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NMR analysis of a model pentapeptide, acetyl–Gln–Gln–Gln–Pro–Pro, as an epitope of wheat allergen.

Eri Fukushi,[1]* Soichi Tanabe,[2] Michiko Watanabe[2] and Jun Kawabata[1]

[1] Faculty of Agriculture, Hokkaido University, Kita-9 Nishi-9, Sapporo 060-8589, Japan
[2] Faculty of Education, Tokyo Gakugei University, Koganei-shi, Tokyo 184-0015, Japan

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ABSTRACT: NMR analysis of a model pentapeptide, acetyl (Ac)–Gln–Gln–Gln–Pro–Pro, as an epitope of a wheat allergen was performed. The problem of severe signal overlapping in the ^{1}H NMR spectrum was overcome by elaborate two-dimensional methods using ^{13}C information. HMQC-TOCSY allowed the assignments of the ^{1}H and ^{13}C NMR signals except the γ-glutamylamide parts. E-HSQC-ROESY, which was constructed by modification of the E-HSQC, could serve for discriminating ROEs of protons whose δ_H values were close to each other. Although the sensitivity of HSQC-ROESY itself was low, selecting only CH carbons by adjusting the proper delay and flip angle of a pulse allowed a narrow F_1 spectral width and hence the collection of numerous transients. As a result, the configurations of the amide bonds of the backbone were determined as all-*trans*.

KEYWORDS: NMR; ^{1}H NMR; ^{13}C NMR; Ac–Gln–Gln–Gln–Pro–Pro; E-HSQC-ROESY

INTRODUCTION

The minimum primary structure of the IgE-binding epitope in gluten, a major wheat allergen has been determined as Gln–Gln–Gln–Pro–Pro, and its model pentapeptide, acetyl (Ac)–Gln–Gln–Gln–Pro–Pro (**1**) (Fig. 1), are known to act as a hapten capable of binding to a specific IgE molecule.[1] Since a detailed analysis of the solution structure of this model peptide would be a great help for designing a molecule applicable to functional foods and drugs treating wheat allergy, we started an NMR study on **1** and related compounds. This paper deals with the unambiguous assignments of the ^{1}H and ^{13}C NMR signals, except the γ-glutamylamide parts of **1** and the determination of the configurations of the backbone amide bonds as all-*trans*.

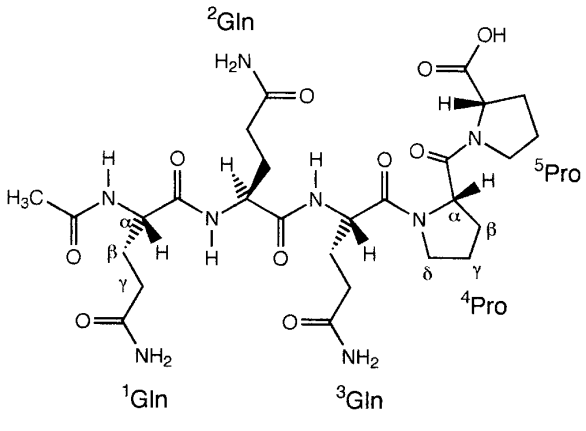

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Figure 1. Structure of Ac–Gln–Gln–Gln–Pro–Pro (**1**).

RESULTS AND DISCUSSION

Since the ^{1}H NMR spectrum of **1** in dimethylsulfoxide (DMSO)-d_6 solution (Fig. 2) gave a pattern closely similar to that in aqueous solution, DMSO solution was used for the NMR analysis. In the ^{1}H NMR spectrum, two of the three α-NH signals of Gln residues were completely overlapped in the same position at 297 K, whereas these signals were separated into a pair of doublets at 303 K. Hence the analytical temperature was set at 303 K throughout the following analysis.

In the ^{1}H–^{1}H COSY spectrum[2] of **1** there were difficulties in analyzing proton networks using only information on proton chemical shifts owing to their severe overlap as follows. First, α-H of two Gln and one Pro residues resonated at almost the same chemical shifts. Second, β- and γ-CH_2 proton signals of all amino acid residues were overlapped at δ_H 1.60–2.20 ppm. Third, a broad signal of the residual water covered the δ-CH_2 proton signals of two Pro residues. Therefore, we chose HMQC-TOCSY[3] in place of ^{1}H–^{1}H COSY for the purpose of the intra-residual assignment, since the α-C signals of five amino acid residues were resolved (Table 1). The HMQC-TOCSY spectrum of **1** allowed all intra-

* Correspondence to: E. Fukushi, Faculty of Agriculture, Hokkaido University, Kita-9 Nishi-9, Sapporo 060-8589, Japan.
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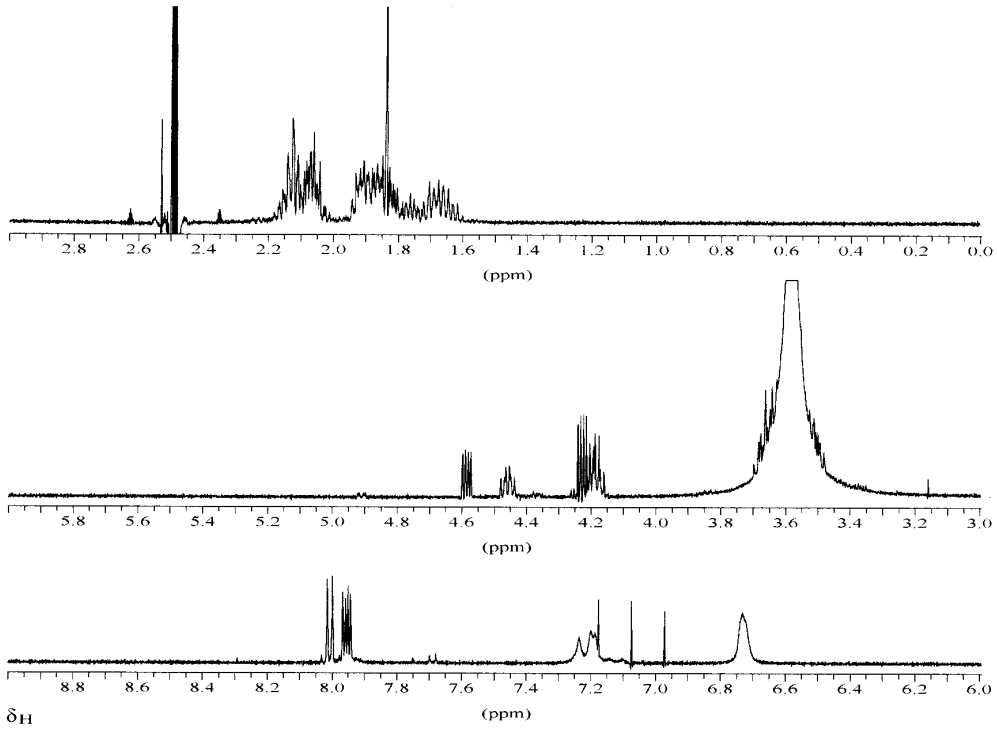

Figure 2. ^{1}H NMR spectrum of **1**.

residual side-chain assignments of ^{1}H and ^{13}C signals from α-CH to NH and to γ-CH_2 of Gln, and from α-CH to δ-CH_2 of Pro residues.

As for the arrangements of these residues, the connectivity from acetyl to ^{3}Gln was sequentially assigned from the HMBC[4] spectrum as follows (Fig. 3). The correlation peak of acetyl protons (δ_H 1.84 ppm) and a carbonyl carbon at δ_C 169.6 ppm led to the assignment of this carbon as acetyl carbonyl. This carbon gave additional correlation peaks with protons at δ_H 4.18 and 8.01 ppm, assignable to α-CH and NH protons of ^{1}Gln, respectively. The α-CH proton of ^{1}Gln was further correlated with a carbonyl at δ_C 171.6 ppm assignable as the α-CO carbon of ^{1}Gln. In the same manner, α-H (δ_H 4.20 ppm), α-NH (δ_H 7.96 ppm) and α-CO (δ_C 171.2 ppm) of ^{2}Gln and α-H (δ_H 4.46 ppm), NH (δ_H 7.95 ppm) and α-CO (δ_C 169.6 ppm) of ^{3}Gln could be assigned sequentially. However, the α-CO carbon of ^{3}Gln unfortunately gave no correlation peaks with both α-CH protons (δ_H 4.23 and 4.59 ppm) of Pro residues. This lack of correlation seemed to be due to a small ^3J(C,H) value between the α-CH proton of Pro residue and the α-CO carbon of ^{3}Gln because of their

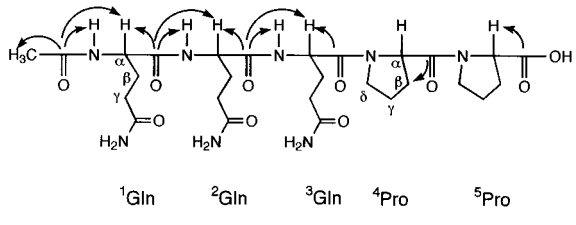

Figure 3. Sequential assignment of **1**. The connectivities by HMBC correlation peaks are indicated by arrows.

dihedral angle being approximately π/2. The assignments of two Pro residues were accomplished from the chemical shift difference between an amide carbonyl carbon of ^{4}Pro and a carboxyl carbon of ^{5}Pro. As a result, signals at δ_C 170.1 and 173.4 ppm were assigned to carbonyl carbons of ^{4}Pro and ^{5}Pro, respectively. The HMBC cross peaks between carbonyl of ^{4}Pro and β-CH_2 protons at δ_H 1.76 and 2.15 ppm, and between carbonyl of ^{5}Pro and an α-CH proton at δ_H 4.23 ppm allowed the correlation of the proton networks within both Pro residues with the aid of HMQC-TOCSY. The sequence of all residues was thus established, and the assignments of the proton and carbon signals in all amino acid residues except the γ-glutamylamide parts were completed. These assignments were further confirmed by the NOE data mentioned below.

Next, the analysis of proton–proton coupling patterns was performed. The *J*-resolved ^{1}H–^{1}H correlation spectrum[5] was used to clarify the splitting pattern of protons in the crowded region. However, the complex splitting nature of two α-CH protons of ^{1}Gln and ^{2}Gln could not be analyzed by F_1 slices of this spectrum. To confirm the splitting nature of these protons, we adopted better resolved 1D methods. Decoupling difference experiments were the first choice. Irradiation at each NH signal of ^{1}Gln or ^{2}Gln, however, failed to disclose the coupling patterns of the coupled α-CH proton since the δ_H values of two NH protons were also close to each other ($\Delta\nu = 25$ Hz). Homonuclear SPT (selective population transfer)[6] difference experiments, which utilize a weaker irradiation power than the decoupling experiments, were successful for this purpose. Irradiation at the higher frequency transition of NH of ^{1}Gln [Fig. 4(b)] or ^{2}Gln [Fig. 4(c)] clearly

Table 1. ^{1}H and ^{13}C NMR spectral data for **1**

Group		δ_C (ppm)	δ_H (ppm)	Multiplicity	J (Hz)
Ac	CH	22.6	1.84	s	
	C=O	169.6			
^{1}Gln	αNH		8.01	d	7.9
	α	52.5	4.18	ddd	8.4, 7.9, 5.7
	β	27.9	1.85	m	
			1.68	m	
	γ	31.6	2.08	m	
	αC=O	171.6			
^{2}Gln	αNH		7.96	d	8.2
	α	52.2	4.20	ddd	8.9, 8.2, 3.9
	β	28.2	1.85	m	
			1.68	m	
	γ	31.6	2.06	m	
	αC=O	171.2			
^{3}Gln	αNH		7.95	d	7.8
	α	50.0	4.46	ddd	8.5, 7.8, 5.7
	β	27.2	1.87	m	
			1.64	ddd	13, 8.5, 6.9
	γ	30.9	2.12	m	
	αC=O	169.6			
^{4}Pro	α	57.6	4.59	dd	8.8, 4.4
	β	27.8	2.15	dddd	12, 8.8, 7.5, 7.3
			1.76	m	
	γ	24.5	1.87	m	
	δ	46.9	3.67	ddd	9.9, 7.2, 7.2
			3.58	ddd	9.9, 7.8, 5.5
	C=O	170.1			
^{5}Pro	α	58.5	4.23	dd	8.8, 4.2
	β	28.6	2.13	dddd	12, 8.8, 7.0, 5.3
			1.82	m	
	γ	24.7	1.91	m	
	δ	46.4	3.65	ddd	9.8, 7.3, 7.3
			3.50	ddd	9.8, 6.4, 6.4
	C=O	173.4			

extracted the corresponding α-H signal split into a doublet of doublet of doublets; α-H of ^{1}Gln, δ_H 4.18 ppm, J = 8.4, 7.9 and 5.7 Hz; and α-H of ^{2}Gln, δ_H 4.22 ppm, J = 8.9, 8.2 and 3.9 Hz. The assignments of all ^{1}H and ^{13}C signals except the γ-glutamylamide parts are summarized in Table 1.

The NOESY[7] spectrum of **1** gave negative correlation peaks, even though its molecular weight is as low as 638. This phenomenon possibly arose from the high viscosity of the DMSO solution which caused its molecular correlation time to be long enough to give negative NOEs at a 500 MHz magnetic field. Since negative NOE and chemical exchange peaks cannot be discriminated in the NOESY spectrum, we used ROESY[8] for the stereochemical assignments instead. The ROESY spectrum of **1** disclosed clear ROE correlations between α-H of ^{3}Gln and δ-H of ^{4}Pro and between α-H of ^{4}Pro and δ-H of ^{5}Pro. These ROE correlations supported the sequence ^{3}Gln–^{4}Pro–^{5}Pro, and also indicated that the configurations of the two amide bonds of ^{3}Gln–^{4}Pro and ^{4}Pro–^{5}Pro were *trans*.[9] The configurations of other amide bonds included in the sequence ^{1}Gln–^{2}Gln–^{3}Gln, however, could not be determined from the ROESY spectrum since two α-NH signals of ^{2}Gln and ^{3}Gln were close to each other, and so were two α-H signals of ^{1}Gln and ^{2}Gln, as mentioned above. Even though ROE peak(s) had been observed between these protons, it would be impossible to tell that which α-H signal gave the ROE to which NH signal.

To separate these α-H signals using well resolved δ_C, we tried to measure the HMQC-ROESY[10] spectrum. However, a satisfactory signal-to-noise ratio to observe the cross peaks was not attained, probably because ROESY was much less sensitive than TOCSY. This problem was overcome by using CH-selected editing (E)-HSQC-ROESY instead of HMQC-ROESY. Since **1** has only five CH carbons whose δ_C values are concentrated in the range 50.0–58.5 ppm, the F_1 spectral width of the CH-selected E-HSQC-ROESY spectrum of **1** requires only 10 ppm at most. The narrower the F_1 spectral width which can reduce the data points on the F_1 axis (t_1 increments), the larger is the number of transients that can be accumulated in a certain total measuring time, expecting a sufficient signal-to-noise ratio. We selected HSQC rather than HMQC for the ^{13}C chemical shift labeling, since in the latter spectrum the

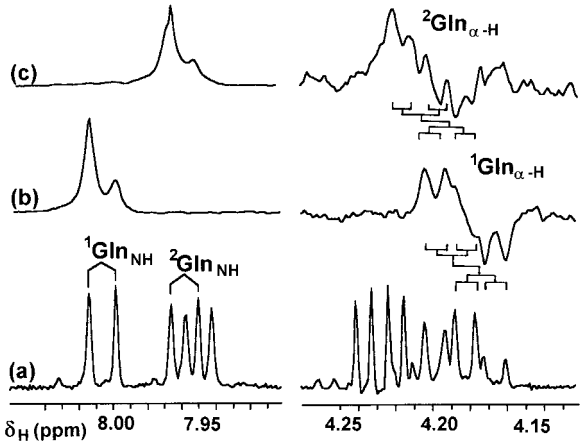

Figure 4. SPT difference experiments of **1**. (a) NH and α-H region of the normal ^{1}H NMR spectrum. (b) and (c) SPT difference spectra irradiating at higher frequency transients of NH protons of (b) ^{1}Gln and (c) ^{2}Gln. Irradiation was performed by DANTE pulse sequences.[12] The DANTE sequence was made up of total of 4762 pulses of duration 0.05 ms separated by the pulse interval of 0.16 ms from a decoupler attenuated by 40 dB. The total irradiation time was 2.0 s. Subtracting each 64 scans accumulated by irradiation at off-resonance from those at on-resonance was repeated eight times, and the resulting total measuring time was *ca.* 50 min. Exponential multiplication (*LB* = 1.0) was performed prior to Fourier transformation.

contribution of proton–proton J couplings brings about broadening of the correlation peaks in the F_1 dimension and, in consequence, lowers the F_1 resolution. The original E-HSQC pulse sequence[11] [Fig. 5(a)] includes an editing period consisting of {delay $\tau/2 = 1/2[^1J(C,H)]$} − {^{1}H-pulse of flip angle $\beta = \pi$, ^{13}C-π-pulse} − {delay $\tau/2$}, which gives the CH_2 signals in opposite phase to the CH and CH_3 signals in a similar manner

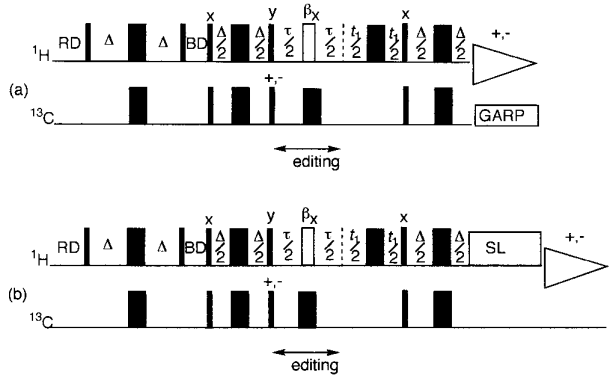

Figure 5. Pulse sequences of (a) E-HSQC and (b) E-HSQC-ROESY. Narrow, wide and open bars represent $\pi/2$, π and a variable pulse with a flip angle of β, respectively. ROESY mixing was achieved by a spin-lock (SL) composed of two CW pulses (shifted in phase by + and −). The delays RD, BD, Δ and τ represent a repetition delay, a BIRD delay for elimination of ^{12}C-bonded proton signals, $1/2[^1J(C,H)]$, and a variable delay for editing, respectively.

as seen in DEPT or INEPT. The intensity modulations of CH, CH_2 and CH_3 signals depending on the τ and flip angle of pulse β were estimated using product operator analysis.[12] The resulting coefficients of the terms containing τ and β contributed to the final spectrum are as follows; for simplicity, ^1J(C,H) is represented as J:

$$\begin{aligned}
\mathrm{CH}\quad & 1-(1-\cos\beta)\cos^2(\pi J\tau/2)\\
\mathrm{CH_2}\quad & 1/2[\sin^2(\pi J\tau)+\cos^2(\pi J\tau)\cos\beta\\
& +1/2\sin^2(\pi J\tau)\cos^2\beta]\\
\mathrm{CH_3}\quad & 1/4[3\sin^2(\pi J\tau)\cos^2(\pi J\tau/2)\\
& +\cos^2(\pi J\tau/2)\{1-3\cos(\pi J\tau/2)\}^2\cos\beta\\
& +\sin^2(\pi J\tau/2)\{1+3\cos(\pi J\tau/2)\}^2\cos^2\beta\\
& +3\sin^2(\pi J\tau)\sin^2(\pi J\tau/2)\cos^3\beta]
\end{aligned}$$

Hence the intensity modulations of the CH, CH_2 and CH_3 signals by variable τ under the fixed condition of $\beta = \pi$ are as follows:

$$\begin{aligned}
\mathrm{CH}\quad & -\cos(\pi J\tau)\\
\mathrm{CH_2}\quad & -\cos(2\pi J\tau)\\
\mathrm{CH_3}\quad & 2\cos(\pi J\tau)-3\cos^3(\pi J\tau)
\end{aligned}$$

On the other hand, the intensity modulations of the CH, CH_2 and CH_3 signals by variable β under the fixed condition of $\tau = 1/J$ are as follows:

$$\begin{aligned}
\mathrm{CH}\quad & 1\\
\mathrm{CH_2}\quad & \cos\beta\\
\mathrm{CH_3}\quad & \cos^2\beta
\end{aligned}$$

These functions are plotted in Fig. 6. From the experiments using simple model compounds, the τ and β dependences of the intensity modulations of the CH, CH_2 and CH_3 signals were confirmed (data not shown). As indicated in Fig. 6(b), setting the delay τ to $1/[^1J(C,H)]$ and the ^{1}H pulse flip angle β to $\pi/2$ gave only CH signals in the spectrum. We then constructed an E-HSQC-ROESY sequence by the insertion of ROESY spin locking immediately before acquisition [Fig. 5 (b)], in which the ROESY correlation peaks were expected to be in opposite phase to the direct coupling peaks.

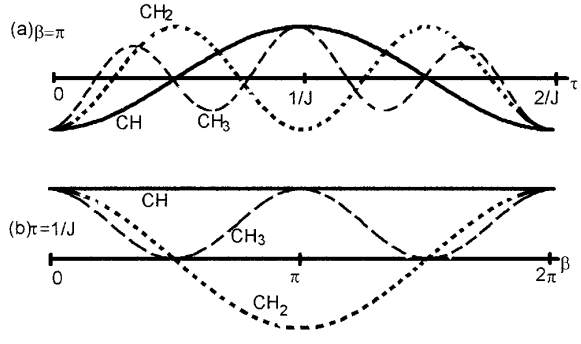

Figure 6. Intensity modulations of CH, CH_2 and CH_3 signals in E-HSQC. (a) τ dependence under fixed β (π) and (b) β dependence under fixed τ ($1/J$). Solid, dotted and dashed lines represent intensities of CH, CH_2 and CH_3 signals, respectively.

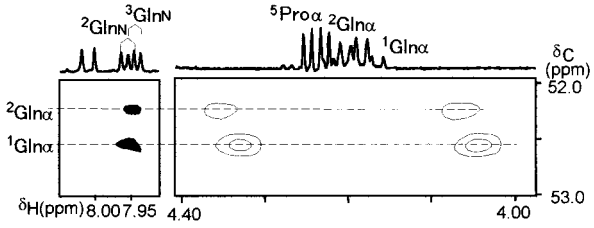

Figure 7. Part of the CH-selected HSQC-ROESY spectrum of **1**. The editing flip angle β and the delay τ were set to $\pi/2$ and 7.2 ms [^{1}J(C,H) = 139 Hz], respectively. The delays RD, BD, Δ and τ were set to 2.0 s, 0.4 s, 3.6 ms and 7.2 ms, respectively. The ROESY mixing (0.24 s) was achieved by a spin-lock composed of two CW pulses (shifted in phase by + and −). The F_1 and F_2 spectral widths were 1384 and 3759 Hz, respectively. For each 64 t_1 increments, 1280 transients (with four dummy scans) were accumulated in 2K data points. Zero-filling to 256 for F_1 and multiplication with squared sine-bell windows shifted by $\pi/4$ and $\pi/8$ in the F_1 and F_2 dimensions, respectively, were performed prior to 2D Fourier transformation. The resulting data matrix was 1K × 128. The total measuring time was *ca.* 64 h. Positive and negative peaks are drawn with open and filled contours, respectively.

In the CH-selected E-HSQC-ROESY spectrum of **1** (Fig. 7), ROEs between α-H of ^{1}Gln and NH of ^{2}Gln and between α-H of ^{2}Gln and NH of ^{3}Gln were separately observed. These ROEs indicate that two amide bonds of ^{1}Gln–^{2}Gln and ^{2}Gln–^{3}Gln are *trans*. Considering the coupling constant between NH and α-H within each Gln residue (^{1}Gln, 7.9; ^{2}Gln, 8.2; ^{3}Gln, 7.8 Hz) mentioned above, it is concluded that the ^{1}Gln–^{2}Gln–^{3}Gln part adopts an extended β-strand-like conformation.[13] As for the backbone conformation of the remaining part of **1**, the ROEs between α-H of ^{3}Gln and δ-H of ^{4}Pro and between α-H of ^{4}Pro and δ-H of ^{5}Pro obtained from the ROESY spectrum indicate that two amide bonds of ^{3}Gln–^{4}Pro and ^{4}Pro–^{5}Pro are *trans*. As a whole, two Pro residues make a small turn following the extended β-strand-like ^{1}Gln–^{2}Gln–^{3}Gln part. Further stereochemical analysis involving the side-chain conformation using NOEs and coupling constants by computer-assisted structural calculation and structure analysis of other related peptides having a similar IgE-binding ability is in progress.

EXPERIMENTAL

Peptide

The model peptide, Ac–Gln–Gln–Gln–Pro–Pro (**1**), was purchased from the Peptide Institute Inc. (Japan).

NMR sample and instrument

The peptide **1** (4 mg) was dissolved in 0.5 ml of DMSO-d_6. NMR spectra were recorded at 303 K with a Bruker AM 500 spectrometer (^{1}H 500 MHz, ^{13}C 125 MHz) equipped with a 5 mm diameter C/H dual (1D ^{1}H and ^{13}C spectra) or H/X inverse probe (SPT and 2D spectra). Chemical shifts (δ) in ppm were determined relative to the residual proton (2.49 ppm) and carbon (39.7 ppm) signals of the solvent.

1D normal ^{1}H NMR spectra

For the 1D ^{1}H spectrum, 64 FIDs were accumulated in 32K data points for a spectral width of 8064 Hz at 500.135 MHz. Multiplication with $\pi/8$-shifted squared sine-bell function was performed prior to Fourier transformation.

1D normal ^{13}C NMR spectrum

For the ^{13}C spectrum, complete proton decoupling was derived by attenuation of the high-power output of the decoupler (the $\pi/2$ pulse duration was 100 μs) and 4416 FIDs were accumulated in 64K data points for a spectral width of 31 250 Hz at 125.759 MHz. Exponential multiplication ($LB = 1.0$) was performed prior to Fourier transformation.

HMQC-TOCSY spectrum

The phase-sensitive ^{13}C-coupled HMQC-TOCSY spectrum was determined by the sequence proposed by Lerner and Bax[3] without ^{13}C decoupling during acquisition. The TOCSY mixing time (0.1 s) was performed by MLEV-17 composite pulses guarded by trim pulses (2.5 ms) derived from the high-power output of the decoupler channel attenuated by 12 dB ($\pi/2$ pulse duration, 40 μs). The repetition and BIRD delays were 2.0 and 0.4 s, respectively. The spectrum was measured with the F_2 spectral width of 3759 Hz in 2K data points using 256 transients (with two dummy scans) for each of 220 t_1 increments of the F_1 spectral width of 5030 Hz. Zero-filling to 0.5 K for F_1 and multiplication with squared cosine-bell windows in both dimensions were performed prior to 2D Fourier transformation. The resulting data matrix was 1K × 0.25K. The total measurement time was *ca.* 37 h.

HMBC spectrum

Since the ^{13}C signals of **1** concentrated in two separate regions of δ_C 22–59 and 169–175 ppm in the ^{13}C NMR spectrum, the HMBC spectrum was measured setting the F_1 region to δ_C 73–125 ppm, the signals at δ_C 22–59 and 169–175 ppm were folded to appear at δ_C 74–111 and 117–123 ppm, respectively. The repetition and evolution delays were 2.0 s and 60 ms [^{LR}J(C,H) = 8.3 Hz], respectively. The spectrum was measured covering the F_2 spectral width of 3759 Hz in 1K data points using 512 transients (with two dummy scans) for each of 124 t_1 increments. Zero-filling to 0.25K for F_1 and the

Lorenz–Gauss transformation ($GB = 0.1$, $LB = -2.0$) in F_2 and multiplication with squared cosine-bell windows in the F_1 dimension were performed prior to 2D Fourier transformation. The resulting data matrix was 0.5K × 0.25K. The total measurement time was *ca.* 42 h.

J-resolved ^{1}H ^{1}H correlation spectrum

The 2D *J*-resolved ^{1}H–^{1}H correlation spectrum was measured with an F_2 spectral width of 3703 Hz in 2K data points using 128 transients (with two dummy scans) for each of 124 t_1 increments of the F_1 spectral width of 60 Hz. Zero-filling to 0.25K for F_1 and multiplication with sine-bell windows in both dimensions were performed prior to 2D Fourier transformation. The resulting data matrix was 1K × 0.25K. The total measuring time was *ca.* 10 h.

SPT (selective population transfer) difference spectra

The 1D SPT difference spectra were obtained by irradiation with DANTE pulse sequences.[14] The DANTE sequence was made up of total of 4762 pulses of duration 0.05 ms separated by a pulse interval of 0.16 ms from the decoupler attenuated by 40 dB. The total irradiation time was 2.0 s. Subtracting each 64 scans accumulated by irradiation at off-resonance from those at on-resonance was repeated eight times, and the resulting total measuring time was *ca.* 50 min. Exponential multiplication ($LB = 1.0$) was performed prior to Fourier transformation.

ROESY spectrum

For the 2D phase-sensitive ROESY spectrum, all pulses were derived from the high-power output of the decoupler attenuated by 19 dB (the $\pi/2$ pulse duration was 100 μs, a 2500 Hz spin-lock field). The ROESY mixing (0.2 s) was achieved by a spin-lock composed of two CW pulses (shifted in phase by + and −).[15] The spectrum was measured with a spectral width of 3759 Hz in 1K data points using 160 transients (with two dummy scans) for each of 242 t_1 increments. Zero-filling to 0.5K for F_1 and multiplication with squared cosine-bell windows in both dimensions were performed prior to 2D Fourier transformation. The resulting data matrix was 0.5K × 0.25K. The total measurement time was *ca.* 26 h.

E-HSQC-ROESY spectrum

The 2D phase-sensitive CH-selected HSQC-ROESY was measured by the sequence shown in Fig. 5(b). The editing flip angle β and the delay τ were $\pi/2$ and 7.2 ms [^1J(C,H) = 139 Hz], respectively. The delays RD, BD, Δ and τ were set to 0.2 s, 2.0 s, 0.4 s, 3.6 ms and 7.2 ms, respectively. The ROESY mixing (0.24 s) was achieved by a spin-lock composed of two CW pulses (shifted in phase by + and −). The F_1 and F_2 spectral widths were 1384 and 3759 Hz, respectively. For each 64 t_1 increments, 1280 transients (with four dummy scans) were accumulated in 2K data points. Zero-filling to 256 for F_1 and multiplication with squared sine-bell windows shifted by $\pi/4$ and $\pi/8$ in the F_1 and F_2 dimensions, respectively, were performed prior to 2D Fourier transformation. The resulting data matrix was 1K × 128. The total measuring time was *ca.* 64 h.

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