpha CH}$	β-CH		Others
pGlu-1	7.79	4.05	0	2.21	1.89	γ-CH_2 2.10
Gln-2[a]	8.13	4.21	8.6	1.87	1.73	γ-CH_2 2.10
Arg-3	8.19	4.28	7.8	1.53		γ-CH_2 1.54 δ,δ'-CH_2 3.07 ϵ-NH 7.48
Leu-4	7.96	4.30	8.0	1.44		γ-CH 1.58 δ-CH_3 0.85 δ'-CH_3 0.82
Gly-5	8.20	3.77 3.69	6.0			
Asn-6[a]	8.11	4.57	7.8	2.56	2.44	
Gln-7[a]	8.28	4.08	8.4	1.82	1.68	γ-CH_2 2.03
Trp-8	8.10	4.49	8.1	3.15	3.00	NH 10.79 H-2 7.09 H-4 7.58 H-5 6.95 H-6 7.08 H-7 7.32
Ala-9	7.90	4.35	6.7	1.21		
Val-10	7.60	4.12	8.2	1.98		γ-CH_3 0.86 0.84
Gly-11	8.20	3.77 3.66	5.5			
His-12	8.14	4.61	7.0	3.08	2.95	H-4' 7.16 H-2' 7.27
Leu-13	8.13	4.25	8.3	1.49		γ-CH 1.56 δ-CH_3 0.85 δ'-CH_3 0.89
Met-14	8.07	4.28	7.8	1.94	1.79	γ-CH_2 2.42 2.37 ϵ-CH_3 2.05

[a] The side chain NH_2 protons of Asn-6, Gln-2 and Gln-7 were observed at 6.79, 7.32; 7.08, 7.51; 6.79, 7.17 ppm but were not specifically assigned

Table 2

Chemical shifts δ (ppm) of carboxy-terminal nonapeptide and amino-terminal hexapeptide of bombesin in DMSO-d_6

Amino acid	NH		α-CH	
	Nona-peptide	Hexa-peptide	Nona-peptide	Hexa-peptide
pGlu-1		7.81		4.08
Glu-2		8.24		4.22
Arg-3		8.08		4.25
Leu-4		8.10		4.28
Gly-5		8.26		3.71
Asn-6		8.05	4.09	4.47
Gln-7	8.21		4.24	
Trp-8	8.19		4.51	
Ala-9	8.09		4.38	
Val-10	7.74		4.15	
Gly-11	8.21		3.70	
His-12	8.15		4.62	
Leu-13	8.16		4.25	
Met-14	8.12		4.27	

assignments of the amino acid system from Asn-6 to Leu-13 to be made (table 1). NOE effects are observed for the peptide units going from pGlu-1 to Gly-5. At any rate Leu-4 and Gly-5 can be identified because the positions of Leu-13 and Gly-11 are known. Therefore, there are still ambiguities concerning the assignment of pGlu-1, Gln-2 and Met-14. These were assigned by analogy with the C-terminal nonapeptide Asn-Met (6–14) and the N-terminal hexapeptide pGlu-Asn (1–6), on the assumption that the chemical shifts for N-terminal and C-terminal peptide units (pGlu-1, Gln-2 and Met-14) are almost invariant (table 2).

4. DISCUSSION AND CONCLUSION

A comparison with the published data [5] reveals several differences in the NH and α-CH assignments. In particular, in the NH region the resonance at 8.27 ppm was assigned to the pGlu-1

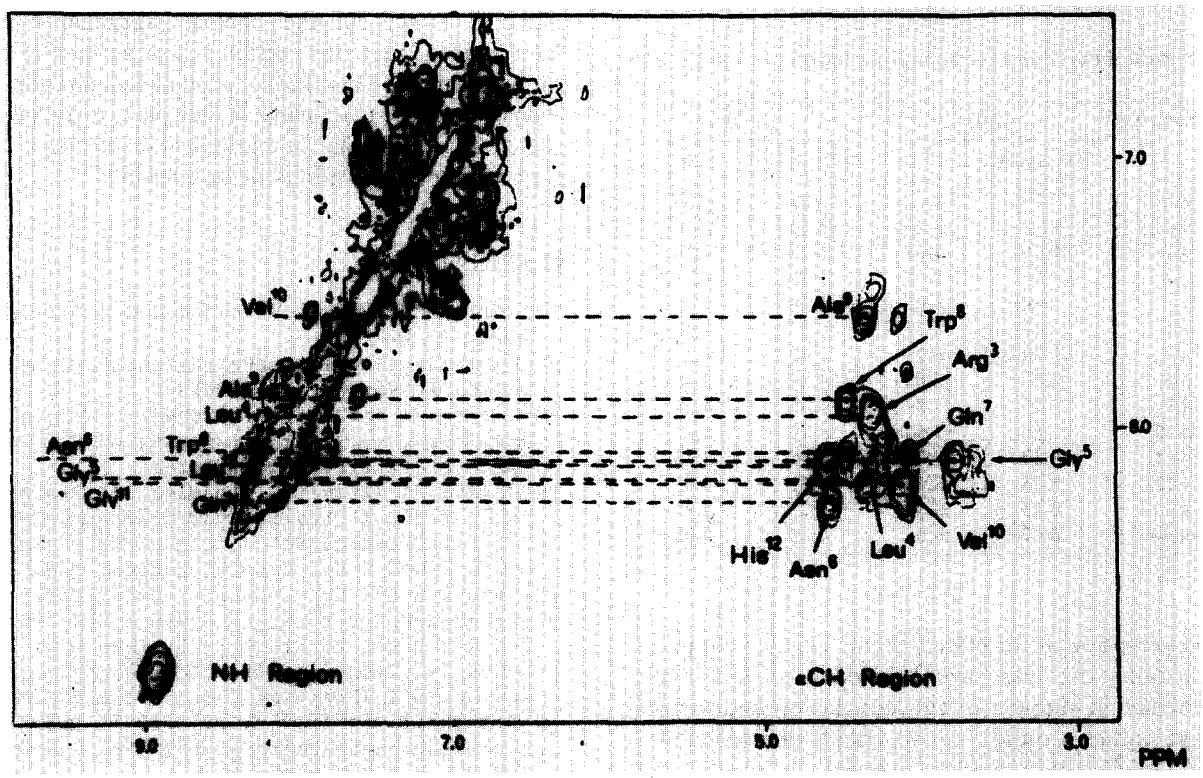

Fig.4. NOESY spectrum of bombesin in DMSO-d_6. Only the expansion of the region including NH and α-CH cross-peaks is presented. Spectral parameters as in fig.2. Mixing time for polarization transfer, 200 ms.

system [5]. We find, instead, that the pGlu NH is located at 7.79 ppm (singlet with $J_{NH\text{-}\alpha CH} = 0$ Hz) (fig.1). The three resonances around 8.20 ppm are instead assigned to the two glycines and to Arg-3 whereas in [5] they were assigned to Gly-5, Gly-7, Asn-6 and Gln-2. We found Gln-2 and Asn-6 NH resonances at 8.13 and 8.11 ppm; Arg-3 NH assigned in [5] at 8.08 ppm occurs instead at 8.19 ppm. Leu-13 NH, assigned at 8.19 ppm, is found instead at 8.13 ppm. The remaining NH resonances, i.e. Leu-4, Trp-8, Ala-9, Val-10, His-12 and Met-14, show differences in chemical shift that vary between 0.01 and 0.04 ppm. It is worth pointing out that Ala, Met, Leu and Val NH resonances are the only ones to be observed separately from the rest of the NH envelope. A comparison of our assignments and those of [5] is given in table 3. The data shown in table 1, obtained not only at a higher magnetic field (500 MHz) but also with a wide range of different 2D NMR techniques, e.g. COSY, COSYRCT, DQ, *J*-resolved, ^1H-^{13}C HETCOR, NOESY experiments, can be clearly considered more reliable.

From a structural point of view the NMR parameters such as chemical shifts and coupling constants demonstrate that bombesin does not possess a defined structure in solution. Peptide bonds are characterized by coupling constants that are of the order of 7.8–8.2 Hz (table 1). These values being almost constant indicate an average over many possible conformations [8,9]. The peptide chain then seems to be quite mobile and in fact amide NH temperature coefficients ($3\text{–}4 \times 10^{-3}$ $\Delta\delta/\Delta T$) demonstrate [10,11] that there is no great tendency to form intramolecular hydrogen bonds which are indicative of secondary structures. For these reasons, it is not surprising that despite the chemical shift differences, we agree with the conclusions in [5]. Furthermore, the side chain protons do not show the substantial chemical shift differences found in rigid or cyclic peptides and in fact β-protons have very similar chemical shift values with

Table 3

Chemical shifts δ (ppm) of bombesin compared to those of [5]

Amino acid	NH		α-CH	
	This work	[5]	This work	[5]
pGlu-1	7.79	8.27	4.05	4.09
Glu-2	8.13	8.23	4.21	4.26
Arg-3	8.19	8.08	4.28	4.28
Leu-4	7.96	8.00	4.30	4.31
Gly-5	8.20	8.23	3.69	3.75
Asn-6	8.11	8.26	4.57	4.59
Gln-7	8.28	8.23	4.08	4.26
Trp-8	8.10	8.08	4.49	4.48
Ala-9	7.90	7.89	4.35	4.34
Val-10	7.60	7.64	4.12	4.12
Gly-11	8.20	8.23	3.66	3.75
His-12	8.14	8.14	4.61	4.62
Leu-13	8.13	8.19	4.25	4.25
Met-14	8.07	8.08	4.28	4.28

the exception of the pGlu system. The $J_{\alpha\text{-}\beta}$ coupling constants that can be measured provide further evidence in support of this conclusion and in fact have values around 5.5–7.0 Hz [8] demonstrating an even distribution of the possible conformational rotamers. Nevertheless, although coupling constants and backbone chemical shifts do not offer evidence of preferred structures in solution, the relaxation data indicate that a different mobility along the peptide chain exists. In fact, the existence of NOE effects in the peptide fragment 6–13 demonstrates that this section is not as mobile as the N-terminus. This finding may reflect a tendency to a possible structuring of this part of the peptide chain, indicating the biological importance of the nonapeptide terminus of bombesin.

REFERENCES

[1] Anastasi, A., Erspamer, V. and Bucci, M. (1971) Experientia, 27, 166–167.
[2] Walsh, J.H. (1983) in: Brain Peptides (Krieger, D.T. et al. eds) pp. 941–960, Wiley, New York.
[3] Erspamer, V. and Melchiorri, P. (1983) in: Neuroendocrine Perspectives (Muller, E.E. and McLeod, R.M. eds) pp. 37–106, vol.2, Elsevier, Amsterdam.
[4] Di Bello, C., Gozzini, L., Tonnellato, M., Corradini, M.G., D'Auria, G., Paolillo, L. and Trivellone, E. (1987) in: Peptide Chemistry 1987 (Shiba, T. and Sakakibara, S. eds) pp. 63–66, Protein Research Foundation, Osaka.
[5] Carver, J.A. (1987) Eur. J. Biochem. 168, 193–199.
[6] Stewart, J.M. and Young, J.D. (1984) in: Solid Phase Peptide Synthesis, 2nd edn, Pierce, Rockford, IL.
[7] Di Bello, C., Lucchiari, A., Buso, O., De Castiglione, R. and Gozzini, L. (1986) Gazz. Chim. Ital. 116, 221–228.
[8] Deslaurier, R. and Smith, I.C.P. (1980) in: Biological Magnetic Resonance (Berliner, J. and Reuben, J. eds) pp. 243–344, vol.2, Plenum, New York.
[9] Goodman, M., Ribeiro, A.A. and Naider, F. (1978) Proc. Natl. Acad. Sci. USA 75, 4647–4651.
[10] Kopple, K.D., Ohnishi, M. and Go, A. (1969) Biochemistry 8, 4087–4095.
[11] Llinas, M. and Klein, M.P. (1975) J. Am. Che. Soc. 97, 4731–4737.