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NK-2의 Antagonist인 cyclo-[Gln-Trp-Phe-βAla-Leu-Met]의 형태에 관한 연구

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Conformation of cyclo-[Gln-Trp-Phe-βAla-Leu-Met], a NK-2 Tachykinin Receptor Antagonist

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요 약. 새로운 NK-2 antagonist이며 고리상 펩티드인 cyclo-(Gln[1]-Trp[2]-Phe[3]-βAla[4]-Leu[5]-Met[6])의 DMSO용액 중에서의 형태를 2차원 핵자기공명과 분자동력학적인 계산에 의하여 결정하였다. 조건을 만족시키는 25개의 구조는 모든 아미노산 잔기의 골격원자들(N, C^{α}, C')의 자승 평균 평방근이 0.02Å이내로 수렴하였다. 이 고리상 펩티드의 구조는 Met[6]NH와 Ala[4]CO, Ala[4]NH와 Met[6]CO, Phe[3]NH와 Met[6]CO의 사이에 분자내 수소결합이, Gln과 Trp에 type-I β-turn, 그리고 Leu에서 γ-turn을 취하고 있었다. 알려져 있는 고리상 펩티드인 cyclo-(Gln[1]-Trp[2]-Phe[3]-Gly[4]-Leu[5]-Met[6])의 Gly이 βAla으로 바뀜에 따라 βAla의 여분의 메틸렌이 고리의 골격의 반발력을 완화시키고 수소결합들이 형태의 안정에 기여하고 있다고 생각된다.

ABSTRACT. Solution conformation of cyclo-(Gln[1]-Trp[2]-Phe[3]-βAla[4]-Leu[5]-Met[6]), new NK-2 antagonist in dimethyl sulfoxide solution, has been determined by the use of two-dimensional nuclear magnetic resonance spectroscopy combined with simulated annealing calculations. The peptide exhibited converged structures with the atomic root-mean-square difference for the backbone atoms (N, C^{α}, C') of all residues being 0.02Å in the 25 annealed structures. The analysis of the structures indicated that the cyclic peptide has three intramolecular hydrogen bonds between Met[6]NH and βAla[4]CO, βAla[4]NH and Met[6]CO, Phe[3]NH and Met[6]CO, and contain a type-I β-turn with Gln and Trp and a γ-turn with Leu. The addition of an extra methylene group to Gly, i.e. β-Ala residue, may relax some unfavorable restraints in the cyclic backbone structure, hence enabling an additional hydrogen bond, which results in stabilizing one conformation.

INTRODUCTION

The mammalian tachykinin peptides are a class of spasmogenic peptides having the common carboxy-terminal sequence of Phe-Phe/Val-Gly-Leu-Met-NH_2 and exhibiting very similar biological profiles.[1,2,3,4] Three of these peptides, substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) have been identified to exist in the peripheral and central nervous systems.[5,6,7,8] In addition, a variety of their actions are known to be mediated through the activation of three distinct receptors, called NK-1, NK-2, and NK-3, which preferentially bind to SP, NKA, and NKB, respectively.[4] Since NKA in the respiratory system is suspected to be a major cause of bronchoconstriction,[9,10] this strongly suggests the therapeutic value of NKA antagonists. In the linear carboxy-terminal heptapeptide NKA(4-10), replacement of the Gly residue at position 8 with βAla, [βAla[8]]NKA(4-10), is known to give a potent and selective NK-2 receptor agonist.[11] Based on this fact, c(QWFβLM) was synthesized as the biologically active analog of c(QWFGLM) by

replacing Gly residue to βAla, and it was found that this compound still possesses potent antagonist activity against NK-2 receptor. The conformation of this new antagonist in solution has been studied by proton NMR spectra and molecular annealing.

MATERIALS AND METHODS

Peptide synthesis. Tert-butyloxycarbonyl (Boc)-amino acid derivatives and 4-chloromethyl polystyrene resin were purchased from Peptide Institute, Inc. (Osaka, Japan). Coupling reagents and Boc-βAla were from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Other chemical reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Deuterated dimethyl sulfoxide (DMSO-d_6) was from Aldrich Chemical Co. (Milwaukee, WI). The linear peptide, H-Met-Gln-Trp-Phe-βAla-Leu-OH, was synthesized by manual and conventional solid-phase methodologies using Boc-HF strategy, and peptide coupling was afforded by N,N'-dicyclohexylcarbodiimide (DCC)/N-hydroxybenzotriazole (HOBt) in N,N'-dimethylformamide (DMF). Its purity was analyzed by reversed phase-high performance liquid chromatography (RP-HPLC) (GL Science, inertsil ODS-2, 4.6×150 mm; A: 99.9% water, 0.1% trifluoroacetic acid, B: 80% aqueous acetonitrile, 0.1% trifluoroacetic acid; Gradient 0.100% B in 40 min; retention time 23.9 min). Cyclization of this peptide was achieved with benzotriazolyl N-oxy-tris (dimethylamino) phosphonium hexafluorophosphate (BOP) reagent.[12] The retention time of purified c(QWFβLM) was 26.5 min using the same conditions. The identity of the resultant cyclic peptide was confirmed by fast atom bombardment mass spectrometry (Fab-mass [(M+H) 777.4]) and the amino acid analysis [Glx 1.06 (1); Trp nd (1); Phe 1.04 (1); βAla 0.83 (1); Leu 1.00 (1); Met 0.96 (1)]. The antagonist c(QWFGLM) was similarly prepared. The NK-2 selective agonist, [βAla^8]NKA(4-10), was synthesized per Rovero *et al.*[11]

Biological activities. The peptide-induced contraction of guinea pig trachea (GPT) was measured as described by Shanab *et al.*[13] Briefly, Hartley guinea pigs (250~300 g) were sacrificed by blow on the head and their trachea were taken out rapidly. To measure isometric tension, tracheal rings approximately 2-3 mm in diameter were cut and vertically mounted in water-jacketed (37°C) organ baths (5 ml capacity), i.e., two rings were jointed in series, suspended by a steel hook into the organ bath filled with Krebs solution (NaCl 119.0 mM, KCl 3.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.5 mM, $CaCl_2$ 1.25 mM, $NaHCO_3$ 25.0 mM, and glucose 11.0 mM (pH 7.4), and gassed with O_2-CO_2 (95:5, v/v) at 37°C. The prepared rings were initially placed under 1 g of tension and left to equilibrate for 1 h while being washed every 20 min with the Krebs solution. Next they were excised by the adding 10^{-6} M carbachol. Contractions were recorded isotonically under a resting tension of 1 g via FD-pick up (TB-611T, Nihon Koden) connected to an amplifier (AP601G, Nihon Koden) and recorder (WI-645G, Nihon Koden). Peptides were dissolved in saline with the aid of a small amount of DMSO. Antagonist activity of peptides was determined using [βAla^8]NKA(4-10) as a selective NK-2 agonist.[11] In all preparations, full concentration-response curves to the [βAla^8]NKA(4-10) were determined in the absence and presence of various concentrations of antagonists. Schild plot[14] analysis was performed for c(QWFβLM) and c(QWFGLM). The pA_2 value, negative logarithm of the antagonist concentration that doubles the agonist concentration needed to give the same physiological response as in the absence of the antagonist, was estimated according to Van Rossum.[15]

NMR measurements. All ^{1}H-NMR experiments were carried out on a Bruker AMX-500 spectrometer at a probe temperature of 313 K unless otherwise specified. The concentration of the peptides were 15 mM in DMSO-d_6. No symptom of self-aggregation was detected such as a concentration dependence of chemical shifts. The temperature coefficient of NH resonances was determined by plotting the NH resonance chemical shiftfrom 298 to 318 K in 5 K increment. The $^3J_{NH}$ coupling constants were obtained from one-dimensional ^{1}H-NMR spectrum. Chemical shifts are reported in reference to residual methyl proton resonances of DMSO-d_6, being set at 2.49 ppm. The following spectra was obtained in a phase-sensitive mode[16] using two-dimensional double-quantum-filtered correlation spectroscopy (DQF-COSY),[17] homo nuclear Hartmann-Hahn spectroscopy (HOHAHA)[18] with a mixing time of 83 ms, and ROESY[19] with a mixing time of 200 ms. The spectral width in both dimensions was 6944 Hz and data points

Table 1. ^{1}H Chemical shifts[a], coupling constants, and temperature coefficients of chemical shifts for c(QWFβLM) in DMSO-d_6 solution at 313 K

residue	NH	C$^\alpha$H	C$^\beta$H	C$^\gamma$H	others	$^3J_{NH\alpha}$(Hz)	ΔδNH/ΔT(ppb/K)
Gln[1]	8.30	3.83	1.77[b]	2.01[b]	NH_{amine} 7.06, 6.66	4.8	−4.5
					C2H 6.94 C4H 7.36		
Trp[2]	8.15	3.96	3.11 2.93		C5H 6.91 C6H 7.00	5.6	−5.0
					C7H 7.27 NH_{indole} 10.74		
Phe[3]	7.76	4.37	3.10	2.82	H_{arom} 7.18	8.3	−4.4
βAla[4]	7.42	3.51, 3.11	2.38 2.21			11.4[c]	−3.0
Leu[5]	8.08	4.04	1.48[b]	1.60	CH_3 0.85, 0.79	6.4	−5.3
Met[6]	7.61	4.37	2.04 1.85	2.41[b]		8.4	−0.4

[a]Chemical shifts are reported relative to the methyl group of DMSO-d_6 set at 2.49 ppm.
[b]Two methylene protons are degenerated.
[c]$^3J_{NH\alpha}+{}^3J_{NH\alpha}$.

in time domain were 2048 (t_2)×512 (t_1). Real data points in the frequency domain were 2048(t_2) x 1024(t_1). Before Fourier transformation, time domain data were multiplied by a squared sine bell function for t_2 dimension and a sine bell function for the t_1 dimension. A $\pi/2$ phase was applied in both dimensions. Temperature coefficients of NH proton resonances were determined in from 298 ~318 K with results being summarized in *Table* 1. Note all the NH resonances shifted linearly with temperature; a phenomenon indicating that the molecule does not undergo any conformational changes in this temperature range.

Structure calculations. Energy minimization and simulated annealing calculations were carried out using the XPLOR program[20] with a Iris 35 workstation (Silicon Graphics Inc., Mountain View, CA). Initial structures were generated using random Φ, Ψ, and χ_1 angles. For simulated annealing calculations, the YASAP protocol[20] was employed. The form of the target function to be minimized was

$$F_{total}=F_{bond}+F_{angle}+F_{impr}+F_{repel}+F_{ROE}+F_{tor}$$

where F_{bond} and F_{angle} represent the covalent energy terms for maintaining correct bond lengths and bond angles, respectively. F_{impr} the energy involving chirality and planarity, F_{repel} the repulsion term for preventing unduly close contacts of atoms, and F_{ROE} and F_{tor} the respective square-well potentials for introducing penalties when the interproton distances and torsion angles deviate from the acceptable value ranges that were experimentally determined (see Clore *et al.*[21] for details). The interproton distances without stereospecific assignments were taken as single $(<r^{-6}>)^{-1/6}$ average distances so that no corrections were necessary for pseudo atom treatments. Analysis of structures was also carried out by XPLOR, while their displaying and plotting of structures were carried out with QUANTA the code (version 3.2, Molecular Simulations, Waltham, MA). For a quantitative assessment of the calculational convergence, a partial sum, $F_3=F_{repel}+F_{ROE}+F_{tor}$, was computed for each structure. The 25 annealed structures having the smallest F_3 values were selected from 200 calculations. For quantitative comparisons of different structures, minimum

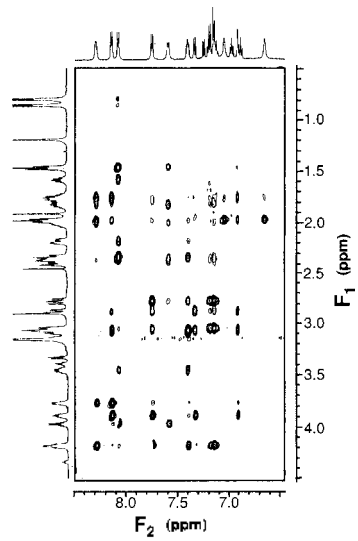

Fig. 1. 500 MHz ROESY spectrum (aliphatic/NH region) of c(QWFβLM) in DMSO-d_6 solution at 313K with a mixing time of 200 msec.

Table 2. Observed ROEs for c(QWFβLM)[a]

	ROE	upperbound distance (Å)		ROE	upperbound distance (Å)
Gln^1	NH-C_αH	3.0	βAla^4	NH-C_αH	3.0
	NH-$C_\beta H_2$	3.0		NH-C_αH'	2.5
	NH-$C_\gamma H_2$	3.0		NH-C_3H	3.0
	NH-Trp^2NH	4.0		NH-C_3H'	4.0
	NH-βAla^4NH	4.0		NH-Gln^1C_αH	4.0
	NH-Leu^5NH	4.0		NH-$Gln^1C_\beta H_2$	4.0
	NH-Met^6NH	4.0		NH-$Gln^1C_\gamma H_2$	4.0
	NH-Met^6CH	3.0		NH-Trp^2C_αH	3.0
	NH-Met^6CH'	4.0		NH-Phe^3C_βH'	3.0
	NH-Met^6CH_2	4.0		NH-Leu^5NH	4.0
	$C_\beta H_2$-C_εNH	4.0		CH-C_βH	3.0
	$C_\beta H_2$-Trp^2C2H	3.0		CH-C_βH'	3.0
	$C_\gamma H_2$-C_εNH	3.0		CH-$Gln^1C_\gamma H_2$	3.0
	$C_\gamma H_2$-Trp^2C2H	3.0	Leu^5	NH-C_αH	3.0
Trp^2	NH-C_αH	2.5		NH-$C_\beta H_2$	3.0
	NH-C_3H	3.0		NH-C_γH	3.0
	NH-C_βH'	4.0		NH-$C_\delta H_3$	4.0
	NH-C2H	3.0		NH-$C_\delta H_3$	4.0
	NH-C4H	4.0		NH-βAla^4C_αH	3.0
	NH-Gln^1C_αH	2.5		NH-βAla^4C_βH	2.5
	NH-$Gln^1C_\beta H_2$	3.0		NH-βAla^4C_βH'	3.0
	NH-$Gln^1C_\gamma H_2$	3.0		NH-Met^6NH	3.0
	NH-Phe^3NH	3.0		C_αH-$C_\beta H_2$	3.0
	NH-βAla^4NH	4.0		C_αH-C_γH	3.0
	NH-Leu^5C_αH	4.0		C_αH-$C_\delta H_3$	3.0
	C_αH-C_βH'	2.5		C_αH-$C_\delta H_3$	3.0
	C_αH-C2H	2.5		$C_\beta H_2$-Met^6C_αH	4.0
	C_αH-C4H	3.0		$C_\beta H_2$-$Met^6C_{\alpha\gamma}$H	4.0
	C_βH-C2H	2.5	Met^6	NH-C_βH	4.0
	C_βH-C4H	3.0		NH-C_βH'	3.0
	C_βH-C2H	3.0		NH-$C_\gamma H_2$	3.0
	C_βH-C4H	3.0		NH-Phe^3C_βH'	4.0
	C1H-C7H	3.0		NH-Leu^5C_αH	3.0
Phe^3	NH-C_3H	3.0		NH-$Leu^5C_\beta H_2$	3.0
	NH-C_3H'	3.0			
	NH-Gln^1C_αH	4.0			
	NH-$Gln^1C_\beta H_2$	4.0			
	NH-$Leu^5C_\beta H_2$	3.0			
	C_αH-C_βH	3.0			
	C_αH-C_βH'	2.5			
	C_αH-$C_\beta H_2$	3.0			
	C_αH-$Trp^2C_{\beta\chi}$H	3.0			
	NH-βAla^4NH	2.5			
	$C_{\alpha\beta}$H-C_βH	2.5			

[a]Since stereospecific assignments were not carried out, protons at lower and upper fields were respectively designated as X and X'.

root-mean-square differences (RMSDs) were calculated for the backbone atoms (N, C^α, C') of residues 1-6. A mean structure was obtained for each peptide by averaging the coordinates of the structures that had been

superimposed in advance on the best converged structure. Because such an averaged structure was poor in geometry, it was subjected to a restrained energy minimization[21]. The final coordinates of individual annealed structures were obtained by fitting them to the mean structure for the backbone atoms of residues 1-6.

RESULTS AND DISCUSSION

Biological activities. Although c(QWFβLM) showed no agonist activity in an assay of guinea pig trachea, it demonstrated potent antagonist activity against [βAla^8] NKA(4-10), giving pA_2=6.4, whereas c(QWFGLM) gave pA_2=7.0.

Proton NMR resonance assignments. The proton NMR resonances of c(QWFβLM) were assigned to individual protons according to the method established by Wüthrich.[22] Briefly, resonances were assigned to the spin systems of specific amino acid residues using DQF-COSY and HOHAHA spectra. Though some cyclic peptides have multiple conformations which are in slow exchange with each other on a NMR chemical shift scale and give two (or more) sets of spin systems for each amino acid residue[23], only one set of spin systems were observed for c(QWFβLM) (*Fig.* 1). The identified spin systems were then aligned along the primary structure on the basis of the distance information obtained from the ROESY spectrum shown in *Fig.* 1.

Structure calculations. A simulated annealing calculation was carried out to find conformations consistent with distance constraints obtained from the cross-peak intensity of ROESY[24] spectrum. These ROEs were classified into three distance constraints: ≤2.5, ≤3.0, ≤4.0, corresponding to the strong, medium, and weak intensity of ROESY cross peaks at 313K and 200 ms of mixing time. At this mixing time, spin diffusion effects were not observed. For the structure calculations, 42 intra-residue, 24 sequential, and 14 non-sequential ROE constraints were used as input data. *Table* 2 lists all ROEs observed for c(QWFβLM) in which this classification was

Table 3. Structural Statistics[a]

	25 structures	mean structure
RMSDs from experimental distance restraints (82)	0.080±0.002	0.078
number of violation >0.3Å	1.88 (max. 0.40Å)	1 (max. 0.36Å)
RMSDs from experimental dihedral angle restraints (3)	10.1±0.5	
number of violation > 5.00 deg	1 (max. 18.5 deg)	1 (max. 17.3 deg)
F_{NOE} (kcal mol^{-1})[b]	26.7±1.0	25.3
F_{bar} (kcal mol^{-1})[b]	4.6±0.4	4.6
F_{repel} (kcal mol^{-1})[b]	31.7±0.9	34.4
E_{LJ} (kcal mol^{-1})[c]	45.9±3.2	54.1
RMSDs from idealized geometry		
bonds (Å) (110)	0.0129±0.0003	0.013
angles (deg) (194)	3.17±0.01	3.16
Impropers (deg)[d] (69)	1.22±0.03	1.12

[a]The "25 structures" refers to the 25 annealed structures having the smallest F_3 values; the "mean structure" refers to the restrained minimized structure obtained by restrained minimization of the averaged coordinates of the individual 25 structures; The number of constraints is given in parantheses.

[b]The values of the force constants used for the calculation of the square-well potential are the original values of 50 kcal · mol^{-1} · Å^{-1} and 50 kcal · mol^{-1} · rad^{-2}, for NOE. The value of van der Waals repulsion term is calculated with the original force constant of 4 kcal · mol^{-1} · Å^{-6} with the van der Waals radii scaled by a factor 0.8 times the standard value used in the CHARMm empirical function[29].

[c]E_{LJ} is the Lennard-Jones type van der Waals energy calculated with the CHARMm empirical energy function[29], which was not included in th simulated annealing calculation.

[d]The improper torsion terms are used to maintain the planar geometry as well as the chiralities.

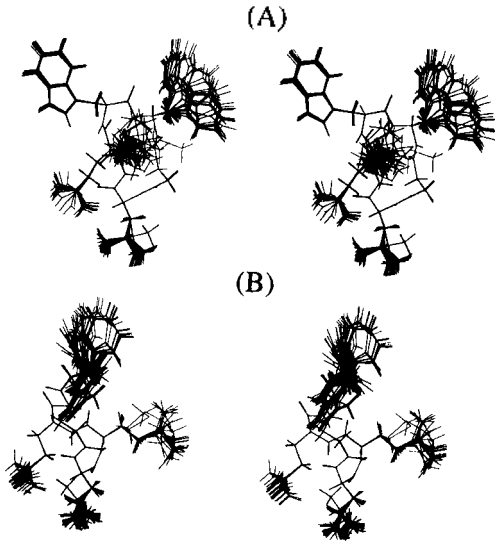

Fig. 2. Best fit superposition of 25 c(QWFβLM) structures calculated by simulated annealing (stereo view). (A): front view, (B): side view.

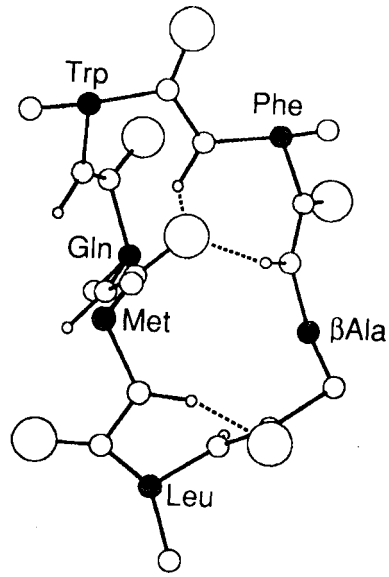

Fig. 3. Bean and stick representation of the mean structure of the c(QWFβLM). Dashed lines indicate possible hydrogen bonds. A type I β-turn with Gln^1 and Trp^2 at the corners is at upper left and a γ-turn with Leu^5 at the corner is at the bottom. Filled, shaded spheres are α-carbons, empty large spheres are carbonyl carbon, middle spheres are carbon and nitrogen and small spheres indicate amide protons, respectively.

applied. Three constraints on the dihedral angle Φ were also introduced according to Gierasch *et al.*[25] Φ of Phe^3 and Met^6 with $^3J_{HN\alpha}$ greater than 8 Hz was constrained in the range of −120±40°, whereas Φ of Gln^1 with $^3J_{HN\alpha}$ less than 5 Hz was constrained in the range of −65±25°. Two hundred solution structures and selected 25 structures were used for calculation as described above. *Fig.* 2 shows the best-fit overlays of the 25 converged structures of c(QWFβLM). Structural statistics for the selected structures are presented in *Table* 3. Their average atomic RMSD around the mean structure was 0.02 Å for backbone atoms and 0.77 Å for nonhydrogen atoms.

Description of structures. The overall shape of c(QWFβLM) looks like three spokes and an axle: namely, the side chains of Trp, Phe, and Leu form the spokes (*Fig.* 2a) and the side chains of Met and Gln form the axle, in the opposite orientation to each other (*Fig.* 2b). This result suggest that it has three intramolecular hydrogen bonds, i.e., Met^6 NH-OC $βAla^4$, $βAla^4$ NH-OC Met^6, and Phe^3 NH-OC Met^6. Supporting this hypothesis, amide protons of Met^6 (−0.4 ppb/K), $βAla^4$ (−3.0 ppb/K) exhibited a small temperature coefficient of chemical shift (*Table* 1). As shown in *Fig.* 3, the hydrogen bond between Phe^3 NH and Met^6 CO defines a type I β-turn with Gln^1 and Trp^2 at the corner positions, whereas the one between Met^6 NH and $βAla^4$ CO defines a γ-turn with Leu^5 at the corner.

Stabilized conformation of a cyclic peptide by Gly→βAla substitution. It is known that cyclization does not always constrain flexible linear peptides to one stable conformation.[25,26] In fact, Gly→βAla substitution can confer conformational freedom to the backbone dihedral angles of a cyclic peptide[27]. Quite unexpectedly, the replacement of Gly by βAla brought about a drastic change in its conformational properties. The resultant stabilization of a single, well-defined conformation by the addition of an extra methylene group at the Gly residue may well be due to loosening of strain in the backbone of the native cyclic peptide. The addition may also enable the formation of another hydrogen bond involving CO of βAla. Although Kessler[28] suggested that the inclusion of βAla in cyclic peptides results in a less defined conformation, such a modification may actually help structure analyses of cyclic peptides when the orig-

inal cyclic peptide is comprised of a mixture of multiple conformations. Since c(QWFβLM) has a single, well-defined conformation in DMSO solution, structure analyses of peptide analogs designed based on this will greatly facilitate the structure-activity relationship study of NK-2 antagonists, and ultimately, the development of a potent as well as selective NK-2 agonist/antagonist. Moreover, such peptides with a defined conformation will be useful probing structure differences existing in different NK receptors.

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