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Original Research Article

Diffusion Measurements vs. Chemical Shift Titration for Determination of Association Constants on the Example of Camphor–Cyclodextrin Complexes

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Abstract

Measurements on camphor–cyclodextrin complexes reveal that precise association constants are more easily determined by chemical shift titration. Diffusion measurements using HR-DOSY allow easy following of the complex composition at different concentration ratios and estimation of the binding energy. Linear dependence of the diffusion coefficients on the molecular mass of free and associated cyclodextrins has been observed in D_2O. The solution structures of α- and β-cyclodextrin complexes of camphor in D_2O were deduced from intermolecular cross-relaxation data. Different preferential orientation in the 2:1 α-CD and 1:1 β-CD species have been derived in contrast to the loose 1:1 complex with γ-CD. Proton NMR chemical shift values proved to be much more sensitive to diastereomeric complex formation than diffusion coefficients.

Introduction

Molecular recognition is an expanding field of research in biochemistry and chemistry. Quantification of the non-covalent interactions is usually achieved by measurement of the respective association constant. NMR Spectroscopy is most suitable for measurement of the lower range of values 10–10^5, due to its typical concentration range [1]. The vast majority of the investigations use the chemical shift titration method, followed by non-linear curve fitting [2, 3]. Its main advantage consists in the possibility to use several independent signals for the evaluation of the association constants so being less prone to misinterpretations caused by minor impurities. The observed shift changes provide at the same time insight into the structure of the complexes, which is difficult to extract from UV or fluorescence titrations and impossible from calorimetric data. Main difficulties arise when the chemical shift changes during the titration are small or not due to the association process (e.g. in acid–base equilibria). Recently there has been growing interest in the use of pulsed field gradient (PFG) methods to estimate binding properties of small organic molecules to suitable host compounds (cyclodextrins, calixarenes, etc.) [4, 5, 6].

Pulsed field gradient spin echo (PFGSE) NMR technique has been applied for the first time by Stilbs [7] for quantification of substrate binding in solution on the example of inclusion complexes of cyclodextrins (CD) with alcohols. Since then dramatic progress has been made, these experiments are now possible on a routine basis. In this respect the following advances on modern NMR instrumentation should be especially mentioned: (i) actively shielded z-gradients probe and gradient amplifying and blanking are now standard NMR hardware accessories for performing gradient experiments, (ii) improved PFGSE pulse sequences allow acquisition of clean baselines, pure phases and line shapes, independent of the field gradient pulse amplitude [8], (iii) the high resolution DOSY concept handles the diffusion like a second dimension in the 2D NMR spectra. Appropriate software has been developed [9], allowing determination of diffusion coefficients in mixtures without tedious linearization procedures.

Determination of association constants using HR-DOSY provides an additional NMR based method and an alternative of the classical chemical shift titration method, particularly convenient for evidence of binding between differently sized species [4, 10]. The equilibrium constant K_a for a complex of n molecule host and m molecule guest, e.g.

$$nH + mG \Leftrightarrow C[H_nG_m]$$

couldbe deduced from Equation (1):

$$K_a = [C]/[H]^n[G]^m = [C]/([H]_0 - n[C])^n([G]_0 - m[C])^m \quad (1)$$

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where$[G]_0$ and $[H]_0$ are the total and [G], [H] and [C] the equilibrium concentrations of the free host (H), of the free guest (G) and of the complex (C). Usually the stoichiometry, i.e. the values of n and m, are determined first, e.g. from Job plots [11]. The association constant is determined using Equation (2) if the mole fraction X_b of the bound species is known.

$$K_a = X_b/[(1 - X_b)([H]_0 - X[G]_0)] \quad (2)$$

For complexes in slow exchange on the NMR scale this is easily done by integration. However, for systems in fast exchange the measured NMR parameter P_{obs} – chemical shift or diffusion coefficient – is a weighted average between the relevant NMR values for the free (P_f) and bound (P_b) species. The mole fraction of the bound molecules could be calculated from:

$$X_b = (P_f - P_{obs})/(P_f - P_b) \quad (3)$$

The NMR value for the free molecule P_f is usually easily obtained. But the NMR parameter for the complex (100% bound) – P_b is principally not directly measurable. To obtain accurate association constants a titration has to be performed, measuring the concentration dependence of the relevant parameter (P_{obs}) [2, 4], followed by a non-linear curve fitting according to Equations (1)–(3). Reliable values could only be defined if the titration is made in the proper concentration range ($\sim 1/K_a$) and when collecting data for species having adequate complexation degrees [12]. This necessitates a good preliminary estimate of the binding energy. Single or several point procedures proved to be useful [13]. These could be chemical shifts, induced at saturation concentrations; complexation induced chemical shifts from analogous measurements as well as diffusion coefficients for the host molecules instead of the complexes. The association constant is then calculated on the basis of only a few spectroscopic observations provided a good guess of the value for P_b is available.

We were interested to compare data obtained by chemical shift determination with diffusion measurements. The main purpose of our investigation was to check systematically the advantages of the diffusion experiments in respect of reliability, speed and ease of performing the experiments that has not been done in the literature so far. The recently published practical guide for the determination of binding constants [1] does not include diffusion measurements. Additionally, we were concerned if both methods give comparable values for the binding energies since considerable differences have been occasionally reported [4, 5]. Due to their different size and molecular mass the native α-, β-, γ-CD cyclodextrin molecules are well suited for diffusion experiments. As a model molecule we chose camphor (**C**), a small chiral organic molecule with many chemically non-equivalent protons. It's complexes with α-CD have been intensively studied by chromatography and chemical shift measurements, especially with respect of the possibilities for enantiomeric differentiation [14]. Based on precise longitudinal and transverse relaxation rates solution structures for the 2:1 complexes have been determined [15]. However, there are still open questions concerning the binding in this system [16]. We did not find quantitative data on the camphor complexation with β-, γ-CD cyclodextrins in water. In this work we present also a systematic investigation of the solution structures of the camphor complexes with the three native cyclodextrins relying on intermolecular cross relaxation.

Experimental

Camphor – natural and racemic (reagent grade quality) and tetramethylammonium bromide (TMAB) were purchased from Aldrich and used without further purification. α-, β- and γ-CD (Aldrich) were dried prior to use overnight.

All NMR experiments have been recorded on a Bruker DRX 400 spectrometer (9.4T) equipped with a pulse gradient unit capable of producing magnetic field pulse gradients in the z-direction of 56 G cm^{-1}. The spectra have been acquired in an inversed probe-head at 300 K in 5 mm tubes. TMAB (1 mM, $\delta = 3.18$, $D = 10.0 \times 10^{-10}$ m^2 s^{-1}) has been used as an internal standard for both the chemical shift and the diffusion measurements. All NMR measurements have been done with standard BRUKER pulse sequences. Proton chemical shifts in the complexes were assigned through GHSQC experiments. Intermolecular proximity has been derived from 1D and 2D ROESY experiments. To minimize TOCSY cross talk a spin locking field consisting of a pair of 180° pulses, each with duration of 180 us with alternating phases have been used [17]. Typical measuring conditions for the 2D spectra were: spectral width 2000 Hz; data size 2K/1K and π/3 shifted squared sine bell windows in the ω2/ω1 direction; relaxation delay 2 s, 8–64 scans, depending on concentration. Phase sensitive spectra were acquired using the TPPI scheme. Selective excitation in the 1D experiment has been achieved through the use of a pulsed field gradient spin echo (program selrogp.2). The duration of the 180° selective pulses with a Gaussian shape was 50 or 100 ms. Sinusoidal-shaped field gradients have been applied with 1 ms duration and ca. 8 G cm^{-1} strength. Nine experiments with 1 K scans and mixing time of 50, 100, 200, 300, 400, 500, 600, 800 and 1000 ms have been performed.

HR-DOSY experiments have been performed using the bipolar longitudinal eddy current delay (BPPLED – Bipolar Pulsed Field Gradient Longitudinal Eddy Delay) pulse sequence [18]. The duration of the magnetic field pulse gradients was 3 ms with 5 ms eddy current delay and spoil gradients of 1 ms with 17:13% ratio. The pulse gradients have been incremented from 2 to 95% of the maximum gradient strength in a linear

ramp. The diffusion times have been optimized between 65 and 75 ms for each sample in order to obtain complete dephasing of the signals with the maximum gradient strength. Typically in each PFG NMR experiment a series of 32 BPPLED spectra on 4 K data points (16 dummy scans and 8 or 32 scans) have been collected. The temperature was set and controlled at 300 K with an air flow of 545 l h^{-1} to avoid any temperature fluctuations due to sample heating during the pulse field gradients. After Fourier transformation and baseline correction, the diffusion dimension has been processed with the Bruker Xwinnmr software package (version 3.0). The diffusion constants are calculated by exponential fitting of the data belonging to individual columns of the 2D matrix. Single components have been assumed for the fitting routine. The software gives the mean value of the diffusion coefficient. The extension of the signals in the indirect dimension reflects the accuracy of these values and can also be obtained digitally. At least two different measurements have been done for the determination of each diffusion coefficient, so a precision of $\pm 0.1 \times 10^{-10}$ m^2 s^{-1} can be assumed.

Job's plots have been made with 2 mM stock solutions, they confirm the 1:2 stoichiometry for α- and 1:1 for the β- and γ-complexes. Final chemical shift titration for β-cyclodextrin has been performed with 0.16 mM camphor and 1.8 mM macrocycle stock solutions with eight additions, corresponding to complexation degree between 20 and 80% according to the well established procedure [3]. A mean value of 6000 ± 600 mol l^{-1} has been derived from separate calculation of the data for the three methyl groups, assuming a 10% average error including weighing, dilution, titration etc. errors.

Results and Discussion

Dependence of the diffusion coefficients on the cyclodextrin solution concentration

Significant variations of the diffusion coefficients of the cyclodextrins in the concentration range from 1 to 100 mM have been measured. To account for the changes in the solvent viscosity we find it useful to use TMAB (tetramethylammonium ion) as an internal reference [19]. It does not show appreciable complexion with cyclodextrins and has been successfully applied for chemical shift referencing purposes so far [20]. The rest water signal could also be used instead; however, this is precluded if water pre-irradiation has to be applied.

As it can be seen from Table 1 and Figure 1, a linear relationship between the corrected diffusion coefficients and the molecular mass is found, that allows interpolation of the diffusion coefficients of the complexes. The measured data for the 2:1 complex between α-CD and camphor fits well to it. At concentration higher then 10 mM, as possible in α- and γ-CD, experimental evidence for cyclodextrin aggregation is provided. If dimerization is assumed as a single association process, the diffusion data indicate stronger association for α-CD (K_a ~57) than for γ-CD (K_a ~10) in accordance with previous experimental [21] and theoretical data [22].

Comparison between chemical shifts and diffusion measurements

In Table 2 the fractions of bound camphor for 1:1 β-CD and 2:1 α-CD complexes at different concentration ratios between camphor and CD using diffusion and chemical shift methods on identical samples are presented. The used values of 2.7 and 2.2×10^{-10} m^2 s^{-1} for the diffusion coefficients of the fully bound species are in consent with the molecular mass of the complexes. The latter could be measured from the HR-DOSY spectrum (see Figure 2). The chemical shift of H-8, corresponding to fully bound camphor in the 2:1 complex has been taken from the ^{1}H NMR spectrum of the same solution and used for the calculation of the mole fractions using chemical shifts. The mole fractions for the different concentration ratios in the β-CD complexes have been calculated from the full titration data – association constant and complexation induced shift. The quite small diffusion coefficient differences between free and complexed macrocycles due to small relative mass changes will not be discussed.

As expected within the experimental error diffusion measurements give virtually the same bonding as chemical shift determinations at all concentration ratios measured. This implies that the decision which method should be used will primarily depend on the specific properties of the molecules involved in the association process taking into account the reliability of the results as well as the ease for experiment and calculation. Prerequisite to obtain accurate results using both methods is the measurement of a full titration curve at the correct concentration and within the appropriate range of complexation degrees (20–80%). Because of the superior sensitivity, an order of magnitude higher

Table 1. Diffusion coefficients of cyclodextins in D_2O at 300 K*

Compound	Molarity (M)	$D*10^{10}$ (m^2 s^{-1})
α-CD	0.1	2.5**
	0.01	2.9
	0.001	2.9
β-CD	0.015	2.9
	0.01	2.8
	0.001	2.8
γ-CD	0.135	2.4
	0.01	2.7
	0.001	2.7
Complex	0.028 α-CD	2.2
$(\alpha\text{-CD})_2$ **C**	0.0012 camphor	

*All values are scaled to 0.001 M TMAB.**3.4, 3.2, 3.0×10^{-10} m^2 s^{-1} for α-, β-, γ-CD at 40°C according to J. Szejtli, *Chem. Rev.* **98**, 1746 (1998).

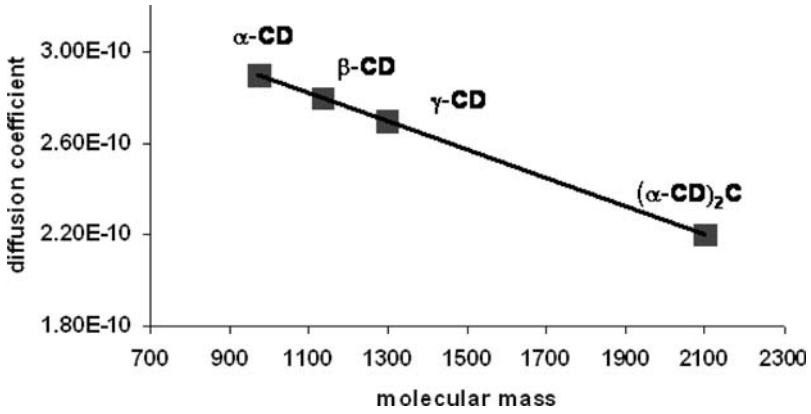

Figure 1. Dependence of the diffusion coefficients on the molecular mass.

association constant could be measured using chemical shift determination than by means of diffusion measurements. Additionally, in order to achieve a high precision the error of the measurement should be small in comparison to the complexation induced parameter. In this respect the data for the 1β:1**C** complex using the chemical shift method should be more than 10 times more accurate. The induced chemical shift for the methyl groups is around 50 Hz with a measurement error of less than ± 0.1 Hz/PT. For the same complex the induced diffusion amounts 4.1×10^{-10} m^2 s^{-1} with an error of $\pm 0.1 \times 10^{-10}$ m^2 s^{-1}. The latter corresponds to a complexation induced shift of 4 Hz that means e.g. 0.01 ppm at 400 MHz. Such a value is usually taken as completely insufficient as a proof of binding and is rarely used to carry out a chemical shift titration. The reason is the sophisticated dependence of the induced chemical shifts on shielding mechanisms comprising different intra- and intermolecular events, some not related to the association process. On the contrary, the diffusion coefficients are intuitively related to the formation of the complexes. If the viscosity is taken into account, a reduction in the diffusion coefficient of an organic compound is a firm indication of an association process. Especially when large differences between the molecular masses of two interacting molecules exist, as e.g. in the case of macrocycles like cyclodextrins etc. the diffusion measurement becomes attractive. Our experience shows that a diffusion check is advisable. It would allow disentangling the extreme sensitivity of the chemical shift for almost "everything", proving that the observed changes are due to complexation. We propose to do it on the samples prepared for the determination of the stochiometry (Job's plot). Then a better estimate of the order of the association constant could be made than solely relying on the chemical shift data. The combined information on both the induced chemical shifts and the induced diffusion coefficients will enhance the reliability of the estimated binding constant and facilitate the choice of a suitable titration method for setting up of the concentration conditions [1]. This is especially important for compounds with exchangeable

Table 2. Mole fractions of bound camphor (X_b), determined by diffusion and chemical shift difference (ΔCS) methods for 2:1 α-CD and 1:1 β-CD complexes

Ratio CD/**C***	[CD] $\times 10^4$	[**C**] $\times 10^4$	D_{CD}* $\times 10^{10}$ (m^2 s^{-1})	D_C* $\times 10^{10}$ (m^2 s^{-1})	$X_b \times 10^2$ (Diffusion)	$X_b \times 10^2$ (ΔCS)	ΔCS (Hz)
1α: 2	6	12	2.8	6.6	4.3	5.5	7.0
1α: 3	10	30	2.6	6.5	6.5	8.5	10.8
1α: 1	10	9	2.7	6.2	13.0	12.9	16.4
1.4α: 1	17	12	2.7	5.7	23.9	**	**
2.8α: 1	34	12	2.7	4.0	60.9	62.1	78.8
4.2α: 1	50	12	2.9	3.5	71.7	80.0	101.5
10α: 1	120	12	3.0	2.6	91.3	97.1	123.2
14α: 1	280	20	2.7	2.2	100	99.3	126.0
28α: 1	330	12	2.8	2.2	100	100	126.9
							K_a***
1β: 3	4.5	15	2.7	5.8	24.4	21.6	3614
1β: 2	6	13	2.8	5.3	36.6	38.2	4651
1β: 1	9	10	2.8	4.4	58.5	62.3	4475
2β: 1	12	6.7	2.7	3.7	75.6	79.9	4619
3β: 1	13	5	2.8	3.5	80.5	84.0	4600

*Diffusion coefficients – camphor (C) 6.8×10^{-10} m^2 s^{-1}; 2:1 α-CD/C 2.2×10^{-10} m^2 s^{-1}; 1:1 β-CD/C 2.7×10^{-10} m^2 s^{-1}. **Not determined, very broad signals due to intermediate exchange rate. ***Determined from the diffusion measurements.

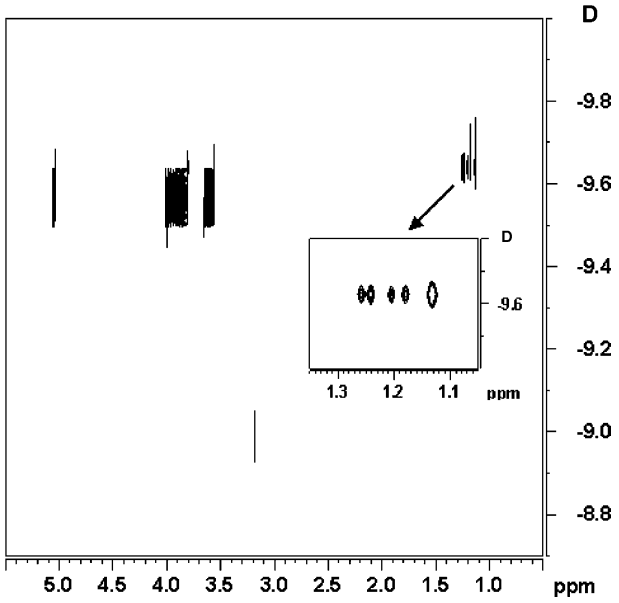

Figure 2. HR-DOSY spectrum of the complex α-CD with racemic camphor at concentrations of 33.0×10^{-3} and 1.2×10^{-3} M l^{-1}.

protons where it would prevent the possibility to confuse acid–base chemistry with binding phenomena.

Solution structure of the camphor complexes

Structural studies have been performed with the aim to discriminate between possible modes of encapsulation and compare the differences between smaller and larger cavities. Most reliable information is obtained by testing the intermolecular proximity of protons in the included specie to the H3 and H5 protons, located inside of the cycloamylose cavities. Nuclear Overhauser enhancements in the rotated frame are best suited for molecules of this size in D_2O. We measured both 1D and 2D ROESY spectra. One-dimensional experiments allow measuring the build-up curves at different mixing times

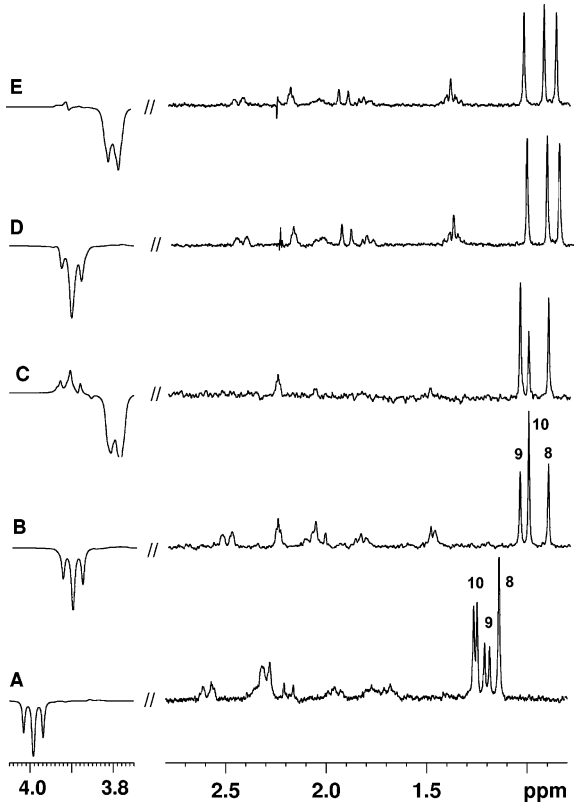

Figure 3. 1D ROESY spectra of α-, β- and γ-CD complexes of camphor, acquired with a mixing time of 300 ms. The selectively inverted protons are (A) H-3 in α-CD, (B) H-3 in β-CD, (C) H-5 in β-CD, (D) H-3 in γ-CD and (E) H-5 in γ-CD.

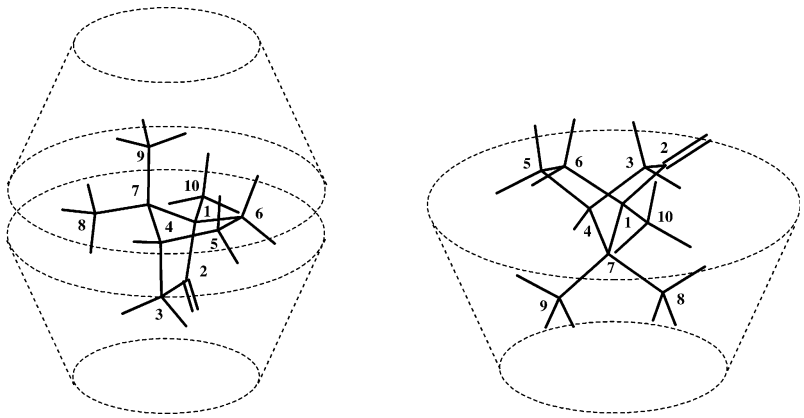

Figure 4. Preferred arrangement of the camphor molecules in the corresponding 2α:1**C** and 1β:1**C** complexes.

in order to assure that the observed effects are due to cross relaxation. Protons H-3 and H-5 inside the CD cavity have been selectively inverted (see Figure 3). Maxima at approximately 300 ms for the α- and 500 ms for the β- and γ-complexes have been observed after a linear part at short mixing times. Due to the symmetry and mobility of the macrocycles no unique individual structures have been sought for. However, evidence of preferred orientation in both the α- and the β-CD complexes is obtained.

The corresponding structures are presented in Figure 4. The lower NOE between H-3 and H-9 methyl camphor protons in comparison with all other protons indicated its deeper location inside the cyclodextrin cavity (see Figure 4). This conclusion is corroborated by the observation of a small but detectable NOE between H-5 (CD) and H-9 (**C**) in the 2D ROESY spectrum, presented in Figure 5. It should be mentioned that the observed enhancements in this 2D spectrum at a concentration ratio of 3C: 1α-CD correspond to a complex mixture, containing 2:1 as well as 1:1 α-CD complexes and free camphor. They indicate the presence of two different 1:1 complexes – one having the methyl group 9 inside the cavity and the other incorporating the other face of the molecule, comprising the C-3 and C-2 carbonyl group. Analogous type of complexation has been postulated for the 2α-CD:1α-pinene complexes [23]. We were not able to detect differences in the ROESY spectra of the single natural molecule and of the racemate mixture. The proposed orientation differs from that derived from relaxation rates [15]. We assume that our structure is in line with the relaxation data and points to a perpendicular and not collinear arrangement of the diffusion tensor and the symmetry axis of the CD capsule.

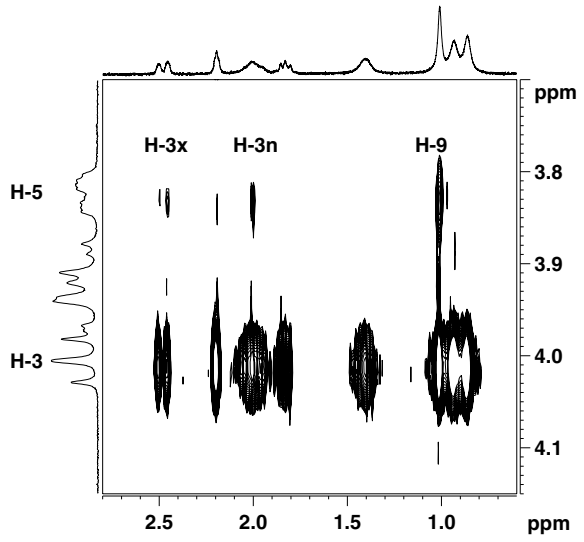

Figure 5. Part of the 2D ROESY spectrum of a solution of 3×10^{-3} M l^{-1} camphor and 1×10^{-3} M l^{-1} α-CD.

The observed intermolecular proximities suggest a sufficiently large β-CD cavity for symmetrical inclusion of the camphor molecule. This arrangement corresponds to the best fit of the two molecules in line with their shape. All camphor protons show interactions with the H-3 proton near the wider rim. Deeper into the cavity are the geminal methyl groups 8 and 9. H-5 CD protons are close also to H-4 and H-10 of camphor.

On the contrary, despite of the relatively high association constant ($\Delta G = 17.5$ as obtained from DOSY measurement of 1 mmol solutions) no favoured position of the terpene moiety within the γ-CD could be revealed, obviously due to sufficient room available for its free mobility inside the cavity. All protons show intermolecular NOE's with both CD protons H-3 and H-5, located inside the cavity.

Enantiodifferentiation

We were not able to obtain reliable differentiation of the two camphor enantiomers using DOSY measurements with any of the macrocycles at any concentration measured. Unlike the differing chemical shifts for the diastereomeric complexes, the diffusion coefficients of both complexes have been the same within the experimental error (see e.g. Figure 2). This is not surprising since the molecular weight and the shape of the complexes are the same whereas the binding differences are too small to be reliably measured by DOSY ($\sim$1.2 kJ

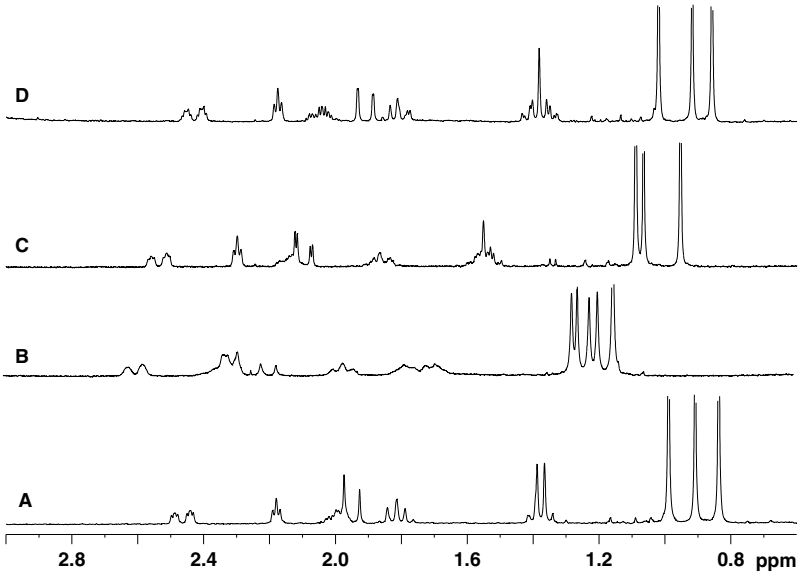

Figure 6. Proton NMR spectra of (A) camphor and its complexes with (B) α-CD, (C) β-CD and (D) γ-CD.

Table 3. Complexation induced shifts and enantiomeric differences in ppb for the complexes of camphor with α-, β- and γ-CD

	H-8	H-9	H-10	H-3x	H-3n	H-4	H-5x	H-6x	H-5,6n
α–CD-**C**(–)	314.2	235.4	351.7	137.8	198.6	145.8	346.2	314.2	235.4
Δ (– – +)		*25.0*	*−16.8*		*−116.6*				*25.0*
β-CD-**C**	122.4	104.2	164.6	72.8	104.5	123.7	149.6	122.4	104.2
Δ (– – +)					*7.3*				
γ-CD-**C**	24.2	31.8	9.5	−33.1	−93.6	−4.7	44.2	24.2	31.8
Δ (– – +)				*−8.4*	*−3.7*				

mol L^{-1} for the α-CD[14]). On the contrary, different complexation induced shifts for many protons have been observed upon association of both natural camphor or its racemic mixture with any of the three cyclodextrins. This parameter appeared to be most sensitive to the minor differences in the diastereomeric complexes. The data are shown in Figure 6 and Table 3. The highest values for the complexation induced shifts as well as the differences between the two diastereomeric complexes are observed for the complexes of α-CD. In the smaller cavities of α- and β-CD only deshielding is observed indicating that the steric interaction term should be dominating in the induced shielding mechanism. In the looser γ-CD complex small positive and negative induced shifts of the camphor protons have been observed pointing at the importance of additional contributions.

Conclusions

Chemical shift and diffusion coefficient measurements are equally well suited for determination of association constants between organic molecules of different size. Chemical shift titrations are usually better suited for precise determination. Since estimation of the binding energy could often be more easily done by HR-DOSY, a diffusion check is highly advisable. Camphor molecules possess preferred orientation in 2:1 alpha and 1:1 beta complexes and move freely inside the γ-CD cavity. Free energies of complex formation of 21.7 and 17.5 kJ mol L^{-1} have been measured for the association with β- and γ-CD. Chiral recognition seems not to be possible with diffusion experiments.

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