Spectroscopic Characterization of the Inclusion Complex of a Luteinizing Hormone-Releasing Hormone Agonist, Buserelin Acetate, with Dimethyl-β-cyclodextrin

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Received August 5, 1996; accepted October 22, 1996

Inclusion complexation of buserelin acetate, an agonist of luteinizing hormone-releasing hormone, with dimethyl-β-cyclodextrin (DM-β-CyD) in aqueous solution was studied spectroscopically and its mode of interaction was assessed. Ultraviolet absorption and circular dichroism (CD) spectroscopies indicate that the aromatic side chains of buserelin acetate, L-tryptophan and L-tyrosine residues, are incorporated into the hydrophobic environment of the DM-β-CyD cavity. Furthermore, proton and carbon-13 nuclear magnetic resonance spectroscopies suggest that in addition to the two aromatic side chains, a tertiary butyl D-serine residue is inserted into the DM-β-CyD cavity from the secondary hydroxyl side. On the other hand, the continuous variation plots for the buserelin acetate: DM-β-CyD system showed a 1 : 1 stoichiometry of the complex. Therefore, the complexation should be initiated by the inclusion of one of the three binding sites on the buserelin molecule into DM-β-CyD, which may in turn prevent the further access of the second cyclodextrin to the other binding sites, probably due to steric hindrance and/or conformational changes of the peptide. These structural features of the complex would account for the stabilizing effect of DM-β-CyD on the enzymatic degradation of buserelin acetate.

Key words buserelin acetate; dimethyl-β-cyclodextrin; inclusion complexation; spectroscopic characterization; stoichiometry; stability constant

We have previously demonstrated that methylated cyclodextrins, especially 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CyD), are effective in improving the rate and extent of nasal bioavailability of buserelin acetate, an agonist of luteinizing hormone-releasing hormone (LHRH) in rats.[1] The absorption enhancement afforded by the cyclodextrins can be attributed primarily to their ability to reduce the permeation barrier of the nasal epithelium to buserelin acetate. In addition, the cyclodextrins inhibited the enzymatic degradation of buserelin acetate in rat nasal mucosa, thus making the peptide more available for nasal absorption. Fluorescence spectroscopic studies indicated that the cyclodextrins formed inclusion complexes with buserelin acetate, which may participate in the protection of the peptide against proteolytic inactivation. This view is supported by the fact that a positive correlation exists between the inhibitory effects of the cyclodextrins on the enzymatic degradation of buserelin acetate and the magnitude of apparent stability constants for its cyclodextrin complexes.[1] In general, molecules of peptides and proteins are too bulky to be wholly included in the cyclodextrin cavity, thus their interaction with cyclodextrins could only be local; that is, accessible hydrophobic moieties may form inclusion complexes with cyclodextrins.[2,3] Building upon these studies, this paper deals in detail with the mode of inclusion complexation of buserelin acetate with DM-β-CyD to gain further insight into the mechanism for the stabilization of the peptide against enzymatic degradation. Several spectroscopies such as ultraviolet (UV) absorption, circular dichroism (CD) and proton (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) were employed in this study.

Experimental

Materials Buserelin acetate (Hoechst Japan, Ltd., Saitama, Japan), and tertiary butyl-D-serine (Hoechst AG, Frankfurt am Main, Germany) were used without further purification. DM-β-CyD was supplied from Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan) and used after recrystallization from methanol and water. All other materials were of reagent grade, and deionized double distilled water was used.

Spectroscopy UV absorption and CD spectra were recorded with a Hitachi U-3200 spectrophotometer (Tokyo, Japan) and a Jasco J-600 recording polarimeter (Tokyo, Japan), respectively. All measurements were carried out in isotonic phosphate buffer (pH 7.4) at 25 °C, with cells of 1 and 10 mm path lengths for the far-UV (190—250 nm) and the near-UV (250—350 nm) ranges, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded at 30 °C on a JEOL JNM EX-400 or JNM-α500 spectrometer operating at 399.78 or 500.0 MHz for ^{1}H-NMR and 100.53 MHz for ^{13}C-NMR, respectively. Deuterium oxide (D_2O) was used as a solvent and the water signal as an internal reference for ^{1}H-NMR, while the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP) was used as an external reference for ^{13}C-NMR. ^{1}H- and ^{13}C-NMR chemical shifts were given in parts per million (ppm) relative to those of the HOD signal and the TSP signal, with accuracies of ±0.005 (^{1}H-NMR) and ±0.015 ppm (^{13}C-NMR), respectively. Two-dimensional (2D) NMR spectra [correlation spectroscopy (COSY) and 2D rotating frame nuclear Overhauser effect spectroscopy (ROESY)] were recorded on a Bruker AMX-500 spectrometer operating at 500.14 MHz. The NMR signals of buserelin were assigned with the aid of 2D ^{1}H–^{1}H and ^{1}H–^{13}C COSY experiments,[4] and those of DM-β-CyD were assigned according to the report of Yamamoto and co-workers.[5]

Continuous Variation Method The stoichiometry of a complex of buserelin acetate with DM-β-CyD in D_2O at 30 °C was determined by a continuous variation method,[6] analyzing the ^{1}H-NMR chemical shift displacement of the H3′ proton of DM-β-CyD. The total concentration of buserelin acetate and DM-β-CyD was kept constant at 10 mM.

Determination of Stability Constant of Complexes The UV absorption changes of L-tryptophan (0.15 mM) and L-tyrosine (0.5 mM) in the presence of DM-β-CyD (varied from 0 to 50 mM) in isotonic phosphate buffer (pH 7.4) at 25 °C were measured at 241 and 278 nm, respectively. Upon an assumption that each amino acid forms an inclusion complex with DM-β-CyD at a 1:1 molar ratio, the apparent stability constants (K_c) for complexes of the amino acids with DM-β-CyD were determined according to the following Scott's equation (Eq. 1)[7]:

$$\frac{a \cdot b}{d} = \frac{1}{K_c \cdot \varepsilon_c} + \frac{b}{\varepsilon_c} \quad (1)$$

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where a is the total concentration of the amino acids, b is the total concentration of DM-β-CyD, ε_c is the difference in molar absorptivities for free and complexed amino acids, and d is the change in absorbance of the amino acids by the addition of DM-β-CyD.

Since tertiary butyl-D-serine has no UV absorption or fluorescence emission at wavelengths longer than 200 nm, the K_c value for a complex of the amino acid with DM-β-CyD was determined by competitive fluorescence spectrometry with 8-anilinonaphthalene-1-sulfonate (ANS) as a fluorophore probe in the buffer solution (pH 7.4) at 25 °C. Under the present condition, the K_c value for a complex of ANS with DM-β-CyD was determined to be $49.0 \pm 7.0\ \text{M}^{-1}$ from changes in the fluorescence intensity of ANS (0.1 mM) at 490 nm (excitation wavelength: 365 nm) in the presence of DM-β-CyD (varied from 0 to 20 mM) according to the Eq. 1. The K_c value for the complex of tertiary butyl-D-serine with DM-β-CyD was determined from changes in the fluorescence intensity of ANS (0.1 mM) at 490 nm (the excitation wavelength: 365 nm) in the presence of tertiary butyl-D-serine (varied from 0 to 50 mM) and DM-β-CyD (1 mM) according to the method of Matsui and co-workers.[8]

Results and Discussion

Figure 1 shows the effects of DM-β-CyD (20 mM) on the UV absorption and CD spectra of buserelin acetate (0.1 mM) in isotonic phosphate buffer (pH 7.4) at 25 °C. In the UV spectrum of buserelin acetate, an absorption maximum at 280 nm with a shoulder at 290 nm is assigned to a π–π^* transition arising from L-tryptophan and L-tyrosine residues; the absorptivity is nearly equal to the sum of absorptivities of corresponding aromatic amino acids.[9] In the presence of DM-β-CyD, the molar absorption coefficient of buserelin acetate around 280 nm increased. Similar spectral changes were observed when the peptide was dissolved in a less polar solvent such as methanol or ethanol, suggesting that these chromophores in buserelin acetate are incorporated into the hydrophobic environment of the DM-β-CyD cavity. Furthermore, fluorescence spectroscopic studies have shown that a single emission peak of buserelin acetate around 350 nm, corresponding primarily to the L-tryptophan residue, was shifted to a shorter wavelength with a concomitant increase in the fluorescence intensity as the concentration of DM-β-CyD increased.[1] These spectral changes also support the incorporation of the aromatic side chains of buserelin acetate into the cavity of DM-β-CyD.

The far-UV CD spectrum of buserelin acetate in the 190—250 nm range showed little or no secondary structure of the peptide (Fig. 1B). This is consistent with a recent NMR spectroscopic study demonstrating that there is no marked preference for a conformer in buserelin acetate, as well as the native LHRH, in aqueous solution, and both the skeleton and side chains of the oligopeptide are highly flexible.[4] Upon the addition of DM-β-CyD, the intensities of a negative maximum at 200 nm and a positive maximum at 230 nm were diminished, probably arising from conformational changes of the peptide due to spatial constraints imposed on the side chains[10] and/or induced Cotton effects through inclusion complexation with DM-β-CyD.

Recent studies have described that the latent secondary structure of a LHRH agonist, leuprolide acetate, is stabilized by the addition of a strong hydrogen bond donor such as trifluoroethanol to the peptide solution.[11] In the same manner, trifluoroethanol stabilized the secondary structure of buserelin acetate, as indicated by the difference in the far-UV CD spectrum of buserelin acetate in the absence and presence of 20% (v/v) trifluoroethanol (Fig. 2). The two outstanding features are a positive maximum at 195 nm and a negative maximum at 220 nm, which are suggestive of type II β-turns.[12] On the other hand, the shape and positions of the extrema in the difference far-UV CD spectrum of buserelin acetate in the absence and presence of DM-β-CyD are somewhat different from those obtained with trifluoroethanol. Furthermore, the intricate pattern of the difference CD spectrum obtained with DM-β-CyD may indicate conformational heterogeneity of the peptide upon binding to DM-β-CyD. However, it is difficult to determine exactly the types and amounts of any secondary structure due to the contribution from the L-tryptophan and L-tyrosine side chains[11] and the induced Cotton effect of these side chains by binding to DM-β-CyD.

As shown in the near-UV CD spectrum of buserelin acetate in Fig. 1B, a negative maximum around 280 nm arises from aromatic side chains, the intensity of which

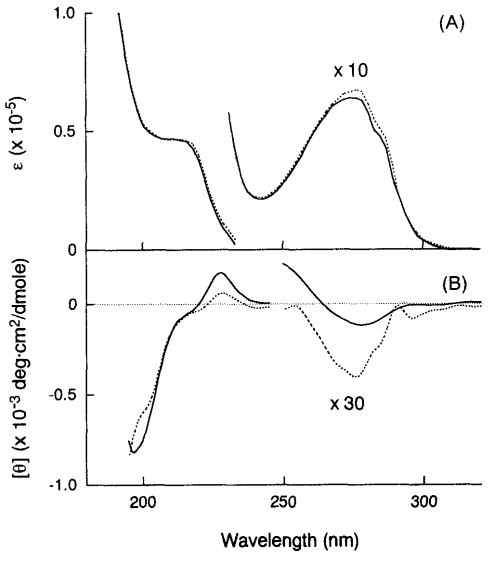

Fig. 1. Effects of DM-β-CyD (20 mM) on UV Absorption (A) and CD Spectra (B) of Buserelin acetate (0.1 mM) in Isotonic Phosphate Buffer (pH 7.4) at 25 °C

——, buserelin acetate alone; ------, with DM-β-CyD.

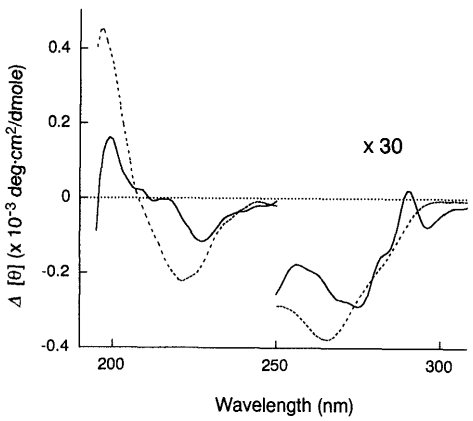

Fig. 2. Difference CD Spectra of Buserelin Acetate (0.1 mM) in the Absence and Presence of Either Trifluoroethanol (20% v/v) or DM-β-CyD (20 mM) in Isotonic Phosphate Buffer (pH7.4) at 25 °C

——, with trifluoroethanol; ------, with DM-β-CyD.

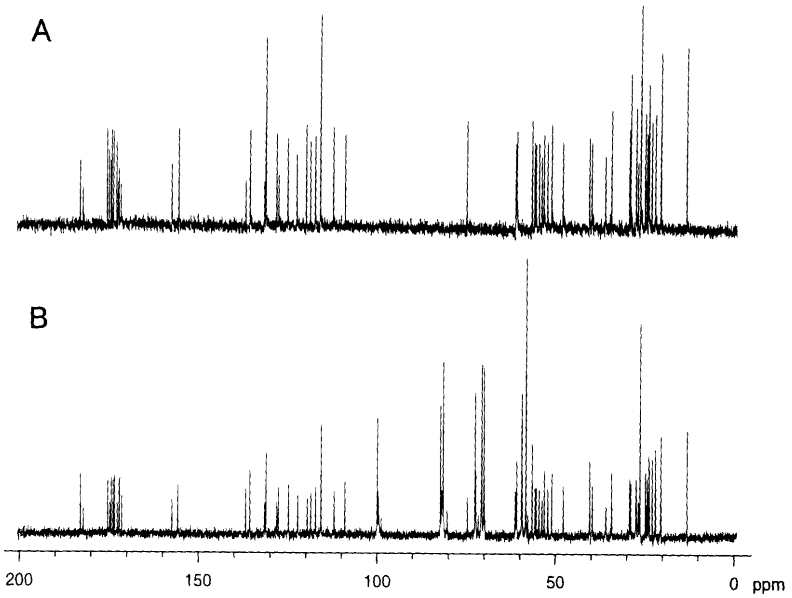

Fig. 3. ^{13}C-NMR Spectra of Buserelin Acetate (20 mM) in the Absence and Presence of DM-β-CyD (20 mM) in D_2O
A, buserelin acetate alone; B, with DM-β-CyD.

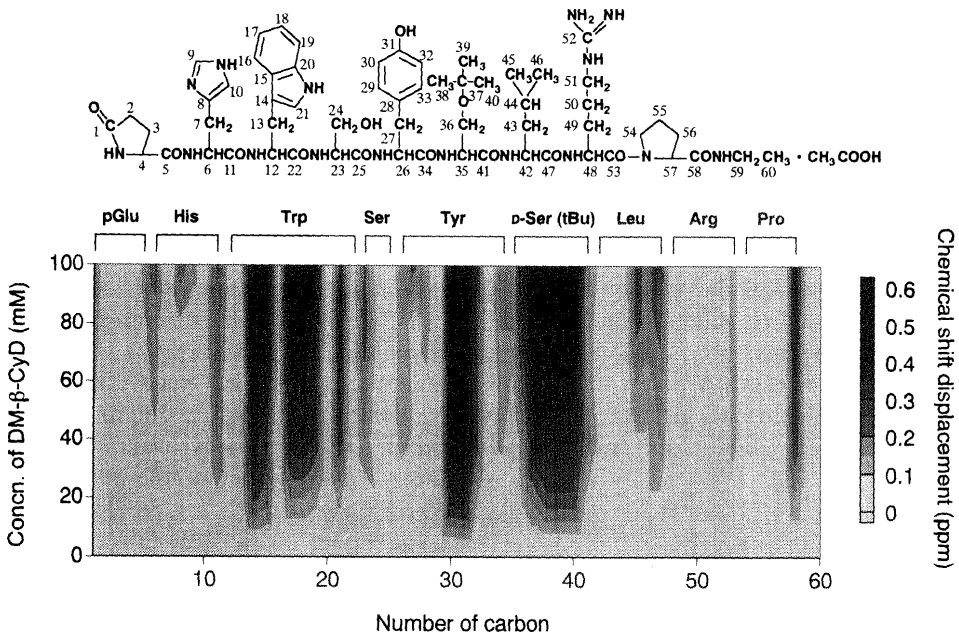

Fig. 4. ^{13}C-NMR Chemical Shift Displacements of Buserelin Acetate (20 mM) as a Function of DM-β-CyD Concentrations in D_2O

would depend on the extent of intermolecular association of the peptide.[13] Under the present condition, buserelin acetate is considered to exist primarily in a monomer form because the molar ellipticity of the peptide at 280 nm and the band shape did not change as the peptide concentration varied from 0.05 mM to 1 mM. Upon the addition of DM-β-CyD, the near-UV CD spectrum of the peptide was markedly changed in shape and intensity. This complexity in the CD pattern suggests that upon the binding to DM-β-CyD, the local environment around the aromatic groups in the peptide differs distinctly, indicating a conformational heterogeneity of the complex formed.

Insight into the inclusion mode of buserelin acetate with DM-β-CyD was gained by employing ^{1}H- and ^{13}C-NMR spectroscopies. Figure 3 shows the ^{13}C-NMR spectra of buserelin acetate (20 mM) in the absence and presence of DM-β-CyD (20 mM) in D_2O, and the ^{13}C-NMR chemical shift displacements of buserelin acetate induced with DM-β-CyD are summarized in Table 1. Figure 4 shows the effects of DM-β-CyD at various concentrations up to 100 mM on the ^{13}C-NMR chemical shifts of buserelin acetate (20 mM) in D_2O, where an absolute value for the chemical shift displacement (ppm) for each carbon of buserelin acetate was plotted against the concentration of DM-β-CyD added. The ^{13}C-NMR signals of L-tryptophan (C14—15 and C17—19), L-tyrosine (C30—32) and tertiary butyl-D-serine (C37—40) residues were largely shifted, with a rise in the DM-β-CyD concentrations, indicating that these hydrophobic side chains in buserelin acetate are preferably included in the cyclodextrin cavity. Further-

Table 1. ^{13}C-NMR Chemical Shift Displacements of Buserelin Acetate (BLA, 20 mM) Induced with DM-β-CyD (20 mM) in D_2O

Amino acid	Carbon number[a]	Chemical shift of BLA (ppm)		
		δ_0[b]	$\delta_{DM\text{-}\beta\text{-}CyD}$[c]	$\Delta\delta$[d]
pGlu	1	182.305	182.275	−0.030
	2	—[e]	—[e]	—[e]
	3	25.324	25.324	0.000
	4	56.805	56.835	0.030
	5	174.807	174.807	0.000
His	6	53.466	53.435	−0.031
	7	29.361	29.361	0.000
	8	131.182	131.213	0.032
	9	135.190	135.220	0.030
	10	117.127	117.157	0.030
	11	173.016	172.925	−0.091
Trp	12	54.801	54.862	0.061
	13	27.267	27.327	0.060
	14	108.808	109.021	0.213
	15	127.236	127.418	0.182
	16	118.462	118.493	0.031
	17	122.227	122.075	−0.152
	18	119.616	119.464	−0.152
	19	112.117	111.996	−0.121
	20	136.374	136.404	0.030
	21	124.716	124.595	−0.121
	22	173.805	173.805	0.000
Ser	23	56.076	56.016	−0.060
	24	61.177	61.268	0.091
	25	171.498	171.498	0.000
Tyr	26	55.742	55.682	−0.060
	27	36.344	36.344	0.000
	28	127.813	127.843	0.030
	29	130.818	130.818	0.000
	30	115.760	115.639	−0.121
	31	154.922	155.226	0.304
	32	115.760	115.639	−0.121
	33	130.818	130.818	0.000
	34	172.196	172.166	−0.030
D-Ser	35	54.042	54.073	0.031
(*tert*-Bu)	36	61.389	61.511	0.122
	37	75.020	74.838	−0.182
	38	26.629	26.872	0.243
	39	26.629	26.872	0.243
	40	26.629	26.872	0.243
	41	171.012	170.921	−0.091
Leu	42	52.433	52.433	0.000
	43	40.837	40.867	0.030
	44	23.441	23.441	0.000
	45	22.500	22.561	0.061
	46	20.952	21.043	0.091
	47	174.291	174.200	−0.091
Arg	48	51.341	51.341	0.000
	49	27.813	27.843	0.030
	50	24.322	24.322	0.000
	51	40.108	40.108	0.000
	52	156.865	156.896	0.031
	53	171.741	171.680	−0.061
Pro	54	48.153	48.153	0.000
	55	24.808	24.868	0.060
	56	29.665	29.695	0.030
	57	61.055	61.086	0.031
	58	173.502	173.350	−0.152
Ethylamide	59	34.674	34.704	0.030
	60	13.727	13.757	0.030

a) The carbon numbering for buserelin acetate is the same as that in Fig. 4. *b*) The chemical shift of BLA alone. *c*) The chemical shift of BLA with DM-β-CyD. *d*) $\delta_{DM\text{-}\beta\text{-}CyD} - \delta_0$. *e*) Could not be determined due to the overlapping with other signals.

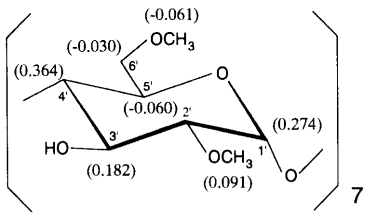

Fig. 5. Effect of Buserelin Acetate (20 mM) on ^{13}C-NMR Chemical Shifts of DM-β-CyD (20 mM) in D_2O

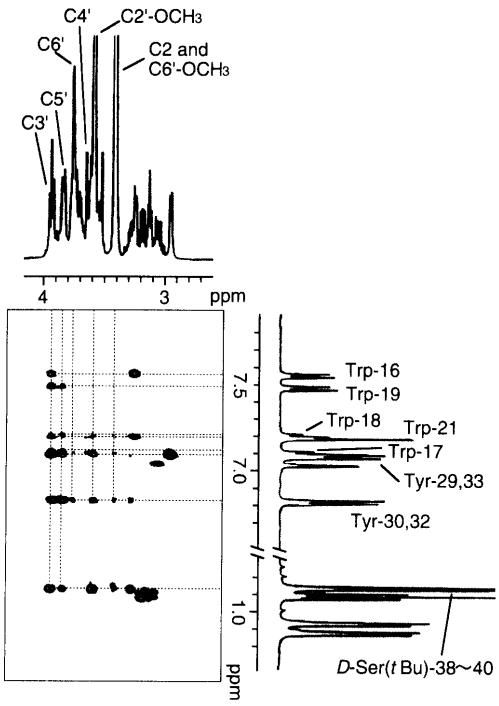

Fig. 6. Partial Contour Plots of ROESY Spectrum of a Buserelin Acetate (20 mM): DM-β-CyD (20 mM) System in D_2O

The cross peaks between buserelin acetate and DM-β-CyD resonances are shown by the dotted lines.

more, upon the addition of DM-β-CyD at concentrations of more than 40 mM, most of the carbonyl carbons in the peptide backbone were shifted upfield, indicating global conformational changes of the peptide, probably through the inclusion of hydrophobic side chains within the cyclodextrin cavity. On the other hand, as shown in Fig. 5, the buserelin acetate (20 mM)-induced ^{13}C-NMR chemical shift displacements of DM-β-CyD (20 mM) were relatively large for the C1′, C3′ and C4′ carbons of the host. The large chemical shift displacements in the C1′ and C4′ carbons may be due to conformational changes around the α-1,4 glycosidic bond through the inclusion of buserelin acetate, because of its high flexibility. Moreover, the large chemical shift change in the C3′ carbon, which is located on the wider secondary hydroxyl side of the cyclodextrin cavity, indicates the formation of an inclusion complex of DM-β-CyD with buserelin acetate, with the side chains inserted into the cavity from the secondary hydroxyl side.

The two-dimensional ROESY spectrum provides information on the orientation of a guest molecule with respect to a cyclodextrin molecule and the depth of the guest insertion into the cyclodextrin cavity.[14,15] Figures 6 and 7 show partial contour plots of the ROESY spectrum

of the buserelin acetate: DM-β-CyD system. The cross peaks connecting the intermolecular protons are listed in Table 2, while the other cross peaks seen in Fig. 6 are due to the intramolecular interaction of buserelin acetate. These cross peaks are due solely to the nuclear Overhauser effect, because they arise between two different molecules, suggesting close contact (<4—5 Å) of the interacting protons. Based on the ROESY cross peaks and the UV, CD and ^{13}C-NMR spectral changes, the most probable location of the three possible binding sites of buserelin acetate within the DM-β-CyD cavity is shown in Fig. 8. The benzene moiety of the L-tryptophan residue is assumed to be present within the hydrophobic center of the DM-β-CyD cavity, and the pyrrole moiety is located around the secondary hydroxyl side of the cavity or oriented out of the cavity (Fig. 8A). Further support for such an inclusion mode of the L-tryptophan: DM-β-CyD complex was gained from the ^{13}C-NMR chemical shift displacement of each carbon of the L-tryptophan residue induced with DM-β-CyD. A recent theoretical study has proven that the carbons included in the hydrophobic cavity of cyclodextrins are largely shielded compared with the deshielded carbons located around the wider secondary hydroxyl rim of the cavity.[16] Indeed, upon the addition of DM-β-CyD, the ^{13}C-NMR signals (C17—C19 and C21) of the benzene moiety were shifted upfield, while the signals (C14, C15) of the pyrrole moiety were shifted downfield (Table 1). In the most probable inclusion mode of the L-tryptophan residue, the long axis of the benzene ring (C16—C19) may cross at some intermediate angle with the axis of the CyD cavity.

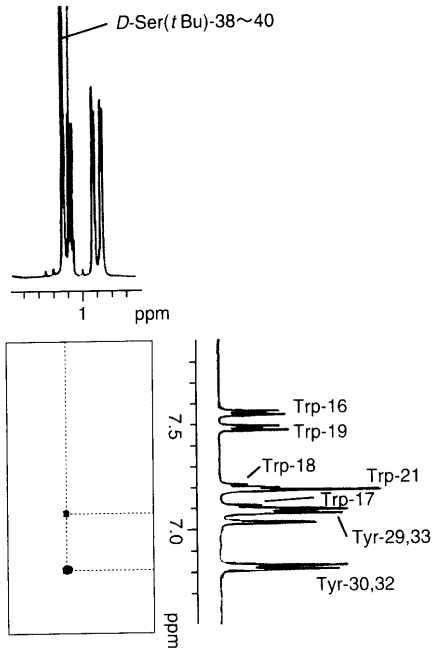

Fig. 7. Partial Contour Plots of the ROESY Spectrum of Buserelin Acetate (20 mM): DM-β-CyD (20 mM) System in D_2O

Cross peaks due to the intramolecular interactions of buserelin acetate are shown by the dotted lines.

Table 2. ROESY Cross Peaks Connecting Intermolecular Protons between Buserelin Acetate and DM-β-CyD in D_2O

Buserelin acetate	DM-β-CyD
Trp-H16	H3′
Trp-H17	H3′ and H5′ (weak)
Trp-H18	H3′ (weak) and H5′ (weak)
Trp-H19	H3′ and H5′ (weak)
Trp-H21	H3′ and C2′-OCH_3 (weak)
Tyr-H29, 33	H3′, H5′ (weak) and C2′-OCH_3 (weak)
Tyr-H30, 32	H3′, H5′, H6′ (weak) and C2′-OCH_3 (weak)
D-Ser(*tert*-Bu)-H38-40	H3′ and H5′ (weak)

The proton numberings for buserelin acetate and DM-β-CyD are the same as those in Figs. 3 and 4.

As shown in Fig. 8B, DM-β-CyD may form an inclusion complex with the L-tyrosine residue which is inserted into the cavity from the more accessible secondary hydroxyl side. This view was assured by large upfield shifts of the ^{13}C-NMR signals (C30, C32) of the L-tyrosine residue upon the addition of DM-β-CyD. Similarly, the tertiary butyl-D-serine residue may be included in the DM-β-CyD cavity (Fig. 8C), where both the ^{13}C-NMR signals (C38—C40) and ^{1}H-NMR signals (H38—H40) were shifted downfield by the addition of DM-β-CyD. In the ROESY spectrum of buserelin acetate alone, there were two cross peaks between the ^{1}H-NMR signal (H38—H40) of the tertiary butyl D-serine residue and the ^{1}H-NMR signals (H29, H33 and H30, H32) of the L-tyrosine residue, indicating close contact of the two adjacent side chains in the buserelin molecule. Therefore, the deshielding of the carbons and protons of the tertiary butyl-D-serine residue indicates that the inclusion of each side chain within the different DM-β-CyD cavities perturbs the intramolecular association of the side chains, which may compensate for the shielding effect due to the inclusion within the cyclodextrin cavity. However, the possibility of the inclusion of both side chains within a DM-β-CyD

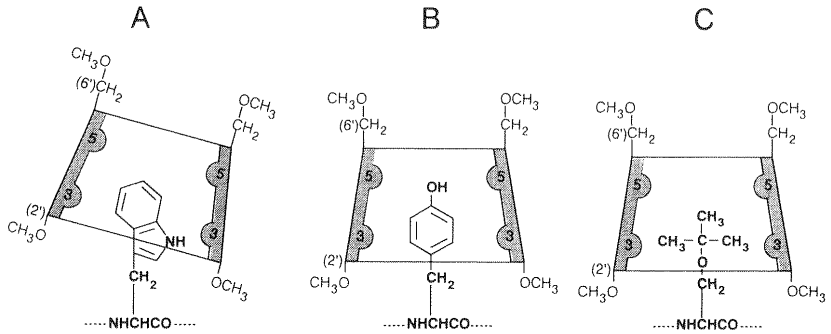

Fig. 8. Proposed Structures of Inclusion Complexes for Three Side Chains in Buserelin Acetate with DM-β-CyD in Solution

A, L-tryptophan residue; B, L-tyrosine residue; C, tertiary butyl-D-serine residue.

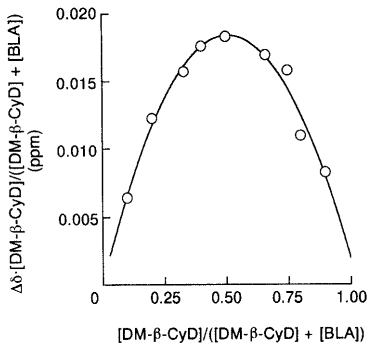

Fig. 9. Continuous Variation Plots of Changes in the ^{1}H-NMR Chemical Shift (H3′) of DM-β-CyD for a Buserelin Acetate:DM-β-CyD System in D_2O

The total concentration of buserelin acetate (BLA) and DM-β-CyD was kept constant at 10 mM.

cavity should not be totally discarded, because the two intramolecular cross peaks were still detectable, even in the presence of DM-β-CyD (Fig. 7), and also based on observations made with the space-filling Corey-Pauling-Koltun molecular models of the inclusion complexes.

Figure 9 shows the continuous variation plots[6] of changes in the ^{1}H-NMR chemical shift (H-3′) of DM-β-CyD for the buserelin acetate: DM-β-CyD system, keeping the total concentration of species constant at 10 mM. Although buserelin acetate has three possible binding sites for DM-β-CyD, the plots gave a peak at a 1 : 1 molar ratio of the guest and host, indicating a 1 : 1 stoichiometry of the complex. A possible interpretation of these results is as follows. The complexation may be initiated by the inclusion of DM-β-CyD into one of the three binding sites in the buserelin molecule, which may prevent access of the second cyclodextrin to the other binding sites, probably due to steric hindrance and/or conformational changes of the peptide. Consequently, the three kinds of the complexes of buserelin acetate with DM-β-CyD at a 1 : 1 molar ratio with a difference in binding site may coexist in the solution. This heterogeneity in the structure of the complex formed is consistent with the CD observations (Figs. 1 and 2).

In general, the peptide backbone represents a bulky substituent, which may prevent the formation of inclusion complexes between the side chains and cyclodextrins through steric strain. Nevertheless, recent studies have shown that oligopeptides containing hydrophobic amino acids form complexes with cyclodextrins of the same stability as the amino acids themselves or of higher stability, indicating that the backbone-caused steric strains are not dominant in the interaction of an oligopeptide with cyclodextrin.[17,18] Therefore, the probability of the three kinds of complexation seems to be proportional to the affinity of DM-β-CyD to the three amino acids themselves. Based on the magnitude of the stability constants of complexes of DM-β-CyD with three amino acids in Table 3, the L-tyrosine residue is considered to be the primary binding site for DM-β-CyD, followed by the L-tryptophan and tertiary butyl-D-serine residues.

Our previous studies have demonstrated that the enzymatic degradation of buserelin acetate in rat nasal mucosal homogenates was decelerated by the addition of DM-β-CyD in a concentration-dependent manner.[1] On the HPLC chromatogram of the reaction mixture after 8 h of incubation with the nasal homogenates, the major metabolites were the 3—9 heptapeptide and the 5—9 pentapeptide fragments, and DM-β-CyD did not alter the degradation pathway of the peptide. On the basis of the inclusion mode of buserelin acetate with DM-β-CyD, DM-β-CyD may protect buserelin acetate sterically from proteolytic enzymes by including hydrophobic amino acid residues within the cavity, because these binding sites identified in the present study are located near the enzymatic cleavage sites of the peptide. Furthermore, conformational changes in buserelin acetate upon the binding to DM-β-CyD may alter the susceptibility of the peptide to the enzymes.

Table 3. Stability Constants (K_c) of Complexes of Amino Acids Consisting of Buserelin Acetate with DM-β-CyD in Isotonic Phosphate Buffer (pH 7.4) at 25 °C

Amino acid	K_c (M^{-1})[c]
L-Tryptophan[a]	21 ± 1
L-Tyrosine[a]	171 ± 9
Tertiary butyl-D-serine[b]	8 ± 0.4

a) Determined from UV absorption changes. *b*) Determined from fluorescence quenching of ANS due to competitive binding. *c*) Mean ± S.D. of at least three experiments.

In conclusion, the present results will provide not only structural information on the interaction between oligopeptides and cyclodextrins but also a rational basis for stabilizing therapeutic peptide and proteins.

Acknowledgements The authors are grateful to Mr. Takashi Tokunaga, Central Pharmaceutical Research Institute, Japan Tobacco, Inc. (Osaka, Japan) for measurements of the 2D NMR spectra and for his helpful discussion. The authors also thank Dr. Kazuya Abe and Miss Miwa Ishibashi for technical assistance.

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