Chiral Recognition by Cyclodextrins: the Interaction of Naringin with β-Cyclodextrin

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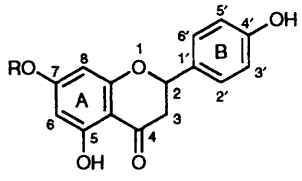
Complex formation between naringin, **1**, the bitter component of grapefruit, and β-cyclodextrin has been studied by ^{1}H NMR spectroscopy. Differential binding of the diastereoisomers of **1** to a polymer supported β-cyclodextrin column allowed partial separation of the isomers and a full assignment of the ^{1}H NMR spectrum of the mixture to be made. In aqueous solution, one diastereoisomer binds to β-cyclodextrin approximately 1.7 times more strongly than the other. The interaction occurs by inclusion of the aromatic ring B, but substantial chemical shift changes are also observed for certain protons in the disaccharide unit of **1**. Comparison of NMR data for the inclusion complex of the aglycone naringenin, **2**, suggests that the disaccharide unit is an important factor in the chiral recognition of **1** by β-cyclodextrin.

The complexing properties of β-cyclodextrin (β-CD) have long been known and many studies of the interaction of this molecule with organic and inorganic compounds have been carried out.[1,2] β-CD is a cyclic oligosaccharide containing seven α-(1→4) linked glucose residues with a central hydrophobic cavity, 650–800 pm in internal diameter. Externally, the hydroxy groups attached to C-2 and C-3 form a ring about the wider mouth of the cavity, with the primary hydroxy groups at C-6 positioned at the narrower end. The inner surface contains the 3-H, 5-H and glycosidic oxygen atoms. The 3-H atoms all lie in one transverse plane, towards the wider end of the cavity, and the 5-H atoms lie in another plane, which is closer to the narrower end.

Because the cyclodextrin molecule is chiral, different enantiomers or diastereoisomers of chiral substrates may form complexes with β-CD which have different association constants. This is the basis for the use of chromatography columns in which β-CD is attached to a solid support.[3] These columns are useful for enantiomer separations (resolution) and for checking the optical purity of pharmaceutical preparations. ^{1}H NMR spectroscopy has also been used to determine enantiomeric composition,[4,5] since the NMR spectra of optical isomers become distinguishable in the presence of β-CD if complexes are formed. Requirements for chiral recognition and stereoselective binding[3,6] include formation of an inclusion complex with a tightly fitting guest molecule, and proximity of the guest's chiral centre to the included group. There should also be an atom or group attached to the chiral centre which is capable of participating in a hydrogen bond with the secondary hydroxy groups at the mouth of the β-CD cavity. Crystal structures of β-CD complexes of (*R*)- and (*S*)-fenoprofen, 2-(3-phenoxyphenyl)propionic acid, have confirmed this general picture.[7] Only the complex with the (*S*)-isomer had the guest molecule in the correct orientation to form a hydrogen bond between the carboxylic acid group at the chiral centre and a secondary hydroxy of the β-CD. A study of the racemate showed that the (*S*)-isomer had the greater affinity for β-CD.

Naringin is the main flavonoid constituent of grapefruit and is also found in certain types of orange. It was first isolated in 1857 but its full structure (**1**) was not established until a century later.[8] Its chief characteristic is an intense bitterness which may be detected[9] at concentrations as low as 10^{-4} to 10^{-5} mol dm^{-3}. This bitterness can adversely affect the flavour of some processed citrus juices when the level of naringin is too high. Methods of reducing the bitterness have therefore been sought, including enzyme treatments[10] and adsorption of the naringin



1 R = α-L-Rha-(1→2)-β-D-Glc
2 R = H

by various resins.[11] In 1982, it was reported that the bitterness of citrus juices could be reduced by addition of β-CD to the juice, whence formation of inclusion complexes of naringin and limonin takes place.[12] Subsequently, it was shown that partial removal of these constituents from fruit juice could be achieved by treatment with insoluble β-CD polymers.[13] Various reagents were used to cross-link the β-CD monomer and the efficiency of different polymers was tested.[14] More detailed knowledge of the interaction between naringin and β-CD is therefore of relevance to fruit juice processors, and is also of interest in the context of studies on the basis of chiral recognition by cyclodextrins. Naringenin (**2**), the aglycone of **1**, contains a chiral centre, but exists in an optically inactive form in the naturally occurring glycoside. In this work, ^{1}H NMR is used to study the differential binding of the (2*R*) and (2*S*) forms of **1** to β-CD in solution and on a chromatography column.

Results and Discussion

Low-field and high-field regions of the ^{1}H NMR spectrum of naringin are shown in Figs. 1(*a*) and 2(*a*). These Figs. show all the resonances which were used to study the complexation of **1** by β-CD. All the carbohydrate resonances, except those indicated in the Figs., lie between δ 3.5 and 5.2. The spectrum of **1** was assigned by a double quantum filtered (DQF) COSY experiment and by comparison with the spectrum of naringenin (**2**). The spectrum of the aglycone part of **1** closely resembles that of **2**, except that in **1**, nearly all the resonances are split into two equally intense lines. The multiplets of some of the carbohydrate protons, *e.g.* 1-H (Glc) and 6-H (Rha), also contain twice the expected number of lines. This is because **1** containing a disaccharide unit with chiral centres, consists of diastereoisomers with (2*R*) and (2*S*) configurations, distinguishable by NMR; the enantiomers of the racemate **2** are indistinguishable.

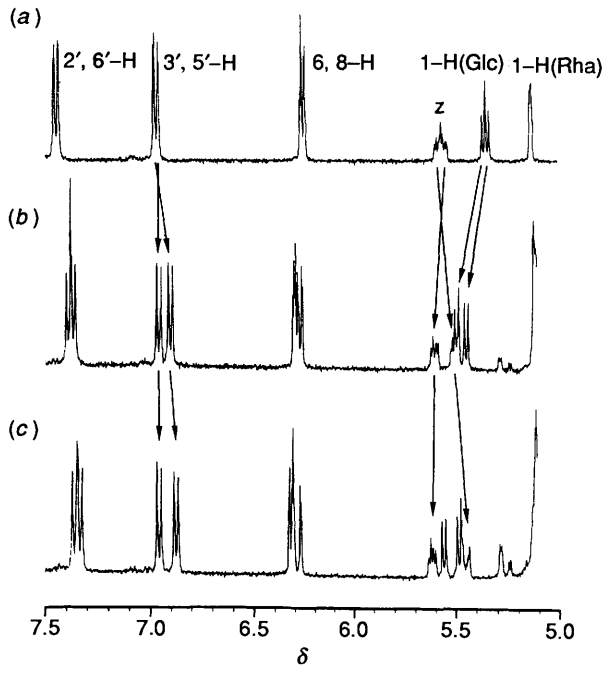


Fig. 1 400 MHz ^{1}H NMR spectra (low field region) of (*a*) **1**, (*b*) **1** + β-CD (1 : 1 molar ratio), (*c*) **1** + β-CD (1 : 3 molar ratio). Arrows indicate direction of displacement of resonances on addition of β-CD.

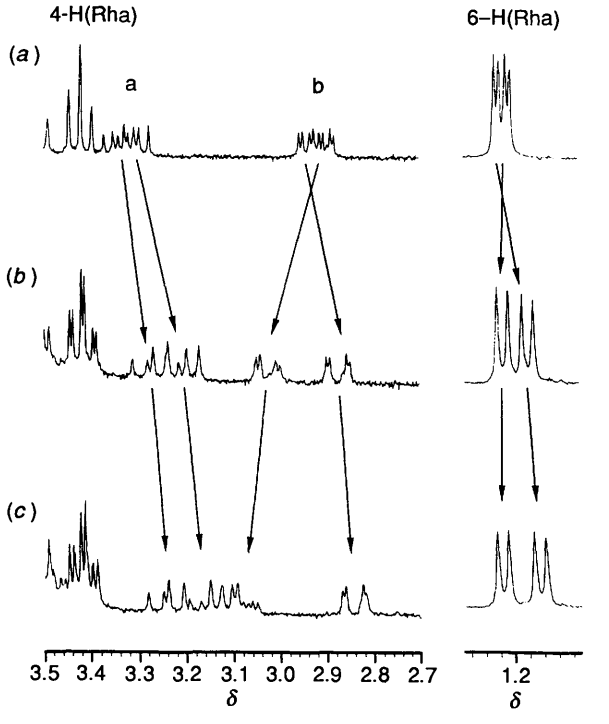


Fig. 2 400 MHz ^{1}H NMR spectra (high field region) of (*a*) **1**, (*b*) **1** + β-CD (1 : 1), (*c*) **1** + β-CD (1 : 3)

In **2**, the protons belonging to rings A and B were readily identified as they gave rise to characteristic AB (6-, 8-H) and AA'XX' (2'-, 6'-, 3'-, 5'-H) type spectra. The two pairs of protons in ring B were assigned by ^{13}C[^{1}H] selective decoupling experiments on **2** (dissolved in [$^2H_4$]methanol) which showed that the C-2', -6' and C-3', -5' resonances at δ 129.1 and 116.3, respectively, were decoupled by irradiation of the ^{1}H multiplets at δ 7.3 (2'-, 6'-H) and 6.8 (3'-, 5'-H). It is assumed that the same assignments hold for **1** and **2** dissolved in water, since little alteration is observed in the chemical shifts on changing the solvent from methanol (Table 1). The assignment agrees with a recent NMR study[15] of **2** in dimethyl sulfoxide. The 2- and 3-H protons gave rise to ABX type spectra in **1** and **2**. For conciseness, the ^{1}H NMR signals due to 3-H_{ax}, 3-H_{eq} and 2-H are referred to as a, b and z. The DQF COSY spectrum showed that in **1**, the high frequency z multiplet was correlated with the high frequency a and b multiplets; measurement of the coupling constants, J_{az} and J_{bz}, (see below) confirmed the assignment of the lines labelled a in Fig. 2(*a*) to 3-H_{ax}.

Table 1 Proton chemical shifts[a] of naringenin, **2**, and the diastereoisomers of naringin, **1**(I) and **1**(II). Samples dissolved in D_2O unless otherwise stated.

Proton	**2**[b]	**2**	**2** + β-CD[c]	**1**(I)	**1**(II)
2-H (z)	5.32	5.52	5.50	5.56	5.58
3-H_{ax} (a)	3.09	3.29	3.16[d]	3.32	3.34
3-H_{eq} (b)	2.68	2.85	3.00[d]	2.91	2.93
6-H[e]	5.89	5.96	5.87	6.25	6.25
8-H[e]	5.87	5.96	5.87	6.26	6.27
2'-,6'-H	7.30	7.46	7.37[f]	7.45	7.45
3'-,5'-H	6.81	6.98	6.90[f]	6.97	6.97
1-H(Glc)				5.34	5.36
1-H(Rha)				5.13	5.13
4-H(Rha)				3.43	3.43
6-H(Rha)				1.22	1.21

[a] δ (±0.01) Relative to tetramethylsilane. [b] In CD_3OD. [c] 1:2 Molar ratio (**2**:β-CD). [d] Resolved signals for two isomers, separation 0.01 δ units. [e] Assignments may be reversed. [f] Resolved signals for two isomers, separation 0.006 δ units.

Once the assignments had been made, the effects of addition of β-CD on the spectra of **1** and **2** were studied. Konno *et al.*[12] used changes in the chemical shifts of the 3-H and 5-H signals of β-CD to demonstrate that a complex was formed between **1** and β-CD. However, this gave no direct information on possible different interactions between the different diastereoisomers of **1** with β-CD; in the fast exchange limit, the chemical shifts of the β-CD protons are merely a weighted average of the shifts in free β-CD and in the two complexes. By observation of the chemical shift changes in the guest molecule, the interactions of the two diastereoisomers of **1** with β-CD can be studied separately.

Figs. 1(*b*), (*c*) and 2(*b*), (*c*) show the effects on the ^{1}H NMR spectrum of increasing the β-CD concentration at a fixed concentration of **1**. It can be seen that, in general, the frequency separation between multiplets assigned to different isomers of **1** increases with β-CD concentration. However, it is not easy to divide the resonances of **1** into two sets corresponding to the two isomers because the protons of **1** are grouped into isolated spin systems and the isomers are present in equal abundance. To overcome this, separation of the diastereoisomers was attempted using an FPLC (fast protein liquid chromatography) system with a column in which β-CD was attached to a sepharose support. Resolution of racemic **2** has previously been reported using cellulose triacetate as the column packing.[16]

The ^{1}H NMR spectrum of the first fraction to be eluted from the column is shown in Figs. 3(*a*) and (*b*), from which it can be seen that partial separation of the diastereoisomers of **1** was achieved. One diastereoisomer (designated I) evidently bound more strongly to the column, to give a set of resonances of reduced intensity compared with isomer II. The distinction between the two sets of signals is more clearly seen in Figs. 3(*b*) and 4(*b*), where β-CD has been added to the fraction to give better spectral dispersion. The lines or multiplets associated with isomer I have been marked with an asterisk. The chemical shifts are given in Table 1. Identification of the resonances associated with isomers I and II allowed plots of the chemical shifts *versus* β-CD concentration to be obtained (Figs. 5 and 6) from solutions containing a fixed concentration of **1**. It is then possible to estimate the dissociation constants for binding between the two isomers and β-CD. As separate peaks are not

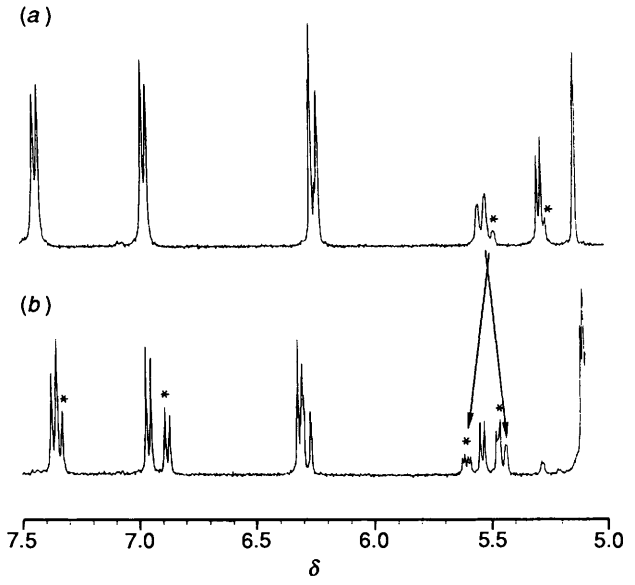


Fig. 3 400 MHz ^{1}H NMR spectra (low field region) of first fraction eluted from the column: (*a*) **1**, (*b*) **1** + β-CD (*ca.* 1:3). Resonances from the less abundant isomer I are marked *.

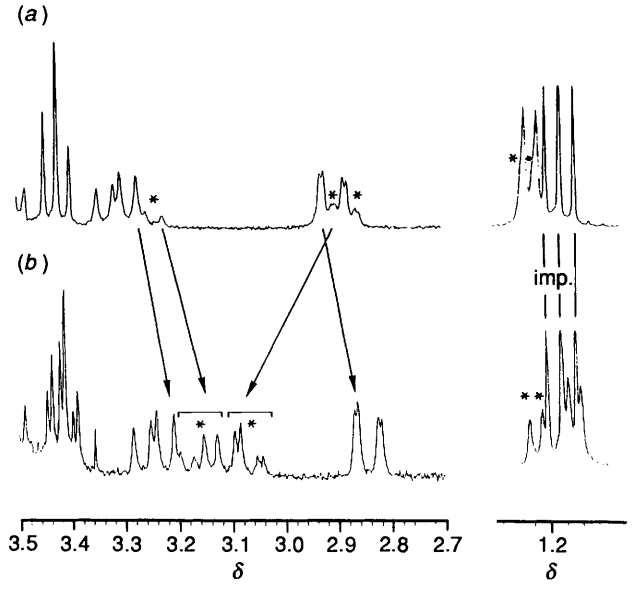


Fig. 4 400 MHz ^{1}H NMR spectra (high field region) of first fraction: (*a*) **1**, (*b*) **1** + β-CD (*ca.* 1:3). Resonances from isomer I marked *. Impurity is residual ethanol from eluent.

observed for the bound and free forms of **1**, exchange is fast on the NMR timescale and so the measured chemical shifts are weighted averages. Assuming that a 1:1 complex, GC, is formed between a guest molecule, G, and cyclodextrin, C, then eqns. (1)–(4) hold, where δ_{obs} is the measured chemical shift, δ_G

$$K_d = \frac{[G][C]}{[GC]} \quad (1)$$

$$[GC] = G_t \frac{\Delta}{\Delta_o} \quad (2)$$

$$\Delta = \delta_{obs} - \delta_G \quad (3)$$

$$\Delta_0 = \delta_{GC} - \delta_G \quad (4)$$

and δ_{GC} are the chemical shifts of a given proton of G in the free

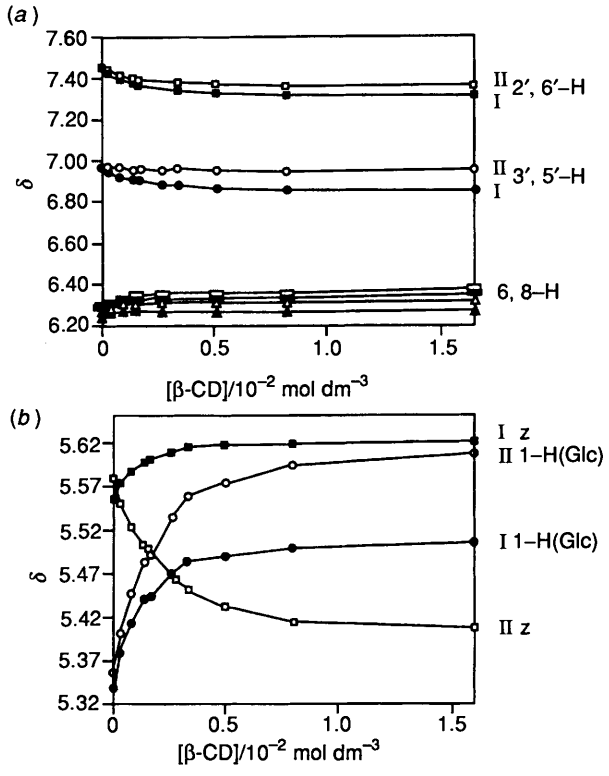


Fig. 5 Chemical shifts of naringin protons *versus* β-cyclodextrin concentration. Concentration of naringin was 1.495 × 10^{-3} mol dm^{-3}. Plots correspond to spectra in Fig. 1: (*a*) aromatic protons, (*b*) z and 1-H (Glc).

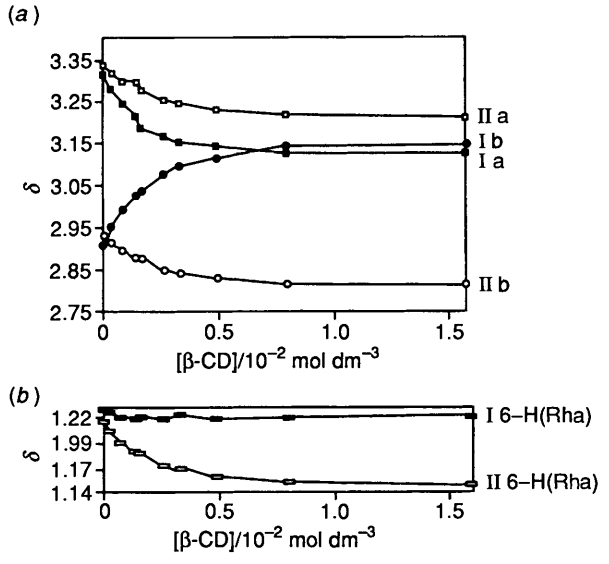


Fig. 6 Chemical shifts of naringin protons *versus* β-cyclodextrin concentration. Concentration of naringin was 1.495 × 10^{-3} mol dm^{-3}. Plots correspond to spectra in Fig. 2: (*a*) a and b, (*b*) 6-H(Rha).

and complexed forms, respectively, and G_t is the total concentration of G. Combining eqns. (1) and (2), it may be shown that the measured chemical shift is related to the total cyclodextrin concentration, C_t, by eqn. (5).[17] The data in Figs. 5

$$\Delta = \Delta_0 \left[\frac{(G_t + C_t + K_d) - [(G_t + C_t + K_d)^2 - 4G_tC_t]^{\frac{1}{2}}}{2G_t} \right] \quad (5)$$

and 6 were fitted to eqn. (5) using the program 'Tablecurve' to estimate values of the unknowns K_d and δ_{GC}. Values of Δ_0 obtained in this way are listed in Table 2. Although reasonable

Table 2 Proton chemical shift displacements (Δ_o) and dissociation constants (K_d) for β-CD complexes of isomers I and II of naringin **1**

	Δ_o[a]		$K_d/10^{-4}$ mol dm^{-3}	
Naringin proton	I	II	I	II
2-H (z)	0.07	−0.19	3.1 ± 0.8	10.5 ± 1.8
3-H_{ax} (a)	−0.20	−0.14	5.9 ± 1.0	11.7 ± 1.2
3-H_{eq} (b)	0.25	−0.14	8.2 ± 1.3	12.4 ± 1.3
6-H[b]	0.02	0.07	n.d.	n.d.
8-H[b]	0.05	0.08	3.3 ± 0.7	6.6 ± 0.5
2′-,6′-H	−0.15	−0.13	7.4 ± 1.0	10.9 ± 1.6
3′-,5′-H	−0.14	−0.02	8.3 ± 1.2	17.0 ± 10.4
1-H(Glc)	0.17	0.26	3.7 ± 0.5	6.6 ± 1.1
6-H(Rha)	−0.01	−0.07	5.1 ± 2.0	10.4 ± 1.0

[a] $\Delta_o = \delta_{complex} - \delta_{free}$ (±0.01). [b] Assignments may be reversed.

fits to the experimental data were obtained, use of this procedure is not strictly justified since naringin contains two isomers, the diastereoisomers I and II, and the equilibrium concentrations are determined by the two connected equilibria of eqns. (6) and (7). K_d^I and K_d^{II} were then calculated for each

$$G^I + C \rightleftharpoons G^IC \quad (6a)$$

$$G^{II} + C \rightleftharpoons G^{II}C \quad (6b)$$

$$K_d^I = \frac{[G^I][C]}{[G^IC]} \quad (7a)$$

$$K_d^{II} = \frac{[G^{II}][C]}{[G^{II}C]} \quad (7b)$$

β-CD concentration, C_t, using relationships similar to eqn. (2) to calculate $[G^IC]$ and $[G^{II}C]$. Values of Δ_o were taken from Table 2 and $G_t^I = G_t^{II} = 0.5\ G_t$. The other equilibrium concentrations were obtained from eqns. (8) and substituted

$$[G^I] = G_t^I - [G^IC] \quad (8a)$$

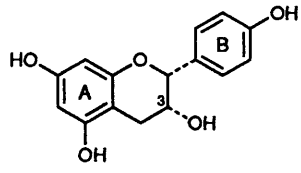
$$[G^{II}] = G_t^{II} - [G^{II}C] \quad (8b)$$

$$[C] = C_t - [G^IC] - [G^{II}C] \quad (8c)$$

into eqns. (7). Values of K_d listed in Table 2 are averages taken over all β-CD concentrations except the lowest.

There are unexpected differences, greater than the experimental errors, between the K_d values for the various protons. For example the values for 2-H (isomer I) and 1-H(Glc) (both isomers) are markedly lower than the average. This may indicate that the simple two-state model is not adequate to describe the system. Nevertheless, the results agree that, for all the protons examined, isomer I, which was found to bind more strongly to immobilised β-CD, also forms the stronger complex in solution. Taking average values for K_d^I and K_d^{II}, it is found that isomer I binds *ca.* 1.7 times more strongly than isomer II. This can be compared with recent reports of relative binding strengths of 1.2–1.6 for L- and D-isomers of *N*-2,4-dinitrophenyl amino acids [18] and 3–6 for several pairs of phenolic flavan-3-ols with (3*S*) and (3*R*) configurations.[19] Commercial treatment of grapefruit juice with β-CD polymers should lead to a change in molar ratio of the naringin diastereoisomers from 1 : 1, and this may be a means of detecting that such treatment has been used.

Previous work on complexation by β-CD [1,2] and consideration of steric factors suggest that complex formation involves inclusion of ring B of **1** in the β-CD torus. In agreement with earlier results,[12] it was found that the chemical shifts of 3-H and 5-H of β-CD moved upfield as the proportion of β-CD in complexed form increased, the change in shift of 5-H being slightly greater. Calculations of ring current effects have shown [20] that this behaviour is expected when an aromatic ring penetrates the CD cavity so that the centre of the ring is positioned between the planes formed by the 3-H and 5-H protons. The protons of ring B in **1** show upfield shifts on complexation (Table 2), although the change is very small for 3′-, 5′-H in isomer II. The direction of chemical shift displacement on inclusion of aromatic rings in β-CD appears to be very sensitive to the exact location of the guest molecule proton and is not readily predictable. For example, the three aromatic protons of the *N*-2,4-dinitrophenyl amino acids showed [18] upfield, downfield and zero displacement on complexation. In the β-CD complex of epiafzelechin (**3**), where the substitution of the B ring is the same as in **1**, the 6′-, 2′-H protons showed a



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downfield displacement and the 5′-, 3′-H protons no displacement.[19] It was considered that complexation of flavan-3-ols, of which **3** is a representative, could also take place by inclusion of ring A in the β-CD cavity. This possibility seems unlikely for **1**, where C-7 is substituted with the disaccharide unit.

3-H_{ax}, 3-H_{eq} and 2-H in the heterocyclic ring of **1** are close to the wider entrance of the β-CD cavity when complexed and their ^{1}H NMR signals show appreciable chemical shift displacements on complex formation. In the case of 3-H_{eq} and 2-H, the corresponding signals b and z are displaced in opposite directions in the two isomers. This suggests that the average position of the guest molecule with respect to the CD ring is strongly affected by the configuration at the chiral centre of the aglycone. (−)-Naringin is known to have the (2*R*) configuration,[21] but since in the present experiments complete separation of the two isomers was not achieved, it is not possible to say whether the (2*R*) or (2*S*) isomer binds more strongly to β-CD. However, the NMR parameters do show that the conformation of the heterocyclic ring is different in the two isomers when bound. Coupling constants for the 3-H_{ax} (a), 3-H_{eq} (b) and 2-H(z) protons are given in Table 3 for **1** and **2** in the free state and in the presence of β-CD (2 : 1 molar ratio of β-CD to **1** or **2**). These values are identical, within experimental error, for free **1** (both diastereoisomers) and **2**. The heterocyclic ring adjacent to ring A is non-planar and C-2 can be above or below the plane containing ring A. Thus the (2*R*) and (2*S*) isomers can both, in principle, have two low energy ring conformations, with aromatic ring B occupying an equatorial or axial position. The coupling constants and a simple form of the Karplus relationship [22] ($^3J_{HH} = 7 - \cos\theta + 5\cos 2\theta$, where θ is the dihedral angle) show, however, that ring B is equatorial both in **1** and **2**; in each case, one of the vicinal couplings, J_{az} is greater than 12 Hz, corresponding to a dihedral angle of approximately 180°.

The values of the coupling constants J_{az} and J_{bz} for the two isomers of naringin **1** change significantly in the presence of β-CD, with greater change from the free state being observed for diastereoisomer I than for II. For free naringenin **2**, the corresponding coupling constants are, of course, identical in its

Table 3 Proton coupling constants[a] for the heterocyclic rings of **1** (isomers I and II) and **2** in the free state and with added β-CD

J^b	**1**(I)	**1**(I) + β-CD[c]	**1**(II)	**1**(II) + β-CD	CD **2**	**2** + β-CD[c]
J_{az}	12.5	9.5	12.1	13.0	12.2	9.5[d]
J_{bz}	3.4	4.7	3.3	2.7	3.2	4.0[d]
J_{ab}	17.6	17.6	17.3	17.2	17.3	17.3[d]

[a] In D_2O. [b] ± 0.3 Hz. [c] 1:2 molar ratio of guest: β-CD. [d] Values identical for both isomers.

two enantiomers and furthermore, in both free and complexed states, these values are similar to those of diastereoisomer I of naringin. Other similarities between the two isomers of **2** and isomer I of naringin are their chemical shift displacements upon binding, of 3-H_{ax} (signal a, upfield) and 3-H_{eq} (signal b, downfield), the small displacement of the 2-H signal (z), and the upfield displacements of both sets of protons in ring B. These results suggest (i), that there is a change in the conformation of the heterocyclic ring on binding and (ii), that there are similarities between the binding of both isomers of the aglycone and isomer I of naringin, but that isomer II binds to β-CD in a different fashion. In turn, (ii) implies that the disaccharide unit may participate in the chiral recognition of **1** by β-CD.

It is interesting to note that the 1-H(Glc) and, to a lesser extent, the 6-H(Rha) resonances experience chemical shift changes on binding. Hydrophilic sugar rings are unlikely to enter the hydrophobic cavity of β-CD, and effects on the chemical shifts of protons so far removed from the proposed complexation site are unexpected. The chemical shift of 1-H(Rha) was found to remain unchanged in both isomers on addition of β-CD, so changes in the internal conformation of the (1→2)-linked disaccharide unit seem unlikely. The substantial downfield chemical shift displacement exhibited by 1-H(Glc) is more likely to result from a new short-range interaction between this proton and the hydroxy oxygen atoms ringing the β-CD cavity, the average distance being shorter for the complex of isomer II than for I. Unusual downfield shifts have been observed for particular protons in oligosaccharides and associated with the close approach of an oxygen atom from another sugar unit.[23]

It is difficult to give more precise details of the structures of the complexes on the basis of the NMR data. However, it is clear from a comparison of **1** and **2** that the presence of the sugar units in **1** is a significant factor in the chiral recognition of its two isomers by β-CD. (The sugar units are also involved in binding of **1** to the taste receptor because the aglycone **2** is flavourless.)[9] Both **1** and **2** appear to satisfy the requirements[3,6] in a guest molecule for chiral discrimination since the included aromatic group (ring B) has an adjacent chiral centre, with a substituent capable of forming a hydrogen bond. However, the differences observed in the NMR parameters upon complexation to β-CD are much greater for **1** than **2**. The effect of the disaccharide unit may simply be to prevent one possible mode of association (inclusion of ring A) but the chemical shift changes seen for 1-H(Glc) and 6-H(Rha) suggest a more direct form of interaction between the Rha–Glc unit and β-CD.

Experimental

A saturated solution of the sparingly soluble naringin (Fluka) was prepared in D_2O by sonicating a suspension and filtering through a 22 μm filter. The concentration of the solution, determined by UV spectroscopy, was 1.495 mmol dm^{-3}. Mixtures of β-CD (Sigma) and naringin for NMR spectroscopy were prepared by adding weighed amounts of solid β-CD to 1 cm^3 of the naringin stock solution. Partial separation of naringin diastereoisomers was carried out using a homemade β-CD column packing, prepared by Vretblad's method,[24] which entails reaction of β-CD with epoxysepharose (Sigma) to form the solid phase of the column. This was connected to an FPLC system (Pharmacia Fine Chemicals) using P500 pumps and a UV-1 detector (288 nm). The mobile phase was ethanol–water (10:90 v/v).

^1H NMR spectra were recorded in 5 mm o.d. tubes on a JEOL GX-400 spectrometer operating at 400 MHz. Sample temperature was regulated at 27 °C. 1000 Scans of 32K data points were collected for each sample, with a spectral width of 5 kHz and pulse delay of 2 s. Solvent presaturation during the pulse delay was used to suppress the residual HDO signal. The water resonance was used as the chemical shift reference (taken as δ 4.75 with respect to tetramethylsilane). The double quantum filtered COSY experiment was carried out in phase-sensitive mode using the method of States *et al.*[25] The data matrix was 2048 × 512 × 2 with a spectral width of 4000 Hz in both dimensions. Plots of naringin chemical shifts versus β-CD concentration were fitted to eqn. (5) using the program 'Tablecurve' (Jandel Scientific, USA) running on a personal computer.

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