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Two-dimensional ^{13}C–^{1}H heteronuclear correlation NMR spectroscopic studies for the inclusion complex of cyclomaltoheptaose (β-cyclodextrin) with a new *Helicobacter pylori* eradicating agent (TG44) in the amorphous state

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Abstract—The interaction of a newly developed *Helicobacter pylori* eradicating agent (TG44, 4-methylbenzyl-4′-[*trans*-4-(guanidinomethyl)cyclohexylcarbonyloxy]-biphenyl-4-carboxylate monohydrochloride) with cyclomaltoheptaose (β-cyclodextrin, β-CyD) in the solid state was studied by high-speed frequency-switched Lee-Goldburg (FSLG) ^{13}C–^{1}H heteronuclear correlation (HETCOR) NMR experiments. The TG44/β-CyD solid complex in a 1:1 stoichiometry was prepared by the grinding method. Powder X-ray diffractometry confirmed that the complex is in an amorphous state. The solid-state ^{13}C signals of TG44 and β-CyD were significantly broadened by the complexation. As the temperature increased, the ^{13}C signals of the aromatic moieties of TG44 were insignificantly influenced, whereas those of the cyclohexyl moiety became sharper. The $T_{1\rho H}$ values of the aromatic moieties of TG44 were almost the same as those of the β-CyD carbons, whereas those of other TG44 carbons gave much smaller values. The ^{13}C–^{1}H HETCOR spectra gave the intermolecular correlation peaks between the aromatic carbons of TG44 and the β-CyD protons or between the biphenyl protons of TG44 and the β-CyD carbons, when measured using longer contact times (500 and 1500 μs). On the basis of these solid NMR spectroscopic data together with aqueous NMR data, we assume that β-CyD includes predominantly the biphenyl moiety of TG44 in the solid state. ^{13}C–^{1}H HETCOR spectroscopy is particularly useful for the determination of inclusion modes of the complexes that occurring in an amorphous form.

Keywords: Cyclomaltoheptaose; β-Cyclodextrin; Inclusion complex; *Helicobacter pylori*; Solid-state NMR

1. Introduction

4-Methylbenzyl-4′-[*trans*-4-(guanidinomethyl)cylohexylcarbonyloxy]-biphenyl-4-carboxylate monohydrochloride (TG44, see Chart 1 for the chemical structure) is a newly synthesized *Helicobacter pylori* (*H. pylori*) eradicating agent with high selectivity to *H. pylori,* compared with other Gram-negative and Gram-positive bacteria.[1,2] In a previous paper,[3] we reported that the *H. pylori* eradicating activity of TG44 is markedly enhanced when it is orally administered in the form of the complex with cyclomaltoheptaose (β-cyclodextrin, β-CyD), compared with TG44 alone. The enhanced antimicrobial activity of the TG44/β-CyD complex can be ascribed to its improved dispersing and dissolving properties through the inclusion complexation. Further, we demonstrated that β-CyD forms a 1:1 inclusion complex with TG44 in aqueous solution, by including preferably the biphenyl group of the drug. However, it was difficult to determine the inclusion mode of the complex in the

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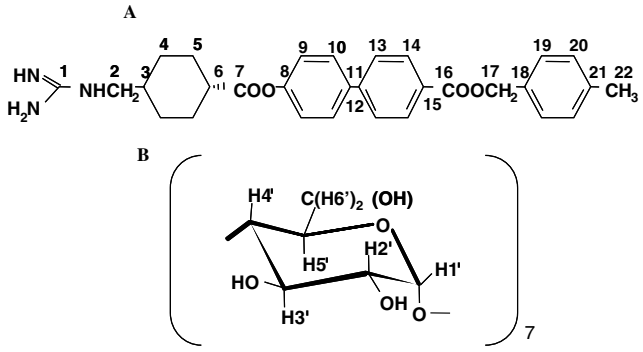

Chart 1. Structures of TG44 (A) and β-CyD (B) and their carbon and proton numberings.

solid state, because the complex was in an amorphous state, resulting in a halo-pattern in the powder X-ray diffractogram, although we demonstrated from the Giordano plot[4] in differential scanning calorimetric studies that TG44 forms a 1:1 complex with β-CyD in the solid state. In this study, therefore, we carried out solid-state two-dimensional ^{13}C–^{1}H-heteronuclear correlation (2D HETCOR) NMR spectroscopic studies to gain insight into the inclusion mode of TG44/β-CyD complex in the solid state, since this 2D method provides through-space correlations between a pair of ^{1}H and ^{13}C nuclei, thereby yielding useful information about intermolecular interaction.[5,6]

2. Results and discussion

2.1. 1D ^{13}C CP/MAS NMR spectroscopic studies

Figure 1 shows 1D ^{13}C CP/MAS NMR spectra of TG44, β-CyD and TG44/β-CyD complex (1:1) prepared by the cogrinding method. The ^{13}C signals of TG44 and β-CyD were assigned on the basis of aqueous NMR spectroscopic data.[3] It was difficult to assign separately each carbon in the biphenyl (C-10, C-13, C-14 and C-15) and tolyl (C-19, C-20 and C-21) moieties of the drug, because these carbons gave a single broad peak, particularly for the complex. Similarly, the C-2′, C-3′ and C-5′ carbons of β-CyD were difficult to assign separately. All carbons of TG44, except for the C-17 carbon, gave ^{13}C signals different from those of β-CyD. The chemical shifts of the guest and host carbons were negligibly changed by the complexation, but the signals were markedly broadened, probably because the complex is in an amorphous state in which various complexes with slightly different conformations, inclusion modes and orientations coexist. In a previous paper,[7] we reported that upon heating, ^{13}C NMR signals of guest moieties outside of the β-CyD cavity became sharper, whereas those included in the cavity do not change significantly because the molecular motions of the included guest moieties are severely restricted. Figure 2 shows the ^{13}C NMR spectra of the TG44/β-CyD complex measured at 25 and 80 °C. It is apparent that the signals of the C-6 carbon of the cyclohexyl group, methylene carbon (C-17) and the C-22 carbon of the tolyl group of the drug became sharper upon heating, whereas those of the biphenyl groups were only slightly changed. These results indicate that the biphenyl moiety of the drug is preferably included and their molecular motion is inhibited in the β-CyD cavity in the solid state. The signals of β-CyD carbons insignificantly changed probably because the cyclic structure of β-CyD is too large to be significantly influenced by heating under these experimental conditions.

The spin-lattice relaxation times in the rotating frame for protons ($T_{1\rho H}$) for solid 1:1 TG44/β-CyD complex were measured, because $T_{1\rho H}$ data are known to give useful information for intermolecular interactions of polymers.[8,9] Table 1 summarizes the $T_{1\rho H}$ values of each carbon of the complex as well as their ^{13}C chemical shifts. The β-CyD carbons (C-1′–C-6′) gave almost identical $T_{1\rho H}$ values (4.2–4.3 ms). It is of interest to note that the $T_{1\rho H}$ values (4.1 ms) of the biphenyl and terminal benzene carbons of TG44 were almost the same as those of the β-CyD carbons, whereas those of other TG44 carbons gave much smaller $T_{1\rho H}$ values (2.4–3.2 ms). These results mean that proton spin diffusion between the aromatic moiety of TG44 and β-CyD occurs sufficiently on the $T_{1\rho H}$ time scale, whereas there exists negligible spin diffusion between the cyclohexyl moiety and β-CyD, suggesting that β-CyD interacts with the aromatic moieties of TG44, predominantly with the biphenyl moiety.[3]

2.2. 2D ^{1}H–^{13}C H HETCOR spectroscopic studies

High MAS frequency of 15 kHz was applied to the HETCOR experiment to reduce the strong ^{1}H–^{1}H homonuclear dipolar interactions, and a ramped spin-lock pulse was applied to the protons during CP to enhance the ^{13}C signal sensitivities under the high MAS frequency. In addition, FSLG decoupling was applied to the protons throughout the evolution period (t_1 in Figure 5B in the experimental section) to suppress the strong ^{1}H–^{1}H dipolar interactions and thereby enhance resolution of the ^{1}H line shape. The scale of the ^{1}H chemical shift (vertical axis) was, therefore, corrected by the scaling factor of $1\sqrt{3}$. Since mixing is achieved with a simple Hartmann–Hahn contact in FSLG-HETCOR experiments, only strong correlations between ^{1}H and ^{13}C such as one-bond ^{1}H–^{13}C correlations can be obtained in the spectra recorded within a fairly short mixing time (<100 μs).[10–12] On the other hand, when the experiments are performed with longer mixing times (100–500 μs), long-range ^{1}H–^{13}C correlations can be observed.[10,12] Figure 3 shows the 2D ^{1}H–^{13}C HETCOR spectra of the TG44/β-CyD (1:1) complex, measured

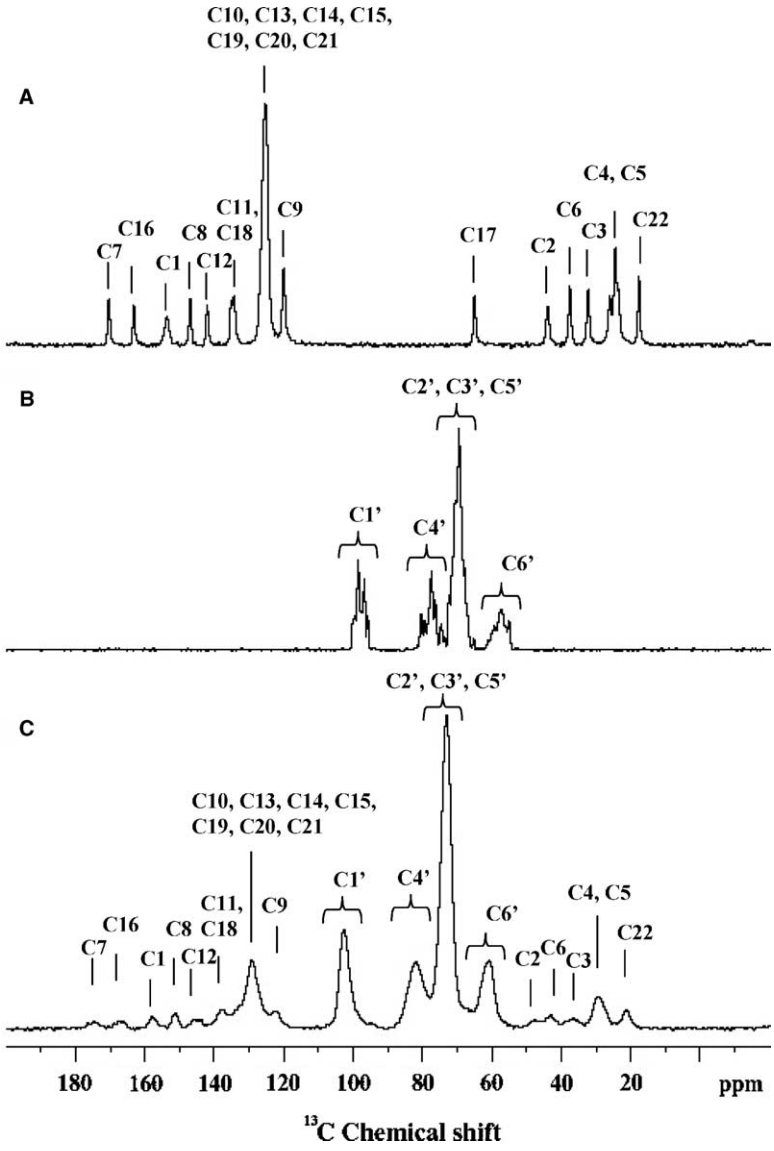

Figure 1. ^{13}C CP/MAS NMR spectra of (A) TG44, (B) β-CyD and (C) TG44/β-CyD (1:1) complex.

using the pulse sequence (Fig. 5B) and different contact times (60, 500 and 1500 μs). The 2D HETCOR experiment recorded with a short mixing time of 60 μs provided only correlations between the directly bonded ^{1}H–^{13}C pairs, enabling us to precisely assign ^{1}H shifts of the protons attached to the carbons of the TG44 and β-CyD, respectively (Table 2). The cyclohexyl (H-4, H-5 and H-6), biphenyl (H-9 and H-10, H-13, H-14) and tolyl groups (H-19, H-20 and H-22) of TG44 gave ^{1}H signals at (1.1–2.8 and 1.9–3.1 ppm), (6.9–8.0 and 7.0–8.4 ppm) and (8.7–9.6 and 1.4–2.2 ppm), respectively, in ^{1}H NMR spectra. β-CyD gave ^{1}H signals at 4.7–6.0 ppm (H-1′), 3.1–5.0 ppm (H-2′, H-3′, H-5′), 3.2–4.6 ppm (H-4′) and 3.3–5.2 ppm (H-6′), respectively. As the contact time increased, new correlation peaks appeared between TG44 carbons (128–130 ppm) and β-CyD protons (2.7–4.1 ppm) and between C-17 carbon (63–66 ppm) of TG44 and β-CyD proton (4.1 ppm), as shown by the dotted lines in Figure 3B, C. The correlation peak between the 30 ppm carbon signal and the 3 ppm proton signal may be attributable to the broadening of the TG44 carbon peaks. These intermolecular correlation peaks appeared much more clearly in the ^{13}C–^{1}H HETCOR spectrum with a constant time of 1500 μs (Fig. 3C), that is, the high intermolecular correlation peaks were observed between the aromatic carbons (biphenyl carbons: C-10, C-13, C-14 and C-15 and tolyl carbons: C-19, C-20 and C-21) and the β-CyD protons. Furthermore, in the 1500 μs spectrum, an additional correlation peak appeared between the biphenyl protons of TG44 (7 ppm) and the β-CyD carbons (C-2′, C-3′ and C-5′, 72–77 ppm). Figure 4 shows

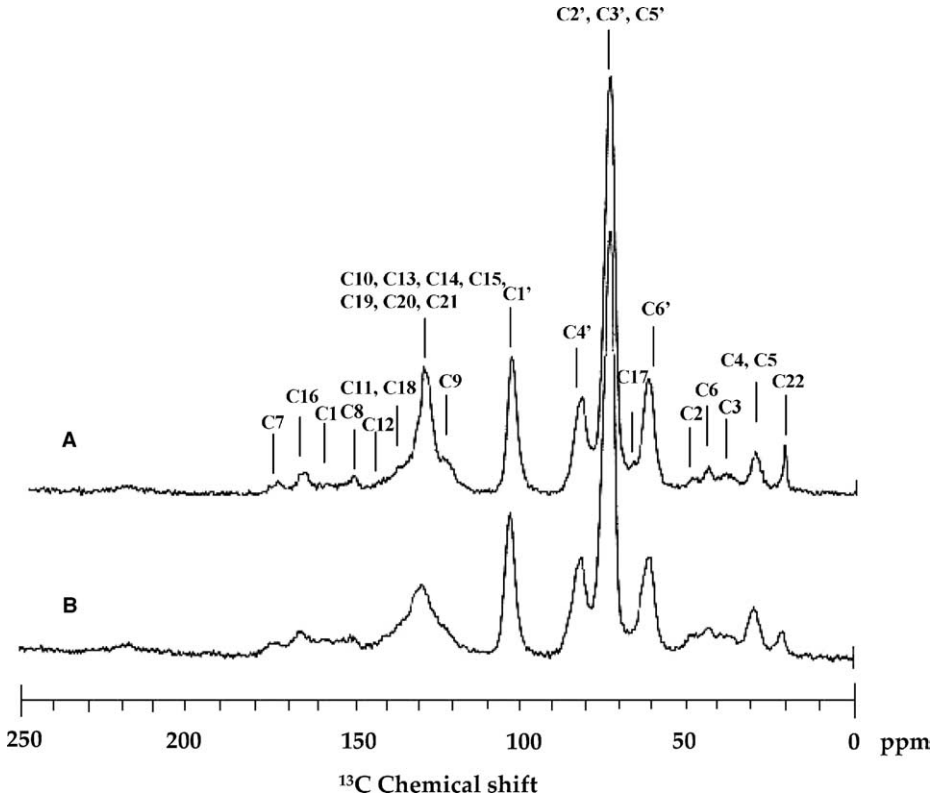

Figure 2. ^{13}C CP/MAS NMR spectra of TG44/β-CyD (1:1) complex at 80 °C (A) and 25 °C (B).

Table 1. $T_{1\rho^H}$ values of each carbon atom of TG44/β-CyD complex

Carbon no.	C-7	C-16	C-1	C-8	C-12	C-11,18	C-10,13,14,15,19,20,21	C-9
Chemical shift/ppm	174	167	158	151	146	138	129	122
$T_{1\rho^H}$/ms	N.D.[a]	N.D.	3.21	3.14	2.97	3.21	4.12	4.10
Carbon no.	C-17	C-2	C-6	C-3	C-4,5	C-22		
Chemical shift/ppm	73	48	43	36	29	21		
$T_{1\rho^H}$/ms	N.D.	N.D.	2.92	2.81	3.77	2.41		
Carbon no.	C-1′	C-4′	C-2′3′5′	C-6′				
Chemical shift/ppm	103	95	82	61				
$T_{1\rho^H}$/ms	4.19	4.22	4.33	4.19				

[a] N.D. These values could not be determined because the intensity of these resonances was low.

the 1D slices along the vertical axis at 7 ppm as shown in Figure 3. It is apparent that the biphenyl protons of TG44 gave the correlation peak with the C-2′, C-3′ and C-5′ carbon of β-CyD at 72–77 ppm. It is reasonable to assume that the biphenyl moiety is included in the β-CyD cavity. If the other part of the drug molecule is included in the cavity or the drug interacts with the outside of the cavity, many other correlation peaks should be observed, but no intermolecular correlation peaks were observed in other regions and even between the terminal methyl carbon (C-22) of TG44 and β-CyD. Chart 2 shows the inclusion mode of TG44/β-CyD (1:1) complex in the solid state, estimated from the present solid-state NMR spectroscopy and those in the aqueous solution reported previously,[3] that is, β-CyD includes preferably the biphenyl moiety of TG44 molecule.

3. Conclusion

It is well known that many drugs form inclusion complexes with CyDs in aqueous solution and in the solid state.[13,14] Inclusion modes of the complexes in aqueous solution can be precisely determined using various spectroscopic techniques such as NMR spectroscopy.[15,16] When the complexes are obtained in single crystals, the three-dimensional structure can be determined by single X-ray analysis.[17,18] On the other hand, when they

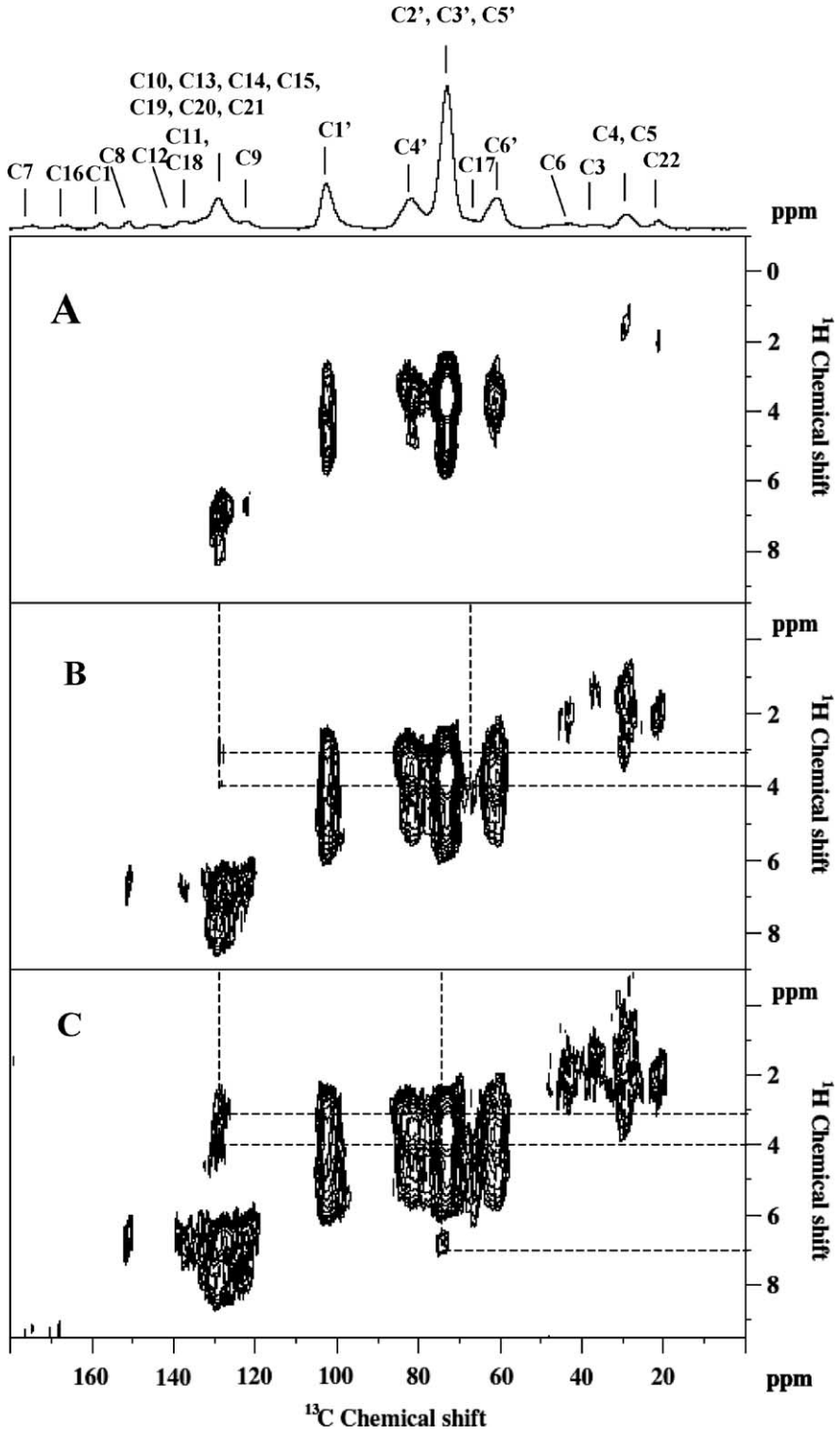

Figure 3. 2D FSLG ^{1}H–^{13}C HECTOR spectra of TG44/β-CyD (1:1) complex, measured with contact times of 60 μs (A), 500 μs (B) and 1500 μs (C).

are in the amorphous form, the structure determination is extremely difficult, because of the absence of diffraction peaks in X-ray analysis.[19,20] In this study, we attempted to estimate the inclusion mode of TG44/β-CyD complex in an amorphous state, using solid-state 2D ^{1}H–^{13}C HETCOR spectroscopy. The 2D ^{1}H–^{13}C HETCOR spectra of TG44/β-CyD complex gave intermolecular correlation peaks between the guest carbons and the β-CyD protons or between the guest protons and the β-CyD carbons, and indicated that the biphenyl

Table 2. ^{1}H Chemical shifts of TG44 and β-CyD

Proton no. of TG44	H-2	H-3	H-4, H-5	H-6	H-9	H-10, H-13, H-14	H-17	H-19, H-20	H-22
Chemical shift/ppm	2.5–3.3	1.2–1.9	1.1–2.8	1.9–3.1	6.9–8.0	7.0–8.4	3.0–5.2	8.7–9.6	1.4–2.2
Proton no. of β-CyD	H1′	H2′, H3′, H5′	H4′	H6′					
Chemical shift/ppm	4.7–6.0	3.1–5.0	3.2–4.6	3.3–5.2					

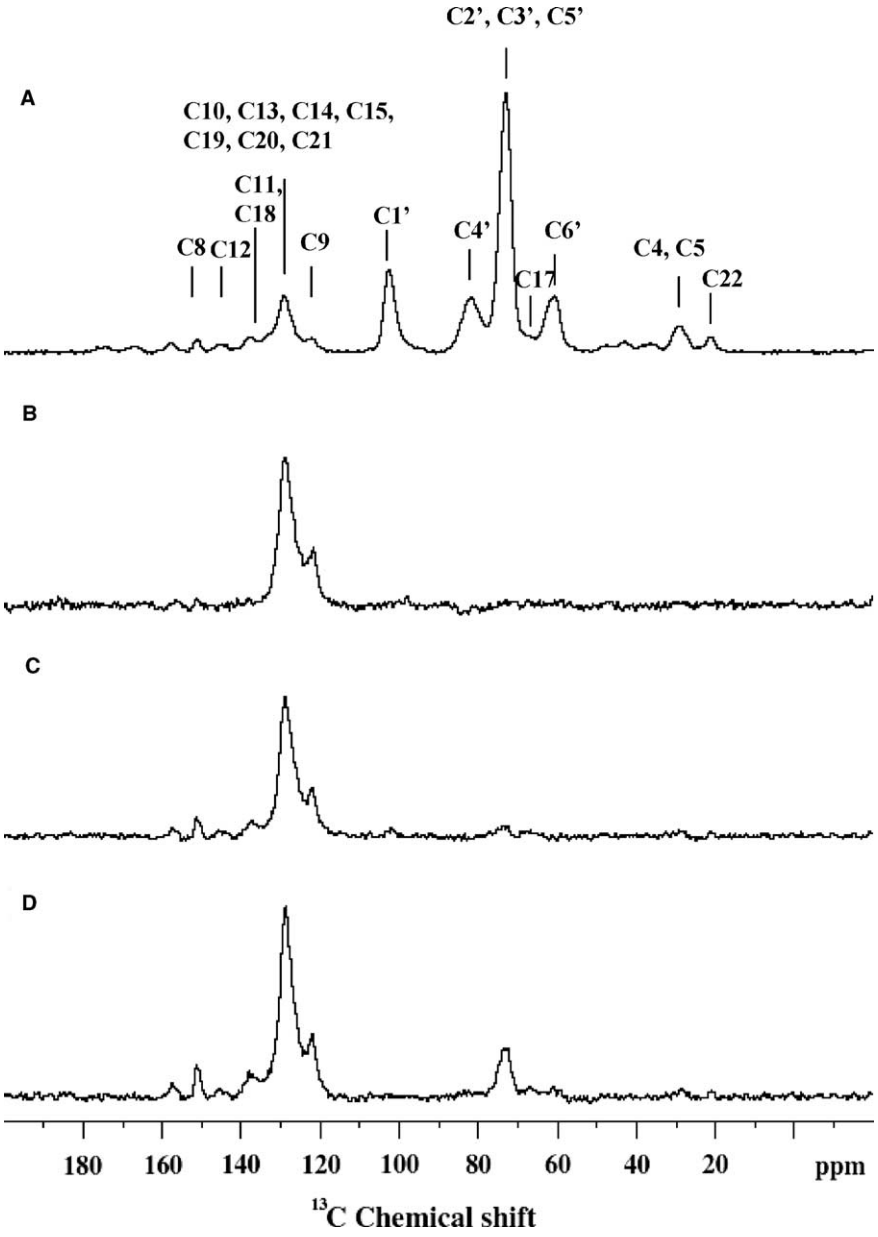

Figure 4. ^{13}C CP/MAS NMR spectrum (A) of the TG44/β-CyD (1:1) complex, and 1D slices along the horizontal axis of the FSLG ^{1}H–^{13}C HETCOR spectra (Fig. 3) taken at 7 ppm on the ^{1}H chemical shift axis, measured at contact times of 60 μs (B), 500 μs (C) and 1500 μs (D).

moiety of TG44 is predominantly included in the β-CyD cavity in the solid state, in a similar manner as that in the aqueous solution. Therefore, solid-state 2D ^{1}H–^{13}C HETCOR spectroscopy is useful in the determination of the inclusion modes of many CyD complexes that occurred in amorphous form.

4. Experimental

4.1. Materials

TG44 and β-CyD were supplied by Nagase ChemteX Corporation (Osaka, Japan) and Nihon Shokuhin Kako

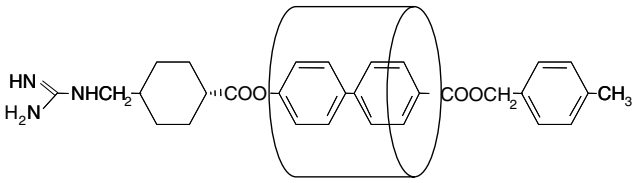

Chart 2. Proposed inclusion mode of TG44/β-CyD complex in the solid state.

Co. (Tokyo, Japan), respectively. All other chemicals and solvents were of analytical reagent grade, and deionized distilled water was used throughout the study.

4.2. Preparation of solid complex

The TG/β-CyD solid complex was prepared by the cogrinding methods,[21,22] that is, TG44 and β-CyD in a 1:1 molar ratio were put in a vibrational mill (Chuo Kakohki Model MB-1, Aichi, Japan) at a total mass amount of about 450 g, and ground for 10 h at 25 °C. The ground product gave a halo-pattern in the powder X-ray diffractogram, confirming that the solid complex is in an amorphous state

4.3. NMR spectroscopic studies

All NMR spectra were measured at B_0 field of 9.4 Tesla using a Bruker AV400 spectrometer (400 MHz of ^{1}H frequency) with 89 mm wide-bore magnet at room temperature (298 ± 1 K). The pulse sequences of the solid-state NMR experiments used in this study are shown in Figure 5 (see Refs. 5 and 23 for detailed procedures).

1D CP/MAS ^{13}C NMR (Fig. 5A) spectra were obtained using a Bruker 4 mm double-tuned MAS probe. Samples for the NMR measurements were set to ZrO_2 rotor (4 mm of outer diameter) and KelF-made cap. Sample volume was ca. 80 μL. The MAS spinning speed was set to 13,000 kHz, regulated by a Bruker MAS II pheumatic MAS controller. In the CP step, the radio-frequency field (rf) strength was set to 78 kHz for ^{13}C, and a 100 → 55% ramped-amplitude spin-lock pulse[24] was used for ^{1}H. During acquisition, the rf strength was set to 78 kHz for ^{1}H using a TPPM decoupling sequence.[25] The phase-modulation angle for the TPPM decoupling was set to 15.0°, and the flip-pulse length was optimized to 5.9 μs. The contact time and the recycle delay were set to 1.0 ms and 4 s, respectively.

2D ^{1}H–^{13}C HETCOR (Fig. 5B) spectra were obtained by the use of a Bruker 4 mm-double tuned MAS probe. The sample volume was restricted to about 25 μL and set at the centre of the rotor to improve the rf homogeneity. The MAS frequency was set at 13,000 Hz. The magic angle (54.7°) pulse length for protons was set at 1.8 μs. During the ^{1}H chemical shift evolution, the

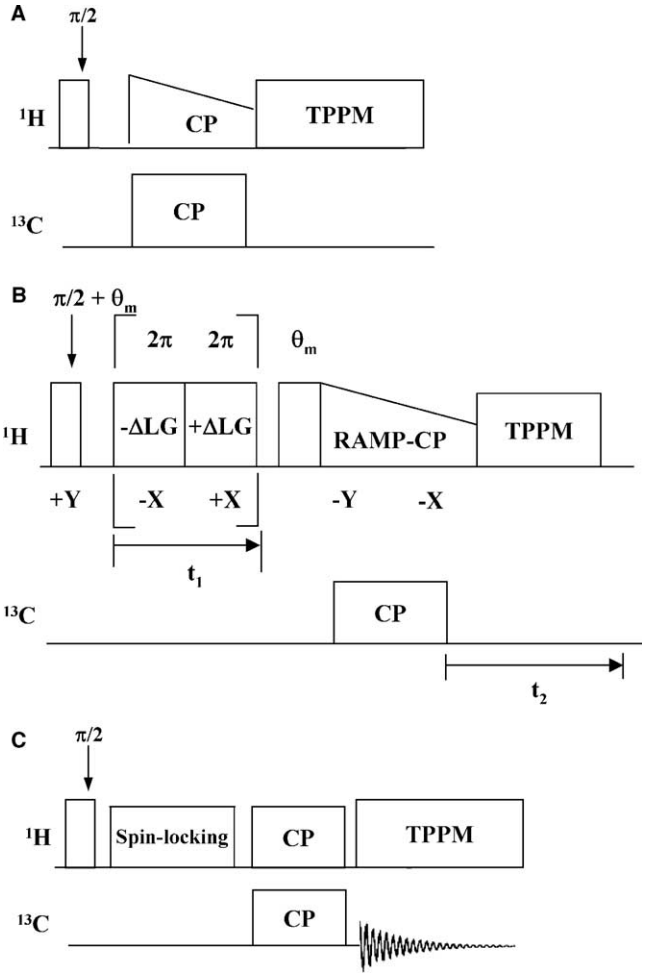

Figure 5. Pulse sequences for 1D CP/MAS ^{13}C NMR spectra (A), for 2D heteronuclear correlation NMR spectra (B) with frequency-switched Lee-Goldburg irradiation during the evolution, and for determination of $T_{1\rho^H}$ values (C).

FSLG sequence[26,27] was applied to protons to efficiently suppress the ^{1}H homonuclear dipolar interactions. Two off-resonance pulses with opposite phases during FSLG decoupling were set at 5.7 μs, and the proton rf field strength was set at 86 kHz. During CP, which corresponds to the mixing time, the rf field strength was set at 64 kHz for carbon, while a ramped rf field was applied to protons. The mixing time was set to 60, 500 and 1500 μs. The recycle delay was set to 4 s. During acquisition, the proton decoupling field strength was set at 78 kHz using a TPPM scheme. Quadrature phase detection was achieved using the States method. A total of 72 t_1 acquisitions with 512 scans each were collected. In all NMR experiments, the ^{13}C chemical shifts were calibrated through the carbonyl carbon resonance of D-glycine as an external reference at 176.03 ppm. In the HETCOR experiment, ^{1}H chemical shifts were referenced by setting the H_β resonance of the L-alanine mixture at 1.0 ppm. The ^{1}H chemical shift scale in the HETCOR spectra was corrected by a scaling factor of

$1\sqrt{3}$, since the ^{1}H chemical-shift dispersion is scaled by a factor of $1\sqrt{3}$ during FSLG decoupling.

The pulse sequence for the determination of $T_{1\rho H}$ values is shown in Figure 5C. The spin-locking time was set to 0.1, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12 and 14 ms. These data were collected, and integral values of ^{13}C resonance were plotted to the spin-locking time. $T_{1\rho H}$ of each resonance was determined by the following equations; $I(t) = I(0)\exp(-t/T_{1\rho H})$, where $I(t)$ is the integral values at t ms of spin-locking time and $I(0)$ is that of the integral value of CP.

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