Two-dimensional Nuclear Magnetic Resonance of Natural Products. Part 4.† Investigation of the Solution Conformation of Luliberin by ^1H, ^{13}C, and 2D ^1H–^{13}C Shift Correlation Nuclear Magnetic Resonance Spectroscopy

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Two-dimensional ^1H–^{13}C shift correlation has been used for an unambiguous assignment of the ^1H and ^{13}C resonances of luliberin (LH-RH). From ^1H and ^{13}C n.m.r. spectra obtained at various pH values no evidence for any strong intramolecular interaction has been obtained.

Luliberin (or LH-RH, luteinizing hormone–releasing hormone) is a neurohormone, which is secreted by cells of the hypothalamus and stimulates the release of luteinizing hormone and follicle-stimulating hormone from the pituitary.[1,2] It has been shown to be a linear basic decapeptide: pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2.[3,4] Many structural variants of this molecule have been prepared and it was noticed that amino acid substitution at most positions on the chain causes a reduction in biological activity. Nevertheless, most of the described analogues do have detectable levels of activity.[5]

In order to gain information about the structure–activity relationship of luliberin, intensive efforts have been made to investigate its conformational structure in solution by high-resolution n.m.r.[6–11] and other spectroscopic techniques.[12,13] The ^1H and ^{13}C resonances have been studied in aqueous and in DMSO solutions and the results do not indicate any strong intramolecular hydrogen bonds. The conformations in the two solvents are very similar, the Pro residue is predominantly in a *trans* conformation and no evidence for stacking of the indole and phenol ring has been obtained.[14] The ^{13}C shifts resemble those calculated from the shifts of the constituent amino acids and ^{13}C T_1 measurements strongly suggest a random coil conformation for luliberin. On the other hand, the measurement of proton nuclear Overhauser effects in luliberin suggests that a high population of a particular set of conformers might exist in aqueous solution (see ref. 10). Results from fluorescence measurements have been interpreted in terms of a charge-transfer interaction between the imidazolium side chain of histidine and the tryptophan residue. Ionization of the histidine quenches the fluorescence of tryptophan.[12,13,15,16] The fluorimetric titration curve reveals an unusually low pK value for His-2 of 5.95.

Recently, conformation calculations[17–20] have resulted in the prediction of several low-energy conformations of luliberin. Some of them have been reported to be in agreement with the nuclear Overhauser measurements.[10]

We have undertaken ^1H and ^{13}C n.m.r. studies with the intention of gaining some insight into the conformational behaviour of luliberin in aqueous solution. As the previously reported ^{13}C assignments differ in some instances, we have carried out two-dimensional ^1H–^{13}C shift correlation experiments at different pH values, thus obtaining an unambiguous assignment of the ^{13}C resonances.

† For Part 3 see: E. Haslinger and H. Kalchhauser, *Tetrahedron Lett.*, 1983, **24**, 2553.

Results and Discussion

^1H and ^{13}C Spectral Assignments.—The assignments presented here differ in four cases from those reported previously (see ref. 6—9). We have used spin-decoupling experiments for assignment of ^1H resonances and measured the pH dependence of ^{13}C and ^1H shifts. Finally, a 2D ^1H–^{13}C shift correlation experiment[21–23] was performed to interrelate the information obtained from ^1H and ^{13}C n.m.r. measurements. The results from the ^1H and ^{13}C spectra are presented in Tables 1 and 2 and Figures 1—3.

The ^1H signal of pyro-Glu-1-H_α is readily found in the ^1H n.m.r. spectrum because it is not overlapping with any of the other resonances. ^1H–^1H Decoupling gives the resonance positions of the protons pyro-Glu-1-H_β, which are diastereotopic and exhibit different chemical shifts. From these, the corresponding ^{13}C resonances could be obtained. The ^{13}C resonances of pyro-Glu-1-C_γ and Pro-9-C_β are coincident. The ^{13}C resonances of His-2 are easily recognizable because, with the exception of the C(4), their shifts are strongly pH dependent giving a pK value of 6.2 for His-2. At pH values smaller than 6.0 no signal for C(2) is observed as a consequence of extensive line broadening.

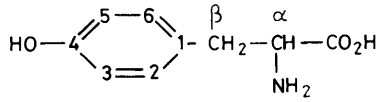
For the unambiguous assignment of the ^{13}C signals of Trp-3 we used the 2D shift correlation together with some additional ^1H–^1H decoupling experiments. From the contour plot of the aromatic region one finds that the ^{13}C signal at δ = 113.0 p.p.m., which corresponds to C(7), is correlated to the high-field doublet in the aromatic region of the ^1H spectrum. The latter is therefore H(7) and the second doublet at slightly lower field must be assigned to H(4) giving δ = 119.3 p.p.m. for the resonance position of C(4) *via* the shift correlation. INDOR[24] experiments with these doublets gave two distinct resonance positions for H(5) and H(6), which are used to assign the ^{13}C resonances of the corresponding carbons (Figure 2). We obtain δ = 120.5 p.p.m. for C(5) and δ = 123.1 p.p.m. for C(6) showing that the assignment from Wessels *et al.*[6] and Deslauriers and Somorjaj[7] is the correct one. In a similar way we obtained the resonance frequencies for the aliphatic carbons of Trp-3. From the contour plot of the aliphatic region one finds that the low-field part of two triplets at δ = 4.7 p.p.m. in the ^1H spectrum belongs to the Trp-3-H_α, thus the high-field part is the resonance of the His-2-H_α.

Ser-4-C_β and Pro-9-C_α are barely resolved and in fact have not been assigned in some previous experiments. However, discrimination of the ^{13}C resonances of Ser-4-C_β and Pro-9-C_α could be achieved despite the almost identical resonance

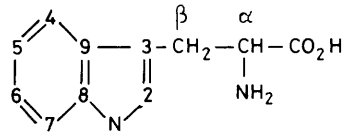
Table 1. ^{13}C Chemical shifts of luliberin in D_2O (pD = 5.85)

Group		Shift (p.p.m.)
pyro-Glu	Cα	57.65
	Cβ	26.16
	Cγ	30.26 [b]
His	Cα	53.97
	Cβ	28.18
	C(2)	135.39
	C(4)	118.14
	C(5)	130.80
Trp	Cα	55.51
	Cβ	28.05
	C(2)	125.55
	C(3)	109.72
	C(4)	119.33
	C(5)	120.45
	C(6)	123.08
	C(7)	112.99
	C(8)	137.25
	C(9)	128.04
Ser	Cα	56.56
	Cβ	62.22
Tyr	Cα	56.59
	Cβ	36.93
	C(1)	129.07
	C(2), C(6)	131.60
	C(3), C(5)	116.50
	C(4)	155.68
Gly	Cα	43.25 [a]
Leu	Cα	53.37
	Cβ	40.92
	Cγ	25.40
	Cδ	23.30
	Cδ′	21.90
Arg	Cα	52.15
	Cβ	28.69
	Cγ	25.09
	Cδ	41.70
	Cε	157.74
Pro	Cα	61.36
	Cβ	30.34 [b]
	Cγ	25.79
	Cδ	49.04
Gly	Cα	43.59 [a]

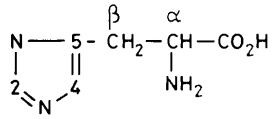
Tyr



Trp



His



[a] Assigned from T_1-measurement (see ref. 9). [b] May be interchanged.

Table 2. ^1H Chemical shifts of luliberin in D_2O (ref. to external Me_4Si)

Group		Shift (p.p.m.)	Group		Shift (p.p.m.)
pyro-Glu	CαH	4.16	Gly	CαH	3.86
	CβH	2.3; 1.6			
	CγH	2.24	Leu	CαH	4.29
				CβH	1.6
His	CαH	4.57		CγH	1.57
	CβH	3.1		CδH	0.91; 0.86
	C(2)H	8.24			
	C(4)H	7.1	Arg	CαH	4.53
				CβH	1.62
Trp	CαH	4.66		CγH	1.6
	CβH	3.1		CδH	3.09
	C(2)H	7.15			
	C(4)H	7.46	Pro	CαH	4.3
	C(5)H	7.1		CβH	1.9; 2.23
	C(6)H	7.2		CγH	1.8—2.0
	C(7)H	7.43		CδH	3.47; 3.7
Ser	CαH	4.29	Gly	CαH	3.86
	CβH	3.65			
Tyr	CαH	4.51			
	CβH	2.81; 3.09			
	C(2)H	6.97			
	C(3)H	6.84			
	C(5)H	6.84			
	C(6)H	6.97			

position of the ^{13}C signals by a *J*-modulated ^{13}C spectrum [25] (Figure 1). (For a similar method see ref. 26.) The corresponding ^1H signals could be obtained *via* the contour plot. An ^1H homo-decoupling experiment gives the resonance position of Ser-4-H_α which in turn yields the assignment of Ser-4-C_α.[27] Comparison of shift data from the literature confirms our conclusion.

The ^{13}C signal of Tyr-5-C_β is found easily by considering chemical shift arguments. Despite extensive overlap of the corresponding proton signals with Arg-8-H_δ, His-2-H_β, and Trp-3-H_β one can readily see the diastereotopic protons of Tyr-5-H_β appearing at different chemical shifts in the 2D-n.m.r. experiment (Figure 3).

C_α of Gly-6 and Gly-10 could not be assigned unambiguously by our experiments, although they have been assigned previously by T_1 measurements (see ref. 9). The ^{13}C and ^1H resonance lines of Leu-7 in position δ are easily assigned in the ^1H and ^{13}C n.m.r. spectrum. The Leu-7-C_γ signal is the only one in this shift region derived from a CH group and is identified in the *J*-modulated ^{13}C spectra (Figure 1). The Leu-7-C_β resonance is immediately found in the contour plot owing to its low proton shift. We have assigned Leu-7-C_α by comparison with shift data given in the literature.[27]

A similar procedure is suitable for the assignment of the ^{13}C resonances of Arg-8 and Pro-9. Some of the Pro-9 ^{13}C signals appear as doublets in the contour plot, distinctly showing different chemical shifts of the attached diastereotopic protons. ^1H Shifts obtained from transverse section of

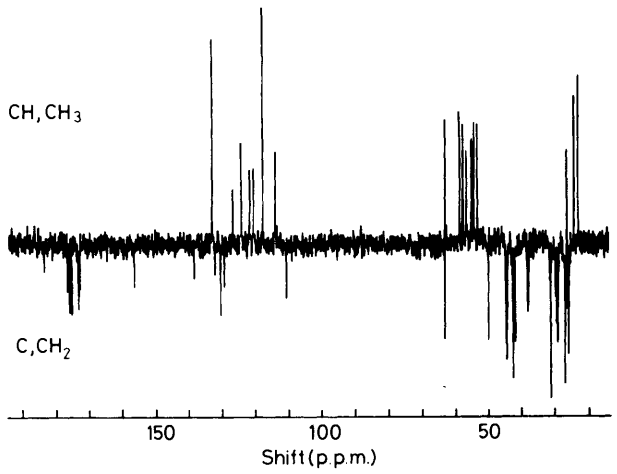


Figure 1. 62.9 MHz *J*-modulated ^{13}C n.m.r. spectrum of luliberin in D_2O (pD = 5.85)

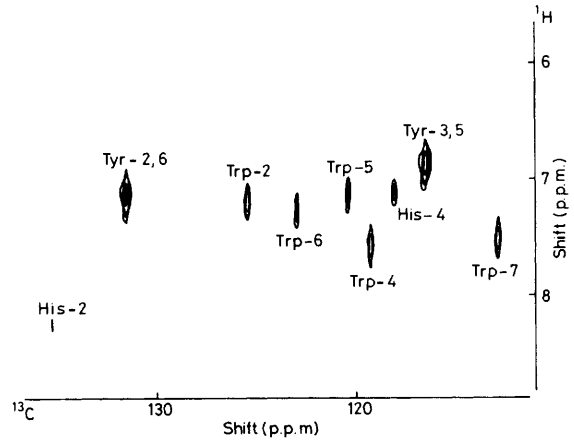


Figure 2. 2D ^1H–^{13}C shift correlation spectrum of the aromatic region of luliberin in D_2O (pD = 5.85)

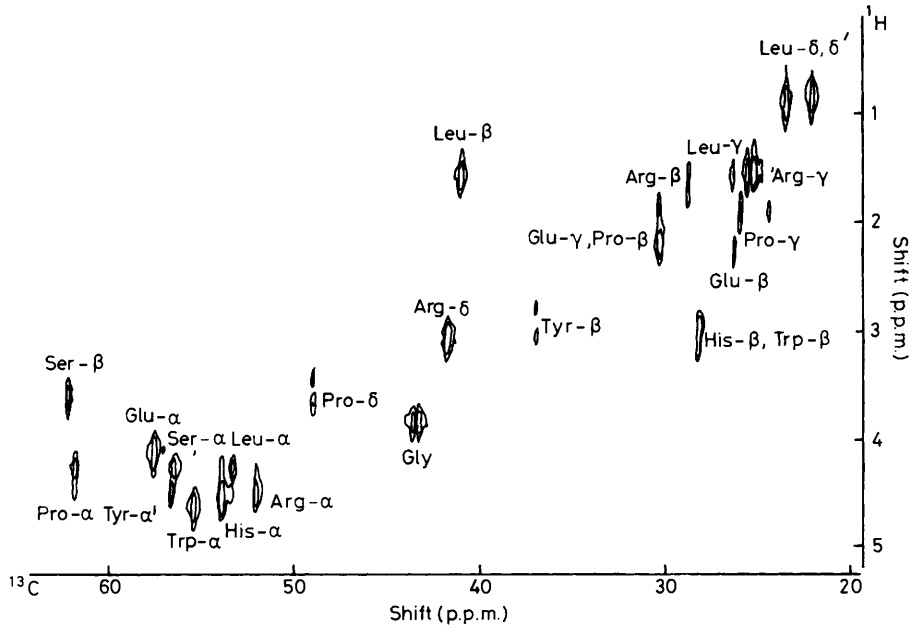


Figure 3. 2D ^1H–^{13}C shift correlation spectrum of the aliphatic region of luliberin in D_2O (pD = 5.85)

the 2D data matrix at the corresponding ^{13}C shifts are given in Table 2.

pH *Dependence of* ^1H *and* ^{13}C *Resonances.*—The sensitivity of chemical shifts to the charge state of ionizable groups has been clearly established and these effects have proved to be a valuable source of information on peptide or protein structure in solution.[28] Histidine was one of the first ionizable residues to be studied in detail in peptides and proteins.[29] In simple peptides it exhibits a p*K* value of 6.8, but in proteins p*K* values of from 3.0 to 8.5 have been found. We have determined the ^1H and ^{13}C chemical shifts at various pH values and computed a p*K* value of 6.2 for His-2 in luliberin,[30] which is smaller than the p*K* value found in His-Trp of 6.8. The resonance of the proton at C(4) in the neighbouring Trp-3 reflects the ionization behaviour of His-2 yielding a p*K* value near 6 (ref. 5). A similar p*K* value has been obtained earlier (refs. 12—14) from fluorescence measurements of the Trp-3 residue in luliberin, showing that His-2 at low pH quenches the Trp-3 fluorescence.

Conclusions

The n.m.r. spectra gave no evidence that there is a strong intramolecular association and no indication for any conformational preference could be obtained. The p*K* value of His-2 is somewhat smaller than in the component peptide His-Trp and the His is protonated predominantly at N(3).[28,31,32] Ring current effects are absent and only a small temperature dependence of some signals has been observed. These results strongly point to a random-coil conformation for luliberin and it might well be that the active conformation of luliberin is established during the binding process to the hormone receptor.

Experimental

Materials.—Luliberin acetate (25 mg) was purchased from The Peptide Research Institute, Osaka, Japan.

Methods.—The n.m.r. experiments were carried out on a Bruker WM-250 n.m.r. spectrometer in sample tubes of 5 mm diameter. Typical parameters for the shift correlation experiments are: 20 mg per 0.4 ml D_2O (pD = 4.30, 5.85, uncorrected pH meter readings). Data matrix: 4 k × 128, SW_1 = 2 000 Hz, SW_2 = 7 700 Hz, 90°-pulse: ^1H: 18 μs, ^{13}C: 16μs, D3 = 3.6 ms, D4 = 2.4 ms. QP-detection in both dimensions, Bruker standard software.[33]

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