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PROTON NMR STUDY OF THE CONFORMATION OF BRADYKININ: pH TITRATION

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SUMMARY: pH titration by ^{1}H-NMR spectroscopy of the peptide hormone bradykinin was carried out in $^{2}H_2O$. Assignment of all α-proton signals and of most of the other resonances permitted the extraction of vicinal coupling constants $^{3}J_{\alpha\beta,\beta'}$ from which side chain conformation of all residues could be followed and analyzed as a function of pH. It is shown that the ionization of the terminal COOH group affects simultaneously the Arg-9 and Phe-8 chemical shifts and side chain orientation, and the non-equivalence of the Gly-4 methylene protons. Cooperative effects along the peptide backbone or a folded structure of the C-terminal part of bradykinin could explain this effect.

INTRODUCTION:

It has been shown on numerous occasions that the study of a peptide's conformational parameters as a function of pH is able to yield rich information about intramolecular interactions, distances and through-space influences. Potentiometric titration, fluorescence, circular dichroism (CD), ^{13}C-NMR and ^{1}H-NMR methods all lend themselves to this kind of investigation.

The conformation of the peptide hormone bradykinin (BK): Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg has been investigated by several authors [1 and references therein] . More specifically, the effects of pH titration on bradykinin were studied by potentiometry (Paiva et al. [2]), by ^{13}C-NMR (London et al. [3]) and by circular dichroism (Lintner et al. [1,4]). The proton-NMR titration described here completes the pi cture obtained from the previous studies in greater detail.

MATERIAL AND METHODS:

The synthesis of bradykinin has been described previously [5]. The peptide was lyophilized twice from $^{2}H_2O$ (99.8% enrichment, Commissariat à l'Energie Atomique, France), then dissolved in $^{2}H_2O$ at a concentration of 10^{-2} M. pH was measured directly in the 5mm NMR tube using a combined glass electrode (Ingold) of 3 mm diameter, and was adjusted by addition of small amounts of ^{2}HCl and $NaO^{2}H$. The pH values reported are the uncorrected meter readings. The ^{1}-H-NMR spectra were recorded at 250 MHz on the Cameca TSN 250 spectrometer of the Laboratoire Interuniversitaire de RMN (Faculté de Pharmacie, Marseille) operating in the FT mode at 22°C. Chemical shifts are given downfield from 2,2,3,3 tetradeutero 3-(trimethylsilyl)propionic acid, sodium salt (Merck) used as internal standard. Below pH 5, the 0.017 ppm shift of the protonated TSP was taken into account.

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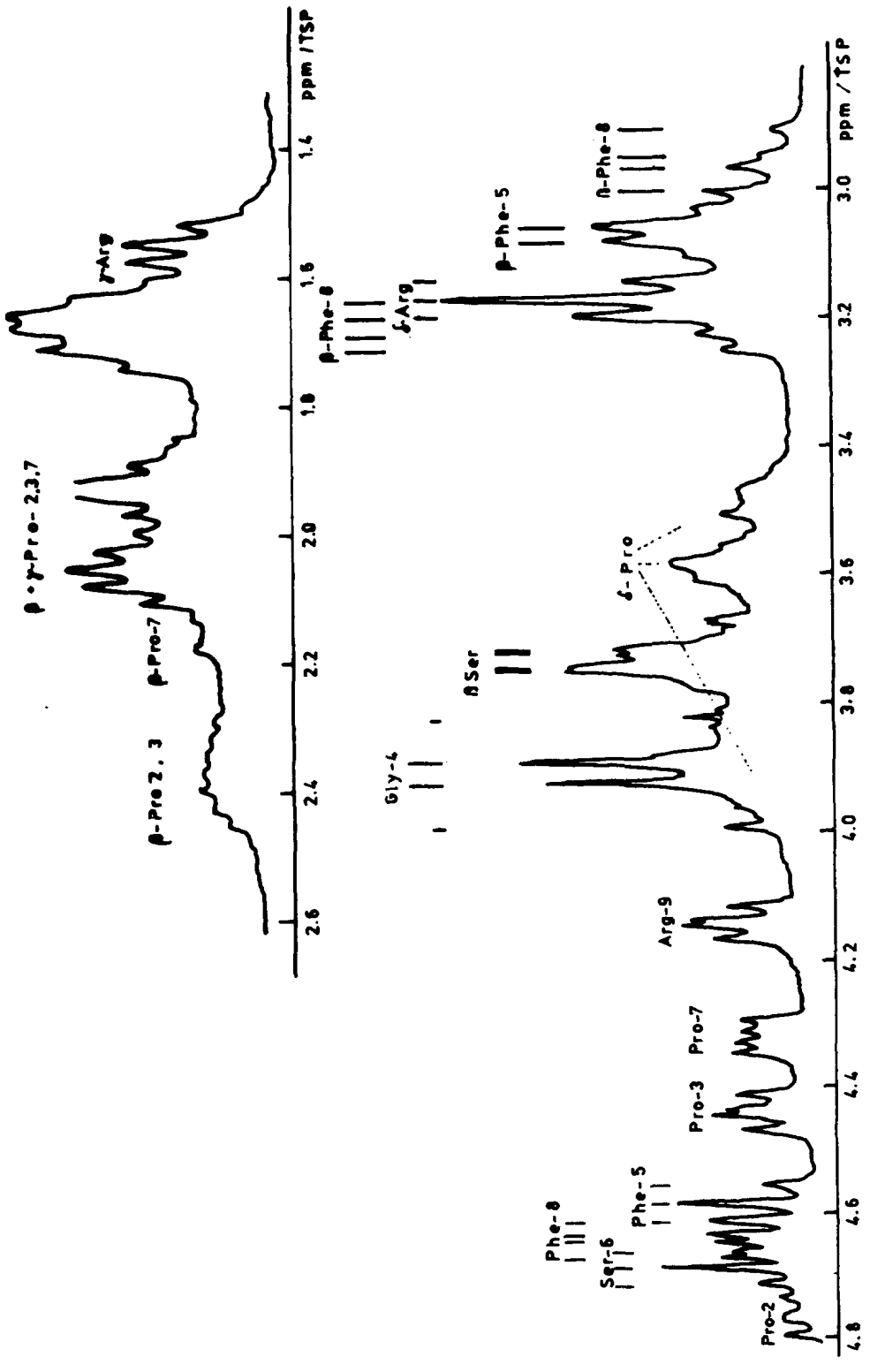

Fig.1: 250 MHz ^{1}H-NMR spectrum of bradykinin in $^{2}H_2O$, pH 8; 1-5 ppm region.

RESULTS: 1) spectral assignment (fig.1&2)

Figure 1 shows the 1-5 ppm region of the 250 MHz spectrum of bradykinin at the pH of dissolution (∿8). The assignment was carried out by combined use of chemical shift values from the literature [6] (glycine, β- and γ-protons of proline, β-protons of phenylalanine and serine), extensive decoupling experiments , titration and amino acid substitution effects.

With 2 arginine, 2 phenylalanine and 3 proline residues, assignment of the signals to individual residues required further analysis. Among the three proline α-proton signals, the one at highest fields was assigned to Pro-7: even if the shifts induced by ring current effects have to be interpreted with caution [7] the presence of the aromatic ring in Phe-8 is most likely to be the cause for the Pro-7 upfield shift (see below: coupling constants and [8]). On the other hand, experience shows that the α-protons of a residue preceding a proline residue is often shifted downfield. Hence the assignment of Pro-2.

Fig. 2a shows the α-proton titration curves from where the Arg-9, the Arg-1 (up to pH 7, above which the signal seems to broaden and to disappear beneath the δ-Pro and Gly resonances) and the Phe-8 signals can be assigned on the basis of their pH sensitivity. The assignment of Pro-2 described above also is confirmed by the amino-group titration dependence.

2) chemical shifts as a function of pH: Fig. 2a, 2b

Figs.2a and 2b show that the titration of the two ionizable groups NH_3^+ and COOH of bradykinin is reflected in the chemical shift values of not only the directly involved Arg-1 and Arg-9 residues but also of residues more or less removed from the N- and C-termini. The most striking effect is produced on the Gly-4 resonance which evolves from a single peak at pH 1 to a well separated AB pattern ($\Delta\delta$= 18 Hz) at pH 5, thus concomitant with the titration of the carboxyl group on Arg-9.

Similarly, the α-resonances of Phe-8, the β and β'signals of Phe-8 and a δ-resonance of proline (probably Pro-7) undergo slight shifts with the titration of COOH (fig. 2b). To a still smaller, but nonetheless distinct degree, the β and β' proton chemical shifts of Ser-6 become increasingly separated (nonequivalent). Finally, the β-protons of Arg-1 and Pro-2 as well as of Arg-9 and Pro-7 undergo shifts related to the titration of the N- and the C-terminal groups (not shown). Only the γ-protons of proline-2,3,7 and the β-protons of Pro-3 seem to be free of any pH dependence, as even the aromatic protons of Phe-8 are shifted downfield by about 4 Hz during the carboxyl titration $COOH\text{-}COO^-$ (pK∿ 3.3).

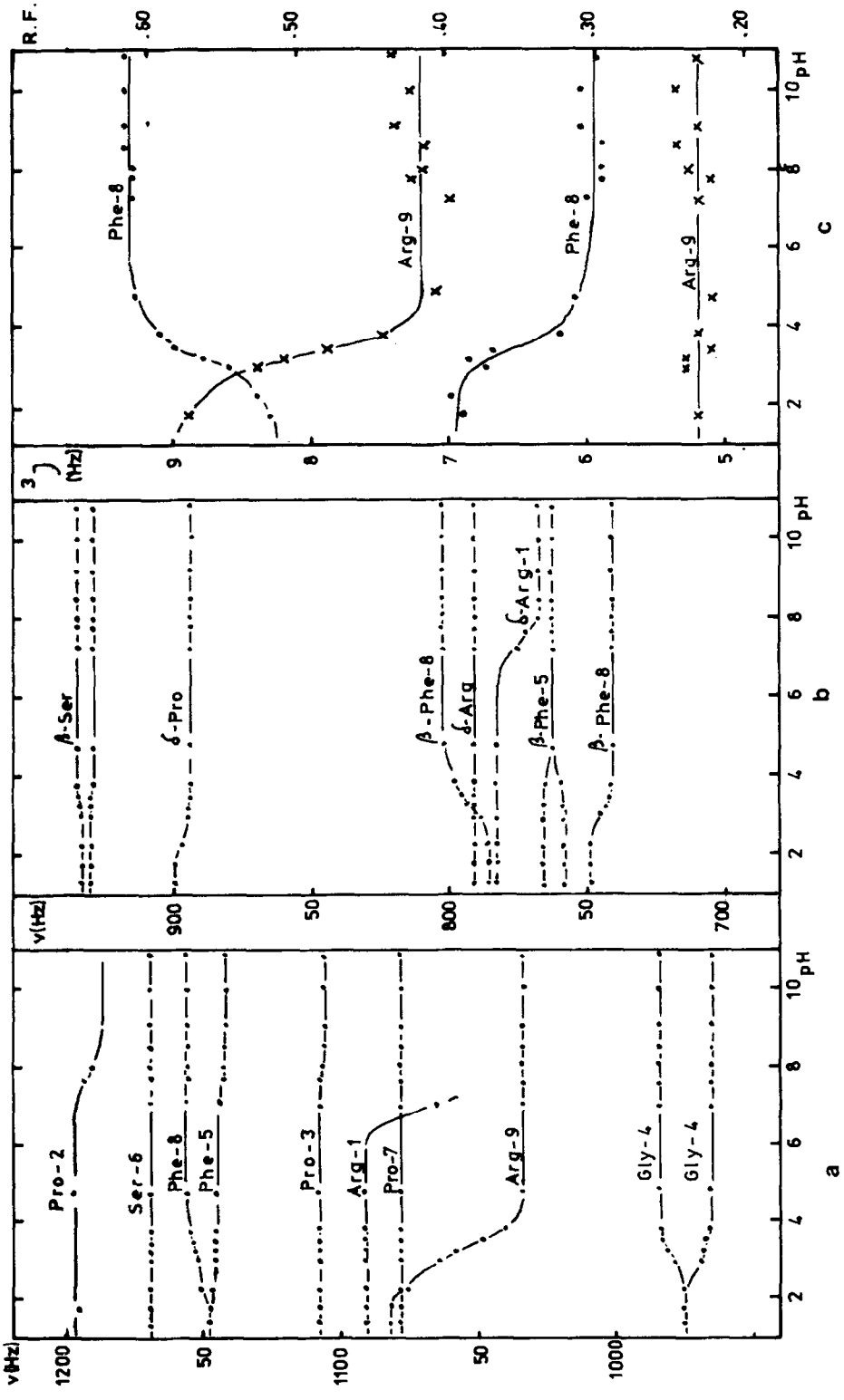

Fig. 2: pH titration curves of bradykinin ^{1}H-NMR parameters; a) α-proton region; b) β- and δ-proton region; c) vicinal coupling constants.

3) Coupling constants:

Table 1 lists the $^3J_{\alpha\beta,\beta'}$ ($^2J_{\alpha\alpha'}$ in case of glycine) coupling constants (at pH 4.7). They were obtained on the α-proton signals in all cases and confirmed by analysis of the AB pattern of the β-protons in the case of Ser-6 and Phe-8. The translation into rotamer distribution according to Pachler (J_t=13.6 Hz; J_g=2.6 Hz) for residues Arg-1, PHe-5, Ser-6, Phe-8 and Arg-9 and into θ angles according to Cung et al. [9] for Pro-2,3,7 is also given in table 1. Fig. 2c shows the pH dependence of $^3J_{\alpha\beta}$ and $^3J_{\alpha\beta'}$ of residues Arg-9 and Phe-8. All other coupling constants are pH invariant.

Items to be noted include:

-The set of $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ coupling constants distinguishes the three proline residues indicating that their ring geometry is different in each position. The coupling constants, however, do not vary with pH.

- Arg-1 and Arg-9 have very different coupling constants: in fact, the Arg-1 α-proton signal is always observed as a pseudotriplet, from which only the sum of $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ can be deduced. This sum is rather small and yields a high fraction of rotamer III population. Unfortunately the signal could not be followed beyond pH > 7.

-The rotamer distribution of the Arg-9 side chain undergoes the effect of the COOH titration and changes from 0.57/0.24/0.19 to 0.41/0.23/0.36, thus toward a more averaged distribution. Simultaneously, the Phe-8 rotamer population changes from 0.52/0.39/0.09 to 0.61/0.30/0.09; thus in the direction of increased stability of rotamer I. Such a change has been observed for other

TABLE 1

	$^3J_{\alpha\beta'}$ a)	$^3J_{\alpha\beta}$ a)	R I; χ_1=-60°	R II; χ_1=180°	R III; χ_1= 60°
ARG	Σ = 12.6		Σ = 0.69		0.31
PRO	8.0	6.5		θ_1 = 30° b)c)	θ_2 = 125°
PRO	8.4	5.6		θ_1 = 25° b)c)	θ_2 = 120°
GLY	^2J = -17.2				
PHE	Σ = 14.0		Σ = 0.80		0.20
SER	7.0	6.2	0.40	0.33	0.27
PRO	8.4	4.3		θ_1 = 25° b)c)	θ_2 = 115°
PHE	9.3	6.1	0.61	0.32	0.07
ARG	7.1	5.1	0.41	0.23	0.36

a) assignment of β,β' (RI and R II) is confirmed by ^{13}C-NMR of ^{13}C-enriched samples and will be published elsewhere. Examples are given in ref. [12].
b) values obtained from the Cung et al. [9] relationship. Single values of χ_1 cannot be established from the combination of θ_1 and θ_2 in each proline residue. This reflects a certain flexibility of the pyrrolidine ring which decreases from Pro-2 to Pro-7.
c) rounded to the nearest 5 degrees.

peptides, too (Phe-4 in Met-Enkephaline, in Phe-Gly [10]). Nevertheless, the increase of rotational freedom of the bulky Arg-9 side chain and the decrease of rotation in the neighboring Phe-8 residue seem to be related.

-The ^3J coupling constants of the Phe-5 side chain cannot be properly extracted from the 250 MHz spectra. The α-proton signal appears as a triplet and yields only $J_{\alpha\beta} + J_{\alpha\beta'}$; at acid pH the β and β' protons do appear to give rise to an AB pattern with very small nonequivalence (∿7 Hz) but at best the central 4 transitions are distinguishable, making it impossible to accurately calculate the full 8 line AB spectrum. With increasing pH, the nonequivalence seems to vanish as the signals are increasingly masked by the δ-protons of Arg-1 (fig. 2b). Only a 400 MHz spectrum allowed us to analyze the AB pattern of the Phe-5 β-protons at pH ∿8, yielding coupling constants of 6.7 and 7.2 Hz, thus confirming the rather free rotation of this side chain.

The coupling constants of the Ser-6 residue do not change with pH. Their values, 7.0 and 6.2 are rather high for a serine residue, usually encountered with ^3J ≤5 Hz. The often cited interaction of the Serine side chain with the peptide backbone (leading to high rotamer III fractions) is possibly replaced by other types of interactions in bradykinin whence the unusual rotamer distribution. We may note in passing that this conformational feature may be reflected in the relative insensitivity of bradykinin biological activity to Ser-Ala substitution [11].

DISCUSSION:

In agreement with the ^{13}C-NMR results the effects of the COOH and NH_3^+ titration are most evident on Arg-1 and Arg-9 as well as on Pro-2 and Phe-8 residues. However, the higher sensitivity of ^1H NMR parameters and the possibility of observing conformationally related coupling constants yield further information:

The phenylalanine side chains have distinctly different behaviour, as the rotamer population of Phe-8 is characterized by a high proportion of R I and R II and almost total absence of R III, whereas Phe-5 approaches the statistical rotamer distribution. The preferred side chain arrangement of Phe-8 is further manifest in the upfield shift of the Pro-7 α and β-signals, and in the enhanced nonequivalence of the Phe-8 methylene protons (from 0.155 to 0.256 ppm) with increasing pH. On the other hand, the Gly-4 resonances preceding Phe-5 are found at 3.86 ppm which corresponds to the range of chemical shifts reported for Gly-2 in the model peptides CF_3CO-Gly-Gly-X-L-Ala-OCH_3 with a non-aromatic residue X: δ=3.8 ppm [7]. As the time averaged ring current effect of Phe-5 does not appear to exert much influence on the Gly-4 proton shift, the Phe-5 side chain must be rather far removed from the glycine moiety.

Two remarks are in order here: 1) This difference of conformational behaviour of Phe-5 and Phe-8 residues in bradykinin has been described by Lintner et al. on the basis of CD studies, where the much stronger ^1L_b dichroic bands of the Phe-8 side chain as compared to the Phe-5 side chain were interpreted as reflecting restricted conformational freedom for the former residue [1]. It was also shown by CD that the Phe-8 side chain and not the Phe-5 side chain feels the influence of the COOH titration; this concerns the coupling constants (and thus the preferred orientation; thus the local asymmetry) more than the δ-values. In fact, ^3J of Phe-8 does depend on pH, whereas ^3J of Phe-5 apparently does not. This leads us to the second remark: 2) How to explain the appearance of the Gly-4 nonequivalence with increasing pH? If the close-by Phe-5 residue does not induce an upfield shift on the glycine protons and moreover does not change its spatial orientation with pH it can hardly be responsible for the splitting of the glycine methylene protons. Considering that along a peptide chain there is a tendency to have side chains alternatively above and below the "plane" of the backbone (most easily visualized in β-pleated sheets or extended conformation) one might imagine the propagation of steric effects over residue side chains two by two: from Phe-8 (itself influenced by the change in rotamer distribution of Arg-9) to Ser-6 to Gly-4. In fact, the β β' nonequivalence of Ser-6 seems to be subjected to the effects of the COOH titration probably resulting from the slight change in Phe-8 rotamer distribution. But the effect is very small and the ^3J coupling constants of Ser-6 do not change with pH. We are then left with the possibility of direct spatial influence of Arg-9 or Phe-8 on Gly-4 which implies a folding back of the C-terminal part of bradykinin in order to bring the two residues into close proximity. An alternative explanation would be to postulate an end-to-end (Arg-1 to Arg-9) interaction which might be pH dependent (ionic bridge): at pH 5 this interaction would stabilize the conformation of the N-terminal part (cf. the proposition of a β-turn Pro-Pro-Gly-Phe [4]) and cause the nonequivalence of the glycine protons; one would then expect, however, to find some carboxyl-titration effects on the chemical shifts and/or the coupling constants in residues Arg-1, Pro-2,3, too, which is not the case.

Previous CD studies [4] have shown the high sensitivity of bradykinin conformation to changes in peptide length (fragments) and sequence (analogs) and solvent and pH parameters. One interesting analog described is Ala^3 BK where the replacement of Pro by Ala leads to changes in the CD pattern and the titration curves.

We have obtained the 400 MHz spectrum of Ala^3 BK at pH 8; its comparison with the spectra of bradykinin yields the following preliminary results:

the substitution of Pro-3 for Ala-3 has an influence on Phe-5 as now the system has become a true A_2X pattern (even at 400 MHz) thus indicating even greater conformational mobility for the Phe-5 side chain. Furthermore, the Pro-2 α-proton appears at 4.480 ppm confirming the assumption that the presence of Pro-3 was responsible for the strong downfield shift of Pro-2 in bradykinin. It is not clear yet if this shift is due only to conformational effects or if it also includes inductive effects of a succeeding proline residue.
In the Ala^3 BK analogue, also the Ser-6 β β' nonequivalence is slightly greater than in bradykinin.

CONCLUSION:

Two main points emerge from the pH titration study:
- The conformation of bradykinin is definitely pH dependent as the chemical shifts and the coupling constants clearly demonstrate (fig. 2). The carboxyl group titration (pK≈3.3) seems to induce a bend in the molecule, bringing Arg-9 and the central Gly-4 into closer spatial proximity. This result is compatible with the folded conformations of bradykinin proposed in the literature [4 and references therein.] .
- Table 1 reveals a gradient of rigidity in the side chains and the backbone, in going from the N- to the C-terminus: the coupling constants of the proline residues reflect decreasing pyrrolidine ring flexibility (tied to the motions in the backbone and the side chain) in going from Pro-2 to Pro-3 to Pro-7; the side chain of Arg-9 is more hindered in its rotation than the one of Arg-1; the same is true of Phe-8 in comparison with Phe-5.
We intend to refine these notions in still more detail by investigating the CD and NMR spectra of a series of Ala^X (x=1,2,...8) analogues of (1-8)-bradykinin(work in progress).

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