Structure of Neohesperidin Dihydrochalcone/β-Cyclodextrin Inclusion Complex: NMR, MS, and X-ray Spectroscopic Investigation

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The inclusion complex between the semisynthetic sweetener neohesperidin dihydrochalcone (**1**) and β-cyclodextrin (βCD) has been investigated in solution, in the gas phase and in the solid state. In aqueous solution they form a 1:1 complex having a $K_{binding}$ of 1.6×10^3 M^{-1} at 315 K. The nuclear Overhauser effects show that the terminal isovanillin ring of **1** is included in the hydrophobic cavity of βCD from the narrower rim of the truncated cone. The peak at *m/z* 1747 corresponding to the supramolecular entity $[\beta CD + \mathbf{1} + H]^+$ was detected by MS−FAB measurements. The fragmentation pattern of **1** is different in the presence and in the absence of βCD, suggesting that the disaccharide moiety of **1** and the external polar surface of the host are tightly associated. In the solid state evidence of the formation of the βCD/**1** complex is obtained from the X-ray powder diffractograms which show diffraction peaks differing in position and intensity from those of native guest **1** and host βCD.

Keywords: *Neohesperidin dihydrochalcone; sweeteners; β-cyclodextrin; inclusion complexes; NMR; FAB−MS*

INTRODUCTION

A characteristic of citrus fruits is their content of flavonoid glycosides, which occur principally in the peel. These include the exceedingly bitter naringin and poncirin, neohesperidin, typical components of bitter orange (*Citrus aurantium*), and hesperidin, devoid of bitter taste, present in lemons and common sweet oranges (*Citrus sinensis*) (Horowitz and Gentili, 1963a). Each of these substances contains a disaccharide unit composed of a molecule of rhamnose and glucose: the rutinose occurring in hesperidin and the neohesperidose occurring in naringin, poncirin, and neohesperidin. The presence or the absence of bitterness seems related to the structure of such disaccharides, although their occurrence is not in itself sufficient to explain the different flavor of these substances. In fact during synthetic studies designed to identify the relationship between structure and bitter taste in the components of this class of glycosides, neohesperidin dihydrochalcone (**1**; 3,5-dihydroxy-4-(3-hydroxy-4-methoxyhydrocinnamoyl)phenyl-2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside; Chart 1) was obtained, showing a pronounced sweet taste (Horowitz and Gentili, 1963b). The material is easily accessible by simple chemical manipulation of naturally occurring naringin or neohesperidin. Subsequent organoleptic investigations revealed that the semisynthetic glycoside **1** could be useful in the food industry, not only as a sugar substitute but also as a flavor enhancer. This behavior seemed particularly important in chewing gum products (Westall et al., 1974), in which the problem of the flavor persistence is crucial due to the length of time the product remains in the mouth.

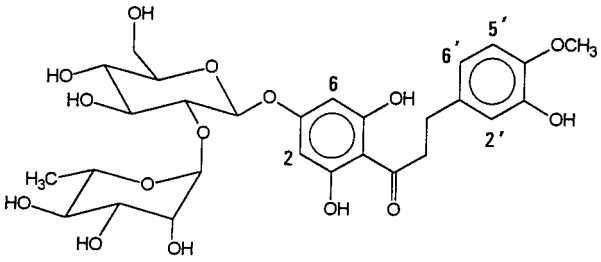
Now, after over 30 years from its discovery, **1** is receiving renewed interest due to its official approval as a food additive (Smith et al., 1996). In this context, in the light of current observations supporting the view that the inclusion into the hydrophobic cavity of cyclodextrins (CDs) causes modifications of solubility, bioavailability, and delivery properties of many bioactive molecules, including the artificial sweetener aspartame (Frömming and Szejtli, 1994), it was decided to characterize by physical means the inclusion product of neohesperidin dihydrochalcone into β-cyclodextrin (βCD). The problem seemed particularly challenging due to the structural complexity of **1**, containing a disaccharide moiety, linked to a diphenolic ketone, linked, in turn, through two methylene groups, to a second oxygen-substituted aromatic ring. By using X-ray, high-resolution NMR, and mass spectrometric (MS) techniques, we provide evidence that the βCD/**1** inclusion complex is formed in the solid state as well as in aqueous solution and in the gas phase. Proton NMR spectroscopy was used to determine the stoiochiometry and the association constant of the complex in water and the intermolecular nuclear Overhauser effects to obtain useful information about the complex geometry in solution. The precise knowledge of the nature of the inclusion product obtained by these means may be useful in the interpretation of the behavior of the complex in different food products (Chung, 1996; Sozzi and Del Viscio, 1997).

MATERIALS AND METHODS

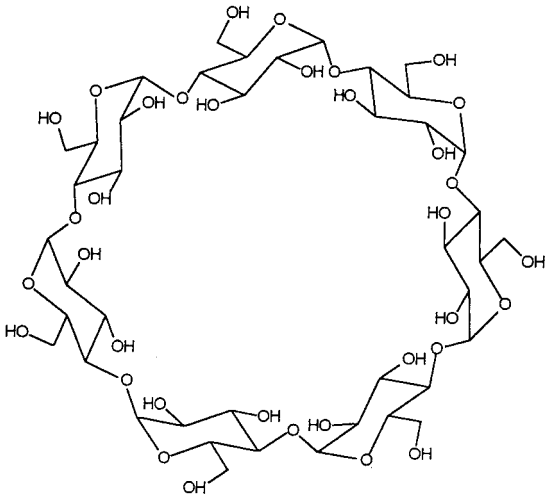
Neohesperidin dihydrochalcone was purchased from Sigma and used with no further purification. βCD was purchased from Roquette and used as received (H_2O content ca. 5% (w/w)). The inclusion complex neohesperidin dihydrochalcone/βCD in the solid state was obtained by freeze-drying a water

S0021-8561(97)00667-5 CCC: $15.00
Published on Web 03/14/1998

Chart 1



Neohesperidin Dihydrochalcone **1**



β-cyclodextrin

solution of the two components (kindly supplied by Dr. Daniel Joulain, Robertet SA, Grasse, France). Commercial 3-mercapto-1,2-propanediol (thioglycerol) was used as the liquid matrix for FAB−MS without any further purification.

The ^{1}H-NMR spectra were recorded on a Bruker ARX 400 at the operating frequency of 400.13 MHz. The chemical shifts are referred to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) in D_2O as external standard. The NMR samples were prepared by dissolving a suitable amount of **1** and βCD or of the complex in 0.5 mL of D_2O. In this solvent the hydrogens of the $-CH_2CO-$ group and the H2 + H6 protons of the tetrasubstituted aromatic ring of free **1** exchange rapidly with deuterium atoms. This exchange process occurs also for the complex, although it is slower than for the free **1**; the aromatic hydrogens H2 and H6 are completely exchanged after about 3 h, while for the methylene protons the process takes more than 24 h (see Figure 1). The assignment of the signals for free **1** and for the complex was made through 1D-TOCSY experiments. The 2D-ROESY (rotating frame nuclear Overhauser effect spectroscopy) experiment was carried out on a Bruker 600 MHz instrument on a 12 mM solution of the 1:1 complex dissolved in the mixture H_2O/D_2O (90:10) at 305 K. In this solvent the exchangeable hydrogens do not desappear during the acquisition of the data, thus preventig the loss of dipolar interactions. The strong H_2O peak was suppressed using a selective presaturation pulse of 2 s. The experiment was performed in phase sensitive mode using the time proportional phase incrementation method (TPPI) with a spin-lock time of 300 ms. The Hartmann−Hann coherence transfer was minimized by using an off resonance frequency for the spin-lock.

The stoichiometry of the complex was determined by the continuous variation method (Job plot) (Connors, 1987). The overall concentration of the species was kept constant ([H] + [G] = 8 mM) and the ratio r = [H]/[H + G]) was varied from 0 to 1 ([H] and [G] are the total concentrations of host and guest, respectively). The quantity ($\Delta\delta$[H]) was plotted against r ($\Delta\delta$ is the difference between the chemical shift of the free H and the observed value of H for a given ratio r). The association constant $K_{binding}$ was determined using the same experimental data of the Job plot. For this purpose the equations developed by Grosenick et al. (1996) were used, and the mathematical treatment is not repeated here. The equation relating the chemical shift of a given proton of the complex with the association constant has been solved by using the iterative procedure Genfit available in the Mathcad 6.0 Plus software package.

Powder X-ray diffraction patterns were taken at 25 °C by using Cu Kα radiation with an Italstructure θ/θ diffractometer equipped with step-scan attachment (step width, 0.05° of 2θ; count time, 8 s/step; scanning speed, 0.375 deg/min; voltage, 40 kV; current, 30 mA), in the $4° \le 2\theta \le 50°$ range. All of the samples have been finely ground in an agate mortar. Each diffraction pattern was collected twice, obtaining reproducible results.

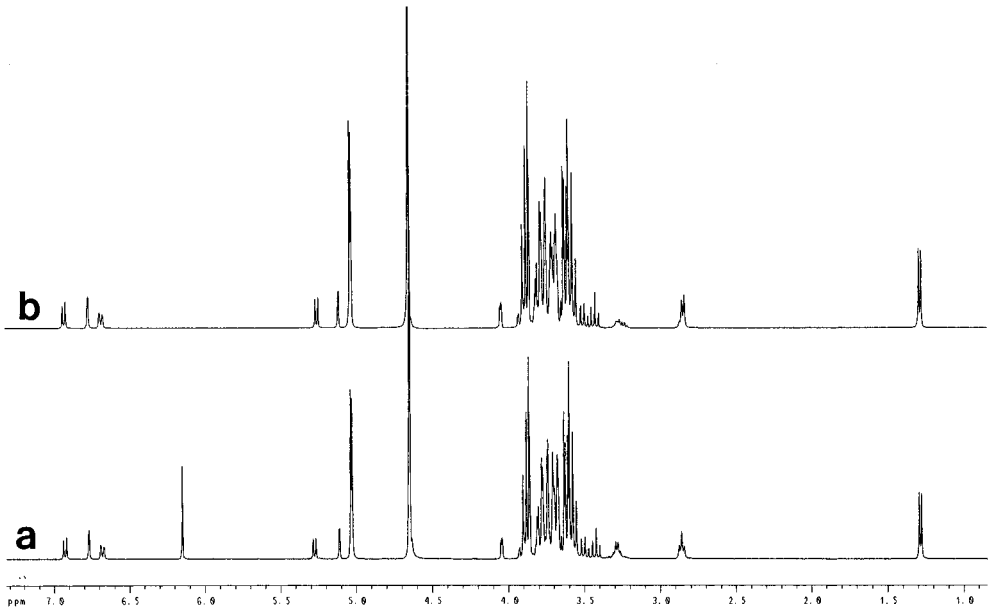


Figure 1. Proton spectrum of a 4 mM 1:1 mixture of the complex neohesperidin dihydrochalcone/βCD in D_2O (a) immediately after dissoution and (b) after ca. 10 h.

Table 1. Chemical Shift Values (δ, ppm) of the Protons of Neohesperidin Dihydrochalcone and βCD in D_2O Solution[a]

proton	βCD	**1**	1:1 complex βCD/**1**	$\Delta\delta$ (complex-free)
H1 βCD	5.057		5.035	−0.022
H2 βCD	3.639		3.621	−0.018
H3 βCD	3.941		3.882	−0.059
H4 βCD	3.564		3.575	0.011
H5 βCD	*b*		3.702	
H6 βCD	*b*		*c*	
H6′ βCD	*b*		*c*	
H1 G		5.277	5.263	−0.014
H2 G		3.666	*e*	
H3 G		*d*	*e*	
H4 G		3.521	3.502	−0.019
H5 G		3.638	*e*	
H6 G		*d*	*e*	
H6′ G		3.942	3.916	−0.026
H1 R		5.158	5.124	−0.034
H2 R		4.072	4.046	−0.026
H3 R		3.729	3.687	−0.052
H4 R		3.448	3.426	−0.022
H5 R		3.826	3.798	−0.028
CH3 R		1.255	1.263	0.08
H6′		6.785	6.688	−0.097
H5′		6.964	6.929	−0.035
H2′		6.82	6.767	−0.053
OCH3		3.847	3.86	0.013
CH_2CO		3.408	3.339	−0.069
CH_2Ph		2.904	2.873	−0.031
H2 + H6		6.049	6.112	0.063

[a] Notation: H*i* G = *i*th proton of glucose of compound **1**; H*i* R = *i*th proton of rhamnose of compound **1**; H*i* βCD = *i*th proton of glucose of βCD. For the other protons, see numbering in Chart 1. [b] Multiplet in the range 3.90−3.81. [c] Multiplet in the range 3.82−3.72. [d] Multiplet in the range 3.79−3.71. [e] Multiplet in the range 3.78−3.57.

The samples for FAB−MS analysis were prepared by dissolving the complex in the liquid matrix in order to obtain an overall complex concentration in the range 5×10^{-2} M and 10^{-2} M. Fast atom bombardment−MS (FAB−MS) and tandem MS spectra were obtained on a Finnigan-MAT TSQ70 triple stage quadrupole machine equipped with an Ion-Tech (Teddington, U.K.) atom gun with Xe as the bombarding gas. The emission current was typically set at 2 mA with an accelerating voltage of 8 keV. For all of the experiments the source was maintained at room temperature. CsI was used for mass calibration. Tandem MS experiments were performed using Ar as the collision gas ($p = 8 \times 10^{-4}$ Torr, 1 Torr = 133.3 Pa; nominal collision energy, $E_{lab} = 10$ eV). All of the spectra were acquired in centroid mode and calculated over an average of 20−60 scans using standard Finnigan software.

RESULTS AND DISCUSSION

NMR Spectroscopy. High-resolution NMR spectroscopy provides one of the most powerful experimental tools for the study of host−guest chemistry in solution (Inoue, 1993). Chemical shift variations of selected host and guest protons reflect the formation of a complex between them. In particular, the entry of an apolar part of the guest into the lipophilic cavity of the host induces a shielding of the inner protons of the glucose units of β-cyclodextrin, namely, H3 and H5. Consequently, H3 and H5 chemical shift variation to higher fields are the first evidence of inclusion. Table 1 reports the chemical shift in D_2O solution of the protons of **1**, βCD, and their 1:1 inclusion complex; the ^{1}H spectrum of the complex is reported in Figure 1. Both H3 and H5 of βCD undergo a high-field shift. The chemical shift variation of H5 could only be estimated qualitatively due to strong peak overlap, while H3 in the complex is shifted 0.059 ppm upfield with respect to native βCD. These data indicate that the interaction of **1** with βCD is a true inclusion. A participation of the external protons of βCD (e.g. H1, H2, and H4) to the binding with **1**, however, cannot be ruled out, as complexation induced chemical shift variation of those signals is also observed (refer to Table 1). The signals of **1** cannot be used to obtain information on the binding since we noticed that the ^{1}H spectrum of **1** is strongly affected by concentration shifts, probably due to self-association of **1** in D_2O solution.

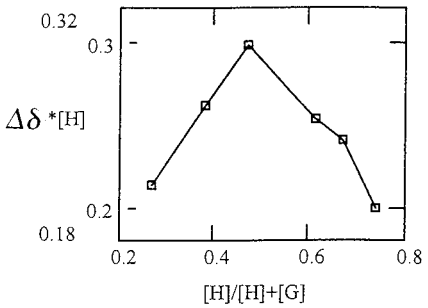


Figure 2. Job's plot for the determination of the stoichiometry of the complex. Abbreviations: [H] = concentration of the host (βCD); [G] = concentration of the guest (compound **1**); $\Delta\delta$ = chemical shift variation of selected proton (H3 of the host). The maximum of the curve is for [H]/[H + G] = 0.5, corresponding to the 1:1 complex.

The complexation induced chemical shift of H3 of βCD has been used to work out the stoichiometry of the complex and the association constant (Connors, 1987). According to the continuous variation method (see Materials and Methods), the resulting stoichiometry is 1:1. The graphical output of the determination (Job's plot) is reported in Figure 2. The association constant in D_2O solution at 315 K was determined via an iterative nonlinear fitting of the chemical shift variation of H3 of βCD and taking into account the 1:1 stoichiometry. For the βCD/**1** inclusion complex a $K_{binding}$ = of 1.6 10^3 M^{-1} was measured.

Deeper insights into the geometry of binding were obtained by measuring intermolecular nuclear Overhauser enhancements (NOEs) between the protons directly involved in the host−guest interaction. The intensity of the observed NOEs is a function of the molecular tumbling parameter $\omega\tau_c$, where ω = the Larmor frequency and τ_c = the rotational correlation time (Neuhaus and Williamson, 1989). Very often $\omega\tau_c$ values for βCD complexes are such that the system is in the region of small to null NOEs, making it difficult to obtain structural information. This problem is normally overcome by collecting the NOEs in the rotating frame (ROEs). The two-dimensional NMR correlation experiments mediated by NOE in the rotating frame (ROESY) are commonly used for the structural characterization of βCD complexes, as reported in the recent literature (Fronza et al., 1996).

The ROESY spectrum performed on the 1:1 complex dissolved in the H_2O/D_2O (90:10) mixture with a mixing time of 300 ms is reported in Figure 3a. Significant cross-peaks due to the intermoleculecular dipolar interactions are observed between the hydrogens H3 and H5 of βCD and several hydrogens belonging to the methylene groups and to the terminal aromatic ring of the guest. An expansion of the ROESY spectrum showing the NOEs of the protons of the isovanilline ring is reported in Figure 3b. Hydrogens H2′ (6.76 ppm) and

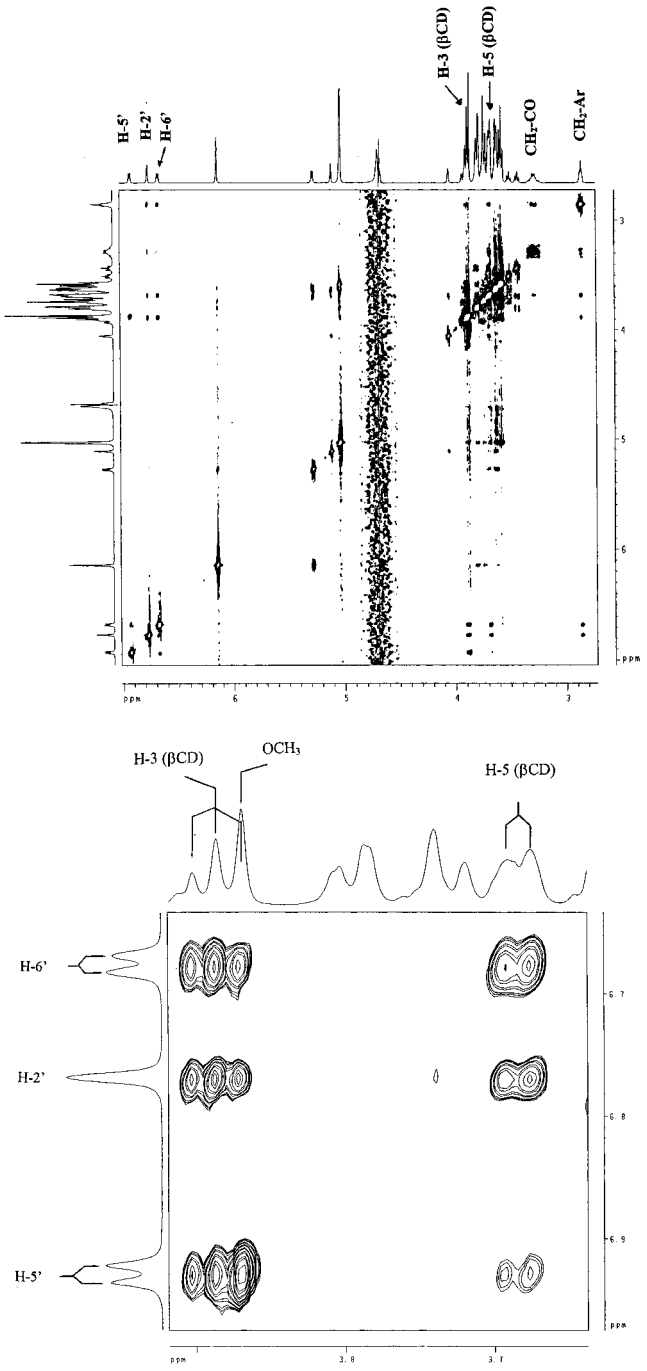


Figure 3. (a, top) 2D-ROESY spectrum performed at 600 MHz on a 12 mM solution in H_2O/D_2O (90:10) of the 1:1 βCD/1 complex at 305 K (mixing time 300 ms). The water signal was suppressed using a presaturation pulse of 2 s. The methyl group of the rhamnose residue is outside the plot limits. Expansion of the ROESY spectrum reported in a, showing the cross-peaks between the hydrogens of the isovanillin ring of neohesperidin and the hydrogens H3 and H5 of βCD.

H6′ (6.69 ppm) show strong dipolar contacts with both H3 (3.89 ppm) and H5 (3.69 ppm) of βCD. On the contrary H5′ (6.93 ppm) displays, in addition to the intramolecular NOE with the *ortho* methoxyl group (3.86 ppm), a strong effect with H3 and a much smaller one with H5 of βCD. In addition to these interactions other important NOEs can be observed between the hydrogens of the methylene group linked to the isovanillin ring (triplet at 2.87 ppm) and both H3 and H5 of βCD and between the $-COCH_2-$ hydrogens (multiplet at 3.34 ppm) with H5 only of βCD. Taken together these

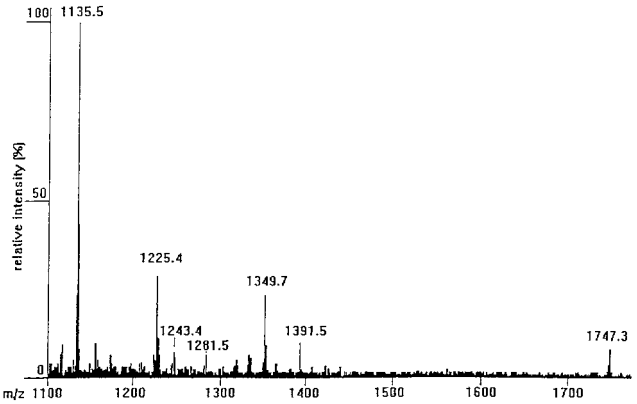


Figure 4. FAB mass spectrum of the 1:1 complex βCD/1. The peak at *m/z* 1747 corresponds to $[\beta CD + Neo + H]^+$, with Neo = compound **1**.

data are clear evidence that **1** and βCD form an inclusion complex with the isovanillin ring penetrating the cavity of βCD. Moreover the observations that the proton H5′ interacts mainly with H3 of βCD and that the hydrogens H2′, H6′ and $-CH_2-$Ar interact with both H3 and H5 of βCD while the $-COCH_2-$ group shows NOE only with H-5 of βCD indicate that the inclusion must occur from the narrower rim of the truncated cone of βCD.

Mass Spectrometry. The use of *soft* ionization techniques such as ionspray (IS), electrospray (ESI), and fast atom bombardment (FAB) has recently allowed the exploitation of mass spectrometry for the study of gaseous non-covalent association of cyclodextrins with a large variety of guest molecules (Selva et al., 1996). In the present case, the 1:1 gaseous protonated non-covalent association of **1** with βCD by FAB−MS using 3-mercapto-1,2-propanediol (thioglycerol) as the liquid matrix was studied. The FAB−MS spectrum of a sample of the 1:1 complex βCD/**1** is shown in Figure 4. The base peak at *m/z* 1135 is due to protonated βCD; the other spectral peaks observed in the acquisition mass range can be grouped in two families: (i) host−matrix clusters and (ii) host−guest associations. To the first group belong to the signals at *m/z* 1225 $[\beta CD + thioglycerol - H_2O + H]^+$ (rel intens, 27%), 1243 $[\beta CD + thioglycerol + H]^+$ (rel intens, 6%), 1333 $[\beta CD + 2thioglycerol - H_2O + H]^+$ (rel intens, 4%), 1349 $[\beta CD + 2thioglycerol - H]^+$ (rel intens, 22%), and 1391 $[\beta CD + 2thioglycerol + H_2O + Na]^+$ (rel intens, 5%). The formation of a large number of protonated or cationized CD−matrix associations is not uncommon in FAB−MS. A critical discussion of the possible role of the liquid matrix in the study of gaseous non-covalent associations has been reported elsewhere (Mele et al. 1997) and will not be repeated here. The signals at *m/z* 1747 and 1281 can be associated with the host−guest complex. Indeed, *m/z* 1747 corresponds to the protonated 1:1 host−guest association $[\beta CD + \mathbf{1} + H]^+$, while *m/z* 1281 is a fragment of 1741, as confirmed by tandem MS experiments on the parent ion *m/z* 1741. The structure of the fragment ion at *m/z* 1281 can be reasonably attributed to βCD linked, either covalently or non-covalently, to a fragment of neohesperidin containing the ion of nominal mass of 147 Da. This latter one is originated by the cleavage of the rhamnose−glucose glycosidic linkage and can be represented as $[rhamnose - H_2O + H]^+$. It is interesting to observe that this fragment is not obtained under collision activated decomposition (CAD) of native protonated neohesperidin dihydrochalcone (*m/z*

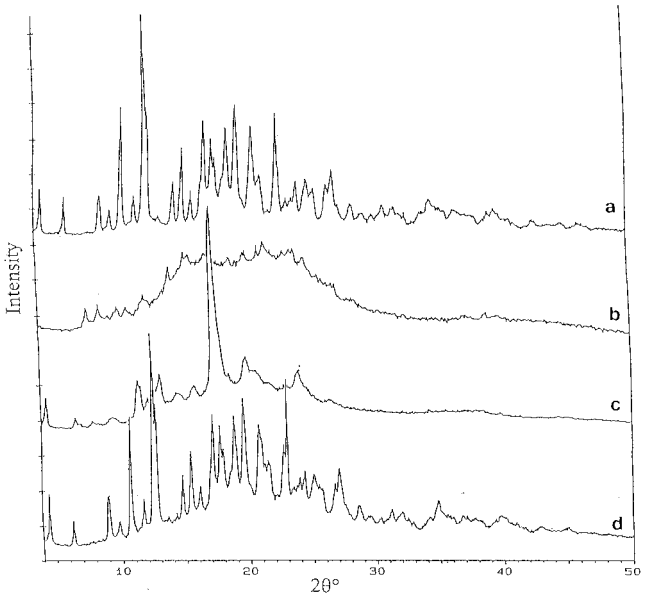


Figure 5. X-ray diffraction patterns of (a) crystalline βCD, (b) neohesperidin dihydrochalcone, (c) the 1:1 complex βCD/1; and (d) the 1:1 mechanical mixture of βCD and **1**.

613), namely, in the absence of the host βCD. This means that the presence of the host βCD can alter the fragmentation pattern of the guest, according to a tight association of the host with the guest involving not only the apolar aglicon of **1** but also the sugar moiety, likely to interact with the external polar surface of the host βCD. This result is consistent with the aglycon of **1** interacting with the apolar cavity of βCD, leaving the sugar residue of **1** outside the βCD cavity, free to interact with the surface of βCD itself, possibly via a hydrogen bonds network.

X-ray Powder Diffractometry. The analysis of the X-ray powder diffraction patterns of cyclodextrins inclusion compounds is a powerful and very well assessed method for the characterization of the complexes in the solid state (Saenger, 1980). The solid state of the inclusion compound has been investigated also with the X-ray powder diffractometry, and the complex formation has been tested by the comparative analysis of four diffractograms (Figure 5a−d): (a) βCD, (b) neohesperidin dihydrochalcone (**1**), (c) 1:1 complex βCD/**1**, and (d) 1:1 mechanical mixture of βCD and **1**.

The diffraction pattern of βCD (Figure 5a) is typical of a rather well crystallized compound. The unit cell parameters have been determined through an X-ray single-crystal analysis. The results can be summarized as follows: orthorhombic; $a = 15.115(3)$, $b = 30.780(5)$, $c = 41.589(6)$ Å. The diffractogram of **1** shows a wide halo above the otherwise linear background (in the region $6° < 2\theta < 50°$; see Figure 5b) and, superimposed on the halo, some representative peaks in the region $7° < 2\theta < 25°$. The presence of the halo reveals a structurally disordered or amorphous product, even if the presence of the sharp peaks indicates some degree of crystalline order.

The diffractogram of the 1:1 complex βCD/**1** (Figure 5c) reveals the presence of both a disordered (amorphous) and crystalline phase, indicating that the product is partially crystalline. The shape of the amorphous halo is similar to that observed for compound **1** and can be then associated to this latter. On the other hand, the diffraction peaks differ in position and intensity from those of native βCD and can be attributed to a new crystalline phase, evidently originated by the molecular interaction of the host βCD and the guest **1**.

Finally, the diffractogram of a 1:1 mechanical mixture of βCD and **1** (Figure 5d) was collected; the profile fits very well the sum of the diffraction patterns shown in Figure 4a,b. The diffraction patterns displayed in Figure 4c,d are remarkably different, thus confirming that the 1:1 complex is a genuine non-covalent compound between the two components βCD and **1**.

CONCLUSIONS

βCD complexes find the most promising applications in pharmaceutical formulations (Frömming and Szejtli, 1994), but potentially they may be of wide use also in the food industry. Many patents claim the application of CDs for stabilization of food flavors and improvement of food quality (Szejtli, 1988). For instance βCD, for its ability to make complexes with the flavonoid glycosides, may be useful in reducing the level of the bitter naringin and limonin components from orange and grapefruit juices (Wilson et al., 1989; Spalding, 1987) or in preventing the precipitation of the flavonoid glycosides in soft drinks (Szejtli, 1988). Recently a study was conducted to examine the changes of taste quality of neohesperidin dihydrochalcone solutions using βCD as a taste modifier (Chung, 1996). It was found that βCD produced a significant effect on the reduction of aftertaste and sweetness of neohesperidin. Despite these observations the complex βCD/**1** was successfully applied as bitter-taste reducing agent for the antibacterial drugs dequalinium chloride and dequalinium acetate in a chewing gum formulation (Sozzi and Del Viscio, 1997). All of these potential applications are clear evidence of the occurrence of nonbonding interactions between the flavonoid glycosides and βCD. Our results discussed above for neohesperidin dihydrochalcone (**1**) provide a detailed picture of the nature of these interactions. We have shown that **1** forms a 1:1 complex in water solution, with the terminal isovanillin ring penetrating the hydrophobic cavity of βCD from the side of the primary hydroxyl groups. The complex is present in the solid state as shown by X-ray powder diffractometry, and it has been detected also in the gas phase by FAB−MS measurements. Very interestingly the MS fragmentation pattern suggests that a specific interaction should exist, at least in the gas phase, between the terminal rhamnose saccharide unit of **1** and the external surface of βCD, most probably through the formation of intermolecular hydrogen bonds.

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Received for review August 5, 1997. Revised manuscript received December 31, 1997. Accepted January 22, 1998.

JF970667D