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A Study of Multiple Complexation of α-, β- and γ-Cyclodextrins: Surprisingly Differing Stoichiometries of β- and γ-Cyclodextrin Complexes

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Chromatographic, NMR and molecular modelling studies indicate that benzene-*sym*-tris-*N,N,N*-carbonyl-triglycylglycine *N′*-1-adamantylamide forms the strongest complex with β-cyclodextrin (β-CD), encapsulating the terminal adamantyl group. The analogous complex with γ-CD is weaker with a deeper penetration of the guest into the host cavity, while the complex with α-CD is very weak. Remarkably, longitudinal relaxation times T_1 for the complexes with β- and γ-CD exhibit considerable differences in the signals for the adamantyl protons while no differences in the corresponding signals for the branch protons were found. Chromatographic measurements for the complexes with β- and γ-CD with the dendrimer revealed interesting differences in the stoichiometry of the dendrimer complexes in mixed solvents chosen because of the low solubility of the dendrimer under study and its CD complexes. Namely, in a 60:40 (v/v) methanol:water mixture the latter complex was undoubtedly of 1:1 stoichiometry while for the former the most plausible stoichiometry was 1:3. The binding constants of the multiple dendrimer complexes with β-CD (in 20:80(v/v) ethanol:water) of 1:1, 1:2 and 1:3 stoichiometry were estimated to be equal to *ca* $4 \times 10^2\,M^{-1}$, $1 \times 10^2\,M^{-1}$ and $25 \times 10^3\ M^{-1}$ while only a 1:1 complex with a binding constant of *ca* $6 \times 10^2\,M^{-1}$ (correlation factor $R = 0.97$) was found for the dendrimer complex with the larger γ-CD (in 60:40 (v/v) methanol:water). (Attempts to fit the experimental curve assuming a mixture of complexes of 1:1 and 1:2 stoichiometry resulted in a value for the constant K_2 three orders of magnitude less than that for K_1.) The considerably larger value for K_3 compared with K_1 and K_2 seems to indicate that the dendrimer complex formation is cooperative. Such behaviour may be due to the more hydrophilic environment of the third adamantyl group in the 1:2 complex favouring its complexation. Chromatographic measurements for the compound mimicking one dendrimer branch yielded a 1:1 complex with γ-CD with a binding constant of $1 \times 10^2\,M^{-1}$ while it was shown to complex at both adamantyl and benzene ends by β-CD with the respective constants of $20 \times 10^2\,M^{-1}$ and $2 \times 10^2\,M^{-1}$.

Keywords: Cyclodextrin complexes; Dendrimer; Stoichiometry; Chromatography; NMR; Molecular modelling

INTRODUCTION

Dendrimer [1–4] studies are becoming of increasing interest because of the unusual structure of dendrimers, characterized by their high end-group functionality and dynamic character on the one hand, and current (in medical diagnostics) [5] and prospective (as drug-delivery systems, DNA biosensors, light-harvesting antennae, etc.) [1–4] applications on the other. Studies of dendrimers containing amino acid moieties are of particular interest, although few dendrimers incorporating amino acids as end-groups or in their branches or even as a core have been reported [6–9]. Gadolinium dendrimer complexes (produced by Schering AG, Berlin) are used as contrast agents in magnetic resonance imaging (MRI) diagnostics. To the best of our knowledge, only one type of dendrimer involving amino acids in branches and adamantyl end-groups has been reported [10]. Several papers describing cyclodextrin complexes with dendrimers

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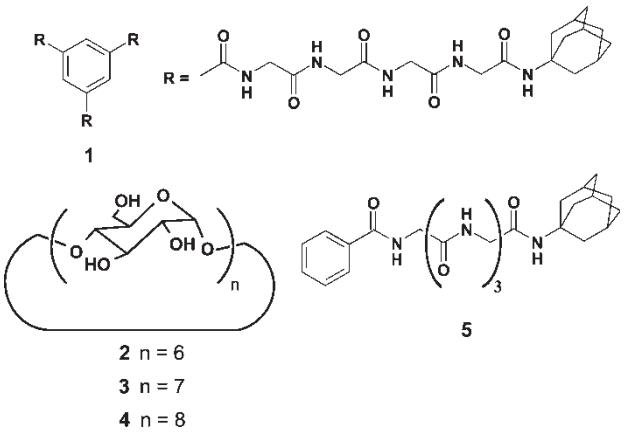
have been published [10–14]. However, the stoichiometry of the complexes could not be determined for the neutral dendrimers because of their low solubility. The only measurements of this kind have been described by Michels *et al.* [10] at pH2 for the more soluble complexes involving completely protonated adamantyl terminated poly(propylene imine) dendrimers that exhibited a fully stretched conformation facilitating the formation of cyclodextrin complexes.

We have recently described the synthesis of benzene-*sym*-tris-*N*,*N*,*N*-carbonyltriglycylglycine-*N*′-1-adamantylamide **1** and some preliminary studies of its complexes with α-, β- and γ-cyclodextrins (α-,β- and γ-CDs, **2**–**4**), respectively [15]. In this report, chromatographic, NMR and modelling studies of these complexes and those involving **5** modelling a branch of the dendrimer **1** are presented with the surprising finding of different stoichiometries for the complexes of **1** with **2** and **3**.



RESULTS AND DISCUSSION

NMR Studies

The signals of both adamantyl protons and carbons of free and CD complexed **1** in mixed 2:1 DMSO-d_6:D_2O solution revealed small but definite complexation-induced shifts (CIS) [15]. The atom numbering for the dendrimer **1** is given in Fig. 1 and its ^{1}H spectrum is presented in Fig. 2. The inclusion character of

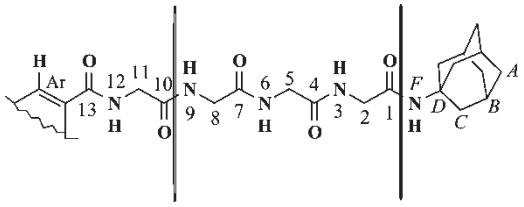


FIGURE 1 Atom numbering of dendrimer **1**.

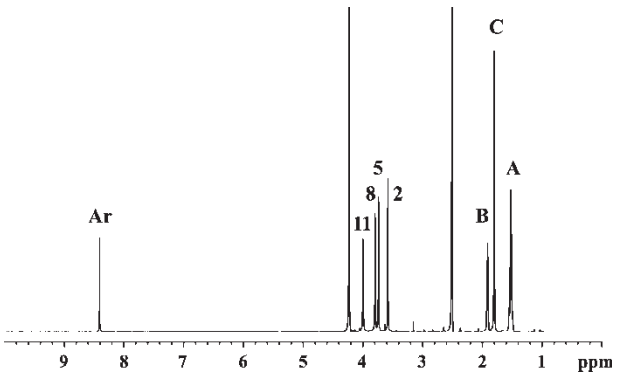


FIGURE 2 ^{1}H spectrum of dendrimer **1** in 2:1 DMSO-d_6:D_2O.

the complexes with β-CD **3** and γ-CD **4** is unequivocally revealed by the NOESY spectrum presented in Fig. 3, clearly exhibiting cross-peaks between the adamantyl protons of the guest and H3′ and H5′ CD protons directed into the cavity. Interestingly, the cross-peaks in the complex with **3** are equal while for the complex with **4** the cross-peaks involving H3′ protons are considerably weaker than those involving H5′ protons, indicating a deeper penetration of the guest into the γ-CD cavity. No cross-peaks were observed for the complex with α-CD **2**.

Longitudinal relaxation times (T_1) presented in Table I were determined only for D_2O solutions of the complexes of **1** with **3** and **4**. In agreement with expectation, the values measured for both complexes exhibited almost no differences for aromatic and methylene protons while the values for the adamantyl B and C protons differed considerably. The value for the B signal was considerably larger for the complex with β-CD **3** than the corresponding value for the complex with **4** while the opposite situation was found for the C protons. These differences are understandable in view of the different depth of the guest penetration into the host cavity as well as the strength and stoichiometry of the complexes of **1** with **3** and **4** determined by chromatographic measurements and molecular modelling described below.

Molecular Modelling

To mimic the complexation of **1**, molecular mechanics [16] calculations for the complexes of **5** mimicking a dendrimer **1** branch with CDs **2**–**4** were carried out. They revealed that the adamantyl end-group 'sat' on top of α-CD, but entered the cavity of **3** and **4**. In agreement with the NMR results, the penetration for **3** was found to be considerably shallower than that for the γ-CD host.

The results of NMR measurements and molecular mechanics modelling of the complexes suggest the following interpretation: the α-CD cavity is too small

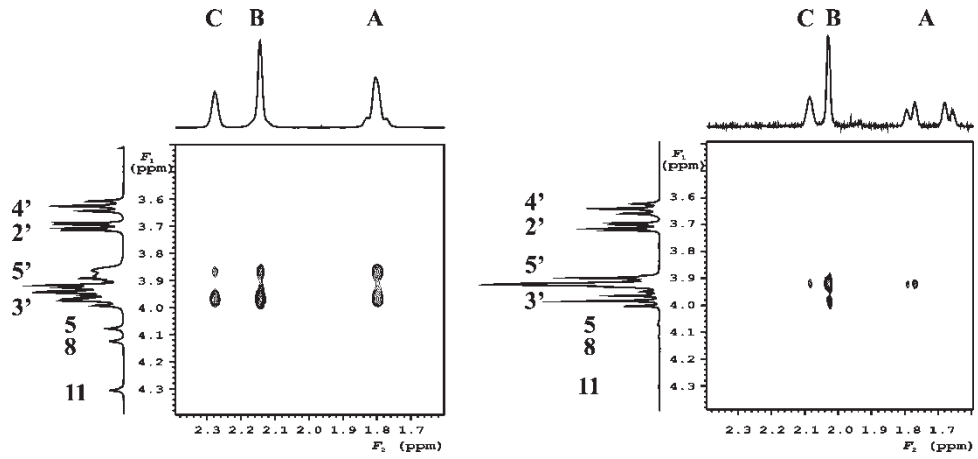


FIGURE 3 NOESY spectra of complexes of dendrimer **1** with β-CD (left) and γ-CD (right).

to host the adamantyl group. Thus this group is located on top of the macrocycle, and NOESY measurements did not exhibit any cross-peaks. The size of the group best fits the β-CD cavity, and in this case the cross-peaks involving both H3′ and H5′ CD protons and all protons of the adamantyl groups are of approximately equal intensity. However, the largest CD studied **4** seems to be slightly too large for this guest, leading to a deeper entrance of the guest into the host cavity and a weaker complex with cross-peaks involving cyclodextrin H5′ protons considerably stronger than those involving H3′ protons.

Chromatographic Studies

The results obtained for the dendrimer complexes are summarized in Table II and Fig. 4. As discussed in detail in the Experimental section, because of the low solubility of the molecules under study the measurements for the complexes involving different CDs could be carried out twice in different mixed solvents. It should also be kept in mind that, in turn, these conditions were different from those used for NMR determinations and the model calculations. Nevertheless, a comparison of the retention times for **1** in the chromatographic systems containing α-, β- and γ-CDs **2**–**4** (at the same concentrations of 10^{-3} M) equal to 363, 18 and 274 min, respectively, with the corresponding value of 443 min for the dendrimer without CDs (all in the mixed solvent MS1 that consisted of 60% MeOH and 40% H_2O) indicates that the complex with **2** is the weakest while that with **3** is the strongest. This conclusion is in full agreement with our NMR and modelling studies.

TABLE I Longitudinal relaxation times (T_1, s) obtained for solutions of the complexes of **1** with excess of **2** or **3**

	2	**5**	**8**	**A**	**B**	**C**	arom
β-CD	0.87	0.87	0.82	0.38	1.00	0.44	3.7
γ-CD	0.87	0.85	0.80	0.42	0.55	0.71	3.7

The dependence of the reciprocal of retention factors k on the CD concentrations for the complexes with **3** and **4** (Fig. 4) differs as a linear relationship has been obtained for the host **4** while a clear parabolic curvature was found for the complex with **3**. Therefore, the data involving **4** in MS1 have been interpreted in terms of a 1:1 complex while those involving **3** in the same solvent clearly indicated a higher stoichiometry. Unfortunately, an analysis of these data did not give unequivocal results for the complex with β-CD **3** in MS1. However, the additional measurements in MS2 (solvent mixture of 20% ethanol + 80% H_2O) could be interpreted in terms of the formation of complexes of 1:1, 1:2 and 1:3 stoichiometry. The considerably larger value of K_3 compared with those of K_1 and K_2 points to the cooperativity of the complexation process in this case. Such behaviour may be due to the more hydrophilic environment of the third adamantyl group in the 1:2 complex favouring its complexation. It should be stressed that, to the best of our knowledge, this is the first estimation of the binding constants for a multiple neutral dendrimer complex with CD and the first to show the differences in the complex stoichiometries of the same compound with different CDs.

The dependence of the reciprocal of the retention factor ($1/k$) on the CD concentration for **5**, mimicking the monomer branch of the dendrimer **1**, is linear ($R = 0.97$), indicating that only a 1:1 complex is formed for γ-CD **4** in mixed solvent MS1 (60% methanol + 40% H_2O) while there is 1:1 and 1:2 complexation with β-CD **3** in mixed solvent MS2 (20% ethanol + 80% H_2O).

TABLE II The apparent stability constants (in M^{-1}) of monomer **5** and dendrimer **1** with β-CD in the mixed solvent MS2 (20% ethanol + 80% H_2O) and γ-CD in MS1 (60% MeOH + 40% H_2O)

	β-CD in MS2			γ-CD in MS1		
	K_1	K_2	K_3	K_1	K_2	K_3
Monomer **5**	20×10^2	2×10^2	–	1×10^2	–	–
Dendrimer **1**	4×10^2	1×10^2	25×10^3	6×10^2	–	–

In agreement with the NMR and modelling results, the values of the stability constants shown in Table II indicate that β-CD complexes the adamantyl group considerably stronger than the aromatic group and that the complexation of the former group with the larger **4** is weaker than that with **3**.

CONCLUSIONS

The NMR, chromatographic and molecular modelling study of dendrimer **1** and CDs **2**–**4** revealed, in agreement with expectation, that the strongest complex is formed with β-CD **3** and the weakest with α-CD **2**. Estimation of stoichiometry and binding constants of the complexes of the dendrimer **1** with **3** and **4** by chromatographic measurements in the mixed solvents indicates that 1:1, 1:2 and 1:3 complexes are formed (in 20% ethanol + 80% H_2O) in the former case (with the 1:3 binding constant much larger than those for 1:1 and 1:2) while only a 1:1 complex is formed with the larger γ-CD in 60% methanol + 40% H_2O. In addition, the chromatographic measurements for the complex with β-CD in the last solvent mixture yielded a stoichiometry considerably greater than 1:1. Taking into account the considerable changes in retention times 443, 363, 18 and 274 min for the dendrimer and its complexes with α-, β- and γ-CD, respectively, we consider that the 1:3 stoichiometry of the complex with β-CD is the most plausible. To our knowledge, no such determination on the dependence of the complex stoichiometry on the host CD has been reported in literature.

EXPERIMENTAL

The synthesis of the titled dendrimer **1** and that of **5** modelling a dendrimer branch were described earlier [15]. All reagents and solvents were of analytical grade and were used as received. CDs **2**–**4** were a generous gift of Wacker Chemie, GmbH.

As mentioned earlier, a low solubility often hampers comprehensive studies of dendrimer complexes with CDs even though the complexation increases their solubility in water. Therefore, some NMR measurements in this work were performed in D_2O solutions while solutions in 1:1 D_2O + DMSO-d_6 had to be applied in others. The chromatographic measurements were in addition limited by the low solubility of CDs **2**–**4** in methanol or ethanol additive to H_2O. The results reported in this work were therefore obtained in different solvents or solvent mixtures.

NMR Studies

All NMR spectra were recorded at 300 K on a Varian Unity Plus 500 spectrometer using a standard 5-mm

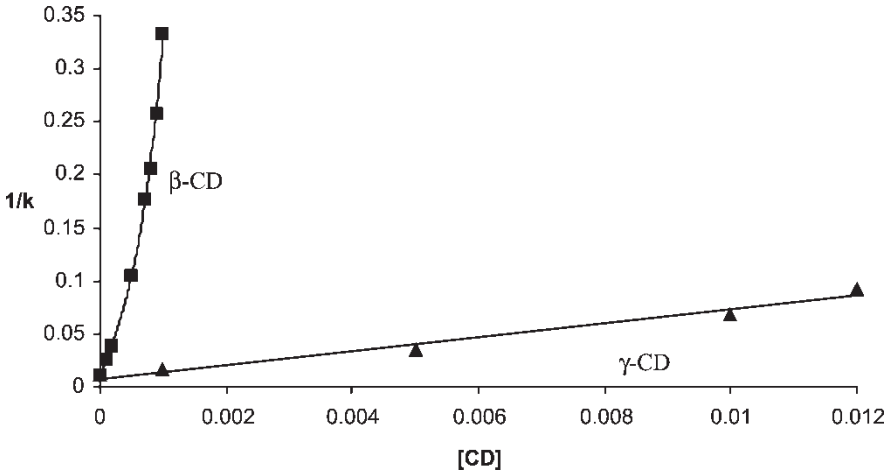


FIGURE 4 Dependence of the reciprocal of the retention factor ($1/k$) for the complexes of dendrimer **1** with β-CD (■) and γ-CD (▲) and the CD concentration (in M), respectively. The eluent consisted of a 60:40 (v/v) methanol:water mixture.

ID_PFG probehead. 7.2 μs high-power ^{1}H $\pi/2$ pulses were employed. Because of the low solubility of dendrimer **1** in water, CIS, in the proton and carbon spectra were measured in a 2:1 DMSO-d_6:D_2O solution (with the concentrations of **1** and β-CD **3** equal to 7×10^{-3} M and 4.2×10^{-2} M, respectively) while the NOESY experiments and T_1 measurements were carried out in D_2O using 1.8×10^{-2} M of **3** and 3×10^{-3} M of **1**. The NOESY experiments were acquired with a 500 ms mixing time, and 96 scans were collected for 256 t_1 increments with a relaxation delay of 4 s. The maximum times t_1 and t_2 were set at 51 and 173 ms, respectively. The data matrix containing 256 × 864 complex points in t_1 and t_2 was zero-filled to 1024 × 1024 complex points. Cosine weighting functions were applied prior to the Fourier transformation of both time domains.

The measurements of T_1 were performed in D_2O by a standard inversion recovery method using a set of eight different recovery delays and interleaved acquisition of 32 scans for each data set. The T_1 times of the aromatic protons were measured in a separate experiment because of their significantly lower relaxation rate.

Molecular Modelling

Molecular mechanics [16] calculations modelling the complexation of the adamantyl group of **1** by **2**–**4** in vacuum were carried out for the branch **5** and the CDs using the HyperChem program [17].

Chromatography

Chromatographic experiments were performed using a Waters (Vienna, Austria) Model 590 pump, a Rheodyne type injector and a Waters UV–VIS detector Model 490 (detection at 220 and 254 nm). The mobile phase was an aqueous solution with an organic modifier (methanol or ethanol) with the concentration of the CD in the range from 10^{-4} M to 1×10^{-2} M. Two sets of chromatographic measurements were carried out using the different solvent mixtures: MS1 (60% methanol + 40% H_2O) and MS2 (20% ethanol + 80% H_2O). The column used was 250 × 1 mm i.d. packed with 5 μm LiChrosorb RP 18. A flow rate of 0.04 ml/min was used. All chromatographic measurements were at the ambient temperature of the air-conditioned room (20°C). Samples of 1 mg/ml of **1** or **5** were dissolved in methanol.

Determination of Stoichiometry and Stability of the Complexes

To establish the stoichiometry and stability of the CD complexes, changes in the retention factors of **1** and **5** with concentration of individual CDs were followed. The data were analyzed on the basis of the following assumptions:

(1) The guest molecules were distributed between the stationary and mobile phases.
(2) The native CD was not adsorbed on the stationary phase [18]. Therefore, it did not change the properties of the RP phase.
(3) The complexation occurred only in the bulk mobile phase solution.

With these assumptions, of two species the one forming more stable complexes with a complexing agent present in the mobile phase should be eluted faster from the column and the following equilibria held in the mobile phase:

$$G_m + CD_m \overset{K_1}{\rightleftarrows} GCD_m$$
$$GCD_m + CD_m \overset{K_2}{\rightleftarrows} G(CD_m)_2$$
$$G(CD_m)_2 + CD_m \overset{K_3}{\rightleftarrows} G(CD_m)_3$$

where K_i ($i = 1$–3) were respective stepwise stability constants.

The solute retention factor k_1 in this chromatographic system could now be defined by [19,20]:

$$k_1 = \frac{k_0}{1 + \sum_{i=1}^{n} \left(\prod_i K_i \right) \cdot [CD]^i} \quad (1)$$

where k_1 and k_0 were the retention factors observed in the systems with or without CD and [CD] was the concentration of CD in the mobile phase.

After rearranging, we obtain:

$$\frac{1}{k_1} = \frac{1}{k_0} + \frac{K_1[CD]}{k_0} + \frac{K_1K_2[CD]^2}{k_0} + \frac{K_1K_2K_3[CD]^3}{k_0} + \ldots \quad (2)$$

The relationship between the reciprocal of k and CD concentrations could then provide information on the stoichiometry and stability constants of the complex under study as, in the case of 1:1 stoichiometry, the reciprocal of k would be a linear function of [CD] while for 1:2 or 1:3 stoichiometry the relationship of $1/k$ and [CD] became parabolic. The stability constants were fitted by non-linear least square procedures according to the model using Eq. (2). As the complexes of methanol or ethanol with β- and γ-CDs were very weak [21], total concentrations of CD were used in the determination of apparent stability constants.

Because of the low solubility of the CDs, two different mixtures of eluting solvents had to be used. The first estimation of CD complexation of **1** was carried out in 60:40 (v/v) methanol:water where the retention time for **1** without CD is 443 min. Only concentrations as low as 10^{-3} M could be obtained for all three CDs under study. Therefore,

the mixture of methanol:water (60:40) for γ-CD and ethanol:water (20:80) for β-CD was used next. These concentrations of the organic solvent prevented adsorption of the CDs on the stationary phase and enabled the use of higher concentrations of **3** and **4** (in the range 10^{-4}M to 1×10^{-2}M) as well as reasonable retention times for both the dendrimer **1** and the monomer **5**.

Acknowledgements

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