Monitoring the Chemical Assembly of a Transmembrane Bradykinin Receptor Fragment: Correlation Between Resin Solvation, Peptide Chain Mobility, and Rate of Coupling

Eliandre Oliveira,[a][‡] Eduardo M. Cilli,[b] Antonio Miranda,[b] Guita N. Jubilut,[b] Fernando Albericio,[a][‡] David Andreu,[a][‡] Antonio C. M. Paiva,[b] Shirley Schreier,[c] Mineko Tominaga,[b] and Clovis R. Nakaie*[b]

Keywords: Bradykinin / EPR spectroscopy / Membranes / Peptides / Resins

A combined resin solvation-peptide chain motion and kinetics of coupling reaction approach was applied to monitor details of the synthesis of TM-34, a 34-residue transmembrane segment of the bradykinin receptor. The dynamics of resin-bound peptide fragments attached to a stable free radical amino acid were examined by EPR spectroscopy. In agreement with an abrupt decrease (from 83 to 43%) in peptide purity occurring in the 12–16 region when DMF was used, a much more strongly immobilized chain population was detected, especially at the 12-mer stage. Conversely, faster couplings and improved synthesis were observed in 20% DMSO/NMP, probably due to the higher chain mobility in this mixed solvent. In addition, findings relating to solvation of peptide resins seemed to corroborate the previously advanced proposition that the 1:1 sum of electron acceptor and electron donor properties of a solvent can be considered to be an alternative and more appropriate parameter for its polarity.

Introduction

Some decades ago, the use of beaded-form cross-linked polymers was mostly restricted to stationary phases in column chromatography. The landmark development that launched such polymeric materials towards more specialized levels of application in many different fields occurred in the early 1960s[1] with the introduction of the solid-phase peptide synthesis (SPPS) method.[2] The concept of performing chemical processes on an insoluble polymer matrix has been successfully extended to the development of efficient synthetic methodologies for oligonucleotides[3] and polysaccharides.[4] More recently, solid phase-based combinatorial chemistry methods have again proven fruitful not only in the generation of peptide libraries,[5] but also in the development of solid-phase organic synthesis strategies, with a remarkable impact on drug development.[6]

As a consequence of this increasing trend in the use of polymer-dependent experimental procedures, a large number of different resins have been developed,[7] together with analytical methods aiming to clarify the potential of each polymer-based methodology. In this latter case, many efforts have been made, involving the use of NMR,[8] IR,[9] fluorescence,[10] and CD[11] spectroscopy. Although applied comparatively less often, EPR[12] has been of great value, as it provides relevant information about the solvated polymeric network. In this context, the use of the paramagnetic amino acid probe TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid),[13] initially used for peptide labeling[14] and later for structural investigation of solvated or peptide-supporting polymers,[15] has also been actively pursued.

In the spectroscopic evaluation of polymeric material, the solvent system plays a crucial role. Solvent molecules may affect the average distance between chains (and, as a result, the degree of chain-chain association), or control the rate of motion of components and the kinetics of reaction. For this reason, polymer solvation has been intensively studied by a variety of experimental procedures.[16] In this regard, a study of the solvation characteristics of model peptide-resins in about 30 single and mixed solvents of different polarity led us to propose the (AN+DN) parameter,[17] the sum of Gutmann's electron acceptor (AN) and electron

[a] Department of Organic Chemistry, Universitat de Barcelona, Martí i Franquès, 1−11 08028, Barcelona, Spain
[b] Department of Biophysics, Universidade Federal de São Paulo, Rua 3 de Maio 100, 04044−020, São Paulo, SP, Brazil
Fax: (internat.) +55−11/55390809
E-mail: clovis.biof@epm.br
[c] Department of Biochemistry, Institute of Chemistry, Universidade de São Paulo, P. O. Box 26077, 05513−970, São Paulo, SP, Brazil
[‡] Current address: Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Doctor Aiguader 80, 08003, Barcelona, Spain

donor (DN) solvent numbers[18] in a 1:1 proportion, as an alternative polarity scale.

In this context, step-by-step monitoring of physicochemical variations that may occur during the resin-bound elongation of a transmembrane fragment bound to a polymer structure may be a distinct and valuable strategy through which to address this issue. This is due to the inherent propensity of long, hydrophobic peptide sequences to undergo aggregation/association processes throughout the swollen resin network. Moreover, it can be of great value in the detection of minor, but crucial, microenvironmental details relevant for monitoring of peptide-resin elongation during SPPS. Thus, taking into account our previous work[19] showing the difficulty of synthesizing and purifying a very hydrophobic 34-residue peptide (TM-34, CTVAEIYLGNLAGADLILASGLPFWAITIANNFD) corresponding to a transmembrane segment (residues 69−97) of the rat bradykinin B2 receptor,[20] this report presents an investigation of the dynamics of the association properties of shorter peptides from the TM-34 sequence. The work focuses on peptide-resin solvation (swelling measurement of bead sizes), peptide chain mobility (EPR study of TOAC-labeled peptide-resins), and the kinetics of the coupling reaction. The purpose was to obtain relevant physicochemical data regarding the swelling properties of the polymer backbone during peptide elongation and the dependence on the solvent system used for the coupling reaction. Special emphasis was placed on the TM-34 region, in which a pronounced difference in bead solvation was observed.[19]

Results

Solvation Studies

To monitor the solvation behavior as a function of chain length, the degrees of swelling of peptide-resins containing different portions of the TM-34 sequence were determined in DCM, DMF, and 20% DMSO/NMP. Table 1 shows a sharp decrease in the peptide-resin solvation at the 12-mer stage in DMF, but not in DCM or in 20% DMSO/NMP (Figure 1), revealing considerable solvent-dependent peptide chain aggregation at this position. Much better swelling (about 80% of swollen bead volume occupied by the solvent) was found in 20% DMSO/NMP; a smaller improvement was seen with the less polar solvent DCM. These findings seem to be in accordance with a preliminary study of the TM-34 assembly[19] in which a strongly solvent-dependent synthesis yield was observed, together with a critical region located at the 12−16-residue segment. A sharp decrease in the yield (from 83 to 43%) was observed for this region when DMF was used for coupling. However, the yield was considerably improved when the mixed 20% DMSO/NMP solvent system was employed instead.

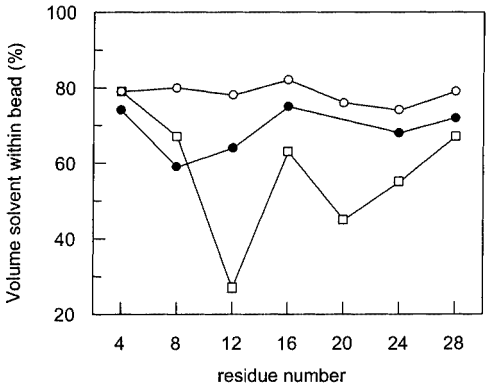

Figure 1. Degree of swelling of TM-(4, 8, 12, 16, 20, 24 and 28)-resins in DCM (·), DMF (□) and 20% DMSO/NMP (o)

The (AN+DN) solvent parameter has been proposed as a novel polarity parameter in two complementarily reports.[17] In order to compare this scale with that based on the dielectric constant ε, the swelling behavior of two peptide-resins, TM-12 and TM-24, was investigated in several solvent systems (Table 2) commonly used in some steps of a cycle of the SPPS method. In close agreement with these earlier reports, which focused on other polymers,[17] the dispersion of points in the swelling versus solvent polarity plot when the (AN+DN) parameter was employed was less for both peptide-resins than that obtained when the ε term was used (Figure 2).

Table 1. Degrees of swelling of TM-(4, 8, 12, 16, 20, 24 and 28) resins in different solvents

	DCM		DMF		20% DMSO/NMP	
Peptide resin	Diameter of swollen bead (μm)	Solvent within bead (%)[a]	Diameter of swollen bead (μm)	Solvent within bead (%)[a]	Diameter of swollen bead (μm)	Solvent within bead (%)[a]
TM-4	77	74	82	79	83	79
TM-8	70	59	75	67	91	80
TM-12	76	64	60	37	90	78
TM-16	87	75	78	63	97	82
TM-20	−	−	67	45	88	76
TM-24	82	68	73	55	88	74
TM-28	89	72	84	67	97	79

[a] [(swollen volume − dry volume)/swollen volume] × 100 using the following values for measured diameters of dry beads: Resins: TM-4 = 49 μm, TM-8 = 52 μm, TM-12 = 54 μm, TM-16 = 55 μm, TM-20 = 55 μm, TM-24 = 56 μm, TM-28 = 58 μm.

Table 2. Degrees of swelling of TM-12 and TM-24-resins in different solvents

Entry	Solvent	Solvent parameter		Solvent within bead[a] (%)	
		ε	(AN+DN)	TM-12	TM-24
1.	TOL	2.4	3.4	66	60
2.	DCM	8.9	21.4	64	68
3.	$CHCl_3$	4.7	27.1	75	72
4.	NMP	33.0	40.6	80	76
5.	DMF	36.7	42.6	37	55
6.	DMSO	46.7	49.1	52	62
7.	TFE	26.7	53.5	15	32
8.	EtOH	24.3	69.1	0	38
9.	MeOH	32.6	71.3	5	25
10.	Formamide	109.5	63.8	10	12
11.	50% TFE/TOL	14.6	28.5	59	71
12.	20% TFE/DCM	12.5	27.5	60	80
13.	50% TFE/DCM	17.8	37.5	58	72
14.	80% TFE/DCM	23.1	47.4	26	39
15.	20% DMSO/NMP	35.7	42.3	78	74
16.	50% DMSO/THF	27.1	38.6	56	63
17.	65% NMP/THF	24.1	36.1	76	73
18.	50% DCM/DMF	22.8	32.0	64	63
19.	50% DMSO/DCM	27.8	35.3	49	64
20.	50% DMSO/MeOH	39.7	60.2	28	39
21.	50% TFE/DMF	31.7	48.1	10	29
22.	50% TFE/DMSO	36.7	51.3	7	24
23.	10% TEA/DCM	8.3	25.1	76	81
24.	10% TEA/DMF	33.3	44.5	60	59
25.	10% TEA/DMSO	42.3	50.4	46	53

[a] [(swollen volume − dry volume)/swollen volume] × 100.

The maximum solvation region occurred at low (AN+DN) values both for the TM-12 and for the TM-24 resins (see C and D in Figure 2). The fact that this region corresponds to those of more apolar solvents is in accordance with the hydrophobicity of these sequences and, more significantly, with the strong and dominant characteristic of the polystyrene structure of the methylbenzhydrylamine-resin. Figure 2 (see C) also shows the lower degree of solvation of TM-12-resin in DMF (solvent 5), as compared to other polar aprotic solvents such as DMSO (solvent 6) or NMP (solvent 4). Swelling in the mixed solvents 21 and 22 (open circles) was slightly less than predicted by their polarity values and is discussed further below in the light of the acidity and basicity of their components.

EPR Studies

EPR spectra of TOAC-labeled peptide-resins containing 8, 12, 16, 20, and 28 residues of the TM-34 sequence in DMF and in 20% DMSO/NMP are shown in Figure 3. Whereas the spectral line-shapes for all peptide-resins are similar in 20% DMSO/NMP (see A in Figure 3), indicating a relatively high mobility, the spectra reveal different mobility for the sequences in DMF (see B in Figure 3). While TM-8 and TM-16 exhibit considerable freedom of motion, the other sequences give rise to spectra indicative of two populations, one strongly (broad triplet) and one weakly (narrow triplet) immobilized. The proportion of the immobilized component is greatest for TM-12.

Table 3 presents some spectral parameters previously employed to assess the dynamics of labeled sites in the polymer network.[15] The central peak linewidth (W_o) contains the contributions of both the weakly immobilized and the strongly immobilized chain populations and the h_{-1} term of the ratio of heights of the high- and mid-field lines (h_{-1}/h_0) corresponds essentially to the more mobile component. Thus, the lower the values of W_o, or the higher the h_{-1}/h_0 ratio, the faster the motion of the labeled resin sites. The EPR parameters in Table 3 corroborate the differentiated solvent-dependent behavior of some of the sequences, especially the TM-12-resin. While the variation of both W_o and h_{-1}/h_0 is small in 20% DMSO/NMP, these values vary considerably in DMF. The W_o value increases in the order TM-8 < TM-16 < TM-20 < TM-28 < TM-12. Accordingly, the h_{-1}/h_0 ratio decreases in the same order, except for the first two peptide-resins. It can also be seen that the W_o (h_{-1}/h_0) values for TM-28 and TM-12 in DMF are much larger (smaller) than for the other peptide-resins; these values are also larger (smaller) than those found in 20% DMSO/NMP for all peptide-resins.

Study of the Rate of Coupling

In view of the striking differences with regard to the TM-12-resin's solvation properties and chain mobility inside resin beads, the kinetics of coupling reactions of this peptide-resin in DMF and in 20% DMSO/NMP were compared. The yield of coupling of the next incoming amino

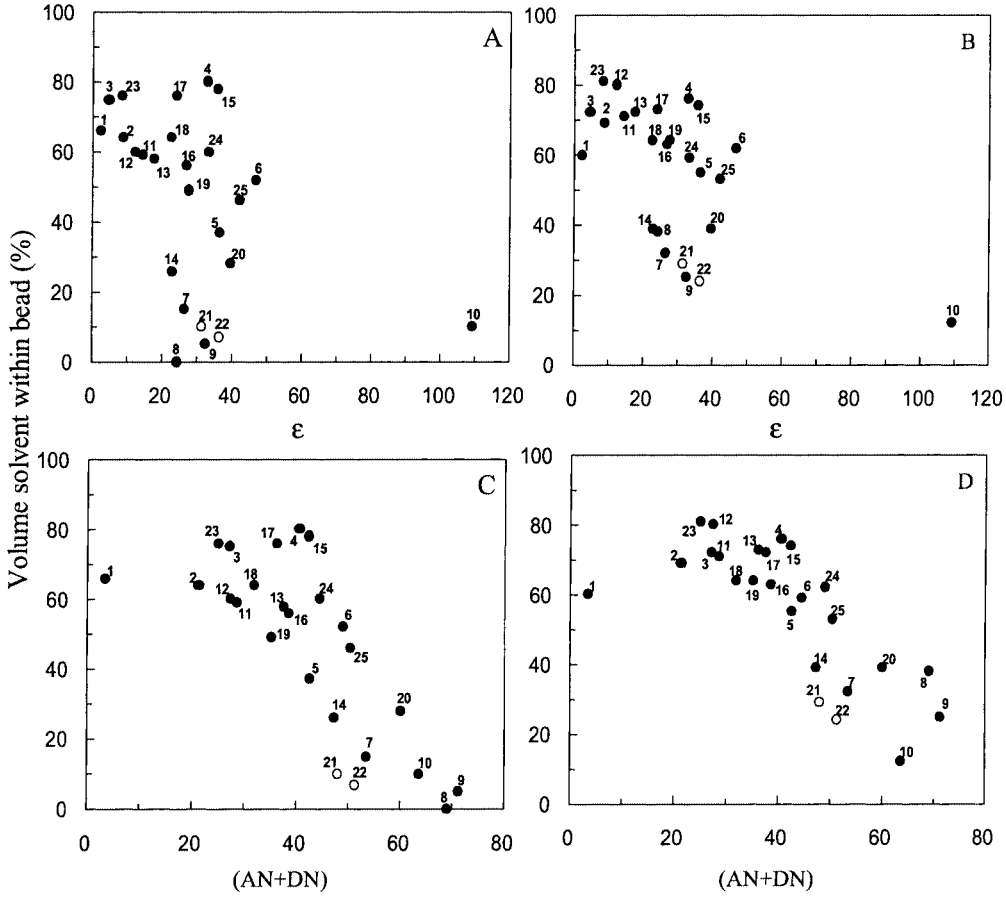

Figure 2. Degree of swelling of TM-12 [A, C] and TM-24 [B, D] resins as a function of parameters ε (A, B) and (AN+DN) (C, D) values, in 25 solvents

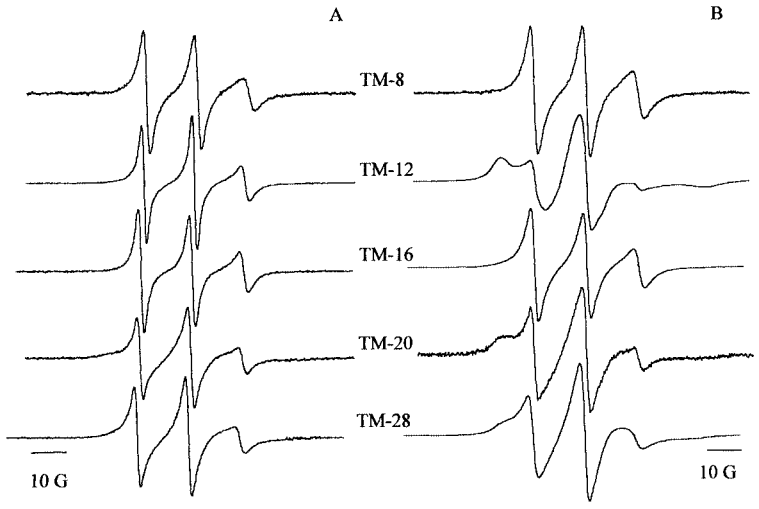

Figure 3. EPR spectra of TM-(8, 12, 16, 20 and 28)] resins in 20% DMSO/NMP (A) and DMF (B)

acid in the sequence (Leu[13]) was determined under equivalent acylating conditions, by the picric acid method. The acylation reaction was faster in 20% DMSO/NMP than in polar, aprotic DMF. While the coupling yield reached 90% in one hour in the former solvent system, it was 69% in the latter.

Discussion

Solvation Properties of Resins. Correlation with Solvent Polarity

The combined swelling/EPR monitoring strategy, applied to the progressive growth of the TM-34 chain bound to a

Table 3. Effect of solvent upon EPR spectral parameters of peptide-resins swollen in DMF and 20% DMSO/NMP

Peptidyl resin	Solvent			
	DMF		20% DMSO/NMP	
	W_o (G)	h_{-1}/h_0	W_o (G)	h_{-1}/h_0
TM-8	2.41	0.36	2.21	0.29
TM-12	3.62	0.07	1.92	0.25
TM-16	2.53	0.43	2.10	0.32
TM-20	2.64	0.21	2.10	0.27
TM-28	3.02	0.15	2.18	0.22

solid support, reveals some details of the resin backbone in the swollen state. The swelling data for seven peptide-resins (Table 1 and Figure 1) indicate that the bead solvation range extends from 37% (TM-12 in DMF) to 82% (TM-16 in 20% DMSO/NMP). DMF shows the most pronounced variation in peptide-resin solvation as the sequence is built. The severe shrinking in this solvent for the 12-mer, and to a smaller extent for the 20-mer, emphasizes the complexity of solute-solvent interactions. The results show that DMF was incapable of disrupting peptide-peptide or peptide-matrix interactions in the 12-mer sequence. In contrast, DCM and, to a greater extent, 20% DMSO/NMP, had the capacity to prevent these interactions.

The special characteristics of DMF, differing from most of the other solvent systems, had already been observed[15] during the synthesis of a well known strongly aggregating octapeptide sequence.[21] These findings suggest caution in the use of this solvent, not only for SPPS, but also for other solid-supported methods. Interestingly, this conclusion seems to be in disagreement with the widely accepted concept that less polar solvents, such as DCM, are more capable of inducing strong peptide chain association.[22] These contradictory results reinforce the notion that there are still open questions concerning the rules governing site-site interactions throughout the polymer network. In this context, the suggestion of the alternative use of other single or mixed polar aprotic solvents such as NMP or 20% DMSO/NMP seems pertinent.[16b,23] When extended to more severe chain aggregation conditions, the use of very strong electron acceptor solvents (such as the polyfluorinated TFE or HFIP) as co-solvents, in association with weaker electron acceptor solvents (such as DCM or chloroform) to disrupt association forces has been suggested.[24]

The purpose of a more complete swelling investigation of TM-12 and TM-24 peptide-resins (Figure 2) was not only to identify solvents more appropriate for their solvation (Table 2), but also to test the applicability of the previously proposed (AN+DN) polarity parameter as carried out with other polymeric materials.[17] Both peptide-resins displayed improved solvation in less polar solvents, characterized by low ε or (AN+DN) values (Figure 2). As already stressed, DMF (solvent 5) induces strong shrinking of the TM-12 resin beads (see C in Figure 2), for which a low degree of swelling (37%) was measured. This effect was not observed for the 24-mer (see D in Figure 2). Nevertheless, the enhanced solvation attained by both resins in apolar solvent systems is clearly indicative of the dominant influence of the hydrophobic styrene-resin matrix in association with the presence of peptide segments.

Another objective of this study was to verify the relationship between the swelling properties of the peptide-resins and the polarity of the medium, measured either by the dielectric constant or by the (AN+DN) parameter. Analysis of Figure 2 shows better correlation with the (AN+DN) scale than with ε. Therefore, the amphoteric character of the sum of the solvent's Lewis acid and Lewis base properties (in a 1:1 proportion) seems to yield a more adequate parameter with which to monitor solute-solvent interactions. In contrast, the macroscopic parameter ε yields a worse fit of the solvation behavior, since only electrostatic interactions are taken into account, while the alignment of the solvent and the solute dipoles is not considered.[25]

These findings thus endorse the appropriateness of the amphoteric (AN+DN) parameter for scaling polarity and are in accordance with the concept that the "two-parameter scale"[26] is better than the "single-parameter" one, as demonstrated,[17] for instance, when the Dimroth-Reichardt Et30 solvent term[27] or Hildebrand's solubility parameter δ[28] were also compared. With this novel (AN+DN) acid-base polarity scale (or "amphoteric constant"), which ranges from zero to 129,[17b] the slightly reduced swelling capacity of the mixed solvents 21 and 22 (Figure 2) can be interpreted in terms of the strength of association between their two components. The strong electron acceptor TFE tends to associate with a strong electron donor (DMF or DMSO) and not with the solute (peptide chains inside the bead), thus inducing less swelling. Such self-neutralizing effects of components are known, and characterize heterogeneous solvents.[21] When the chain-chain interaction is strong, the reduction in the swelling capacity of solvents 21 and 22 is more pronounced, as already demonstrated for other peptide-resins containing aggregating sequences.[17] In analogy to this effect, the reduced solvation capacity of DMF relative to 20% DMSO/NMP towards the aggregating resin-bound TM-12 sequence might therefore be credited to a much weaker chain-chain disruption capacity of the former polar aprotic solvent. Collectively, these findings reinforce the assumption that, under some circumstances, additional factors such as the characteristic inter- or intramolecular association forces of solute molecules must be taken into account when the appropriate concept of solvent polarity is to be applied. Efforts to clarify this issue are currently in progress.

Solvation and Degree of Chain Motion − The Spin Labeling EPR Approach

The pronounced difference in the swelling of TM-12-resin in DMF and in 20% DMSO/NMP can be analyzed in the light of the EPR data. A more strongly immobilized peptide chain population appears in DMF. Conversely, chain mobility is higher (narrower lines) and essentially constant throughout the TM-34 sequence elongation in 20% DMSO/NMP. The use of the W_o or the h_{-1}/h_0 parameters to assess the dynamics of labeled sites proved to be appropriate. For

the TOAC-labeled TM-12-resin, a more pronounced variation in peptide chain mobility was seen in DMF (Table 3). The W_o term, which encompasses the contribution of the more and the less immobilized components, ranged from 2.4 to 3.6 G. In addition, the peak height ratio h_{-1}/h_0 varied from 0.07 to 0.43. This, and other spectral findings, provide evidence of the potential of spin labeling EPR for examination of the dynamic properties of neighboring labeled sites in the polymer network. Clearly, the potential of this approach extends to the optimization of, for instance, combinatorial chemistry methods, which depend on appropriate polymeric materials for chemical reactions.

Spin labeling EPR has been widely used, especially in the study of biological systems.[29] Different spin labels have been employed,[30] varying in the way in which they are introduced into the system under study: either by covalent binding, as in the case of proteins or nucleic acids, or by physical intercalation, as in the case of micelles and bilayers. With regard to the TOAC probe, a variety of applications have been described,[31] including study of the structure-function relationships of partially[14b,32] or fully[33] biologically active peptides, of the conformation of model segments,[34] and of membrane protein fragments,[35] and also fluorescent peptides making use of the nitroxide's quenching effect.[12d,32,36] More recently, a second type of paramagnetic amino acid probe containing an alternative pyrrolidine-type structure (POAC) was also reported in the literature.[37]

Concerning SPPS, these results demonstrate the relevance of investigating the solvation behavior of peptide sequences by the combination of bead swelling studies with EPR spectral analysis. The study of the kinetics of coupling demonstrates the importance of peptide chain freedom for this polymer-supported technique. As expected, coupling was faster in 20% DMSO/NMP than in DMF. Moreover, emphasizing the direct relationship between these factors, the most prominent contaminant at the 16-residue stage was a peptide with deleted Leu^{13} and Ala^{16} residues (identified by mass spectrometry and amino acid analysis).

Although nearly four decades have elapsed since the inception of SPPS,[1] the assembly of long, aggregating peptide sequences is still a challenge, with unknown factors precluding the complete control of this method. The inherent complexity of such heterogeneous products, typically represented by transmembrane segments, still leaves questions to be answered. The difficulties involved not only in the chemical synthesis itself, but also in the appropriate purification strategy have already been addressed.[19,38] Various strategies to improve the critical coupling step have been proposed, involving different acylation components,[39] solvent systems,[16,23,24] addition of chaotropic agents,[40] increase of the temperature,[41] or the use of alternative protecting groups to avoid aggregation.[42] For the relevant issue of purification of insoluble fragments, different sequence-dependent approaches have been already proposed.[43]

In order to explain each degree of expansion of the network, it was recognized early in the polymer field that the swelling effect is subject to rules involving different thermodynamic properties.[16a,44] The combined approach described here, focusing on the stepwise monitoring of the growth of a long, hydrophobic transmembrane sequence, may represent a valid and useful strategy by which: i) to improve knowledge of the chemical processes occurring throughout the complex polymeric network, and ii) to facilitate the planning of alternative experimental conditions for the successful synthesis of difficult peptide sequences.

Conclusion

Solvent-dependent variation in the physicochemical characteristics of the polymer backbone was monitored during the chemical elongation of a resin-bound 34-mer transmembrane fragment (TM-34) of the B2 bradykinin receptor. The solvation behavior of peptide-resins containing minor TM-34 segments was gauged by measuring the swelling of beads and the degree of chain motion by means of EPR spectra obtained by labeling the peptides with the paramagnetic amino acid 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC). Pronounced chain aggregation in DMF at the 12-mer stage of the sequence was observed either by the prominent shrinking of beads or by the appearance of a significant amount of strongly immobilized chain population in this solvent. Accordingly, a slower coupling reaction and a lower yield of the synthesis was observed in DMF than in the mixed solvent 20% DMSO/NMP, which induced constant and optimized solvation throughout the TM-34 assembly in the resin. In addition, a more complete swelling investigation of two resin-bound segments of the TM-34 sequence (TM-12 and TM-24) allowed the potential of the simple sum of the solvent electron acceptor and electron donor properties, in 1:1 proportion, to be confirmed as an optional and sensitive polarity scale. In summary, the combined bead solvation-chain motion study developed here seemed to be sensitive and valuable not only for overcoming possible difficulties in some resin-supported reactions but also for enhancing knowledge of multiple factors that may govern the complex polymer network solvation phenomenon.

Experimental Section

General Remarks

Materials: Reagents and solvents for solid-phase peptide synthesis were of analytical grade and used from recently opened containers, without further purification. Boc-amino acids were purchased from Bachem (Torrance, CA), and the following side chain-protected amino acid were used: Asp (β-OcHex), Cys (MeBzl), Glu (γ-OcHex), Ser (Bzl), Thr (Bzl), Trp (For), and Tyr (2-Cl-Z).

Methods. Peptide Synthesis: TM-34 was synthesized manually by standard Boc-chemistry on a methylbenzhydrylamine-resin (0.34 mmol/g). The synthesis scale was 0.5 mmol/g, and the Boc protecting group was removed from the amine group with 30% trifluoroacetic acid in DCM (30 min), followed by washings with *i*PrOH containing 2% anisole and 10% DIEA in DCM for deprotonation of the peptide amine function. Coupling was performed with a 2.5-

fold excess of Boc-amino acid/TBTU/HOBt (1:1:1) in the presence of excess DIEA (5 equiv.) in DMF or 20% DMSO/NMP. Recouplings were performed under similar conditions when needed. All couplings were monitored by qualitative ninhydrin test and when positive, acetylation was performed with 50% acetic anhydride in DCM (15 min). Small aliquots of several shorter resin-bound TM-34 fragments were cleaved from the resin in HF/*o*-cresol/DMS/EDT (8.5:0.5:0.5:0.5,v/v). The reaction time was 90 min at 0 °C and the excess HF and scavengers were eliminated under vacuum. The peptide-resins were washed with ethyl acetate and dried, and the peptides were extracted with 5 to 50% acetic acid in water and lyophilized. Details of peptide synthesis yields, purification and analytical characterization by HPLC, amino acid analysis, and mass spectrometry were published previously.[19] Crude peptides characterization: TM-8 ESI-MS: m/z = 905.92 [M + H$^+$]. $C_{40}H_{63}N_{11}O_{13}$ (906.01): calcd. AAA: Asp 2.49 (3); Thr 1.08 (1); Ala 1.26 (1); Ile 2.20 (2); Phe 0.97 (1); TM-12 ESI-MS: m/z = 1407.16 [M + H$^+$]. $C_{68}H_{94}N_{16}O_{17}$ (1407.59): calcd. AAA: Asp 3.25 (3); Thr 1.15 (1); Pro 0.96 (1); Ala 2.04 (2); Ile 1.85 (2); Phe 1.75 (2); Trp 0.92 (1); TM-16 ESI-MS: m/z = 1735.06 [M + H$^+$]. $C_{82}H_{118}N_{20}O_{22}$ (1735.96): calcd. AAA: Asp 3.01 (3); Thr 0.97 (1); Ser 1.04 (1); Pro 0.93 (1); Gly 1.01 (1); Ala 3.03 (3); Ile 1.91 (2); Leu 1.09 (1); Phe 2.11 (2); Trp 1.03 (1); TM-20 ESI-MS: m/z = 2190.05 [M + H$^+$]. $C_{104}H_{156}N_{24}O_{28}$ (2190.53): calcd. AAA: Asp 4.00 (4); Thr 1.04 (1); Ser 1.09 (1); Pro 0.97 (1); Gly 1.00 (1); Ala 3.11 (3); Ile 2.45 (3); Leu 3.18 (3); Phe 2.00 (2); Trp 0.92 (1); TM-24 ESI-MS: m/z = 2502.06 [M + H$^+$]. $C_{118}H_{180}N_{28}O_{32}$ (2502.90): calcd. AAA: Asp 4.11 (4); Thr 1.08 (1); Ser 1.00 (1); Pro 0.96 (1); Gly 1.93 (2); Ala 4.77 (5); Ile 2.79 (3); Leu 4.00 (4); Phe 2.07 (2); Trp 0.98 (1); TM-28 ESI-MS: m/z = 2949.90 [M + H$^+$]. $C_{139}H_{209}N_{33}O_{38}$ (2950.39): calcd. AAA: Asp 5.24 (5); Thr 1.13 (1); Ser 0.95 (1); Pro 0.99 (1); Gly 2.70 (3); Ala 4.80 (5); Ile 3.57 (3); Leu 4.59 (5); Tyr 0.95 (1); Phe 2.01 (2); Trp 0.99 (1); TM-34 ESI-MS: m/z = 3792.18 [M + H$^+$]. $C_{173}H_{256}N_{40}O_{52}S_2$ (3792.32): calcd. AAA: Asp 5.30 (5); Thr 2.27 (2); Ser 1.36 (1); Glu 1.15 (1); Pro 0.95 (1); Gly 3.31 (3); Ala 6.04 (6); Val 0.88 (1); Ile 3.50 (4); Leu 4.72 (5); Tyr 0.75 (1); Phe 2.30 (2); Trp 0.84 (1).

Measurement of Peptide-Resin Swelling: Swelling studies of the narrowly sized bead populations were performed as published elsewhere[16a,17,45] after the resins had been dried under vacuum using an Abderhalden-type apparatus. Briefly, 150 to 200 dry and swollen beads of each resin, allowed to solvate overnight, were spread over a microscope slide and measured directly with an Olympus model SZ11 microscope coupled with Image-Pro Plus 3.0.01.00 version software. The values of bead diameter distribution were estimated by geometric means and geometric standard deviations as published elsewhere.[46]

EPR Studies: EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 spectrometer at room temperature (22 ± 2 °C) in Wilmad flat quartz cells. Labeled peptidyl-resins were pre-swollen overnight in the solvent under study. The magnetic field was modulated with amplitudes less than one-fifth of the line widths, and the microwave power was 5 mW to avoid saturation effects. Details of the procedure for TOAC−labeling of resins have been reported.[15]

Yield of the Coupling Reaction: The TM-12-resin (50−100 μmol) was elongated with the subsequent residue of the sequence (Boc-Leu-OH, 2.5 equiv.), in a reaction vessel thermostatted at 25 °C, by the BOP (2.5 equiv.)/DIEA (5 equiv.) coupling method in DMF or 20% DMSO/NMP (0.2 mM for Boc-Leu-OH and BOP, and 0.4 mM for DIEA) as solvent. The rate of rotation of the reaction flask was 20 rpm. The acylating reagents were dissolved in the solvent under investigation and added to the reaction vessel containing peptide-resin pre-swollen in the same solvent. The yield of coupling was monitored by the picric acid method[47] and each experiment was performed in duplicate.

Abbreviations: Abbreviations for amino acids and nomenclature of peptide structure follow the recommendations of the IUPAC-IUB (Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1971**, *247*, 997). Other abbreviations are as follow: AAA: amino acid analysis; Boc = tert-butyloxycarbonyl; Bzl = benzyl; BOP = benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; 2-Cl-Z = chlorobenzyloxycarbonyl; CD = circular dichroism; DCM = dichloromethane; DIEA = diisopropylethylamine; DMF = *N*,*N*′-dimethylformamide; DMS = dimethyl sulfide; DMSO = dimethyl sulfoxide; EDT = ethanedithiol; EPR = electron paramagnetic resonance; EtOH = ethanol; HFIP = hexafluoro2-propanol; HOBt = 1-hydroxybenzotriazole; Fmoc = 9-fluorenylmethyloxycarbonyl, HPLC = high-performance liquid chromatography; For = formyl; IR = infrared; MeBzl = methylbenzyl; MeOH = methanol; NMP = *N*-methylpiperidinone; NMR = nuclear magnetic resonance; β-OcHex = cyclohexyl; *i*PrOH = 2-propanol; SPPS solid-phase peptide synthesis; TBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA = triethylamine; TFA = trifluoroacetic acid; TFE = trifluoroethanol; TOAC = 2,2,6,6-tetramethypiperidine-1-oxyl-4-amino-4-carboxylic acid.

Acknowledgments

This work was funded by grants from the Brazilian Scientific Agencies Fapesp, CNPq and Capes, and from the Instituto de Cooperación Iberoamericana and by the Generalitat de Catalunya (Centre de Referència en Biotecnologia −E.M.C.A.M. Spain). C.M.P. A. S.S. and C.R.N. are recipients of CNPq research fellowships.

[1] R. B. Merrifield, *J. Am. Chem. Soc.* **1963**, *85*, 2149−2154.
[2] [2a] G. Barany, R. B. Merrifield, *The Peptides*, Academic Press Inc.: New York, **1979**, vol. 2, pp 1−284. [2b] J. M. Stewart, J. D. Young, *Solid Phase Peptide Synthesis*, Pierce Chemical Company. Rockford, III, **1984**. [2c]S. B. H. Kent, *Ann. Rev. Biochem.* **1988**, *57*, 957−989. [2d] E. Atherton, D. I. J. Clive, R. C. Sheppard, *J. Am. Chem. Soc.* **1975**, *97*, 6584−6585. [2e] G. B. Fields, R. L. Noble, *Int. J. Peptide Protein Res.* **1990**, *35*, 161−214. [2f] S. A. Kates, F. Albericio, In *Solid-Phase Synthesis. A Practical Guide*, Marcel Dekker, Inc. New York, Basel, 275−330, **2000**.
[3] [3a] R. L. Letsinger, J. L. Finnan, G. A. Heavner, W. B. Lunsford, *J. Am. Chem. Soc.* **1975**, *85*, 3278−3279. [3b] V. Amarnath, A. D. Broom, *Chem. Rev.* **1977**, *77*, 183−217.
[4] [4a] J. M. Fréchet, C. Schuerch, *J. Am. Chem. Soc.* **1971**, *93*, 492−498. [4b] U. Zehavi, A. Patchornik, *J. Am. Chem. Soc.* **1973**, *95*, 5673−5678.
[5] [5a] G. Jung, A. G. Beck-Sickinger, *Angew. Chem. Int. Ed. Eng.* **1992**, *31*, 367−383. [5b] K. S. Lam, M. Lebl, V. Krchanak, *Chem. Rev.* **1997**, *97*, 411−448. [5c] K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, R. J. Knapp, *Nature* **1991**, *354*, 82−84.
[6] [6a] M. Fridkin, A. Patchornik, E. Kachalski, *J. Am. Chem. Soc.* **1966**, *88*, 3164−3166. [6b] J. M. J. Frechet, *Tetrahedron* **1981**, *37*, 663−683. [6c] P. H. H. Hermkens, H. C. J. Ottenheim, D. Rees, *Tetrahedron* **1996**, *52*, 4527−4554. [6d] L. A. Thompson, J. A. Ellman, *Chem. Rev.* **1996**, *95*, 555−600.
[7] [7a] S. A. Kates, B. F. McGuiness, C. Blackburn, G. W. Griffin, N. A. Solé, G. Barany, F. Albericio, *Biopolymers* **1998**, *47*, 365−380. [7b] M. Lebl, *Biopolymers* **1998**, *47*, 397−404. [7c] J. W. Labadie, *Curr. Opin. Chem. Biol.* **1998**, *2*, 346−352. [7d] M. Meldal, in *Methods in Enzymology: Solid Phase Peptide Synthesis*, (Ed.: G. Fields), Academic Press, 83−103, **1997**.
[8] [8a] C. M. Deber, M. K. Lutek, E. P. Heimer, A. M. Felix, *Peptide Res.* **1989**, *2*, 184−188. [8b] W. T. Ford, T. Balakrishanan, *Macromolecules* **1981**, *14*, 284−288. [8c] A. G. Ludwick, L. W. Jelinski,

D. Live, A. Kintamar, J. J. Dumais, *J. Am. Chem. Soc.* **1986**, *108*, 6493−6496. [8d] R. Warrass, M. Wieruszeski, C. Bouitillon, G. Lippens, *J. Am. Chem. Soc.* **2000**, *122*, 1789−1795. [8e] E. Bayer, K. Albert, H. Willisch, W. Rapp, B. Hemmasi, *Macromolecules* **1990**, *23*, 1937−1940.

[9] [9a] J. C. Hendrix, K. J. Halverson, J. T. Jarret, P. T. Lansbury, Jr., *J. Org. Chem.* **1990**, *55*, 4517−4518. [9b] S. S. Rahman, D. J. Busby, D. C. Lee, *J. Org. Chem.* **1998**, *63*, 6196−6199. [9c] B. Yan, *Acc. Chem. Res.* **1998**, *31*, 621−630. [9d] R. C. L. Milton, S. C. F. Milton, P. A. Adams, *J. Am. Chem. Soc.* **1990**, *112*, 5039−5046.

[10] [10a] Y. H. Li, L. M. Chan, L. Tyler, R. T. Moody, C. M. Himel, D. M. Hercules, *J. Am. Chem. Soc.* **1975**, *97*, 3118−3126. [10b] K. J. Shea, D. Y. Sasaki, G. J. Stoddaard, *Macromolecules* **1989**, *22*, 1722−1730. [10c] A. R. Vaino, K. D. Janda, *J. Comb. Chem.* **2000**, *2*, 579−596.

[11] V. N. R. Pillai, M. Mutter, *Acc. Chem. Res.* **1981**, *14*, 122−130.

[12] [12a] D. B. Chesnut, J. F. Hower, *J. Phys. Chem.* **1971**, *75*, 907−912. [12b] T. C. Ward, J. T. Brooks, *Macromolecules* **1974**, *7*, 207−212. [12c] S. L. Regen, *J. Am. Chem. Soc.* **1974**, *96*, 5275−5276. [12d] A. R. Vaino, D. B. Goodin, K. D. Janda, *J. Comb. Chem.* **2000**, *2*, 330−336.

[13] A. Rassat, P. Rey, *Bull. Soc. Chim. Fr.* **1967**, *3*, 815−815.

[14] [14a] C. R. Nakaie, S. Schreier, A. C. M. Paiva, *Braz. J. Med. Biol. Res.* **1981**, *14*, 173−180. [14b] C. R. Nakaie, S. Schreier, A. C. M. Paiva, *Biochim. Biophys. Acta* **1983**, *742*, 63−71. [14c] R. Marchetto, S. Schreier, C. R. Nakaie, *J. Am. Chem. Soc.* **1993**, *117*, 11042−11043.

[15] [15a] E. M. Cilli, R. Marchetto, S. Schreier, C. R. Nakaie, *Tetrahedron Lett.* **1997**, *38*, 517−520. [15b] E. M. Cilli, R. Marchetto, S. Schreier, C. R. Nakaie, *J. Org. Chem.* **1999**, *64*, 9118−9123. [15c] S. C. F. Ribeiro, S. Schreier, C. R. Nakaie, E. M. Cilli, *Tetrahedron Lett.* **2001**, *42*, 3243−3246.

[16] [16a] V. K. Sarin, S. B. H. Kent, R. B. Merrifield, *J. Am. Chem. Soc.* **1980**, *102*, 5463−5470. [16b] G. B. Fields, C. G. Fields, *J. Am. Chem. Soc.* **1991**, *113*, 4202−4207. [16c] K. C. Pugh, E. J. York, J. M. Stewart, *Int. J. Peptide Protein Res.* **1992**, *40*, 208−213. [16d] R. Santini, M. C. Griffth, M. Qi, *Tetrahedron Lett.* **1998**, *39*, 8951−8954.

[17] [17a] E. M. Cilli, E. Oliveira, R. Marchetto, C. R. Nakaie, *J. Org. Chem.* **1996**, *81*, 8992−9000. [17b] L. Malavolta, E. Oliveira, E. M. Cilli, C. R. Nakaie, *Tetrahedron* **2002**, *58*, 4383−4394.

[18] [18a] V. Gutmann, *Electrochim. Acta* **1976**, *21*, 661−670. [18b] V. Gutmann, *The Donor-Acceptor Approach to Molecular Interactions*, Plenum Press, New York, **1978**.

[19] E. Oliveira, A. Miranda, F. Albericio, D. Andreu, A. C. M. Paiva, C. R. Nakaie, M. Tominaga, *J. Peptide Res.* **1997**, *49*, 300−307.

[20] A. E. McEarchen, R. R. Shelton, S. Bhakta, R. Obernolte, C. Bach, P. Zuppan, J. Fujisaki, R. W. Aldrich, K. Jarnagin, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 7724−7728.

[21] M. Narita, S. Honda, H. Umeyama, S. Obana, *Bull. Chem. Soc. Jpn.* **1988**, *61*, 281−284.

[22] [22a] M. Mutter, K. H. Altman, D. Bellof, A. Florsheimer, J. Herbert, M. Huber, B. Klein, L. Strauch, T. Vorherr, H. U. Gremlich, *Peptides: Structure and Function*, (Eds.: C. M. Deber, V. J. Hruby, K. D. Kopple), Pierce Chemical Co Rockford, Illinois, pp 423, **1985**. [22b] S. B. H. Kent, *Ann. Rev. Biochem.* **1988**, *57*, 957−989. [22c] W. S. Hancock, D. J. Prescott, P. R. Vagelos, G. R. Marshall, *J. Org. Chem.* **1973**, *38*, 774−781.

[23] G. B. Fields, K. M. Otteson, C. G. Fields, R. L. Noble, *Innovation and Perspectives in Solid Phase Synthesis*, (Ed.: R. Epton), 241−249, **1990**.

[24] [24a] D. Yamashiro, J. Blake, C. H. Li, *J. Am. Chem. Soc.* **1972**, *94*, 2855−2859. [24b] M. Narita, H. Umeyama, T. Yoshida, *Bull. Chem. Soc. Jpn.* **1988**, *62*, 281−284. [24c] Y. Nishiuchi, T. Inui, H. Nishio, J. Bodi, T. Tsuji, T. Kimura, S. Sakakibara, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 13549−13554.

[25] A. J. Parker, *Chem. Rev.* **1969**, *69*, 1−35.

[26] [26a] T. M. Krygowski, W. R. Fawcett, *J. Am. Chem. Soc.* **1975**, *97*, 2143−2148. [26b] W. R. Fawcett, *J. Phys. Chem.* **1993**, *97*, 9540−9546. [26c] C. G. Swain, *J. Org. Chem.* **1984**, *49*, 2005−2010.

[27] [27a] K. Dimroth, C. Reichardt, T. Siepmann, F. Bohlman, *Justus Liebigs Ann. Chem.* **1963**, *661*, 1−37. [27b] C. Reichardt, *Chem. Rev.* **1994**, *94*, 2319−2358.

[28] J. H. Hildebrand, *Chem. Rev.* **1949**, *44*, 37−45.

[29] [29a] H. M. McConnell, B. G. McFarland, *Quart. Rev. Biophys.* **1970**, *3*, 91−136. [29b] S. Schreier, C. F. Polnaszek, I. C. P. Smith, *Biochim. Biophys. Acta* **1978**, 515, 395−436. [29c] D. Marsh, *Biosci. Rep.* **1999**, *19*, 253−259. [29d] W. L. Hubbell, D. S. Cafiso, C. Altenbach, *Nat Struct. Biol.* **2000**, *7*, 735−739. [29e] P. P. Borbat, A. J. Costa-Filho, K. A. Earle, J. K. Moscicki, J. H. Freed, *Science.* **2001**, *291*, 266−269.

[30] [30a] J. F. W. Keana, *Chem. Rev.* **1978**, *78*, 37−64. [30b] E. G. Rozantsev, *Free Nitroxyl Radicals,* Plenum Press, New York, **1970**.

[31] C. Toniolo, M. Crisma, F. Formaggio, *Biopolymers* **1998**, *47*, 153−158.

[32] C. R. Nakaie, E. G. Silva, E. M. Cilli, R. Marchetto, S. Schreier, T. B. Paiva, A. C. M. Paiva, *Peptides* **2002**, *23*, 65−70.

[33] [33a] S. R. Barbosa, E. M. Cilli, M. T. Lamy-Freund, A. M. L. Castrucci, C. R. Nakaie, *FEBS Lett.* **1999**, *446*, 45−48. [33b] C. R. Nakaie, S. R. Barbosa, R. F. F. Vieira, R. M. Fernandez, E. M. Cilli, A. M. L. Castrucci, M. A. Visconti, A. S. Ito, M. T. Lamy-Freund, *FEBS Lett.* **2001**, *497*, 103−107.

[34] [34a] M. L. Smithe, C. R. Nakaie, G. R. Marshall, *J. Am. Chem. Soc.* **1995**, *117*, 10555−10562. [34b] C. Toniolo, E. Valente, F. Formaggio, M. Crisma, G. Pilloni, C. Corvaja, A. Toffoletti, G. V. Martinez, M. P. Hanson, G. L. Millhauser, C. George, J. Flippen-Anderson, *J. Pep. Science* **1995**, *1*, 45−57. [34c] J. C. McNulty, Silapie, J. L. M. Carnevali, C. T. Farrar, R. G. Griffin, F. Formaggio, M. Crisma, C. Toniolo, G. L. Millhauser, *Biopolymers* **2000**, *55*, 479−485. [34d] Corvaja, C.E. Sartori, A. Toffoletti, F. Formaggio, M. Crisma, C. Toniolo, *Biopolymers* **2000**, *55*, 486−495.

[35] [35a] T. A. Pertinhez, C. R. Nakaie, R. S. H. Carvalho, A. C. M. Paiva, M. Tabak, F. Toma, S. Schreier, *FEBS Letters* **1995**, *375*, 239−242. [35b] T. A. Pertinhez, C. R. Nakaie, A. C. M. Paiva, S. Schreier, *Biopolymers* **1997**, *42*, 821−829.

[36] B. Pispisa, Palleschi,L. Stella, M. Venanzi, C. Toniolo, *J. Phys. Chem. B.* **1998**, *102*, 7890−7898.

[37] M. Tominaga, S. R. Barbosa, E. F. Poletti, J. Zukerman-Schpector, R. Marchetto, S. Schreier, A. C. M. Paiva, C. R. Nakaie, *Chem. Pharm. Bull.* **2001**, *49*, 1027−1029.

[38] [38a] R. Bollhagen, J. Knolle, H. Betz, E. Grell, *J. Chromatogr. A* **1995**, *711*, 181−186. [38b] S. Y. Kassim, I. M. Restrepo, A. G. Kalivretenos, *J. Chromatography A* **1998**, *816*, 11−20. [38c] C. M. Topham, L. Moulédous, J. C. Meunier, *Prot. Eng.* **2000**, *13*, 477−490.

[39] [39a] L. A. Carpino, G. Y. Han, *J. Am. Chem. Soc.* **1970**, *92*, 5748−5749. [39b] J. Castro, L. E. Nguyen, G. Evin, C. Selve, *Tetrahedron Lett.* **1975**, *14*, 1219−1221. [39c] Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397−4398. [39d] L. A. Carpino, A. El-Faham, C. A. Minor, F. Albericio, *Chem. Commun.* **1994**, 201−203. [39e] R. Knorr, A. Trzeciak, W. Bannworth, D. Gillessen, *Tetrahedron Lett.* **1989**, *30*, 1927−1930.

[40] W. A. Klis, J. M. Stewart, In *Peptides: Structure and Biology*, (Eds.: J. E. Rivier, G. R. Marshall), Escom, Leiden, Netherlands, 904−906, **1990**.

[41] [41a] J. P. Tam, *Int. J. Peptide Protein Res.* **1987**, *29*, 421−431. [41b] A. K. Rabinovich, J. E. Rivier, *Peptides: Chemistry, Structure and Biology*, (Eds.: R. S. Hodges, J. A. Smith), Escom, Leiden, **1993**, 71−73. [41c] L. M. Varanda, M. T. M. Miranda, *J. Peptide Res.* **1997**, *50*, 102−108.

[42] T. Johnson, M. Quibell, D. Owen, R. C. Sheppard, *Chem. Commun.* **1993**, 369−372.

[43] [43a] P. Lloyd-Williams, F. Albericio, E. Giralt, *Tetrahedron* **1993**, *49*, 11065−11123. [43b] M. Gairi, P. Lloyd-Willians, F. Albericio, E. Giralt, *Int. J. Peptide Protein Res.* **1995**, *46*, 119−133. [43c] L.

E. Fisher, D. M. Engelman, *Anal. Biochem.* **2001**, *293*, 102−108. [43d] M. Goetz, F. Rusconi, M. Belghazi, J. M. Schimitter, E. Dufourc, *J. Chromatography B* **2000**, *737*, 55−61.
[44] [44a] P. J. Flory, *Macromolecules* **1979**, *12*, 119−122. [44b] P. J. Flory, J. Rehner, *J. Chem. Phys.* **1943**, *11*, 521−526. [44c] A. M. F. Barton, *Chem. Rev.* **1975**, *75*, 731−753.
[45] [45a] R. Marchetto, A. Etchegaray, C. R. Nakaie, *J. Braz. Chem. Soc.* **1992**, *3*, 30−37. [45b] J. P. Tam, Y. A. Lu, *J. Am. Chem. Soc.* **1995**, *117*, 12058−12063.
[46] R. R. Irani, C. F. Callis, *Particle Size: Measurement, Interpretation and Application*, John Wiley & Sons, New York, **1963**.
[47] B. F. Gisin, *Anal. Chim. Acta* **1972**, *58*, 248−249.

Received May 6, 2002
[O02239]