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Use of ^{1}H-^{15}N PEP-HSQC-TOCSY at Natural Abundance to Facilitate the Structure Elucidation of Naturally Occurring Peptides

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Abstract: The application of ^{1}H-^{15}N PEP-HSQC-TOCSY at natural abundance is demonstrated here on a 13 mM sample (0.048 mM ^{15}N/position) of the biologically active linear tetradecapeptide bombesin (MW=1619.9) as a way to expedite the structural characterization of peptidic natural products. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: NMR, Peptides and polypeptides, Natural products, Alkaloids, Toxins

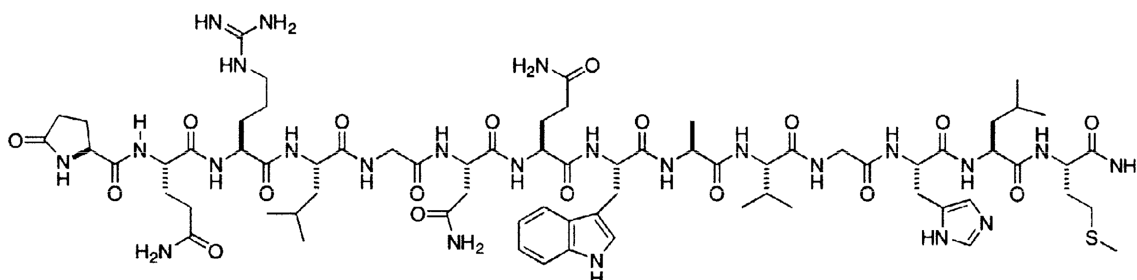

Figure 1. Structure of bombesin.

Naturally occurring peptides have become a topic of interest for many natural products chemistry laboratories owing to their interesting biological activities, such as antimicrobial, cytotoxic, and immunosuppressive, and their complex molecular structures.[1, 2] However, the elucidation of these complex structures is often quite challenging due to spectral overlap of the $^{1}H^{N}$ protons, the $^{1}H^{\alpha}$ protons and carbons, and aliphatic resonances in the NMR spectrum. This is especially true in peptides containing several aliphatic amino acid residues. Previously, a common approach to this problem was the methodical and reiterative analysis of ^{1}H-^{1}H TOCSY, ^{1}H-^{1}H COSY, HMQC or HSQC, and HMBC data, a time consuming and tedious process. An improvement to this strategy has been provided by application of ^{1}H-^{13}C HMQC-TOCSY,[3] or ^{1}H-^{13}C HSQC-TOCSY[4] experiments in an effort to resolve overlapping proton resonances, sorting them by their respective carbon chemical shift. Unfortunately, many peptides contain multiple aliphatic amino acids and exhibit multiple conformations in solution. The chemical shifts of the α-carbons as well as many of the carbons in the side chains of these aliphatic amino acids are still quite congested, rendering the ^{1}H-^{13}C heteronuclear-TOCSY experiments of little help. Herein we propose and demonstrate a different approach to this problem which takes advantage of the larger spectral dispersion of ^{15}N as compared to that of ^{1}H and ^{13}C.[5] The fact that nitrogen atoms appear at regular intervals throughout any peptide and have a chemical shift

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range of 20-50 ppm makes them ideal reference points in the backbone of a peptide. By contrast, α-carbons, the major focus of the above ^{13}C-edited experiments, appear with the same regularity in peptides but normally comprise a much narrower chemical shift range of only 5-10 ppm.

Previously, detection of ^{15}N at natural abundance was not routinely possible because of the low receptivity of ^{15}N (2.2 x 10^{-2} as compared to ^{13}C).[6] This problem has been reduced dramatically by the use of high field strengths (500 MHz and greater), the greater field stability of modern spectrometers, and especially by the use of pulsed field gradients which eliminate t_1 noise from a 2D spectrum. This allows the acquisition of data with much higher overall S/N than was previously possible even with the normal 50% loss in sensitivity associated with the use of pulsed field gradients. In large part, the gain in sensitivity is because the receiver gain can be increased in the B_0 field gradient-based experiments. Additionally, Rance and co-workers devised a method of recovering up to a 'square root of two' of the lost sensitivity by incorporating an additional refocusing section at the end of the sequence which preserves both orthogonal N and P type pathways resulting from the phase modulated experiment.[7] Moreover, the sensitivity gains through the use of inverse detection of ^{15}N are even higher than those associated with ^{13}C because of the larger magnetogyric ratio between ^{1}H and ^{15}N.[8]

The inverse detection of ^{15}N at natural abundance has been demonstrated by Martin and Crouch,[9a] Fukuzawa, Matsunaga, and Fusetani,[9b] and others,[9c, d, e] through the use of ^{1}H-^{15}N HMBC for the structure elucidation of alkaloids and by our lab for the sequential analysis of a novel linear peptide and an anti-inflammatory cyclic peptide.[9f, g] However, most of the applications reported for these experiments, excluding the latter two, were on samples with a concentration of >100 mM.[9h]

Here we demonstrate another application of inverse detected ^{15}N NMR spectroscopy through the use of an HSQC experiment with sensitivity improvement by preservation of equivalent pathways (PEP)[7] coupled with homonuclear isotropic mixing provided by the DIPSI-2 sequence (Figure 2).[10] In effect, this experiment provides TOCSY transfer originating from $^1H^N$ spin to the $^1H^\alpha$ spin and continuing through the sidechain ^{1}H spin system of the constituent amino acid. Each of these TOCSY spin systems is sorted by the chemical shift of the ^{15}N atom attached to $^1H^N$. Because of the relatively large spectral dispersion of ^{15}N, the chemical shifts of these amide nitrogens are almost always resolved even in molecules as large as medium sized proteins (e.g. ubiquitin ~8.5 kD).[11] This technique provides an ideal way to analyze the chemical shift and connectivity of the often overlapped protons in the backbone and sidechains of a peptide. The utility of the ^{1}H-^{15}N HSQC-TOCSY experiment has been demonstrated by its use in the assignment of ^{15}N-labeled medium sized proteins,[12] but up until now its use in the structure elucidation of natural products at ^{15}N natural abundance has not been realized.

The experiment is demonstrated here by complete ^{1}H spectral assignment as well as the assignment of all protonated carbons and nitrogens in bombesin (Figure 1). Bombesin is a neuroactive peptide first isolated from amphibian skin[13] and subsequently shown to be a potent growth factor for human small-cell lung-carcinoma cell lines.[14] The ^{1}H chemical shifts for bombesin in DMSO-*d6* were first published in 1984[15] and

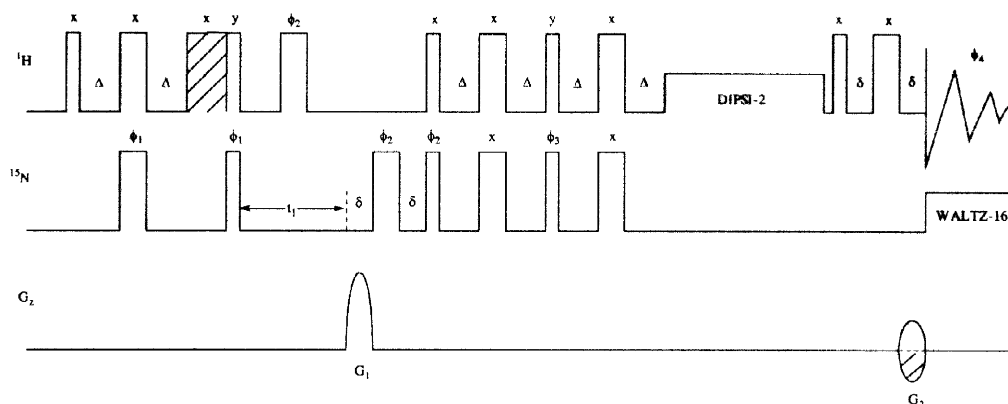

Figure 2. Pulse sequence for ^1H-^{15}N PEP-HSQC-TOCSY. Narrow bars indicate a 90° pulse, wide bars indicate a 180° pulse, and the cross-hatched bar indicates a 2.5 msec high power trim pulse (ϕ_1 = x, -x; ϕ_2 = x, x, -x, -x; ϕ_3 = y, y, -y, -y; ϕ_4 = x, -x, -x, x).

later revised in 1988 but with an incomplete assignment of the sidechain protons.[16] Herein we report further revision and clarification of the ^1H shifts as well as the complete assignment of the observable protonated ^{13}C and ^{15}N resonances in bombesin dissolved in DMSO-*d6* (Table 1) .

As can be seen in the data presented below, it is possible to assign the backbone side chain protons of each amino acid simply by starting with the correlation of each particular $^1H^N$ and following the spin system along the F_2 dimension (Figure 3, 4). Most amino acids will have a characteristic set of correlations, facilitating their identification (Figure 3). As shown in Figure 3, virtually all expected TOCSY correlations for the 14 amino acids are present with the exception of the side chain protons of p-Glu. This can be attributed to the very small coupling between $^1H^\alpha$ and $^1H^\beta$ as evidenced by a singlet observed for the $^1H^\alpha$ resonance.[17] Also, the β proton of valine and the $^1H^\gamma$ of the leucine residues are of rather small intensity because of the highly coupled multiplet pattern of the ^1H resonance which decreases the magnitude of the response for these signals. Precise ^1H chemical shifts can be easily obtained for each residue from slices extracted along the F_2 dimension (Figure 4). The complete ^1H, ^{13}C, and ^{15}N shifts of bombesin are shown in Table 1. By these experiments, we note significant revisions (> 0.05 ppm) in the assigned chemical shifts (relative to those published in reference 16) for protons at positions Gln-2 H^N; Arg-3 H^β and $(H^\gamma)_2$; Trp-8 H^β and $H^{\delta1}$; Gly-11 H^α; His-12 H^β, $H^{\delta2}$, and $H^{\varepsilon1}$; Leu-13 H^γ; and Met-14 H^γ. Additionally, we have assigned ^1H NMR shifts for several of the aromatic protons in Trp-8 ($H^{\zeta2}$, $H^{\eta2}$, $H^{\zeta3}$, $H^{\varepsilon3}$) for the first time.

This experiment provides a valuable new tool for elucidating the structure of complicated peptide natural products. It should be noted that it can be usefully applied to any compound that contains a protonated nitrogen, such as most aliphatic or alicyclic alkaloids. Once this experiment is correctly set up on a NMR spectrometer (e.g. pulse widths determined and delays optimized), it requires little further optimization and can therefore be used in a transparent manner to the routine user.

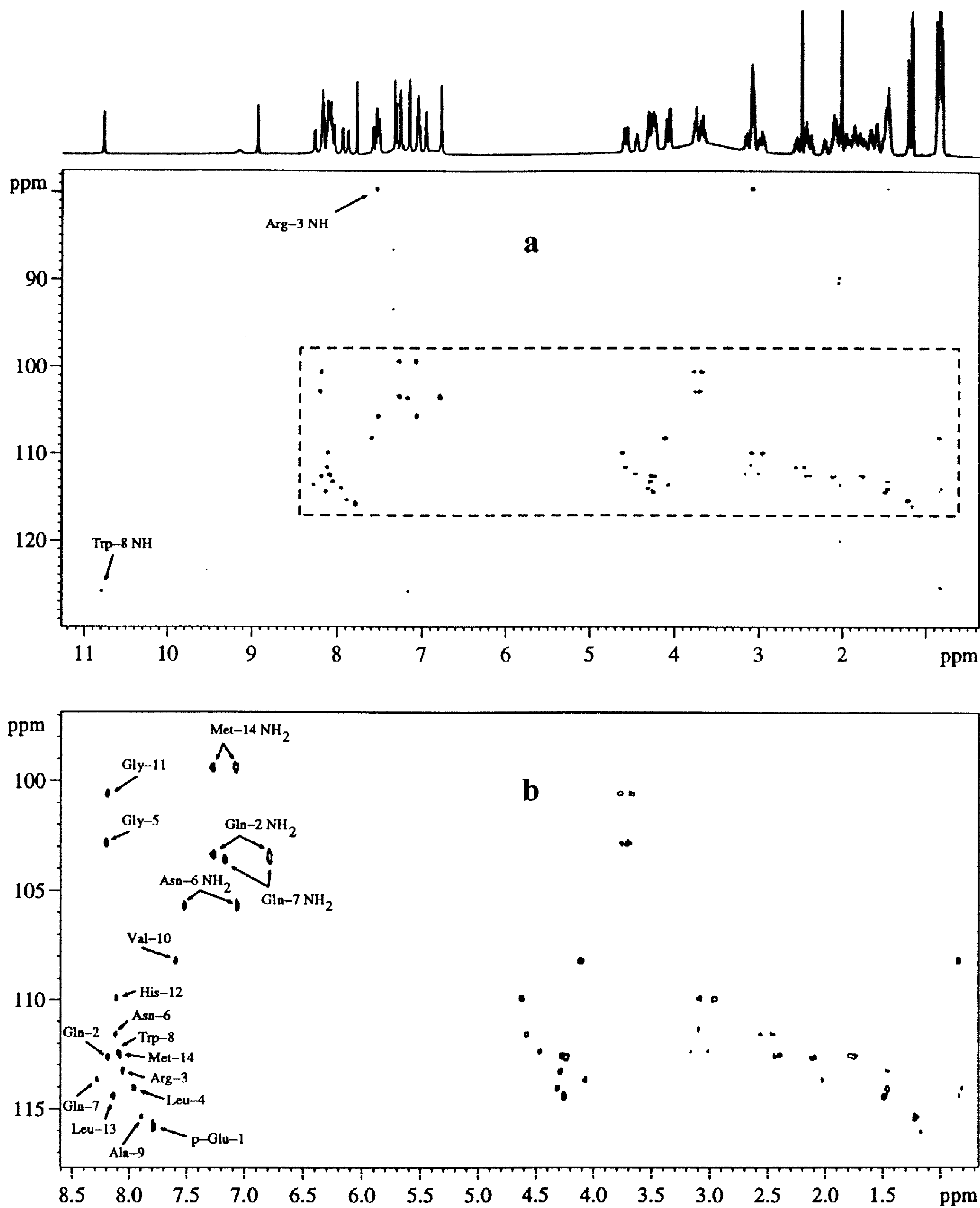

Figure 3. a) Entire spectrum from the ^{1}H-^{15}N PEP-HSQC-TOCSY experiment. b) Expansion of $^{1}H^{\alpha}$ region of bombesin.

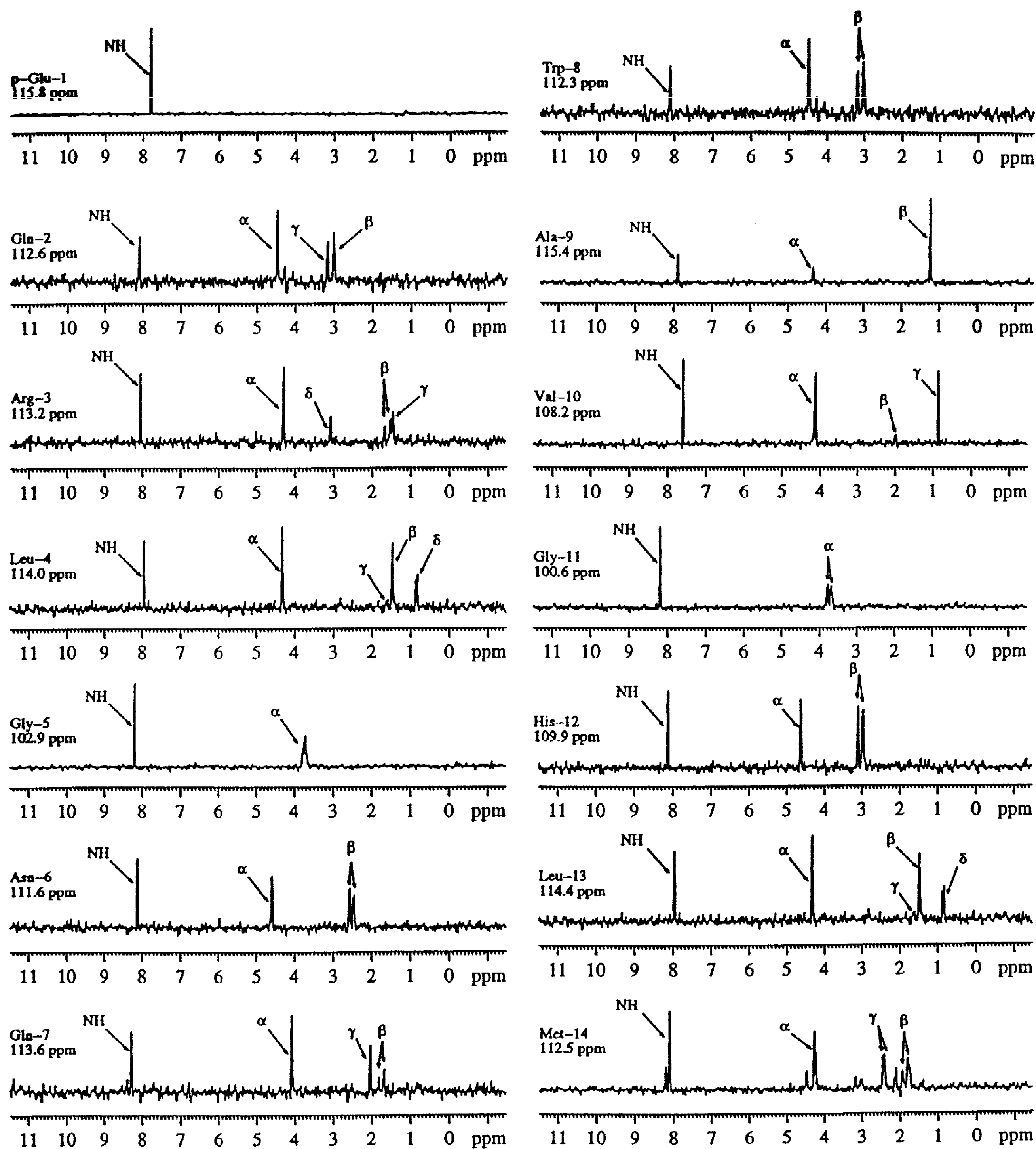

Figure 4. Slices taken through the F_2 dimension for the backbone ^{1}H-^{15}N response for each amino acid residue in bombesin, arranged by residue number.

Table 1. NMR Assignments of Bombesin (Figure 1) in DMSO-*d6*
(600.08 MHz ^{1}H, 150.91 MHz ^{13}C, 60.81 MHz ^{15}N, 298 K)[a]

Residue	Position[b]	^{1}H (Ref 16)	^{1}H (ppm)	^{13}C (ppm)	^{15}N (ppm)
p-Glu-1	H^{N}	7.79	7.79		
	N^{H}				115.8
	H^{α}	4.05	4.07[c]	55.4	
	H^{β}	2.21	2.22[c]	25.1	
	H^{β}	1.89	1.86[c]	25.1	
	$(H^{\gamma})_2$	2.1	2.12[c]	29.2	
Gln-2	H^{N}	8.13	8.19		
	N^{H}				112.6
	H^{α}	4.21	4.23	51.8	
	H^{β}	1.87	1.86	27.1	
	H^{β}	1.73	1.75	27.1	
	$(H^{\gamma})_2$	2.10	2.11	30.8	
	C^{δ}			173.8	
	H^{ε}		6.78		
	H^{ε}		7.26		
	$N^{\varepsilon 2}$				103.3
Arg-3[d]	H^{N}	8.19	8.06		
	N^{H}				113.2
	H^{α}	4.28	4.29	52.0	
	H^{β}	1.53	1.67	28.4	
	H^{β}	1.53	1.54	28.4	
	$(H^{\gamma})_2$	1.54	1.48	24.3	
	$(H^{\delta})_2$	3.07	3.08	40.0	
	H^{ε}	7.48	7.54		
	N^{ε}				79.6
	C^{ζ}			156.9	
Leu-4	H^{N}	7.96	7.96		
	N^{H}				114.0
	H^{α}	4.30	4.32	50.4	
	$(H^{\beta})_2$	1.44	1.48	39.8	
	H^{γ}	1.58	1.62	23.5	
	$(H^{\delta})_3$	0.85	0.87	20.9	
	$(H^{\delta})_3$	0.82	0.82	20.9	
Gly-5	H^{N}	8.20	8.20		
	N^{H}				102.9
	H^{α}	3.77	3.77	41.8	
	H^{α}	3.69	3.66	41.8	
Asn-6	H^{N}	8.11	8.12		
	N^{H}				111.6
	H^{α}	4.57	4.59	49.5	
	H^{β}	2.56	2.57	37.1	
	H^{β}	2.44	2.46	37.1	
	C^{γ}			171.4	
	H^{δ}		7.06		
	H^{δ}		7.52		
	$N^{\delta 2}$				105.7
Gln-7	H^{N}	8.28	8.29		
	N^{H}				113.6
	H^{α}	4.08	4.08	53.0	
	H^{β}	1.82	1.82	26.5	
	H^{β}	1.68	1.67	26.5	
	$(H^{\gamma})_2$	2.03	2.03	30.7	
	C^{δ}			173.4	
	H^{ε}		6.78		
	H^{ε}		7.16		
	$N^{\varepsilon 2}$				103.6

Residue	Position[b]	^{1}H (Ref 16)	^{1}H (ppm)	^{13}C (ppm)	^{15}N (ppm)
Trp-8	H^{N}	8.1	8.09		
	N^{H}				112.3
	H^{α}	4.49	4.47	53.1	
	H^{β}	3.00	3.17	26.5	
	H^{β}	3.00	3.02	26.5	
	C^{γ}			110.0	
	$H^{\delta 1}$	7.09	7.15	123.4	
	$C^{\delta 2}$			136.1	
	$N^{\varepsilon 1}$				125.8
	$H^{\varepsilon 1}$	10.79	10.77		
	$C^{\varepsilon 2}$			127.2	
	$H^{\zeta 2}$		7.31	111.0	
	$H^{\eta 2}$		7.04	120.6	
	$H^{\zeta 3}$		6.96	118.0	
	$H^{\varepsilon 3}$		7.55	118.0	
Ala-9	H^{N}	7.90	7.88		
	N^{H}				115.4
	H^{α}	4.35	4.33	47.8	
	$(H^{\beta})_3$	1.21	1.24	16.8	
Val-10	H^{N}	7.60	7.59		
	N^{H}				108.2
	H^{α}	4.12	4.11	57.9	
	H^{β}	1.98	1.99	30.4	
	$(H^{\gamma})_3$	0.86	0.82	17.4	
	$(H^{\gamma})_3$	0.84	0.85	18.5	
Gly-11	H^{N}	8.20	8.19		
	N^{H}				100.6
	H^{α}	3.77	3.77	57.9	
	H^{α}	3.77	3.67	57.9	
His-12[e]	H^{N}	8.14	8.11		
	N^{H}				109.9
	H^{α}	4.61	4.62	50.9	
	H^{β}	3.08	3.09	26.5	
	H^{β}	3.08	2.96	26.5	
	C^{γ}			129.0	
	$H^{\delta 2}$	7.16	7.33	116.4	
	$H^{\varepsilon 1}$	7.27	8.95	133.1	
Leu-13	H^{N}	8.13	8.13		
	N^{H}				114.4
	H^{α}	4.25	4.26	50.7	
	$(H^{\beta})_2$	1.49	1.49	40.4	
	H^{γ}	1.56	1.63	23.5	
	$(H^{\delta})_3$	0.89	0.89	22.5	
	$(H^{\delta})_3$	0.85	0.85	22.5	
Met-14	H^{N}	8.07	8.19		
	N^{H}				112.5
	H^{α}	4.28	4.28	51.6	
	H^{β}	1.94	1.92	31.2	
	H^{β}	1.79	1.78	31.2	
	H^{γ}	2.42	2.46	28.9	
	H^{γ}	2.37	2.42	28.9	
	$(H^{\varepsilon})_3$	2.05	2.05	14.2	
	C^{O}			172.4	
	H^{N}		7.07		
	H^{N}		7.27		
	N^{H}				99.4

a. ^{15}N referenced to external DMF at δ103.2; ^{13}C to DMSO at δ39.51; ^{1}H to DMSO at δ2.50.
b. Positions named in accordance with reference 18.
c. Assignments by ^{1}H-^{13}C PEP-HSQC-TOCSY.
d. $H^{\eta 11,12}$ and $H^{\eta 21,22}$ signals were not observed due to multiple resonance forms.[19]
e. $H^{\delta 1}$ and $H^{\varepsilon 2}$ signals were not observed due to multiple resonance forms.[19]

EXPERIMENTAL

The sample was prepared by dissolving 10 mg bombesin (MW=1619.9 American Peptide Company, Inc.) as the TFA salt in 460 μl 99.9% DMSO-*d6* to give a final concentration of 13 mM (0.048 mM ^{15}N per position). The ^{1}H-^{15}N PEP-HSQC-TOCSY shown was recorded on a Bruker DRX 600 with a 5 mm ^{1}H-X inverse probe at 298 K using the standard Bruker pulse program "invidigssi" modified by the addition of a 50 μsec delay at the end of the isotropic mixing period. The 90° pulse widths were 35.0 μs for ^{15}N and 7.5 μs for ^{1}H. Gradient values were set to ratios of 80 : 8 : 80 : -8 respectively in the PEP-HSQC segment of the sequence. These values should be acceptable if one uses ratios of 80 : 20.1 : 80 : -20.1 for ^{1}H-^{13}C PEP-HSQC using double inept transfer. The echo-antiecho method was used to provide fully phase sensitive data.[20] The F_1 spectral window was set from 75-130 ppm and the data were acquired with 288 scans per t_1 increment for a total of 198 increments and a total experiment time of 10 hr. The relaxation delay was 1 s and the acquisition time in F_2 was 132 ms. Waltz-16 decoupling was used for decoupling during acquistion. An 80 ms mixing time was used for the DIPSI-2 isotropic mixing sequence. The F_1 dimension was linear predicted to 512 points and zero filled to a total of 1 K points. In the F_2 dimension, 2 K data points were acquired and processed. Data were processed using a 90° phase shifted $\sin^2$ apodization in both dimensions. Further work has shown that a spectrum comparable to that presented in figures 3 and 4 could be acquired on a 10 mmol sample in ~3 hrs with no special considerations other than an inverse detected probe (128 scans per increment with a total of 64 increments and a sweep width of 60 ppm in F_1). These experiment times could be shortened even further by the use of Shigemi® microcells or by the use of an inverse detected microprobe or Varian's gXH-Nano-probe®.

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