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Carbohydrate Research 337 (2002) 1737–1743

CARBOHYDRATE RESEARCH

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Three-dimensional structure of the inclusion complex between phloridzin and β-cyclodextrin

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Received 23 November 2001; received in revised form 23 June 2002; accepted 8 August 2002

Abstract

The inclusion of phloridzin into β-cyclodextrin was studied as a model of molecular recognition in membranes. Effects on ^{1}H NMR spectra and NOE correlational peaks between phloridzin and β-cyclodextrin were observed in the complex. Strong NOEs were observed between hydrogens of a phenol group in phloridzin and β-cyclodextrin. The three-dimensional structure of the inclusion complex between phloridzin and β-cyclodextrin was simulated with distance constraints estimated by the intensity of NOE peaks using the DADAS90 programs. Two inclusion possibilities were suggested—the large rim of β-cyclodextrin as an entrance of the inclusion and the small rim of β-cyclodextrin as the entrance. In both cases, the phenol group of phloridzin was included in the hydrophobic space of β-cyclodextrin.

Keywords: phloridzin; β-cyclodextrin; NOE; distance geometry; inclusion

1. Introduction

Phloridzin (Phz, Fig. 1(a)) acts as a glucose transport inhibitor in kidneys, intestines, lungs, and many other organs. The mechanism of the inhibition reaction has been discussed with respect to the Na-dependent D-glucose cotransporter in membranes.[1–3] The fact that Phz is a glucose transport inhibitor has been used in numerous investigations to clarify the mechanism of glucose transport through membranes in organisms. Interest has been expressed about the interaction of Phz with saccharide chains, which exist on membrane surfaces, leading to studies of the molecular interaction of Phz with cyclodextrins. Here, cyclodextrin is regarded as a molecule of saccharide chains at the brush border membrane of the renal tubule in the kidney.[4,5] From the large changes in the chemical shift of characteristic signals of Phz and cyclodextrin in ^{1}H and ^{13}C NMR spectroscopy, and from the induced circular dichroism spectra, it can be deduced that an inclusion complex forms between Phz and cyclodextrin in aqueous solution.[6,7] The complex formation constant of β-cyclodextrin (β-CD, Fig. 1(b)) with Phz was estimated to be larger than that of γ-cyclodextrin from the analysis of induced circular dichroism spectra, and α-cyclodextrin

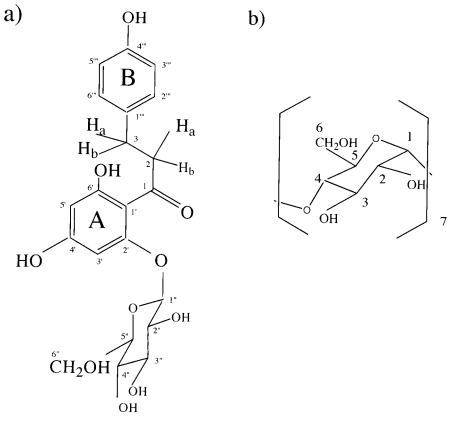

Fig. 1. (a) Phloridzin (Phz), (b) β-cyclodextrin (β-CD).

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0008-6215/02/$ - see front matter
PII: S0008-6215(02)00279-3

Table 1
Chemical shift in ^{1}H signals (ppm)
(a) Phz

	Phz	Phz + β-CD (1:2)
2a	3.353	3.336
2b	3.504	3.533
3a	2.944	2.881
3b	2.944	3.055
3′	6.268	6.395
5′	6.133	6.197
1″	5.252	5.118
2″	3.56	3.686
3″	3.609	3.621
4″	3.462	3.569
5″	3.671	3.686
6a″	3.753	3.829
6b″	3.956	3.985
2‴	7.160	6.951
3‴	6.841	6.697
5‴	6.841	6.697
6‴	7.160	6.951

External DMSP was used as a reference. At 25 °C, in D_2O.

(b) β-CD

	β-CD	Phz + β-CD (1:2)
1	5.081	5.057
2	3.661	3.641
3	3.976	3.921
4	3.596	3.590
5	3.877	3.725
6a	3.886	3.834
6b	3.903	3.837

External DMSP was used as a reference. At 25 °C, in D_2O.

did not form a complex. The three-dimensional structure of the inclusion complex between Phz and β-CD remains, however to be clarified.

This report aims to construct a three-dimensional structure of the complex between Phz and β-CD from the observed NOE between Phz and β-CD in ^{1}H NMR and changes in a long-range coupling constant of Phz corresponding to a torsion angle of the glycosidic linkage in Phz. The hydrogen–deuterium (H–D) exchange of Phz is also discussed.

2. Experimental

NMR spectra were recorded with a Bruker DMX 750 (750.13 MHz for ^{1}H) and a JEOL ECP800 (800.08 MHz for ^{1}H) NMR spectrometer at 25 °C in a deuterium oxide solution. NOE was detected in 2D phase-sensitive NOESY spectra with mixing times from 400 ms to 1 s and also in 2D phase-sensitive ROESY spectra with two spin-lock times, 50 and 100 ms. 2D *j*-HMBC spectra were recorded at several developing times to estimate the heteronuclear coupling constant. 1D ^{1}H high resolution spectra were periodically recorded to observe H–D exchange reactions at 35 °C. External sodium-2,2-dimethyl-2-silapentonate-d_4 (DMSP) was used as a reference for the chemical shifts.

Three-dimensional structures were simulated with a DADAS90 program[8–10] in the MolSkop program suite (JEOL Ltd.). The target function consists of harmonic potentials for NOE constraints, angle constraints, hydrogen bonding constraints, and soft repulsion potentials for volume exclusion between atoms, but it does not contain any energy functions. In the first stage of calculation, to ensure the chemical structure of Phz and β-CD, initial dihedral angles were randomly generated within the range allowed by dihedral angle constraints individually and followed by forming a 1:1 complex using the multi chain method in MolSkop. Starting from 100 randomly generated structures, individual structures were forced to make a complex by minimizing the target function.

Phloridzin (Phz, Sigma Chemicals Co. Fig. 1(a)) was recrystallized from water and β-cyclodextrin (β-CD, Nakarai Chemicals LTD. Fig. 1(b)) was used without further purification. The concentration of Phz was 11 mM for 1D ^{1}H NMR and 2D NOESY spectra. Twice-molar β-CD was added for complex formation. For the 2D *j*-HMBC experiment, we used 16 mM Phz and 32 mM β-CD.

3. Results and discussion

Effects on ^{1}H NMR spectra on complex formation

(a) Shifts of ^{1}H signals on complex formation. Complete signal assignments using 2D DQF-COSY, TOCSY, NOESY and HMBC spectra are shown in Table 1. Fig. 2 shows the shifts of signals on complex formation. In addition to strong high field shifts with protons in the aromatic ring B, medium signal shifts were observed with protons in the aromatic ring A: H-3′ and H-5′, glucose protons: H-2″, H-4″, H-6″, and one methylene proton H-3b. Small signal shifts were observed with other methylene protons: H-2a, H-2b, H-3a, and glucosyl protons, H-3″ and H-5″. The shift to a lower field of the four signals of the glucosyl group in Phz suggests a change in the distance between the pyranose ring and aromatic ring A or between the pyranose ring and aromatic ring B on the formation of the complex. Two methylene protons, H-3a and H-3b, observed as a simple triplet at the same chemical shift in a free molecule, changed to two multiplets with a chemical shift difference of 0.17 ppm in the complex.

Fig. 3 shows the chemical shifts of β-CD on the complex formation. A considerably large high-field shift was observed in the H-5 signal and a moderately large high-field shift was observed in the H-3 signal as shown in Fig. 3. These protons are located on the inner side of the cyclodextrin ring. Thus, the ring current effect of an aromatic ring included in the inner space of β-CD causes these signals to higher field.[11,12] The high-field shifts of two H-6 signals were comparable with that of H-3 (Fig. 3). The shifts of two H-6 signals suggests two possibilities of complex formation—piercing of the B aromatic ring of Phz through the inner space of β-CD from either the large rim of the cyclodextrin or the small rim of β-CD.

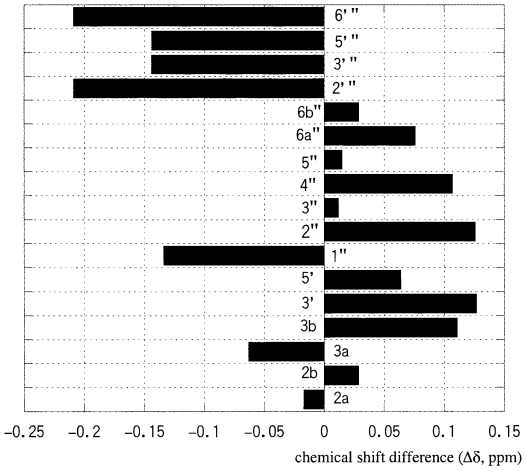


Fig. 2. ^{1}H NMR chemical shift difference of Phz after mixing with β-CD (Phz:β-CD = 1:2).

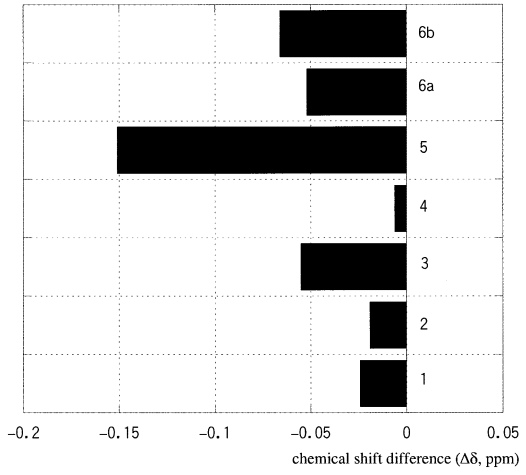


Fig. 3. ^{1}H NMR chemical shift difference in β-CD after mixing with Phz (Phz:β-CD = 1:2).

Table 2
^3J in glucosyl group of Phz (Hz)

	Phz	Phz + β-CD (1:2)
$J_{1''2''}$	7.8	7.9
$J_{2''3''}$	9.3	9.3
$J_{3''4''}$	9.5	9.1
$J_{4''5''}$	9.5	8.8
$J_{5''6a''}$	6.0	4.5
$J_{5''6b''}$	2.2	1.8
$J_{6a''6b''}$	12.5	12.4

Table 3
Coupling constant in *j*-HMBC experiments (Hz)

	Phz	Phz + β-CD (1:2)
$^3J_{C2'H1''}$	5.5	1.5

(b) Coupling constants. Small changes were observed in the three-bond homonuclear coupling constants of the glucosyl group of Phz (Table 2), which suggested slight deformation of the pyranose ring. Among the coupling constants, $J_{5'',6a''}$ and $J_{4'',5''}$ showed relatively large changes. Since the glucosyl group of Phz was not included in the inner space of β-CD as discussed below, hydrogen bondings with hydroxyl groups of β-CD may cause slight deformation of the glucosyl group of Phz.

$^3J_{C2'H1''}$ of Phz in 2D *j*-HMBC experiments were calculated with different developing times[13] (Table 3). The coupling constant decreased on complex formation, suggesting a change in a glycosidic linkage between aromatic ring A and glucose of Phz. Since C-2′ was an aromatic carbon, it was inappropriate to estimate the torsion angle from the Karplus curve for the glycosidic linkage.[14]

In free Phz, two H-3 signals showed one triplet with coupling constant, 7.4 Hz. In the complex, equivalence of the two H-3 atoms was broken. Complex multiplet signals corresponding to two H-3 protons and two H-2 protons made it impossible to analyze their coupling constant. There was no change in the coupling constants of the aromatic rings A and B, except for the change to a singlet from a doublet by the H-D exchange reaction in the aromatic ring A.

(c) H–D exchange of hydrogens in Phz. The H-5′ proton has been reported to be exchangeable.[6] Additionally, it was clarified with experiments at temperatures higher than 20°C that the H-2 protons were also exchangeable. Fig. 4 shows changes in signal intensity of H-5′ and H-2a at 35°C. It is obvious from Fig. 4 that β-CD inhibits the H-D exchange reaction of H-2 in Phz. The mechanism of the H–D exchange reaction of H-2 in Phz is explained as follows: The intermediate state for the H–D exchange reaction (Fig. 5(b)) is formed from one type of conformation about the carbonyl group of Phz (Fig. 5(a)). Since oxygen in the carbonyl group and hydroxyl group OH-6′ form hydrogen bondings with hydroxyl groups of β-CD in the

complex (Fig. 5(c) and (e)), a change to an intermediate state is inhibited. In general, two conformations about the carbonyl group and aromatic ring A (Fig. 5(a) and (d)) exist in a solution, although one conformation (Fig. 5(a)) has been found in the crystal.[15] Only one type of conformation is responsible for the H–D exchange of H-2 in Phz, which is a consequence of a weak reaction.

Through space interactions described via NOE.—Intra-molecular NOE of free Phz are shown in Table 4. These NOE peaks were all observed in three NOESY spectra with three mixing times and two ROESY spectra with two spin-lock times. The intensity of each NOE correlational peak was graded into four groups—strong, medium, weak, and very weak. As the intensity of NOE peaks were slightly different among the NOESY and ROESY spectra, the intensity of NOESY spectra with short mixing time is listed in the table. NOE signals evaluated as strong and as medium were reasonably expected from the chemical structure of Phz. The weak and very weak NOE correlational peaks in the table mutually contradicted and were not applicable in a three-dimensional structure of Phz using a space filling model.

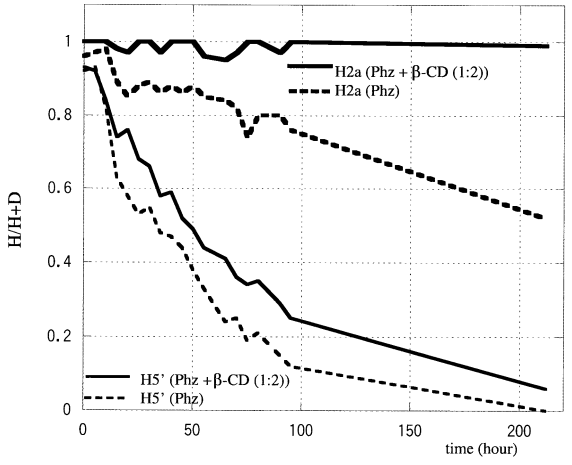


Fig. 4. Change in signal intensity of H-2a and H-5′ of Phz in D_2O at 35 °C.

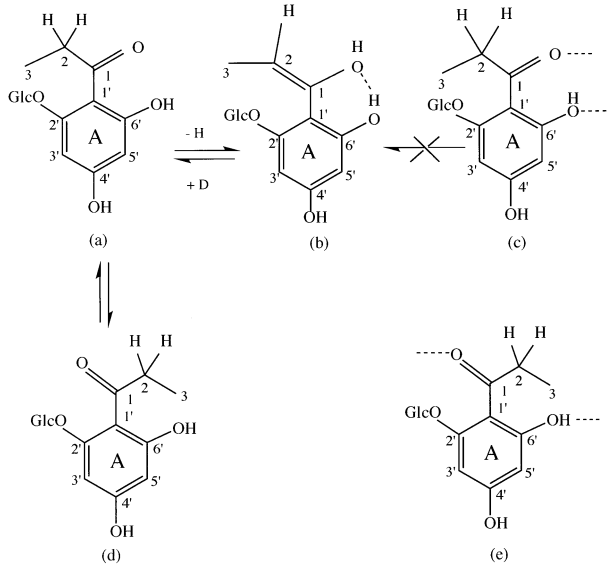


Fig. 5. Mechanism of H–D exchange reaction of H-2 in Phz. Two types of conjugation (a and d) are in equilibrium in free Phz in a solution. An intermediate state for H–D exchange reaction (b) is formed from one of the conjugated conformation (a). Since the carbonyl group and hydroxyl group OH–6″ form hydrogen bonds with β-CD in the complex (c and e), no intermediate state forms from these two types of complex.

Table 4
Intra-molecular NOE in free Phz in a solution

Proton in Phz	Proton in Phz	NOE Intensity
2a	2‴,6‴	m
2b	2‴,6‴	m
3	3′	vw
3	1″	w
3	2″	w
3	3″	vw
3	5″	w
3	2‴,6‴	s
3′	1″	s
3′	4″	vw
3′	5″	vw
3′	6″b	vw
2″	2‴,6‴	w
3″	3‴,5‴	w

s: Strong, m: medium strong, w: weak, vw: very weak.

Table 5 shows NOE signals detected in all three NOESY and two ROESY spectra in the mixture of Phz and β-CD. NOEs are classified to three categories—intra-molecular NOE of Phz (Table 5(a)), intra-molecular NOE of β-CD (Table 5(c)), and intermolecular NOE between Phz and β-CD (Table 5(b)). There were four intra-molecular NOEs of Phz compared to fourteen for free Phz. Some conformations possible in free Phz in solution were diminished by the complex formation. A new NOE correlation peak was observed between H-3″ and H-3′ in the complex coincident with a change of hetero-nuclear coupling constant $^3J_{C2'H1''}$. An intra-molecular NOE in β-CD remained unchanged on complex formation.

Ten intermolecular NOE peaks were observed between Phz and β-CD (Table 5(b)). An especially strong NOE was detected between protons in aromatic ring B of Phz and inner protons of β-CD. This fact proved clearly the inclusion of aromatic ring B in the hydrophobic space of β-CD. No NOE was detected between aromatic ring A and β-CD. There are only two non-hydroxyl-protons in aromatic ring A—one exchangeable and the other is scarcely exchangeable in D_2O. Thus,

Table 5
NOE detected in Phz and β-CD mixture
(a) Intramolecular NOE of Phz

Proton in Phz	Proton in Phz	NOE Intensity
1″	3′	s
2‴,6‴	3a	w
2‴,6‴	3b	w
3′	3″	m

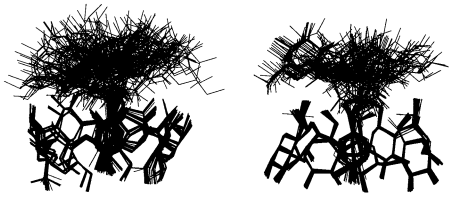
(b) Intermolecular NOE between Phz and β-CD

Proton in Phz	Proton in β-CD	NOE Intensity
2‴,6‴	3	s
2‴,6‴	5	s
3‴,5‴	3	m
3‴,5‴	5	s
2‴,6‴	6	m
3‴,5‴	6	m
3a	5	w
3b	5	w
3a	3	w
3b	3	vw

(c) Intramolecular NOE of β-CD

Proton in β-CD	Proton in β-CD	NOE Intensity
1	4	s
1	6	s

s: Strong, m: medium strong, w: weak, vw: very weak.



(a) (b)

Fig. 6. Two families of complexes between Phz and β-CD obtained with the dihedral angle of C-2′–C-1′–C-1–O-1 was set to 180°. (a) Aromatic ring B enters from a large rim of β-CD; 85 structures are superposed. (b) Aromatic ring B of Phz enters from a small rim of β-CD; 63 structures are superposed. Figures are superposed mainly at β-CD and displayed with carbon and oxygen atoms.

the number of detectable proton in ring A was one. Considering the case in which ring A is located in front of the large entrance of hydrophobic space of β-CD as a stopper of the entrance suggested from the results of induced circular dichroism spectra,[7] no NOE between ring A and β-CD is reasonable.

Four protons in aromatic ring B, H-2‴, H-3‴, H-5‴, and H-6‴, showed NOE with H-6 of β-CD, suggesting two directions of insertion of Phz in β-CD. Further, the possibility of aromatic ring B penetrating through the cavity of β-CD cannot be ruled out, considering the existence of the weak NOE of two H-3 protons of Phz with inner protons of β-CD.

Three-dimensional structure of the complex between Phz and β-CD

(a) Distance constraints. To determine the three dimensional structure of the complex between Phz and β-CD, we simulated three-dimensional structures of 1:1 complex with distance geometry method using three distance constraints for Phz molecule, 14 distance constraints for β-CD and 16 distance constraints as intermolecular constraints. The number of distance constraints differed from the number of detected NOE peaks for three reasons: (1) An introduction of pseudo atoms in simulations. For undistinguishable protons in ^{1}H NMR spectra, pseudo atoms were used to define constraints, i.e., a pseudo atom was defined for one methylene group (H-3a, H-3b) and two pseudo atoms for two pairs of protons in aromatic ring B in Phz. (2) An intermolecular NOE between Phz and β-CD caused two distant constraints, because of ring structure of β-CD. (3) An intra-molecular NOE of β-CD caused seven distance constraints. Distance constraints were estimated to be 3.5, 4.5, and 5.5 Å as upper limits from NOE peaks classified into strong, medium and weak classes, respectively. The upper limit of the distances for a pseudo atom for methylene protons was appropriately corrected. Minimization was conducted for two conformations of Phz, setting the dihedral angle of C-2′–C-1′–C-1–O-1 to either 180 or 0°.

(b) Two possible structures. After 500 structures were simulated, two families of structures of the complex were obtained, with relatively low violation for two conformations of Phz. Fig. 6 shows the result for one conformation of Phz. The difference between two families was the insertion of ring B into β-CD. In one family, the aromatic ring B enters from a large rim of β-CD (Fig. 6(a)). In another, the ring B enters from a small rim of β-CD (Fig. 6(b)). The average RMSD of two families for all atoms were 2.1 and 1.7 Å, respectively. Fig. 7 shows the results about two conformations of Phz. Since no constraint was set for the distance between aromatic rings A and B, the Phz molecule was not well superposed in the figures. The difference between two conformations in Phz was small. The tilt of aromatic ring A and the distance between

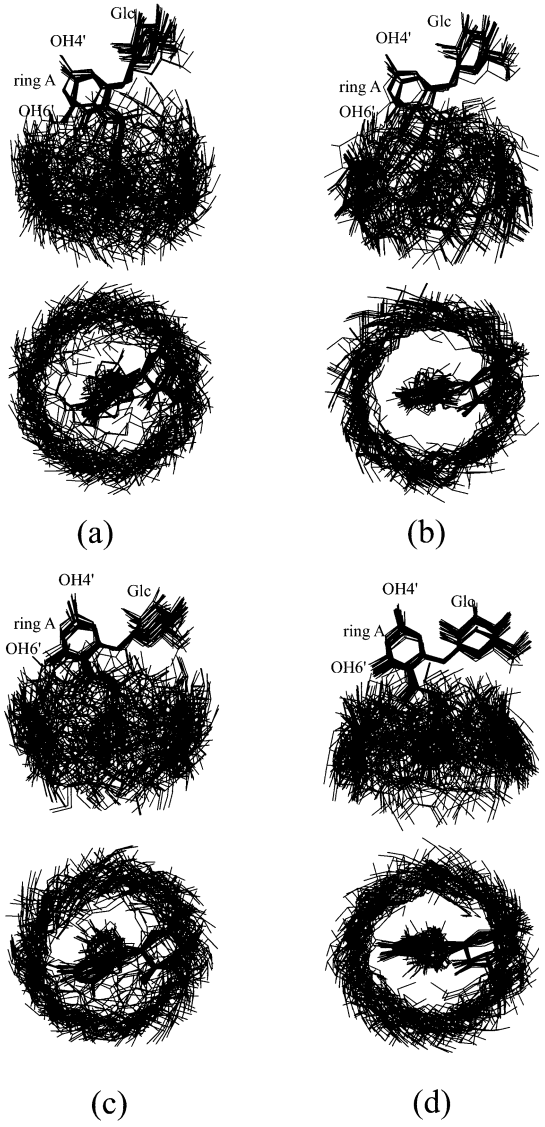


Fig. 7. Side view and overview of the two simulated families of complexes with two different dihedral angles of Phz, C-2′–C-1′–C-1–O-1. 30 Structures are superposed mainly at Phz and displayed with carbon and oxygen atoms in each figures. (a) The dihedral angle was set to 0°, one family with a large rim of β-CD as an entrance. (b) The dihedral angle was set to 0°, another family with a small rim of β-CD as an entrance. (c) The dihedral angle was set to 180°, one family with a large rim of β-CD as an entrance. (d) The dihedral angle was set to 180°, another family with a small rim of β-CD as an entrance.

aromatic ring A and glucose in Phz were differently shown as in Fig. 7.

It is reasonable that a guest enters into the inner space of β-CD from the wide rim because of the low barrier to insertion. From the shift of ^{1}H and ^{13}C signals of a guest and host, for example, it was suggested that a guest enters into the inner space of CD from the wide rim.[16,17] Goschel et al. reported a complex between phosphatidyl-inositol and α-CD, and concluded from the results of molecular dynamics that the guest molecule was inserted into the CD ring from the narrow rim.[18] In our case, as shown in Figs. 6 and 7, both cases were possible.

Considering the structures in Fig. 7, it is difficult for another β-CD to approach the aromatic ring A. Since strong circular dichroism was induced at the absorption band of aromatic ring A, aromatic ring A was estimated previously to be included into another β-CD.[7] From the three-dimensional structures of the complex in Fig. 7, the distance from the center of ring A to the center of β-CD was 6–8 Å. According to this distance, circular dichroism spectra will be satisfactorily induced on the absorption band of aromatic ring A.

The dihedral angle of the *O*-glycosidic linkage in Phz, C-2′–O-1–C-1″–H-1″, was estimated at about −34° for Phz in the two possible complex shown in Fig. 7. From the simple minimization of free Phz using only one distance constraint estimated by NOE between H-1″ and H-3′, the dihedral angle was estimated to be about 60°(Fig. 8(a)). A difference in the dihedral angle coincides with the change in $^{3}J_{C2'H1''}$ on the complex formation.

(c) Retardation of the H–D exchange reaction. The H–D exchange reaction of H-2 is controlled by the hydrogen bondings between OH-6′ group and a hydroxyl group in β-CD and between a carbonyl group of Phz and a hydroxyl group of β-CD. Further, H-D exchange of H-5′ is also controlled by the same hydrogen bondings. As shown in Fig. 7, both OH-6′ group and the carbonyl group of Phz exist near the two rim of β-CD bucket in all case and easily contact the hydroxyl groups of β-CD. Retardation of the H–D exchange reaction on complex formation was supported by the simulated structures of complex.

4. Conclusion

The solution structure of the inclusion complex between Phz and β-CD was simulated with distance ge-

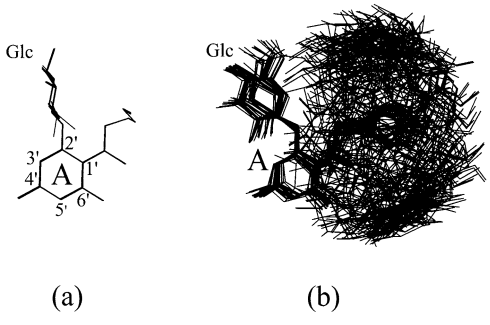


Fig. 8. *O*-Glycosidic linkage of Phz in free Phz (a) and in the complex (b). Propanon group and aromatic ring B in Phz were neglected in (a).

ometry method using distance constraints estimated from NOE correlational peaks between Phz and β-CD. The phenol group of Phz, i.e., the aromatic ring B, enters into the hydrophobic space of β-CD. There are two inclusion possibilities for insertion of the host molecule. Hydrogen bond formation between the hydroxyl group of the aromatic ring A and the hydroxyl group of β-CD changed the acidity of H-2 and H-5′ protons of Phz, retarding the H–D exchange reaction on complex formation.

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