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# A Structural Study of the Naringin-$\beta$-Cyclodextrin Complex

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**Abstract.** The structure of the inclusion complex formed between naringin (naringenin-7-*O*-$\beta$-neohesperidoside) and $\beta$-cyclodextrin (BCD) was studied in detail by UV and NMR spectroscopic techniques and potentiometry. A binding constant value of $1016 \pm 150 M^{-1}$ was arrived at from UV studies. Potentiometric studies showed that pK values of 4′-OH and 5-OH were affected by $\alpha$ and $\beta$-cyclodextrins. One-dimensional difference NOE and spin-lattice relaxation time ($T_1$) measurements indicated that the aglycone portion was affected more than the neohesperidoside portion. The $T_1$ values analysed for local motions indicated that $\tau_c$ values of complexed naringin was higher than that of free naringin. The internal rotation calculated for different groups showed $\tau_i$ values for the phenolic and dihydrobenzopyran portion decrease by a factor of 2. Also a $\xi$ value of 0.12–0.17 observed for the aglycone portion indicated that the coupling between guest and host is weak. All the studies have shown that the disposition in which the phenol group at 2 is inside the BCD cavity with 4-keto and 5-OH hydrogen bonded to the secondary hydroxyl groups at the rim of the wider end of the BCD cavity is the most probable one.

**Key words:** Naringin–BCD, 1DNOE, $T_1$, aglycone, phenyl ring.

## 1. Introduction

Flavanoids and limonoids are two major constituents of the bittering principles in grapefruit and navel oranges [1, 2]. Naringin is the major flavanoid and limonin and nomilin are the principal limonoids responsible for the bitterness in the fruit juices. $\beta$-cyclodextrin was shown to reduce the bitterness of both naringin and limonin through the formation of inclusion complexes [3–6]. A new process involving an immobilized, chemically modified $\beta$-cyclodextrin polymer was also used to debitter grapefruit juice [7]. The naringin complexing ability between free cyclodextrin and its monomer in the polymer assembly was not found to be very much different [8]. Besides, the amount of naringin retained by the BCD polymer was found to be higher than that of polystyrene XAD-4 polymers.

The structure of naringin (naringenin-7-*O*-$\beta$-neohesperidoside) is shown in Figure 1. The aglycone part, naringenin, consists of a 2,3-dihydrobenzopyran-4-one unit with a phenol group attached at position 2. Out of three phenol hydroxyl groups, 4′ and 5-OH are free and 7-OH is $\beta$-linked to a neohesperidoside unit (7-*O*-$\alpha$-rhamnosyl (1 $\rightarrow$ 2)-$\beta$-*O*-glucopyranoside).

Since naringin possess several moeities capable of interacting with BCD, the

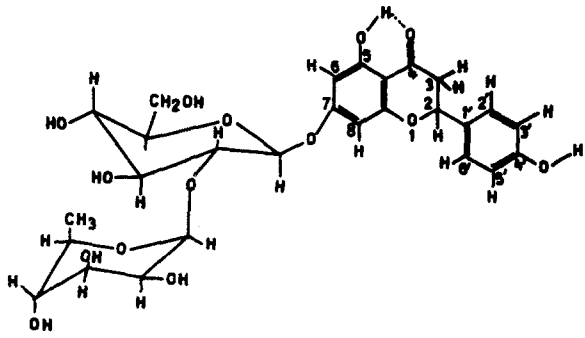

Fig. 1. Structure of naringin.

orientation of naringin with respect to BCD was investigated by a detailed spectroscopic and potentiometric study and the results are presented here.

## 2. Materials and Methods

Naringin and $\alpha$-cyclodextrin purchased from Sigma Co. (USA) were used. $\beta$-Cyclodextrin used was a gift from Amaizo Inc. (USA). All NMR measurements were carried out using DMSO-$d_6$ purchased from Aldrich Chemical Co. Inc. (USA).

Proton NMR measurements were carried out on a continuous-wave Varian EM-390 (operating at 35°C) and Bruker AM 300 (operating at 20°C) NMR spectrometers, the latter fitted with a Spectrospin magnet and Aspect 3000 computer. The concentrations of solutions used for NMR measurements were 0.036–0.067 M. One-dimensional difference nuclear Overhauser enhancement (1DNOE) and proton spin-lattice relaxation time measurements were carried out on a Bruker AM 300 NMR spectrometer as described previously [9, 10].

A Control Dynamics pH meter fitted with an Ingold combination electrode was used for measuring the pH. The p$K$ values were determined from a plot of log[salt]/[acid] against pH [11] and were found to be reproducible to within $\pm 0.02$.

The binding constant value for the naringin–$\beta$-cyclodextrin complex was determined by monitoring the change in absorption at 285 nm during the addition of increasing amounts of BCD to naringin in DMSO. To avoid error due to dilution the BCD solution was made up with naringin solution. A Varian Superscan 3 UV spectrometer was used for monitoring the changes in absorbance. The method of Formoso [12] was employed for determining the binding constant value using the relation:

$$1/\Delta A = 1/(\Delta A_{AB})K[BCD] + 1/(\Delta A_{AB})$$

where $\Delta A$ = difference in absorption between free naringin and the complex at a given concentration of BCD, $\Delta A_{AB}$ = difference in absorption between free naringin and the complex and $K$ = binding constant value for the complex.

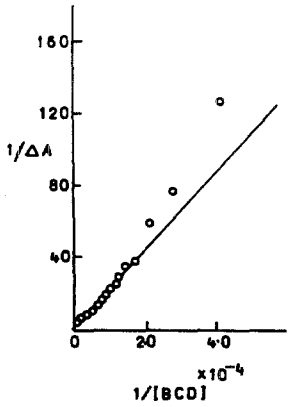


Fig. 2. Plot of $1/\Delta A$ versus 1/[BCD] to evaluate the binding constant from UV measurements. Concentrations: [Naringin] = 103.0 $\mu$M and [BCD] = 1.824 mM.

Naringenin was prepared by refluxing 500 mg of naringin with 50 mL 1:1 2N HCl and methanol for about 1 h at 60°C. The reaction mixture was extracted with ether and the extracts were washed with dil. NaOH and evaporated. Recrystallized naringenin exhibited a melting point of 247–250°C as reported in the literature. The purity was also checked by $^1$H NMR in DMSO-$d_6$.

## 3. Results and Discussion

### 3.1. ULTRAVIOLET SPECTROSCOPY

Naringin in DMSO was found to exhibit UV absorption maxima at 285 nm ($\pi$–$\pi^\star$ transition of the phenolic OH at 4′ and 5, $\epsilon$ = 19 950) and 335 nm ($n$–$\pi^\star$ transition of the hydrogen bonded keto group at 4, $\epsilon$ = 3588). DMSO was chosen because the solubility of naringin was better in DMSO than in water. The band at 285 nm was found to show an increase in absorption on adding increasing amounts of BCD indicating that both the phenolic OH groups are affected by BCD. The stoichiometry of the complex was found to be 1:1 (Figure 2) and a binding constant value 1016± 150 $M^{-1}$ determined agreed well with the value obtained by Konno *et al.* [5] (Figure 2).

### 3.2. POTENTIOMETRY

Titration of ionisable groups in naringin in the presence of both $\alpha$ and $\beta$-cyclodextrins were monitored by pH meter, Table I. Titration against NaOH indicated the presence of three phenol groups, one of them resulting from the cleavage of the glycosidic bond $C_{1\beta}$-$O_7$. However, the tritation of an aqueous solution of naringin in the presence of cyclodextrins showed only two titrating phenolic OH groups, one with a slightly higher p$K$ value than the other. Both $\alpha$ and $\beta$-

TABLE I. p$K$ values of naringin phenolic groups in the presence of $\alpha$ and $\beta$-cyclodextrins[a].

| System | p$K_1$ | p$K_2$ |
|---|---|---|
| Naringin | 9.68 | 9.98 |
| Naringin +$\alpha$-cyclodextrin | 9.4 | 10.24 |
| Naringin +$\beta$-cyclodextrin | 9.64 | 10.09 |

[a] Equimolar solutions (0.01M) of naringin and $\alpha$- and $\beta$-cyclodextrin containing NaCl ($I = 0.2$) were employed. pH adjusted with 0.1M NaOH and HCl.

cyclodextrins change the p$K$ values of the groups (Table I). While one showed a decrease in p$K$ value, the other showed an increase in the presence of cyclodextrins. An increase or decrease in p$K$ values can be expected for groups in different environments. While hydrogen bonding can be expected to increase the p$K$ value for one, the presence of an apolar environment can be expected to decrease the p$K$ value for the other [13]. Hence, the group whose p$K$ value is higher in the presence of cyclodextrins can be ascribed to that at 5, and the one whose p$K$ value was lowered by cyclodextrin can be assigned to that at 4′.

## 3.3. $^1$H NMR STUDIES

### 3.3.1. *Chemical Shift*

Quite a large amount of literature on NMR studies is available on the inclusion of aromatic rings in the cyclodextrin cavity [14, 15]. Similar studies were attempted to test this aspect in the current investigation.

The chemical shift values of naringin and BCD in free and complexed states obtained at 300 MHz in DMSO-$d_6$ are shown in Table II. Although the solubility of naringin is low in $D_2O$, the presence of an HDO signal makes it difficult to observe all the naringin signals. Hence most of the NMR work was carried out using DMSO-$d_6$ although DMSO is not a very good solvent for studying complexation. While the BCD protons showed slightly different chemical shift values between complexed and uncomplexed forms, the naringin protons showed very little changes in its chemical shift values. However, coupling constant values of some of the naringin protons were altered due to complexation, the most significant among them being the phenyl ring protons, namely H-2′, H-3′, H-5′ and H-6′ protons. At alkaline pH, naringin was observed to show opening of the dihydrobenzopyran ring, resulting in the formation of chalcone derivatives. $^1$H NMR spectra of naringin obtained at 300 MHz in DMSO-$d_6$ indicate sharp signals with well defined coupling, ruling out any possible aggregation for naringin at such high concentrations.

Even at very low concentrations, addition of naringenin (aglycone) to BCD in

TABLE II. Chemical shift values of naringin and naringenin-β-cyclodextrin complexes

| Signal | Naringin | | | | Naringenin[b] | |
|---|---|---|---|---|---|---|
| | free | | complex | | complex | |
| | δ (ppm) | J (Hz) | δ (ppm) | J (Hz) | δ (ppm) | J (Hz) |
| 5-OH | 12.08 | | 12.08 | | 12.18 | |
| 7-OH | – | | – | | 10.82 | |
| 4′-OH | 9.64 | | 9.66 | | 9.60 | |
| H-2′ & H-6′ | 7.34 | 11.7 | 7.33 | 5.5 | 7.30 | 10.5 |
| H-3′ & H-5′ | 6.82 | 11.1 | 6.81 | 7.9 | 6.77 | 8.4 |
| H-8 | 6.14 | | 6.1 | | 6.12 | |
| H-6 | 6.10 | | 6.09 | | 5.85 | |
| 2-OH (BCD) | 5.81 | | 5.75 | | 5.73 | |
| 3-OH (BCD) | 5.74 | | 5.70 | | 5.67 | |
| H-2 | 5.51 | 10.95 | 5.51 | 11.1 | 5.43 | |
| H-1 (Rha) | 5.33 | 7.86 | 5.35 | 7.1 | | |
| H-1 (Glu) | 5.15 | 5.49 | 5.14 | 5.6 | | |
| OH (Rha) | 5.10 | 2.8 | 5.10 | 2.8 | | |
| H-1( BCD) | 4.83 | | 4.82 | | 4.82 | |
| OH (Rha, Glu) | 4.74 | 8.94 | 4.74 | 5.6 | | |
| OH (Rha, Glu) | 4.69 | 2.22 | 4.69 | 2.3 | | |
| OH (Rha, Glu) | 4.59 | | 4.60 | | | |
| 6-OH (BCD) | 4.50 | 5.7 | 4.49 | | 4.46 | |
| Rha, Glu CH BCD | 3.66-3.37 | | 3.62-3.29 | | 3.65-3.37 | |
| H-3 (*trans*) | 3.17 | | 3.16 | | 3.10 | |
| H-3 (*cis*) | 2.71 | 15.8 | 2.71 | 14.1 | 2.68 | 16.7 |
| | | 6.7 | | 8.4 | | |
| $CH_3$ | 1.17 | | 1.16 | | | |

[a] Values measured at 300 MHz for $^1H$ at 20°C in DMSO-$d_6$ .

[b] Chemical shift values of narigenin in free and complexed states are not very different.

DMSO-$d_6$ resulted in the disappearance of hydroxyl protons 2-OH, 3-OH, and 6-OH of BCD (Figure 3). The water signal at 3.5 ppm was not only found to shift to 4.26 ppm but also showed an increase in intensity during naringenin addition. Fast exchange between displaced water and OH protons is a strong possibility for the disappearance of hydroxyl protons. Similar effects were also observed for the aspartame-β-cyclodextrin complex [11].

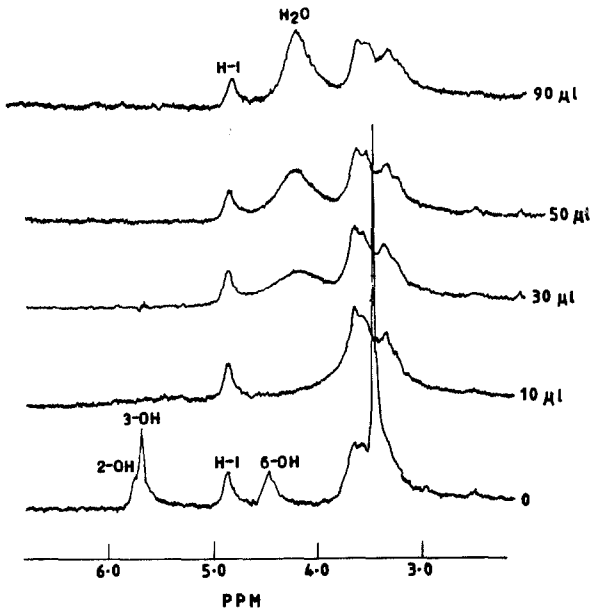


Fig. 3. Effect of addition of naringenin to BCD. Spectra obtained on a CW 90 MHz instrument. [BCD] = 0.21 M. A stock solution of naringenin (0.48 M) was made in DMSO-$d_6$ from which additions to BCD in 0.5 mL DMSO-$d_6$ were carried out.

### 3.3.2. *One-Dimensional Difference NOE*

One-dimensional difference NOE (1DNOE) experiments showed the effect of irradiation of signals from guest on host and *vice versa* (Figures 4 and 5). Limitations in use of DMSO-$d_6$ in 1DNOE experiments and relation between NOE, $\omega$ (Larmor precession frequency) and $\tau_c$ (correlation time) have been discussed elsewhere [9, 10]. Some low negative values were also obtained in addition to positive effects, indicating different correlation times operative in the naringin–BCD complex. However, large positive effects were observed generally for the naringin–BCD complex during intermolecular irradiation. The observed NOE intensities indicate that the non-NOE condition $\tau_c = \omega^{-1}$ does not operate [14]. Under some circumstances the effects of irradiation of naringin protons on BCD protons from both the wider as well as the narrower end could be seen. Hence the effects of spin diffusion could not be totally ruled out.

When the phenyl ring and dihydrobenzopyran protons were irradiated the NOE effects on BCD protons were greater than that observed when the naringin sugar protons were irradiated. Also, the effects of irradiation of BCD protons on naringin protons were small [Figure 4 and 5]. Thus the irradiation of aglycone group signals produced effects on 2-OH, 3-OH, 6-OH and H-1. The aglycone protons whose irradiation was responsible for causing perturbation of BCD protons are the phenyl ring H-2′, H-3′, H-5′, H-6′, 4′-OH, H-6, H-8, 5-OH and H-3 (*cis* and *trans*) protons.

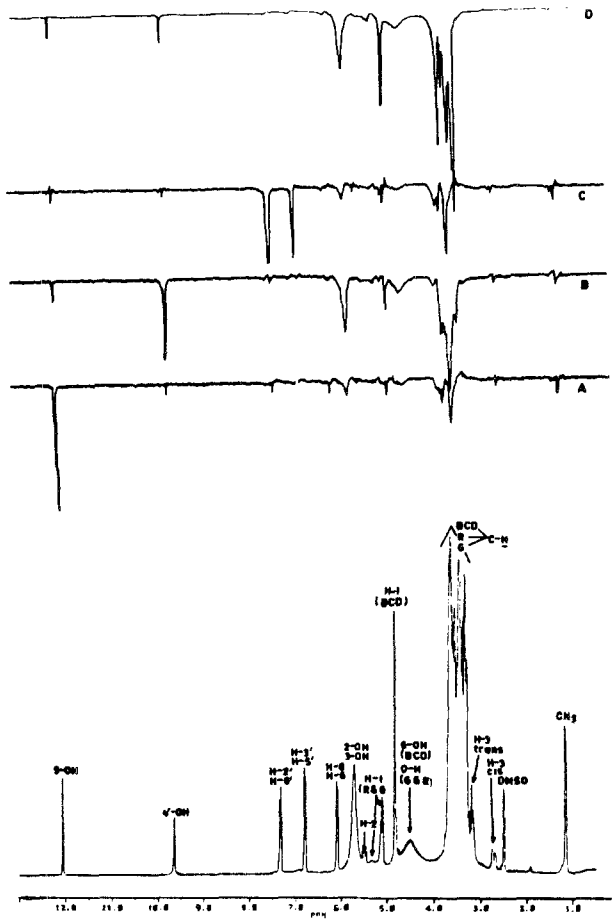


Fig. 4. 1D difference NOE experiments on naringin–BCD (0.05 M) complex obtained at 300 MHz in DMSO-$d_6$. On-resonance irradiation positions are (*A*) 5-OH, (*B*) 4′-OH, (*C*) H-2′, H-6′, (*D*) H-1 (BCD). The results from only few irradiation positions are shown.

Furthermore, the effects of perturbation of dihydrobenzopyran protons on BCD secondary hydroxyl groups at C-2 and C-3 are greater than those of the phenyl protons on the latter. This indicates a greater proximity between dihydrobenzopyran and secondary hydroxyl at the wider end of the BCD cavity.

The sugar backbone protons (CH) from rhamnose, glucose and BCD have merged together and occur as a broad envelope, enabling a study of only the effect of irradiation of few neohesperidoside protons like H-1 (rha), H-1 (glu), OH protons. The rotations between C-2 and C-1′, $C_{1\beta glu}$-$O_7$ and $C_{2glu}$-$C_{1rha}$ bonds bring different portions of the naringin molecule closer to the BCD protons, thus explaining the observed NOEs on some naringin glycosidic protons due to irradiation of the BCD protons.

Some positive values were obtained, as in the irradiation of 2-OH of BCD on

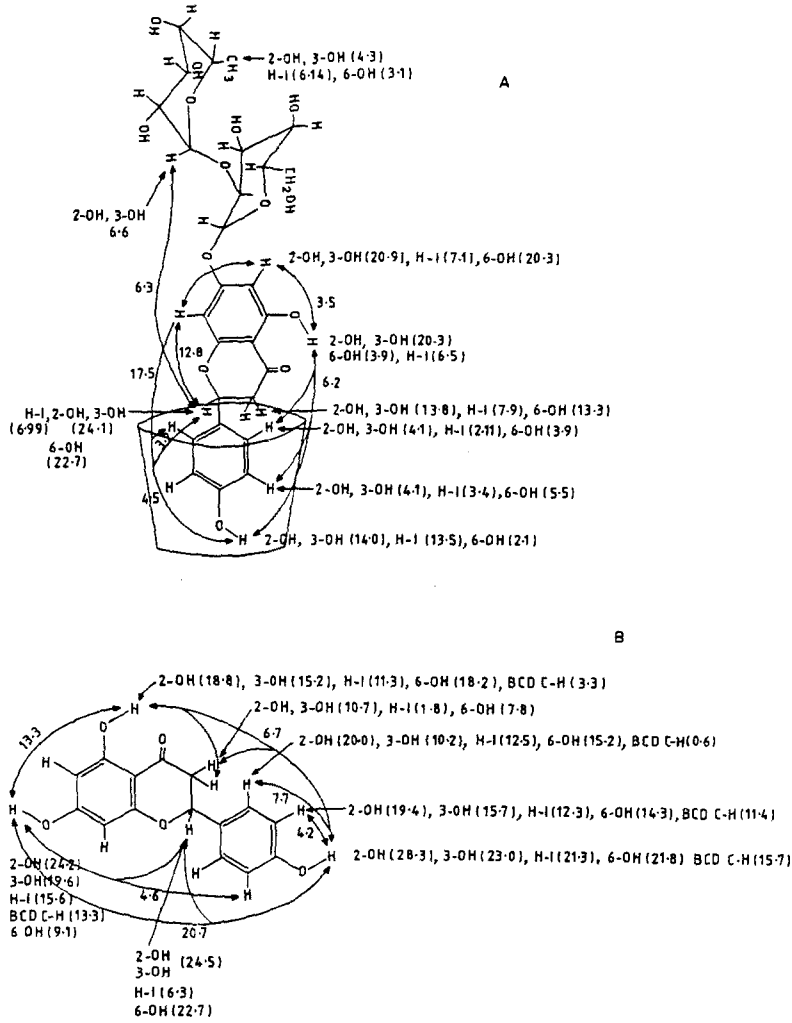


Fig. 5. 1D NOE connectivities of (A) naringin in naringin–BCD and (B) naringenin in naringenin–BCD complexes. Those of free BCD, naringin and naringenin are not shown. Some intramolecular NOE values in the complexes are shown by arrows. Values less than 1% are ignored. Intermolecular NOEs are shown by specifying the group from the host which shows maximum NOE values when the particular groups in question from the guest are perturbed and *vice versa*.

H-2′, H-3′, H-5′ and H-6′ signals in the naringenin–BCD complex. Irradiation of all the three phenolic OH groups (4′, 5 and 7) resulted in perturbation of the 2-OH, 3-OH, H-1, 6-OH and BCD CH protons. The irradiation of BCD protons exerted greater effects on phenyl ring protons than on dihydrobenzopyran protons. The effects were found to decrease from H-2′, H-3′, H-5′ and H-6′ > H-2, H-3 > H-6,

H-8, 5-OH and 7-OH.

The NOE data suggest two possibilities for the disposition of naringin with respect to BCD. The first is that the dihydrobenzopyran portion is hydrogen bonded to the secondary hydroxyl groups at the rim of the wider end of BCD. Such an orientation would leave the phenyl group away from the cyclodextrin molecule, which does not fit with the observed NOE, UV and potentiometric data. On the other hand, the inclusion of a phenyl group inside the cyclodextrin cavity with 4-keto and 5-OH groups of dihydrobenzopyran hydrogen bonded to the secondary hydroxyl groups of BCD fit with the observed data. Further confirmation of this disposition came from the following $T_1$ studies.

### 3.3.3. *Measurements*

There was a reduction in the $T_1$ values of certain groups of naringin in the presence of BCD (Table III). However, certain groups also showed an enhancement of $T_1$. The groups in the vicinity of the keto group in the aglycone portion, namely, 5-OH and H-3 (*cis* and *trans*) showed an increase in $T_1$ in the order 5-OH $\gg$ H-3 *trans* > H-3 *cis*. The other protons of the aglycone portion showed a reduction in $T_1$ in the order H-2 > 4′-OH > H-6, H-8 > H-2′, H-6′ > H-3′, H-5′. The hydroxyl protons of rhamnose and glucose units showed a maximum reduction in $T_1$, probably due to restricted rotation of the two sugar units resulting from complexation. The decrease in $T_1$ indicates that the relative mobilities of groups become altered (mainly reduced) due to complexation. Hydrogen bonding of the keto oxygen with the secondary hydroxyl groups of BCD may be responsible for the increase in $T_1$ values. The presence of free glucose (amount equivalent to BCD) did not alter the $T_1$ values of naringin, indicating that complexation only is responsible for the reduction in $T_1$.

The observed $T_1$ values were uncorrected for viscosity measurements. However, they were corrected for complexation by using the relation

$$1/T_{1\text{ observed}} = \alpha/T_{1\text{ complex}} + (1 - \alpha)/T_{1\text{ free}}$$

where $\alpha$ is the fraction bound. Using a dissociation constant of $9.843 \times 10^{-4}$ M for the naringin–BCD complex, the $T_{1\text{ complex}}$ values obtained are listed in Table IV.

Local molecular motions can be analyzed from $T_1$ measurements [17]. The measured NOE is equivalent to the dipolar contribution to relaxation, $T_{1DD}$. Hence, the $T_{1\text{ complex}}$ values can be used to calculate $\tau_c$ values, namely molecular reorientation time or correlation time [17], by employing the following equation for nuclei of identical spins [18]. For the extreme motional narrowing condition $\omega^2 . \tau_c^2 \ll 1$.

$$1/T_{1DD} = 2\gamma^4\hbar^2 I(I+1)\tau_c/r^6$$

where $\gamma$ = gyromagnetic ratio, $\hbar = h/2\pi$ ($h$ = Planck's constant), $I$ = spin of the nucleus and $r$ = internuclear distance under consideration. Due to uncertainties

TABLE III. $T_1$ values of naringin in the presence of $\beta$-cyclodextrin[a].

| Signal | Free | Complex | Percentage of reduction/ enhancement in $T_1$[b] |
|---|---|---|---|
| 5-OH | 1.13 | 1.91 | +68.8 |
| 4′-OH | 1.12 | 0.85 | -24.0 |
| H-2′ & H-6′ | 1.08 | .097 | -10.2 |
| H-3′ & H-5′ | 1.24 | 1.14 | - 8.1 |
| H-6 & H-8 | 1.02 | 0.97 | -14.6 |
| 2-OH (BCD) | | 0.8 | |
| 3-OH (BCD) | | 0.79 | |
| H-2 | 1.26 | 0.82 | -35.0 |
| H-1 (Rha) | 0.97 | 0.74 | -23.8 |
| H-1 (Glu) | 0.96 | 0.58 | -40.0 |
| H-1 (BCD) | | 0.79 | |
| OH (Rha, Glu) | 1.03 | 0.6 | -41.9 |
| OH (Rha, Glu) | 1.08 | 0.73 | -32.3 |
| 6-OH (Glu) | 1.06 | 0.74 | -30.7 |
| 6-OH (BCD) | | 0.71 | |
| Rha, Glu, BCD | 1.06-0.64 | 0.77, 0.78 | |
| H-3 (*trans*) | 0.62 | 0.69 | +10.9 |
| H-3 (*cis*) | 0.57 | 0.6 | + 6.0 |
| $CH_3$ | 0.53 | 0.46 | -13.02 |

[a] Values measured at 300 MHz by the inversion recovery method [10].

[b] (+) Refer to enhancement and (-) to reduction.

in the distances it was difficult to obtain very accurate values for $\tau_c$. Hence, the values shown in Table IV were calculated for distances between 1.0–2.0 Å. Higher values observed for the sugar protons arise due to assumption of larger distances for the groups. The $\tau_c$ obtained can be related to various local motions, namely $\tau_m$ (overall rotating time) and $\tau_i$ (internal rotational time) [19] of the molecule by the following relation

$$\tau_c = A\tau_m + (B + C)(1/\tau_m + 1/\tau_i)^{-1}$$
$$A = 1/4\,(3\,\cos^2\,\theta - 1)^2$$
$$B = 3/4\,\sin^2\,2\theta$$
$$C = 3/4\,\sin^4\,\theta$$

where $\theta$ is the angle between the relaxation vector of the main field gradient (phenolic C–H bond axis and the principal axis of rotation C-1′–C-4′ of the phenolic group, which is the same as the BCD axis).

From the overall correlation time, $\tau_m$, and effective correlation time, $\tau_c$, the rate of internal rotation, $\tau_i^{-1}$, was calculated, Table IV. From $\tau_m$ and $\tau_i$ it is possible to

TABLE IV. Analysis of local motions in naringin-β-cyclodextrin.

| Group | $T_{1\ \text{complex}}$ | $\tau_c$ (ps) | | $\tau_i$ (ps) | | $\tau_c/\tau_m$ |
|---|---|---|---|---|---|---|
| | | Free | Complex | Free | Complex | |
| Phenolic[a] | | | | | | |
| 4′-OH | 0.84 | 1.37 | 1.57 | 0.85 | 1.85 | 0.12 |
| H-2′ & H-6′ | 0.97 | | | | | |
| H-3′ & H-5′ | 1.14 | | | | | |
| Dihydrobenzopyran | | | | | | |
| 5-OH | 1.95 | | | | | |
| H-6 & H-8 | 0.87 | | | | | |
| H-2 | 0.81 | 2.26 | 2.33 | 0.91 | 1.83 | 0.17 |
| H-3 (*cis*) | 0.6 | | | | | |
| H-3 (*trans*) | 0.69 | | | | | |
| Neohesperidoside | | | | | | |
| H-1 (Rha) | 0.73 | | | | | |
| H-1 (Glu) | 0.57 | | | | | |
| OH | 0.68 | 52.81 | 74.09 | | | |
| $CH_3$ | 0.46 | | | | | |
| BCD | | | | | | |
| H-1 | 0.79 | | | | | |
| 2-OH | 0.8 | | 13.65[b] | | | |
| 3-OH | 0.79 | | | | | |

[a] Values of $\tau_c$ and $\tau_i$ are average values.

[b] This value is equivalent to $\tau_m$.

evaluate the degree of coupling between the two rotamers, BCD and naringin. The coupling coefficient $\xi$, which is the ratio $\tau_c/\tau_m$, explains the efficiency of coupling quite well. A value of 0.17 and 0.12 observed for the dihydrobenzopyran and phenolic portions, respectively, indicate that the coupling between the aglycone portion and BCD is more towards the lower limit value of 0.1 and hence may be considered weak.

The overall correlation time $\tau_c$ for free and complexed naringin indicate that in general the values for the complex are higher due to an increase in size of the molecular assembly. Also, the internal rotations between free and complexed naringin indicate that the rotation of the phenolic and dihydrobenzopyran moieties decreases by a factor of 2 on inclusion inside the cyclodextrin cavity.

Since the aglycone portion was found to be affected, the inclusion of a phenyl group inside the BCD cavity explains the disposition of naringin better than interaction by hydrogen bonding of dihydrobenzopyran and phenyl group with BCD hydroxyl groups. Although DMSO, the solvent used for these studies, would be a

better ligand for BCD than the naringin phenol group, the efficiency of coupling, $\xi$, the binding constant value and the magnitude of the effects observed in various investigations reveal rather a weak nature of the complex.

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