THE CONFORMATIONAL PROPERTIES OF THE PEPTIDE HORMONE SOMATOSTATIN (III)

Assignment and analysis of the ^{1}H and ^{13}C high resolution NMR spectra of somatostatin in aqueous solution

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1. Introduction

The peptide hormone somatostatin (SRIF) possesses a broad range of physiological activities [1], that have been shown to be related to its conformational properties by the synthesis of analogs with restricted mobility [2]. The conformational properties of somatostatin itself have until now mainly been studied by CD [3] although some SRIF fragments have been analysed by a combination of high resolution NMR and semi-empirical energy calculations [4,5]. NMR studies have been hindered by severe line broadening, aggregation at $\gtrsim$15 mM and by signal assignment problems as one can easily see from the sequence containing 7 ABX spin systems:

NH_2-Ala^{1}–Gly^{2}–Cys^{3}–Lys^{4}–Asn^{5}–Phe^{6}–Phe^{7}–

Trp^{8}–Lys^{9}–Thr^{10}–Phe^{11}–Thr^{12}–Ser^{13}–Cys^{14}-OH

A fully assigned ^{1}H spectrum of SRIF in $^{2}H_2O$ has appeared in [6]. However, the resolution of the spectrum did not allow the determination of coupling constants except for those of a few amide proton resonances.

We present here a complete spin system analysis of all amino acids in somatostatin (except for the Lys sidechains) that was derived from a series of ^{1}H spectra obtained by a combination of one- and two-dimensional NMR experiments.

By ^{13}C spectroscopy, ^{13}C-selective $\{^{1}H\}$ decouplings, photo-CIDNP experiments and the use of a few carefully designed analogs we have been able to assign all spin systems except for Phe^{6} and Phe^{7}. The $^{3}J_{NH-C_\alpha H}$ coupling constants and the temperature coefficients ($\Delta\delta/\Delta T$) of all amide proton signals have also been determined.

Our data strongly suggest that in aqueous solution SRIF forms a rapidly exchanging average between several low energy conformations. However, evidence for conformational stability (lower $\Delta\delta/\Delta T$ values) has been found in the range Trp^{8}–Thr^{12}. The $^{3}J_{NH-C_\alpha H}$ coupling constants are incompatible with a turn of type I or III [7] although type II turns in the range Trp^{8} through Thr^{12} could contribute to the conformational equilibrium.

2. Materials and methods

The ^{1}H and ^{13}C NMR data were obtained with a Bruker HX 270 MHz spectrometer working in the FT mode and equipped with an Aspect 2000 computer. ^{1}H homodecoupling experiments were recorded both in the standard way and as difference plots. Two-dimensional J resolved spectra were measured using the Bruker 2-dimensional program (2nd version). In

Abbreviations: SRIF, somatotropin release inhibiting factor or somatostatin; CD, circular dichroism; NMR, nuclear magnetic resonance; CIDNP, chemically induced dynamic nuclear polarisation; LW, Lorentzian linewidth; GW, Gaussian linewidth

† Deceased

all experiments the Lorentz-Gauss line transformation has been used to obtain the best possible resolution or to reduce noise [8]. The amide proton resonances were measured in a peptide solution containing about 30% ^1H_2O and 70% ^2H_2O with uncorrected pH 3.6. All other spectra were recorded in 99.98% ^2H_2O, low in paramagnetic impurities, from Aldrich at pH 4–5.5. Peptide was always 4 mM for ^1H measurements and 14 mM for the ^{13}C measurements. The tetrapeptide acetyl-Trp^8–Lys^9–Thr^{10}–Phe^{11}-amide, somatostatin, [Phg^{11}]SRIF and [HSer^{12}]SRIF were synthesized by the solid phase method in [9]. The peptides were purified by a combination of molecular gel filtration, partition chromatography and high performance liquid chromatography.

3. Results

The ^1H spectra have been divided into four 1 ppm regions covering the aliphatic, the aromatic, the α and the β region, respectively. The aliphatic region (0.9–1.9 ppm) is shown in fig.1 with a very mild and a strong resolution enhancement. The non-equivalence of the Lys^4 and the Lys^9 sidechains is clearly visible and found to be most significant for the γ protons. The assignment of the Lys residues has been based upon a comparison with spectra of the central tetrapeptide acetyl-Trp^8–Lys^9–Thr^{10}–Phe^{11}-amide. In SRIF and in this fragment a Lys γ resonance is found

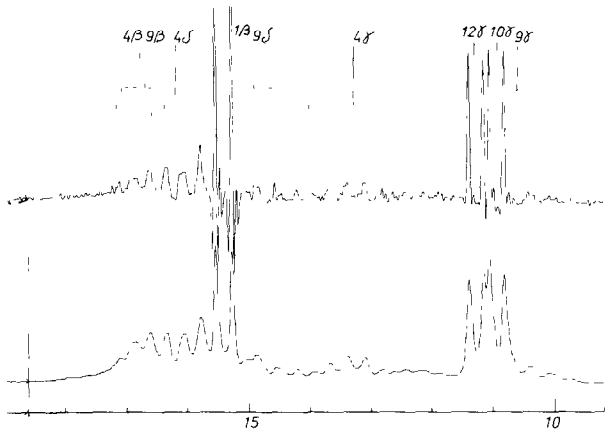


Fig.1. Aliphatic region of somatostatin in D_2O, conditions valid for fig.1–6: pD 4.5; temp. 25°C; concn. 4 mM. The lower spectrum has been obtained by application of a very mild enhancement (assumed LW = 4 Hz, calc. GW = 2.3 Hz). Strong enhancement (LW = 6, GW = 2.2 Hz) has been applied in the upper spectrum.

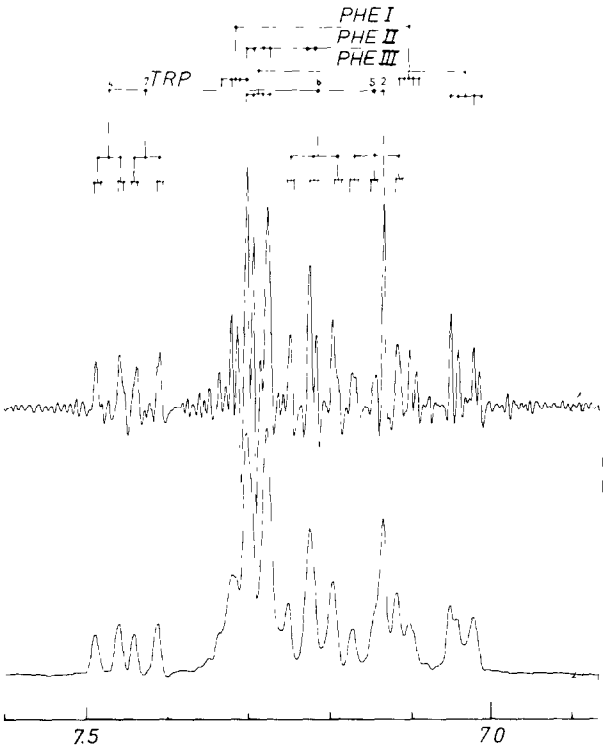


Fig.2. Aromatic region of somatostatin with very mild (LW = 3.5, GW = 2.0 Hz) and strong (LW = 5.5 Hz, GW = 2.1 Hz) enhancements in the lower and the upper spectrum, respectively.

at ~1 ppm. This upfield shift as compared to what has been found in the C-terminal hexapeptide fragment acetyl-Lys^9–Thr^{10}–Phe^{11}–Thr^{12}–Ser^{13}–Ala^{14}-amide [5] is most likely due to a ring current shift by the Trp^8 indole ring on the Lys^9 sidechain. The Thr methyl groups have been assigned by comparison with a spectrum of [HSer^{12}]SRIF. One of the few differences between the two spectra is the disappearance of the Thr α and γ doublets at the lowest fields.

The aromatic region (6.8–7.8 ppm) is shown in fig.2. The Trp^8 resonances 4,6 and 2 have been identified by a photo-CIDNP experiment [10]. The remaining Trp signals were assigned by homo-decoupling experiments that also revealed the separation of the 3 Phe spin systems into intense low field signals and smaller double doublets at higher field. Comparison with the corresponding part of the [Phg^{11}]SRIF spectrum indicates that Phe^{11} has the lowest field resonances.

The α proton region (4.7–3.7 ppm) including the Thr β and Ser β resonances is shown in fig.3 (lower spectrum). The chemical shift projection of a 2-dimen-

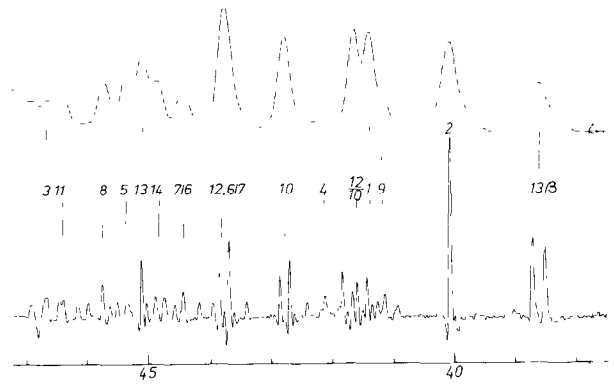


Fig.3. α-Proton region of somatostatin. The lower spectrum is a strongly enhanced (LW = 6 Hz, GW = 2.2 Hz) 1-dimensional spectrum. The upper spectrum is the chemical shift projection of a 2-dimensional *J*-resolved experiment. In this way the chemical shift centra of all α proton multiplets can be assigned in the strongly overlapping 1-dimensional spectrum.

sional *J*-resolved spectrum [11,12] (upper spectrum) indicates very clearly the chemical shift centra of all α protons. Only with this knowledge very selective and accurate 1-dimensional homodecoupling experiments could be done that gave unambiguous results in the β region (2.4–3.4 ppm) shown in fig.4. Here too, a 1-dimensional spectrum and a chemical shift projection from a 2-dimensional *J*-resolved experiment are aligned. In this way the chemical shift centers of most β protons could be found. The others were determined from the decoupling experiments in the α region. The

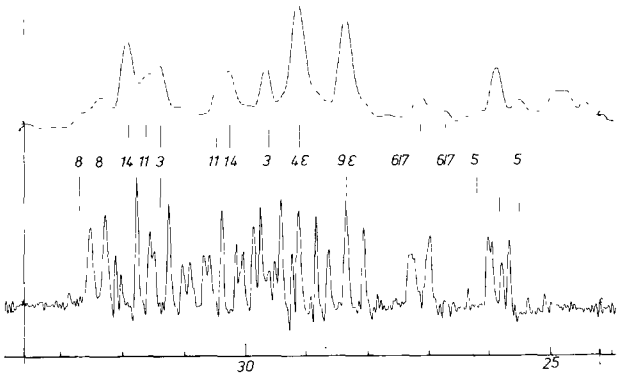


Fig.4. β-Proton region of somatostatin showing a strongly enhanced 1-dimensional spectrum (LW = 6 Hz, GW = 2.2 Hz) and a 2-dimensional chemical shift projection. Most proton chemical-shift centra can be found from this figure. The strongly coupled Asn and Phe[6,7] protons give rise to large artefacts in the 2-dimensional projection.

multiplets of all α protons obtained from cross-sections of the 2-dimensional experiment are shown in fig.5. The β proton region could not be analysed in this way because of strong β–β proton coupling and because of a too strong overlap. Nevertheless a complete analysis of the α and β region was possible from a combination of the 1- and 2-dimensional experiments.

We have solved the assignment problem of the 7 ABX spin systems in somatostatin in the following way: The photo-CIDNP experiment allowed us to identify Trp[8] without any ambiguity. In the spectrum of [Phg[11]]SRIF the second lowest field multiplet disappeared and a (Phe_α) singlet emerged in an otherwise very similar α proton region. This strongly indicates the position of the Phe[11] signals in the SRIF spectrum. To distinguish the Cys[3,14], Asn[5] and the Phe[6,7] spin systems we have done a series of ^{13}C experiments at pH 6. The distinction of the α carbon resonances of these amino acids into 3 groups was possible from their differences in chemical shift. ^{13}C values extracted from [13,14] have been used to do most of the assignments. It appeared quite well possible to do selective and off-resonance $\{^1H\}$ ^{13}C experiments to assign the α protons. To discriminate between Cys[3] and Cys[14] the experiments were repeated at pH 2 (fig.6). The upfield shift of the Cys[14] α carbon upon protonation of the COO^- group [15] is unambiguous. The concomitant shifts occurring in the proton spectrum were also recorded in the same sample in order to be able to do the ^{13}C–^1H correlation properly.

The results of spin system simulations and assignments are given in table 1. Only the Phe[6] and Phe[7]

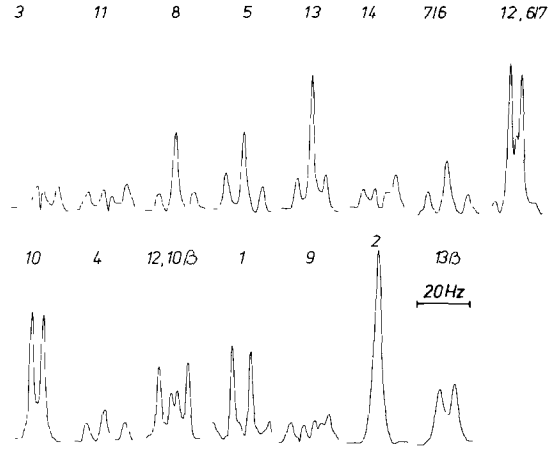


Fig.5. α-Proton multiplets obtained from cross sections of the 2-dimensional experiments.

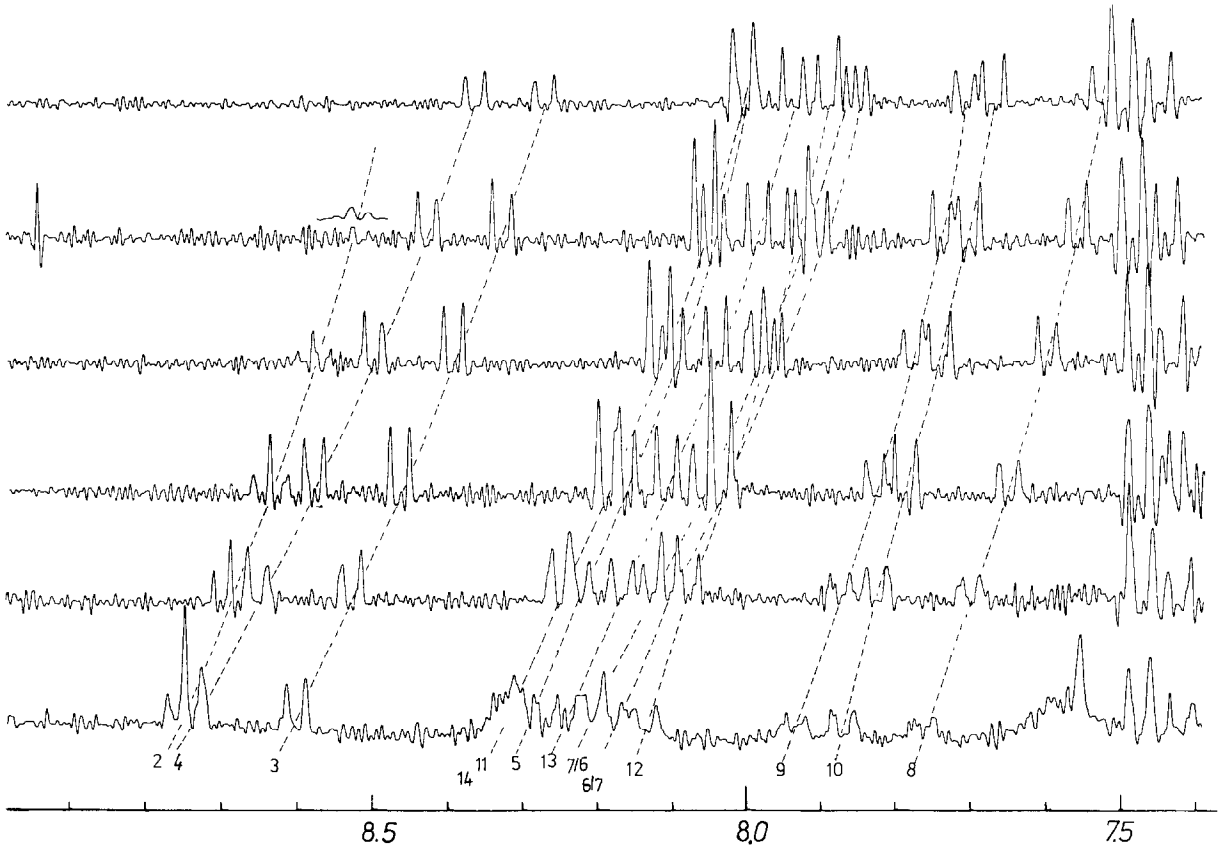


Fig.6. Stacked plot of NH proton region between 5°C (lowest spectrum) and 55°C (upper spectrum). Temperature interval 10°C. Enhancement used in all spectra LW = 4 Hz, GW = 2.3 Hz.

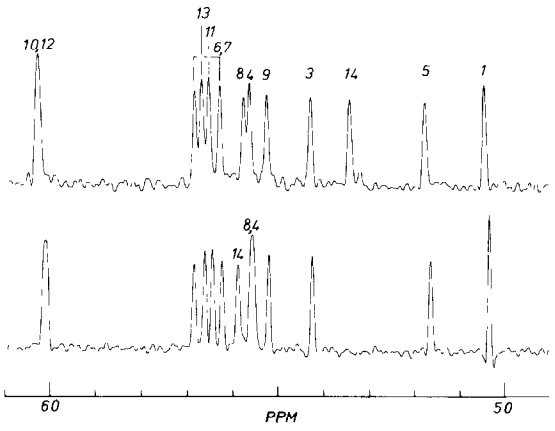


Fig.7. The $^{13}C_{\alpha}$ region of somatostatin in D_2O (30°C, 14 mM). The upper spectrum has been taken at pH 2, lower at pH 6. Enhancement was used both to separate signals and to reduce noise (LW = 8 Hz, GW = 4.7 Hz).

spin systems have not yet been assigned. They will be indicated arbitrarily as Phe[6,7] and Phe[7,6]. The temperature variation of the chemical shift ($\Delta\delta/\Delta T$) of the amide protons and the $^3J_{NH-C_{\alpha}H}$ coupling constants are also given in table 1. The NH signals were measured at pH 3.6 over 5–55°C in a mixture containing ~35% solvent protons. The regular 180–τ–90 solvent peak suppression was used to eliminate the water resonance. Although some overlap between the NH signals exists, it appeared possible to resolve all signals using resolution enhancement (fig.7). By decoupling at 25°C and 45°C all amide protons could be assigned to their corresponding α protons.

4. Discussion

A detailed comparison of our assignments with those in [6] is difficult since their spectra differ markedly from ours at several points. The most conspicuous dif-

Table 1
NMR parameters of somatostatin in D_2O at 25°C (5 mM, pD 4.5)

Amino acid	δ_{NH}	δ_α	δ_β	δ_γ	δ_δ	δ_ϵ	$^3J_{NH-C_\alpha H}$	$^3J_{\alpha\beta}$	$^2J_{\beta_I\beta_{II}}$	$^3J_{\beta\gamma}$	$-\Delta\delta/\Delta T$
Ala[1]		4.137	1.540					6.9			
Gly[2]	8.637	4.007					5.9				5.7
Cys[3]	8.464	4.669	3.136 / 2.967				6.8	5.5 / 7.8 (5.5 / 8.0)	−14.2		6.9
Lys[4]	8.578	4.215	1.659	1.33	1.63	2.91	6.6	– (6.6 / 5.8)	–	–	7.7
Asn[5]	8.164	4.536	2.601 / 2.567				7.4	6.5 / 7.5 (6.7 / 6.9)	−15.6		5.9
Phe[6,7]	8.035	4.369	2.727 / 2.700				7.3	7.1 / 7.6 (6.7 / 8.0)	−14.7		7.0
Phe[7,6]	8.060	4.445	2.975 / 2.938				6.6	7.8 / 6.2 (7.6 / 7.0)	−13.3		6.4
Trp[8]	7.649	4.574	3.246 / 3.231				7.0	5.9 / 6.4 (6.2 / 6.6)	−15.4		4.9
Lys[9]	7.825	4.119	1.648	1.04	1.46	2.83	7.0	– (5.5 / 9.3)	–	–	4.9
Thr[10]	7.786	4.277	4.163	1.093			7.6	4.5 (4.4)		6.6	4.1
Phe[11]	8.185	4.642	3.157 / 3.056				7.3	6.4 / 8.1 (5.8 / 8.4)	−13.9		6.7
Thr[12]	8.033	4.377	4.163	1.126			8.1	4.4 (4.4)		6.3	5.1
Ser[13]	8.107	4.511	3.859				7.7	6.0 (5.7)			6.3
Cys[14]	8.185	4.483	3.185 / 3.031				7.3	5.4 / 8.5 (4.7 / 7.0)	−13.3		6.7

Chemical shifts δ (in ppm ±0.002 ppm) and coupling constants J in Hz ±0.3 Hz were obtained from the spectra in fig.1–6 after simulation of all spin systems (except Lys[4] and Lys[9]). When 2 β protons exist δ- and J-upper values are for the βII protons, the values in parenthesis have taken from the 2-dimensional cross-sections. NH chemical-shift temperature coefficients $\Delta\delta/\Delta T$ are given in ppb/°K

ferences are a $\Delta\delta$ of the Ala[1] α proton of ~0.22 ppm and an inversion of the assignments of the Phe[11] and the Cys[3] α protons. There are also differences in connectivities between α and β protons for our Phe[11] and Phe[7,6], an inversion between the Phe[7,6] and Cys[14] α protons, as well as a misassignment of the Phe[11] NH signal. The influence of pH on several α proton chemical shifts between pH 7–2 (perhaps partly due to conformational changes) might explain some of these differences. The use of deuterated analogs and experiments at much higher fields will be necessary to confirm the assignments in the β proton region.

From the $^3J_{NH-C_\alpha H}$ coupling constants and the amide proton $\Delta\delta/\Delta T$ values it appears impossible that somatostatin exists mainly in one conformation stabilized by internal hydrogen bonds. Our lowest $\Delta\delta/\Delta T$ values (4.9, 4.9, 4.1 and 5.1 ppb/°K for Trp[8], Lys[9], Thr[10] and Thr[12], respectively) are an order of magnitude larger than the ones observed for intramolecularly hydrogen bonded amide protons [16]. Our $^3J_{NH-C_\alpha H}$ values also exclude the existence of β turns of type I and type III. Nevertheless the NMR data do indicate a certain amount of structure: The nonequivalence of the 2 Lys, the 2 Thr and the 3 Phe residues demonstrates interaction between neighbouring residues which gives rise to ring current shifts by aromatic rings. The participation of βII turns in the conformational equilibrium is certainly possible. It is conceivable that βII turns from Phe[7]–Thr[10] to Lys[9]–Thr[12] contribute to the conformational equilibrium. In particular a βII turn from Phe[7]–Thr[10] is consistent with the observed $^3J_{NH-C_\alpha H}$ values and the lower $\Delta\delta/\Delta T$ coefficients. Such a turn will also allow the proximity of Lys[9] and Trp[8] sidechains, which would explain the 0.26 ppm upfield shift of the Lys[9] γ resonances. A much larger upfield shift has been found for D-Trp[8] containing analogs of somatostatin with restricted mobility. In these molecules a large contribution of a βII′ turn (from Phe[7]–Thr[10]) could explain the observed Lys[9] γ shift, that has been found to correlate with the

high biological activity of these analogs [2,17].

Similar to what was observed for SRIF fragments [4,5] the native somatostatin seems to exist in aqueous solution in a conformational equilibrium between several low energy conformations. As has been emphasized recently [18], there is no point in trying to extract an 'average NMR conformation' from the data under such conditions. In a forthcoming publication we will further analyze our NMR results and compare the interpretation carefully with a series of low energy conformations obtained from a extensive series of semi-empirical energy calculations.

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References

[1] Vale, W., Rivier, C. and Brown, M. (1977) Annu. Rev. Physiol. 39, 473–527.
[2] Veber, F. D. and Saperstein, R. (1979) Annu. Rep. Med. Chem. 14, 209–218.
[3] Holladay, L. A., River, J. and Puett, D. (1977) Biochemistry 16, 4895–4900.
[4] Hallenga, K., Van Binst, G., Knappenberg, M., Brison, J., Michel, A. and Dirkx, J. (1979) Biochim. Biophys. Acta 577, 82–101.
[5] Knappenberg, M., Brison, J., Dirkx, J., Hallenga, K., Deschrijver, P. and Van Binst, G. (1979) Biochim. Biophys. Acta 580, 266–276.
[6] Buffington, L., Garsky, V., Massiot, G., Rivier, J. and Gibbons, W. A. (1980) Biochem. Biophys. Res. Commun. 93, 376–384.
[7] Zimmermann, S. S. and Scheraga, H. A. (1977) Biopolymers 16, 811–843; Chou, P. Y. and Fasman, G. D. (1977) J. Mol. Biol. 115, 135–175.
[8] Ernst, R. R. (1968) Adv. Magn. Res. 2, 1–120.
[9] Merrifield, R. B. (1963) J. Am. Chem. Soc. 85, 2149–2154.
[10] Kaptein, R. and Dijkstra, K. (1978) J. Magn. Res. 31, 171–176; Kaptein, R. (1980) Proc. Eur. Conf. NMR of Macromolecules, Sassari Sardinia, 1978, in press.
[11] Nagayama, K., Wuthrich, K., Bachmann, P. and Ernst, R. R. (1977) Biochem. Biophys. Res. Commun. 78, 99–105.
[12] Nagayama, K., Bachmann, P., Wüthrich, K. and Ernst, R. R. (1978) J. Magn. Res. 31, 133–148.
[13] Grathwohl, C. and Wuthrich, K. (1974) J. Magn. Res. 13, 217–225.
[14] Hruby, V. J., Deb, K. K., Spatola, A. F., Upson, D. A. and Yamamoto, D. (1979) J. Am. Chem. Soc. 101, 202–212.
[15] Christl, M. and Roberts, J. D. (1972) J. Am. Chem. Soc. 94, 4565–4573.
[16] Meraldi, J. P., Yamamoto, D., Hruby, V. J. and Brewster, A. I. R. (1975) in: Peptides· Chemistry, Structure and Biology (Walter, R. and Meienhofer, J. eds) pp. 803–814, Ann Arbor Sci., Ann Arbor MI.
[17] Arizon, B. H., Hirschmann, R. and Veber, D. F. (1978) Bioorg. Chem. 7, 447–451.
[18] Jardetzky, O. (1980) Biochim. Biophys. Acta 621, 227–232.