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β-Turns induced in bradykinin by (*S*)-α-methylproline

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The ability of (*S*)-α-methylproline (α-MePro) to stabilise reverse-turn conformations in the peptide hormone bradykinin (BK = Arg[1]-Pro[2]-Pro[3]-Gly[4]-Phe[5]-Ser[6]-Pro[7]-Phe[8]-Arg[9]) has been investigated. Two BK analogues containing α-MePro at position 3 or position 7 were synthesised and their conformations in aqueous solution investigated by NMR spectroscopy. Whereas BK is largely disordered on the NMR time scale both analogues showed ROE connectivities in 2D-ROESY spectra indicative of reverse-turn conformations at both Pro[2]-Phe[5] and Ser[6]-Arg[9], whose formation appears to be cooperative. Some potential applications of α-MePro as a reverse-turn mimetic in the construction of synthetic peptide libraries is discussed.

Peptide; α-Methylproline; NMR; β-Turn; Conformation; Bradykinin

1. INTRODUCTION

Proline residues frequently occur in reverse turns on the surface of globular proteins [1], and similar conformations have been detected in short linear peptides dissolved in water [2]. The inclusion of the proline-N atom into a pyrrolidine ring places severe constraints upon bond rotation about the N–C_α (ϕ-angle) and C_α–C_{CO} (Ψ-angle) bonds, with the result that the ϕ/Ψ-space accessible to the peptide backbone becomes very limited. Compared to other amino acids, proline also appears more frequently in proteins with a *cis* peptide bond [3]. Proline residues thus have a special significance in their effect on chain conformation, which in turn can influence protein folding, and also peptide and protein function [4].

Because of the frequent occurrence of proline residues in biologically active peptides, and in recognition sites on stably folded proteins, proline analogues which act to enhance the stability of turn conformations in small peptides could find many useful applications. One interesting example is (*S*)-α-methylproline (α-MePro). It was shown recently that upon substituting Pro[2] by α-MePro in the peptide antigen Ac-Tyr[1]-Pro[2]-Tyr[3]-Asp[4]-Val[5]-Pro[6]-Asp[7]-Tyr[8]-Ala[9], the peptide adopted a more stable reverse turn conformation around Tyr[1]-Asp[4], and the binding affinity of the antigen to two different anti-peptide monoclonal antibodies was increased by up to a factor of 50 [5,6].

As part of an effort to test whether β-turn conformations can be stabilised by α-MePro in other sequence contexts we have substituted proline by α-MePro at positions 3 and 7 in the peptide hormone bradykinin (BK = Arg[1]-Pro[2]-Pro[3]-Gly[4]-Phe[5]-Ser[6]-Pro[7]-Phe[8]-Arg[9]). The Pro[2]-Pro[3]-Gly[4]-Phe[5] and Ser[6]-Pro[7]-Phe[8]-Arg[9] tetrapeptide sequences have a high predicted likelihood for forming β-turns, based on Chou and Fasman probability factors [7,8], and we report here studies on the conformation of two BK analogues containing α-MePro at positions 3 and 7.

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2. MATERIALS AND METHODS

2.1. *Peptide synthesis*

Peptide syntheses were carried out on an ABI 430A instrument using standard reaction cycles, Fmoc/NMP-methodology, *p*-alkoxybenzyl alcohol (Wang) resin (Bachem, AG) and DCCI/HOBt activation on a 0.25 mmol scale. A fourfold excess of protected and activated amino acid was used in each coupling step. *N*-Fmoc-α-methylproline was prepared as described previously [5]. Residues directly following α-methylproline were double- or triple-coupled, and the last coupling cycle was extended to 8 h. Peptides were purified on LH-20 Sephadex in water + 1% acetic acid, and finally by HPLC on μ-Bondapak-C18 columns (gradient of MeCN + 0.1% TFA vs. water + 0.1% TFA). All peptides were obtained >98% pure by reverse-phase HPLC, and gave satisfactory amino acid analyses, FAB mass spectra (glycerol/thioglycerol/acetic acid matrix), and ^1H, ^{13}C and ^{15}N NMR spectra.

2.2. *NMR spectroscopy*

Two dimensional NMR experiments were carried out on a Bruker AMX-600 in 90% H_2O–10% D_2O at pH 4. Proton chemical shifts are relative to external TSP. All spectra were recorded in phase-sensitive mode with quadrature detection in the F1 dimension using time-proportional phase incrementation [9]. ^1H spectra typically had a spectral width of 5555 Hz (9.25 ppm) in F2 and were collected with 2048 time domain data points. In F1 512 t_1-increments were recorded to yield a size of 1024 × 2048 for the final (real) 2D matrix after zero-filling in each dimension. All data were enhanced in both directions using cosine bell window functions. Baseline flattening was carried out using an automatic third order polynomial baseline correction routine in both dimensions. TOCSY [10,11] spectra were per-

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formed with a 200 ms MLEV17 spinlock ($\gamma B_2/2\pi \approx 7500$ Hz) surrounded by two 2.5 ms purging pulses to remove phase distortions. ROESY Spectra [12] were recorded with 100 ms and 300 ms spinlock times ($\gamma B_2/2\pi \approx 3600$ Hz). Water suppressions for these experiments was achieved with low power pre-irradiation.

3. RESULTS

3.1. *Assignment procedure, chemical shifts and coupling constants*

The strategy used to assign proton spectra [13] relied upon the identification of spin systems for individual amino acid residues in DQ-COSY [14] and TOCSY spectra [10,11], followed by determination of neighbouring amino acids from the observation of sequential NOE connectivities. The ^{1}H assignments are given in Table I. Resonances from the segment Pro^3-Gly^4-Phe^5-Ser^6 were identified readily by an NOE walk through the C_αH-NH connectivities, starting from the Gly^4 amide triplet. The only minor difference between the assignments deduced here for BK at pH 4 (not reported), and those obtained earlier at pH 7.4 in D_2O arise in Arg^1 [7]. For example, at the lower pH the Arg^1 C_αH appears 0.23 ppm to lower field [15].

All the observed $^3J_{\alpha NH}$ values are close to the time-averaged value of 6.3 Hz for free rotation about the N–C_α bond [16], and as the expected values for residues at positions i+1 and i+2 in β-turns in proteins also lie in the range 4–9 Hz [13], no firm conclusions about preferred turn conformations can be drawn from these data. Also, the $^3J_{\alpha\beta}$ coupling constants observed for the analogues (Table I) are mainly consistent with rapid averaging between two or more distinct values of χ_1. Only the side chain of Phe^8 in 3-αMePro-BK and 7-αMePro-BK has $^3J_{\alpha\beta}$ coupling constants which suggest that one of the gauche rotamers or the trans rotamer is favoured [16,17]. At present the favoured χ_1 angle cannot be identified because the β protons have not been stereospecifically assigned.

3.2. *Temperature dependence of NH-chemical shifts*

An amide proton involved in a stable intramolecular hydrogen bond, or one inaccessible to solvent for steric reasons, typically shows a reduced temperature coefficient in the range 0 to -3×10^{-3} ppm/K [18]. The amide temperature coefficients for BK, 3-αMePro-BK and 7-αMePro-BK are shown in Table II. Only the Arg^9 NH shows a reduced temperature coefficient, which is consistent with its involvement in a hydrogen bonded turn conformation. However, both the *cis* and *trans* rotamers at Ser-Pro-Phe in BK show a reduced tempera-

Table I

^{1}H-Chemical shift assignments (δ ppm) and $^3J_{\alpha\beta}$ (Hz) coupling constants for the major *trans* rotamers of the peptides RPP^{Me}GFSPFR and RPPGFSP^{Me}FR in 10% D_2O/H_2O at pH 4.0 and 286 K (P^{Me} = (*S*)-α-methylproline)

	R	P	P^{Me}	G	F	S	P	F	R
N_α-H	----	----	----	8.02	7.83	8.00	----	7.91	7.61
C_α-H	4.14	4.56	----	3.69	4.37	4.49	4.12	4.44	3.96
αMe			1.37						
C_β-H	1.68	2.21	1.99	----	2.89	3.57	1.99	3.03	1.64
		1.69	1.82				1.50	2.79	1.52
C_γ-H	1.47	1.83	1.96	----	----	----	1.70	----	1.34
			1.91						
C_δ-H	2.86	3.52	3.77	----	----	----	3.39	----	2.99
		3.26	3.56						
N_ε-H	7.00	----	----	----	----	----	----	----	7.02
$^3J_{\alpha\beta}$ (Hz)	6.1	nd	- - - -	- - - -	7.4	6.7	8.5	9.1	7.9
							4.4	6.0	5.2

	R	P	P	G	F	S	P^{Me}	F	R
N_α-H	----	----	----	8.37	7.93	8.18	----	7.49	7.68
C_α-H	4.33	4.76	4.41	3.87	4.56	4.60	----	4.65	4.13
α-Me	- - -	- - - -	- - - -	- - - -	- - - -	- - - -	1.39	- - - -	- - - -
C_β-H	1.87	2.42	2.28	----	3.03	3.76	1.70	3.29	1.82
	- - - -	1.87	1.89	- - - -	- - - -	3.67	1.38	2.84	1.71
C_γ-H	1.68	2.00	2.03	----	----	----	1.82	----	1.57
	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	1.59	- - - -	- - - -
C_δ-H	3.10	3.74	3.81	----	----	----	3.57	----	3.16
	- - - -	3.47	3.65	- - - -	- - - -	- - - -	3.52	- - - -	- - - -
N_ε-H	7.16	----	----	----	----	----	----	----	7.15
$^3J_{\alpha\beta}$ (Hz)	5.9	7.5	8.2	- - - -	7.0	7.0	- - - -	10.0	7.6
			5.3					4.7	5.9

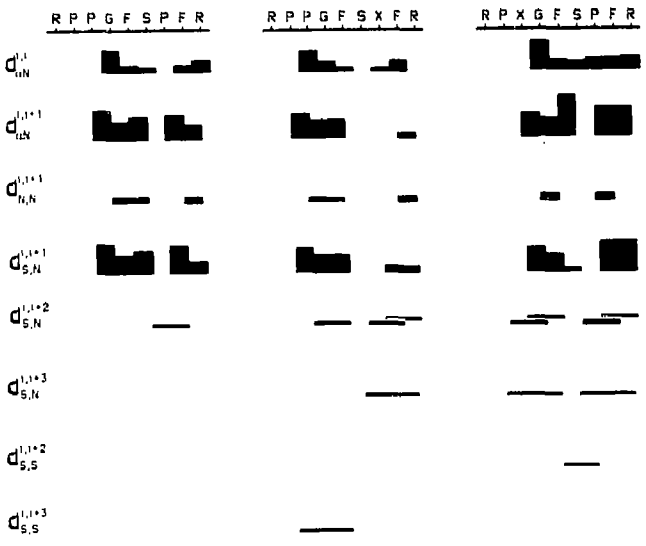

Fig. 1. Schematic diagram showing the magnitude of various ROE connectivities observed in ROESY spectra of the three peptides: RPPGFSPFR (BK); RPPGFSXFR (7-αMePro-BK); RPXGFSPFR (3-αMePro-BK).

ture coefficient for Arg ($\approx -4.0 \times 10^{-3}$ ppm/K) [19], and for the *cis* rotamer, a convenient internal control, a β-turn is not possible. This suggests that the low dδ/dT values observed for Arg[9] in 3-αMePro-BK and 7-αMePro-BK cannot be taken as reliable evidence for the adoption of β-turn conformations at the C-terminal residues in these peptides.

3.3. *NOE information*

The ROE connectivities deduced for each peptide are depicted in Fig. 1. For BK, a set of expected consecutive strong $d_{\alpha N}$ (i, i+1) and weak d_{NN} (i, i+1) ROEs were identified in 2D-ROESY spectra. Numerous other ROEs were observed between protons in neighbouring residues. However, apart from a weak ROE between the Phe[8] amide proton and the Ser[6] $C_\beta H$, whose significance is unclear, no other medium or long range ROEs were observed. These results indicate a largely disordered peptide backbone, which is consistent with previous studies of coupling constants [7] and ^{13}C-relaxation measurements on BK [19].

In the αMePro analogues, however, ROE evidence for β-turn conformations can be found. In the amide NH region of the ^1H-spectra from 3-αMePro-BK, two sets of NH-resonances are evident in a ≈ 10:1 ratio, which suggest again the presence of *trans*- and *cis*-isomers at the Ser[6]-Pro[7] peptide bond. A set of strong $d_{\alpha N}$ (i, i+1) and weak d_{NN} (i, i+1) ROEs for the *trans*-isomer were again identified in ROESY spectra. In addition, medium range ROE connectivities were identified between Phe[5] NH and each of the following protons: Pro[3] C_αMe, Pro[3] $C_\gamma H$ and Pro[2] $C_\beta H$ (Fig. 2). These ROEs represent clear evidence for a significantly increased population of turn-like conformations in the segment Pro[2]-Phe[5]. Consistent with this were other ROEs between Gly[4] NH and a Pro[2] $C_\beta H$ and a Pro[3] $C_\gamma H$. ROEs were also observed between protons in the C-terminal part of this analogue, indicating that a turn conformation was also being populated at Ser[6]-Arg[9]. These include weak ROE connectivities between Arg[9] NH and Pro[7] $C_\alpha H$ and Ser[6] $C_\beta H$, as well as between Phe[8] NH and Ser[6] $C_\beta H$ (Fig. 2). An ROE between the Phe[5] ortho aromatic protons and Pro[7] $C_\alpha H$ was also observed.

The ^1H spectrum of 7-αMePro-BK showed only one major set of NH resonances consistent with the peptide occupying to >95% the all-*trans* configuration. The α-methylproline at position 7 is expected on steric grounds to strongly favour the *trans*-Ser[6]-MePro[7] rotamer. Apart form the normal ROE connectivities between protons within one residue, or in adjacent residues, a number of medium range ROEs were again seen which indicate that turn conformations are stabilised (relative to BK) in both the Pro[2]-Phe[5] and Ser[6]-Arg[9] segments of this peptide. Observed ROE connectivities that are diagnostic for a reverse turn at Ser[6]-Arg[9] include Arg[9] NH to Ser[6] $C_\beta H$, Arg[9] to either Pro[7]-C_αMe or Pro[7]-$C_\beta H$ (coincident resonances), Arg[9] NH to Phe[8] NH, and Phe[8] NH to Ser[6] $C_\beta H$ (Fig. 2). The ROEs observed between Arg[9] and Ser[6], as well as Arg[9] and Pro[7] confirm the presence of a reverse turn formed by residues Ser[6]-Arg[9]. Similar evidence for a turn conformation at Pro[2]-Phe[5] includes ROEs observed between Phe[5] NH and Pro[3] $C_\alpha H$, between Phe[5] $C_\beta H$ and Pro[2] $C_\delta H$, and between Phe[5] NH and Gly[4] NH. Thus the expected stabilisation by α-MePro of a turn conformer at Ser[6]-Arg[9] in this case is accompanied by an additional effect at Pro[2]-Phe[5].

Table II

Temperature dependence of NH chemical shifts in ppb/K

	BK (ref. [19])	3-αMePro BK	7-αMePro BK
Gly[4]	−7.8	−8.8	−9.9
Ser[6]	−6.5	−8.4	−11.3
Phe[8]	−5.6	−9.4	−9.0
Phe[5]	−6.6	−5.6	−7.7
Arg[9]	−3.6	−5.7	−3.5

3.4. *Biological activity*

The biological activity of the analogues is under investigation and the results will be reported in detail elsewhere.

4. DISCUSSION

In agreement with earlier studies, the ROE data reported here suggest that BK does not adopt a stable secondary structure on the NMR timescale in aqueous

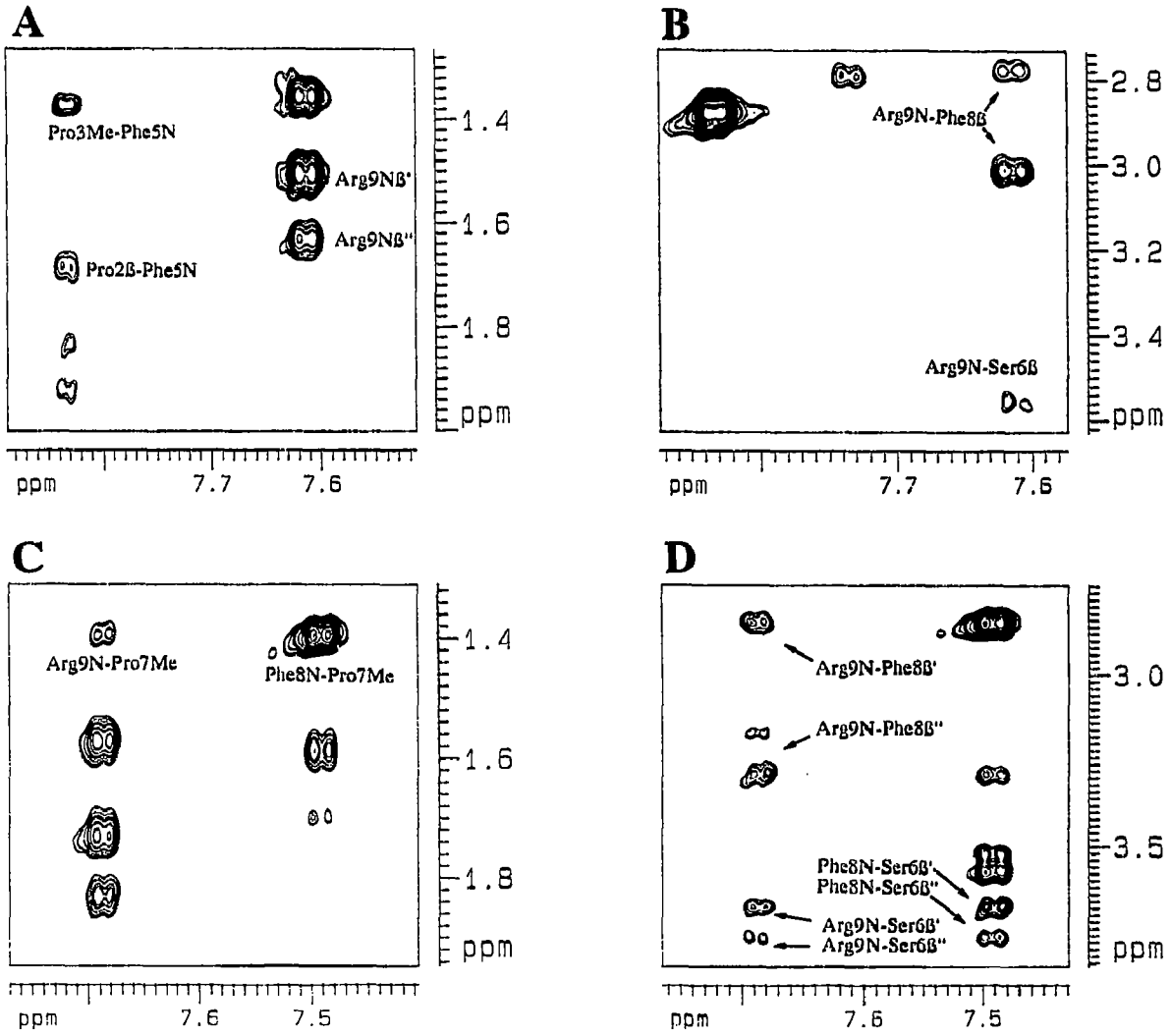

Fig. 2. Portions of 600 MHz phase sensitive ROESY spectra: A and B from 3-αMePro-BK at 286 K; C and D from 7-αMePro-BK at 300 K. Both in 90% H_2O–10% D_2O, pH 4. ROEs from neighbouring residues, as well as longer range effects are indicated. The mixing time for both was 300 ms.

solution [7,19]. However, the 3-αMePro-BK and 7-αMePro-BK analogues show clear evidence for the increased population of reverse-turn conformations in 90% H_2O–10% D_2O. In each peptide, ROE connectivities indicate the presence of two families of turn conformations, one centred on the residues Pro^2-Phe^5 and the other at Ser^6-Arg^9. However, the ROE connectivities do not allow a distinction to be drawn between type-I or type-II turns, nor do they indicate whether both turns are populated simultaneously. Both the Pro^2-Pro^3-Gly^4-Phe^5 and Ser^6-Pro^7-Phe^8-Arg^9 tetrapeptide sequences have a high predicted propensity for forming β-turns, based on Chou and Fasman probability factors [8]. From the outset it seemed likely that the presence of αMePro at position 3 would stabilise a turn at Pro^2-Phe^5. In 3-αMePro-BK, however, a turn is also populated in the region Ser^6-Arg^9. Similarly, in 7-αMePro-BK, evidence for a turn is seen at Pro^2-Phe^5 as well as at Ser^6-Arg^9. This suggests the possibility that these peptides may fold in a cooperative manner in aqueous solution. This would not be surprising since many of the interactions that stabilise protein structures are known to be cooperative in nature.

These and earlier observations [5,6] support the view that substituting proline for α-methylproline may be a general way of stabilising β-turn conformations in linear peptides. Such substitutions are likely to have interesting effects in peptides and proteins where biological activity is intimately linked to conformation. For example, the construction of peptide libraries on surfaces [20], plastic pegs [21], and beads [22,23] is currently attracting great interest. Yet many linear peptides of ≈ 5–15 residues based only on the twenty common proteinogenic amino acids may not adopt well

defined secondary structures. α-Methylproline-containing peptides may be useful in such applications, since this analogue can be incorporated using standard solid-phase methodologies. There are several cases also where proline residues, and reverse turn conformations, have been implicated in peptide immunogenicity [24–26]. Here again α-methylproline substitutions may lead to peptide analogues with altered, and possibly beneficial immunogenic properties. Based on these considerations, α-methylproline might become more widely useful as a synthetic β-turn mimetic.

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REFERENCES

[1] Rose, G.D., Gierasch, L.M. and Smith, J.A. (1985) Adv. Prot. Chem. 37, 1–109.
[2] Dyson, H.J. and Wright, P.E. (1991) Annu. Rev. Biophys. Biophys. Chem. 20, 519–538.
[3] Stewart, D.E., Sarkar, A. and Wampler, J.E. (1990) J. Mol. Biol. 214, 253–260.
[4] MacArthur, M.W. and Thornton, J.M. (1991) J. Mol. Biol. 218, 397–412.
[5] Hinds, M.G., Welsh, J.H., Brennand, D.M., Fisher, J., Glennie, M.J., Richards, N.G.J., Turner, D.L. and Robinson, J.A. (1991) J. Med. Chem. 34, 1777–1789.
[6] Richards, N.G.J., Hinds, M.G., Brennand, D.M., Glennie, M.J., Welsh, J.H. and Robinson, J.A. (1990) Biochem. Pharmacol. 40, 119–123.
[7] Denys, L., Bothner-By, A.A., Fisher, G.H. and Ryan, J.W. (1982) Biochemistry 21, 6531.
[8] Chou, P.Y. and Fasman, G.D. (1979) Biophys. J. 26, 367–383.
[9] Marion, D. and Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. 113, 967–974.
[10] Braunschweiler, L. and Ernst, R.R. (1983) J. Mag. Res. 53, 521–528.
[11] Bax, A. and Davis, D.G. (1985) J. Mag. Res. 65, 355–360.
[12] Bothner-By, A.A., Stephens, R.L., Lee, J., Warren, C.D. and Jeanholz, R.W. (1984) J. Am. Chem. Soc. 106, 811–813.
[13] Wüthrich, K. (1986) in: NMR of Proteins and Nucleic Acids, J. Wiley, New York.
[14] Rance, M., Sørensen, O.W., Bodenhausen, G., Wagner, G., Ernst, R.R., Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. 117, 479–485.
[15] Lintner, K., Fermandjian, S., St. Pierre, S. and Regoli, D. (1979) Biochem. Biophys. Res. Commun. 91, 803–811.
[16] Pachler, K.G.R. (1964) Spectrochim. Acta. 20, 581–587.
[17] Feeney, J. (1976) J. Magn. Res. 21, 473–478.
[18] Hruby, V.J., in: Chemistry and Biochemistry of Amino Acids, Peptides and Proteins (B. Weinstein, Ed.) vol. 3 Marcel Dekker, New York, 1974, pp. 1–188.
[19] London, R.E., Stewart, J.M., Cann, J.R. and Matwiyoff, N.A. (1978) Biochemistry 17, 2270–2277.
[20] Fodor, S.P.A., Read, J.L., Pirrung, M.C., Stryer, L., Lu, A.T. and Solas, D. (1991) Science 251, 767–773.
[21] Meloen, R.H., Amerongen, A.V., Noort, H.-V., Langedijk, J.P.M., Posthumus, W.P.A., Puyk, W.C., Plasman, H., Lentra, J.A. and Langeveld, J.P.M. (1991) Annal. Biol. Clin. 49, 231–242.
[22] Lam, K.S., Salmon, S.E., Hersh, E.M., Hruby, V.R., Kazmierski, W.M., Knapp, R.J. (1991) Nature 354, 82–84.
[23] Houghten, R.A., Pinilla, C., Blondelle, S.E., Appel, J.R., Dooley, C.T., Cuervo, J.H. (1991) Nature, 354, 84–86.
[24] Javaherian, K., Langlois, A.J., LaRosa, G.J., Profy, A.T., Bolognesi, D.P., Herlihy, W.C., Putney, S.D. and Matthews, T.J. (1990) Science 250, 1590–1593.
[25] Chandrasekhar, K., Profy, A.T. and Dyson, H.J. (1991) Biochemistry 30, 9187–9194.
[26] Dyson, H.J., Lerner, R.A. and Wright, P.E. (1988) Annu. Rev. Biophys. Biophys. Chem. 17, 305–324.