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Single and Double Threading of Congo Red into γ-Cyclodextrin. Solution Structures and Thermodynamic Parameters of 1:1 and 2:2 Adducts, as Obtained from NMR Spectroscopy and Microcalorimetry

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Detailed NMR studies of aqueous solutions (pH 7) of γ-cyclodextrin (γCD) and the azo dye Congo Red (CR) show distinct, concentration-independent ^{1}H NMR signals for different species. A very stable 1:1 pseudorotaxane (K_{11} = 38,000 ± 1100 M^{-1}) is formed. In addition, a second complex corresponding to a 2:2 adduct (K_{22} = 13 ± 3 M^{-1}) is produced by dimerisation of the 1:1 species. The structure of the 1:1 pseudorotaxane involves fast motion of the γCD ring along the CR backbone, leaving the outer naphthalene rings free.

The Future of Supramolecular Chemistry

Supramolecular Chemistry, as the chemistry beyond the molecule, has become a basic discipline that combines and uses all methods and techniques of traditional chemistry "divisions", and has also acquired the power and tools to comprehend and bridge other disciplines of science together. In this sense, of course, nearly everything can be characterised as "supramolecular". What I value, however, the most, and I think as the central point, is the fact that Supramolecular Chemistry has brought to the foreground the crucial importance of the weak intermolecular interactions, the dynamic relationships of molecules within interacting systems, and the multitude of factors that drive a process to select one direction (or one structure) over several others available. It has been amazing to realise that such soft interactions make life what it is and even more fascinating to discover similar phenomena in the world of small molecules in solution as described herein. In this sense, Supramolecular Chemistry has a long future ahead, as more tools are becoming available to study systems, and more synthetic methodologies are being developed to prepare them. So, one does not have to despair with "Supra" being outdated and "nano" coming into scientific fashion (and proposal jargon). Supramolecular chemistry has diffused everywhere and underlies all aspects of today's research.

Dina Yannakopoulou graduated from the Chemistry Department, University of Athens, and moved to the USA, where she earned an MS degree with Professor R. A. Abramovitch at Clemson University, SC (1985) and a PhD degree with Professor A. R. Katritzky at the University of Florida (1988). She joined the Institute of Physical Chemistry at NCSR "Demokritos" in 1989, where she still works as a staff scientist. Her interests include the characterisation of host–guest interactions mainly by NMR spectroscopy with emphasis on structure, stability and equilibria of cyclodextrin–guest systems. Another (recent) aspect is synthetic modifications of cyclodextrins, synthesis of new guests, and interactions of both with surfaces.

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This entity undergoes structural reorganisation and dimerises to form the 2:2 adducts. Variable-temperature spectra did not lead to coalescence and allowed for the calculation of K_{11} and K_{22} at each temperature and also of the corresponding thermodynamic parameters. Therefore, formation of the 1:1 complex is favourable ($\Delta G = -26.1 \pm 0.1$ kJ/mol) and exothermic ($\Delta H = -21.7 \pm 1.0$ kJ/mol), whereas formation of the 2:2 entity is also favourable ($\Delta G = -6.36 \pm 0.58$ kJ/mol) but endothermic ($\Delta H = +43.3 \pm 8.7$ kJ/mol). The corresponding values for entropy change are both positive ($\Delta S_{11} = +14.5 \pm 0.7$ J/mol, $\Delta S_{22} = +166 \pm 33$ J/mol). Isothermal titration calorimetry studies confirm the NMR findings. For the 1:1 complexation, the dependence of K upon the concentration is indicative of the dimerisation to form the 2:2 complex. When CR is in excess, aggregation processes involving 2:2 complexes and CR molecules are observed by NMR and calorimetry.

Keywords: Congo Red; γ-Cyclodextrin; 1:1 Pseudorotaxane; 2:2 Pseudorotaxane; Aggregation; NMR; Microcalorimetry; Thermodynamic parameters

INTRODUCTION

The family of the water-soluble cyclic oligosaccharides termed cyclodextrins (CDs) are very well known for their capability to form inclusion complexes with organic molecules [1]. Most of the research [2] and the applications [3,4] involving cyclodextrin complexes refer to βCD, which is, compared with the smaller αCD and the larger γCD (Fig. 1), the least soluble but also the least costly member in the family of natural CDs. Fewer investigations involve the much more expensive and highly water-soluble γCD, which, having a cavity size of 472 Å^3 [4] is capable of including and effectively solubilising large organic molecules.

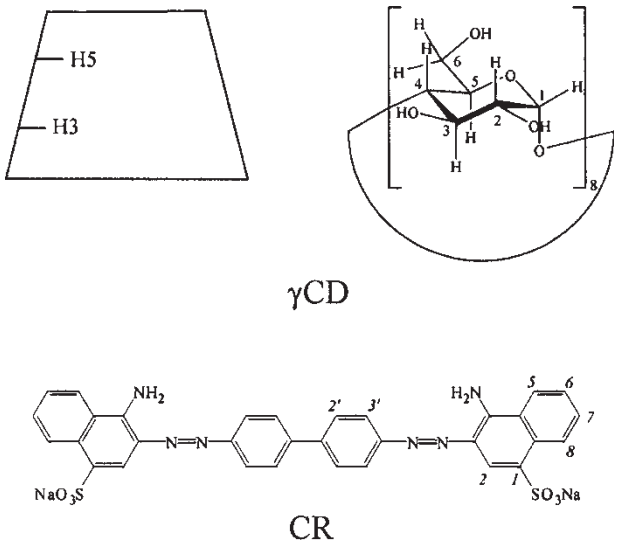

FIGURE 1 Structures of γCD and Congo Red.

Recent experiments using NMR spectroscopy have shown that CDs interact with the side-chain aromatic substituents of proteins [5,6]. X-ray analysis, however, of co-crystallised γCD with glycogen phosphorylase b (GP b) has shown that it binds at the storage site of GP b and inhibits glycogen binding [7]. In that respect, γCD could be used as a vehicle for the targeted delivery of a drug, provided that the association of the latter with γCD is stronger than its association, if any, with the protein itself.

Congo Red (CR, Fig. 1) is a water-soluble azo dye that is known to bind to several proteins [8,9], including those involved in viral recognition and replication [10,11], and also to small peptides [12]. In addition, CR finds wide use as a very specific histological stain for the detection of the β-amyloid strands associated with neurodegenerative conditions such as Alzheimer's disease and scrapie [13–16]. Owing to the interest of our laboratory in developing systems for specific site delivery, we have presently investigated the threading of CR inside γCD as a prototype. We therefore present a detailed examination of the kinds of species present in an aqueous solution of γCD/CR, their structures, the strength of binding, and the values of the thermodynamic parameters that characterise the observed equilibria, as deduced by NMR spectroscopy and microcalorimetry.

RESULTS AND DISCUSSION

NMR Studies in Aqueous Solution

Spectral Changes in the γCD/CR System

CR is a long (25 Å) molecule characterised by an extended conjugated structure (Fig. 1) and high solubility in water. CR, like many azo dyes [17–21], also has a strong tendency to aggregate, as shown by the UV–Vis spectral changes at small concentrations (10^{-5} M), and forms face-to-face aggregates with zero or very little offset [22]. The formation of dimers or higher aggregates does not affect the form of the absorbance bands [22], but the appearance of the ^{1}H NMR spectrum is greatly influenced by such formations. In DMSO, the ^{1}H NMR spectrum of CR [23] is well resolved and shows the expected well-defined multiplets due to the aromatic systems (benzidine and naphthalene), but aggregates still exist. Indeed, in the 2D NOESY spectrum (Fig. 2), in this solvent, we observe many weak but clear interactions of the central benzidine system with the terminal protons *H6–H8* that can only be explained by intermolecular association of CR molecules in well-defined entities, such as dimers,

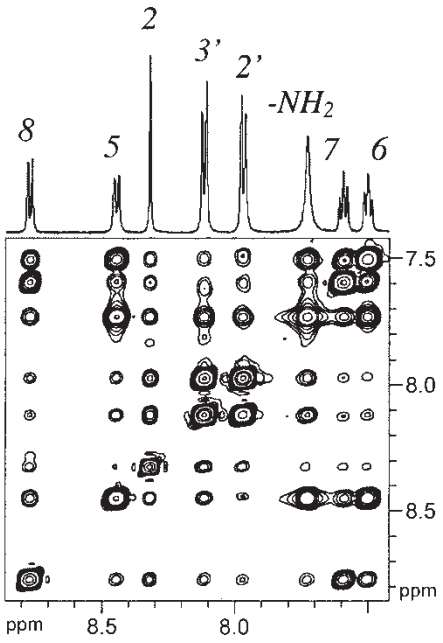

FIGURE 2 2D NOESY spectrum of CR in DMSO, 298 K (unsymmetrised). Cross-peak in-phase with the diagonal.

or trimers, etc. In contrast, the spectrum in D_2O is totally different (Fig. 3) and shows broad resonances arising from aggregates of evidently considerable size. The unbuffered solution is clear and transparent, but addition of buffering salts at pH 6 results in quick formation of apparent aggregates and an ink-like texture of the solution, whereas at pH 7, the same process is much slower. Although the effect of salts on the aggregation of CR has not been reported previously, it is known for other organic salts that increase in ionic strength increases their degree of aggregation [24].

Addition of γCD to a solution of CR results in quick dismantling of the aggregates (Fig. 3), as suggested by the gradual disappearance of the broad peaks and the restoration of the sharp peaks in the ^{1}H NMR spectrum. Conversely, addition of CR into a

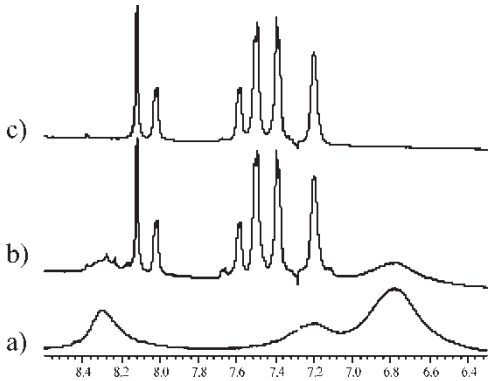

FIGURE 3 ^{1}H NMR spectrum (D_2O, pH 7, 298 K, 500 MHz) of (a) [CR] = 3.5 mM; (b) [CR]/[γCD] = 3.5 mM]/1.5 mM; (c) [CR]/[γCD] = 2.5 mM/2.5 mM.

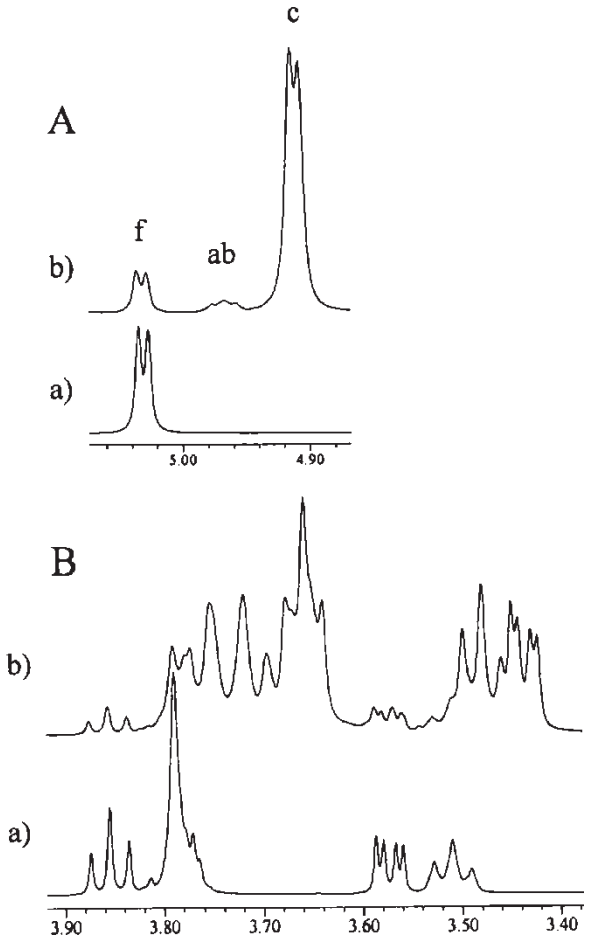

FIGURE 4 ^{1}H NMR, 500 MHz, D_2O, pH 7, 298 K; (a) γCD (2.5 mM), (b) γCD (2.5 mM)/CR (2.5 mM). **A**. anomeric (H1) region; f = free; c = major; ab = minor complex. **B**. γCD region (H2–H6).

γCD solution results in the emergence of new signals (Fig. 4) at frequencies that do not change with the concentration of CR. The frequencies of the original γCD peaks are also concentration-independent. These observations suggest that CR interacts with γCD in a way that its exchange between free and bound states is slow within the time scale of the NMR, and therefore the dynamic exchange process is manifested by a reduced intensity of the original γCD peaks and increased intensity of the new peaks as the concentration of CR increases (Fig. 4).

Although the non-anomeric proton region (Fig. 4b) can be analysed, as will be shown next, the 5.1–4.8 ppm region (Fig. 4a) is more telling since it encompasses only anomeric-type protons. We thus observe, in addition to the doublet of H1 at 5.30 ppm (J = 3.90 Hz) arising from free γCD, two more groups of peaks. The major one at 4.91 ppm is also a doublet peak with the same J value, and can be ascribed to an anomeric proton of a complexed form c, H1c. In between, there exists a small multiplet that, given the 3.90 Hz distance between its peaks, may be

best characterised as two H1-type doublets rather than a triplet, and therefore must arise from one or two additional species of complexed γCD. We observed that these small peaks varied simultaneously upon varying the concentration of CR and rose simultaneously upon heating of the sample. We thus attribute them to a single species, ab, in which two γCD macrocycles, a and b, reside at slightly different parts of the guest CR, thus giving rise to two doublets at very close frequencies, denoted H1ab.

Stoichiometry of the Complexes

Molar ratio titrations as well as continuous variation diagrams were plotted after measurement of the integrals of the various peaks H1. Titrations were carried out by adding solid CR to a γCD solution. A reverse titration could not be done, due to the aggregation of the free CR and cover of the entire aromatic range with broad peaks (Fig. 3). For the continuous variation method, the same problems were encountered, and therefore plots corresponding to the observation of only γCD signals are presented. Figure 5 shows the curves corresponding to the major, c, and the minor, ab, complexes. We observe that in both complexes, the plateau in the molar ratio plots and the inflection maxima in the continuous variation plots appear at [γCD]/[CR] ratio = 1:1. Since the major complex shows a single kind of γCD (only one type of H1) and the minor complex two kinds of γCD rings, we attribute a 1:1 stoichiometry to the major complex c and a 2:2 stoichiometry to the minor complex ab. The latter result is rather unexpected given the considerable length of the guest (~ 25 Å), which could very well accommodate two γCD macrocycles around a single CR molecule giving rise to a 2:1 species, but it seems that the large diameter of γCD permits a more packed arrangement. UV data previously reported for the γCD/CR system [18] have indicated only 1:1 complex formation, while no further information on the structure was presented. Studies of a smaller azo dye, Methyl Orange, with γCD have concluded on the basis of kinetic data that a major 1:2 complex forms (two guests inside one γCD ring) [18] but also a minor 1:1 together with a 2:2 complex [25]. Finally, the naphthylazo dye orange II forms 1:2 and 2:2 complexes with γCD, that were found to self-associate and produce fibroid aggregates [26]. The fact that the proportion of the minor species suddenly increases at a slight excess of the dye, i.e. at [γCD]/[CR] = 45/55 (Job plots), and at a certain

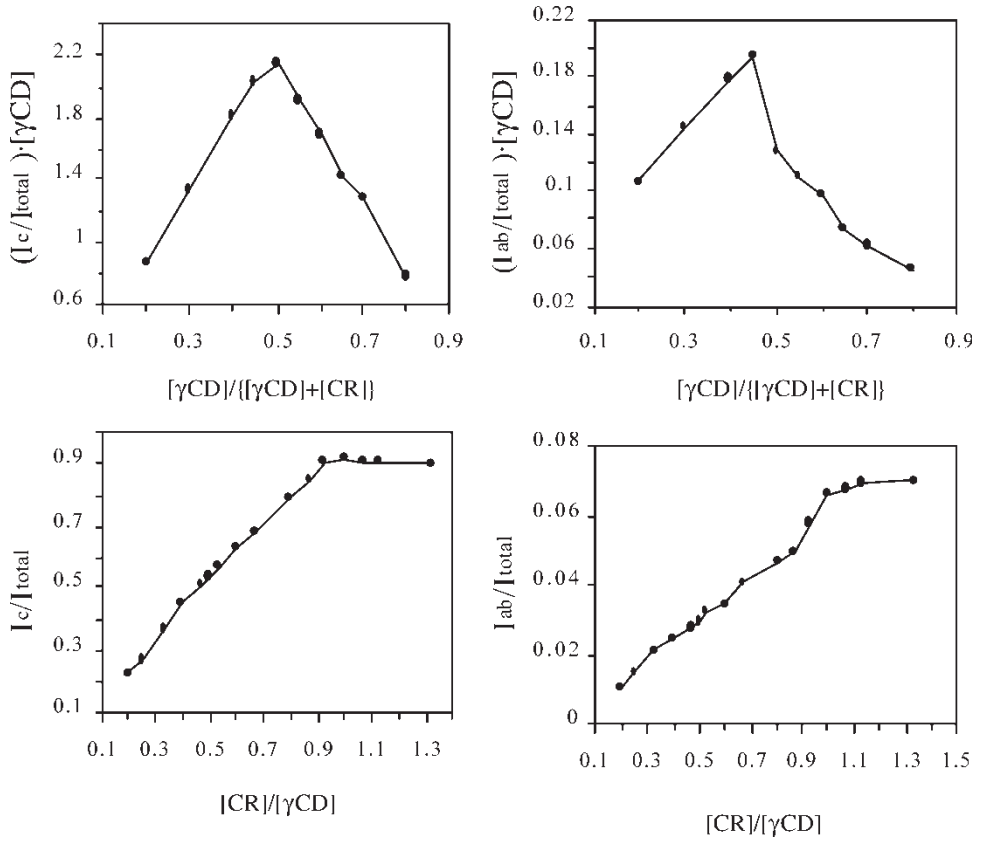

FIGURE 5 Left: continuous variation plot (top) and molar ratio plot (bottom) for the major complex c. Right: continuous variation plot (top) and molar ratio plot (bottom) for the minor complex ab.

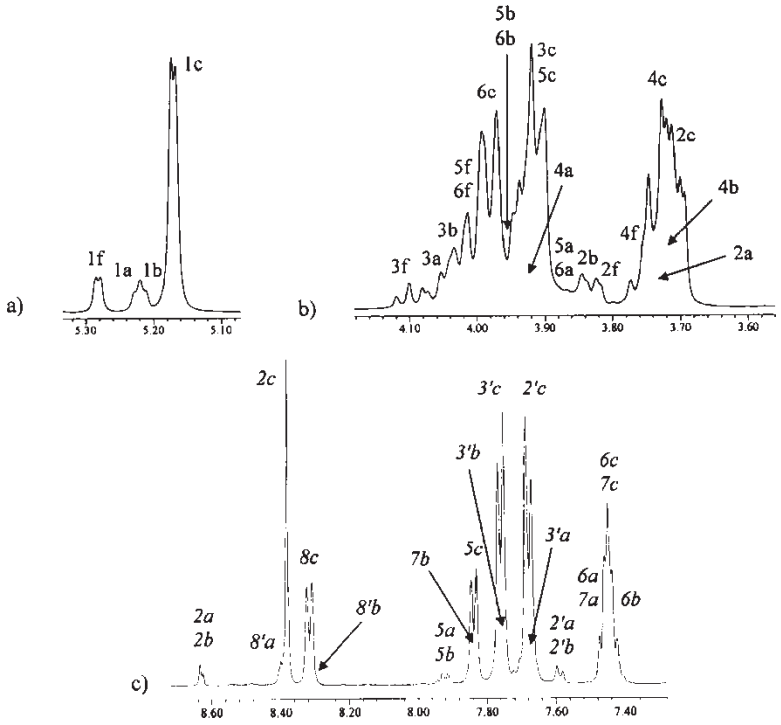

FIGURE 6 ^{1}H NMR, 500 MHz, D_2O, pH = 7, 323 K, γCD (30 mM), CR (28 mM); f = free, c = major complex, ab = minor complex; (a) anomeric (H1) region of γCD; (b) H2-H6 region of γCD; (c) aromatic region, CR.

temperature (343 K), speaks in favour of a dimerisation process involving the 1:1 adduct.

Structures of the Complexes in the Aqueous Solution

The necessary assignment of the proton signals in the region 3.90–3.40 ppm was achieved through the use of 2D COSY, TOCSY and HSQC spectra at 298 and 323 K. The higher temperature favours the formation of the minor complex ab (see below), and the intensities of the small peaks increase. Twenty-eight protons in the cyclodextrin region, seven for each type of γCD, had to be assigned. Fig. 6 presents the assignments at 323 K, as inferred from the 2D spectra. Similarly, the signals of CR were assigned, taking into account the two differently positioned γCD rings in the minor ab complex, which would result in having two formerly equivalent protons now appear at different frequencies, e.g. *H2a*, *H2a′*, *H2b*. The mutual positioning of the molecules in the two types of complexes was deduced from the 2D ROESY spectra, acquired both at 298 and at 323 K. Figure 7 shows that the major complex c involves insertion of CR inside the γCD cavity, since strong cross-peaks of the central benzidine part signals with the cavity protons H3c, H5c and H6,6′c are observed. This would indicate that γCD resides in the central biphenyl portion of CR, as observed for the βCD/benzidine system [27], but interactions of the cavity with some of the naphthalene ring protons of CR are also observed, specifically with the "internal" *H2 and H5* (note that italics are used to symbolise the protons of CR). Since the span of γCD is only 8–9 Å in height, the observed

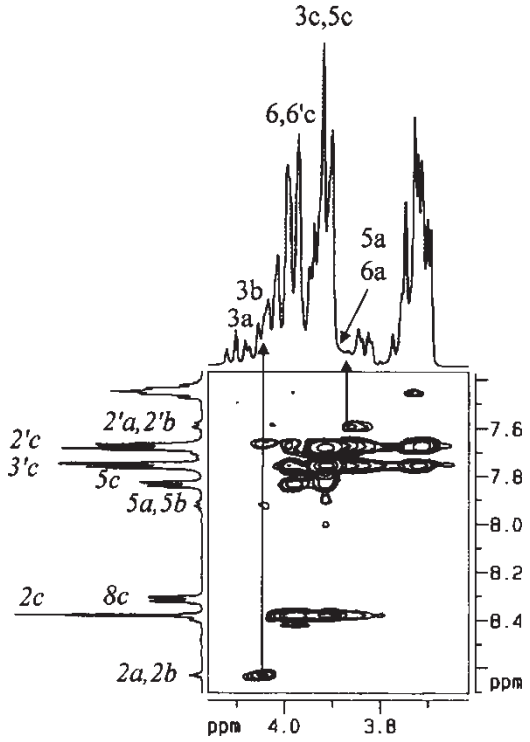

FIGURE 7 2D ROESY spectrum of γCD/CR, 30 mM/28 mM, 323 K, D_2O, pH 7.

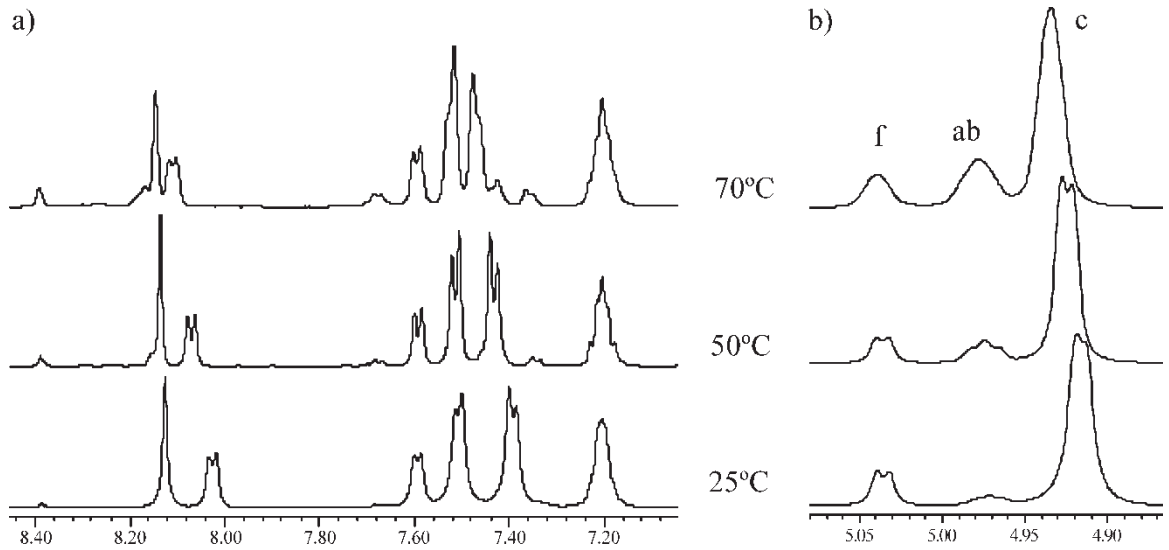

FIGURE 8 Temperature dependence of the ^{1}H NMR spectra of [CR]/[γCD] = 2.5 mM/2.5 mM, D_2O, pH 7, 500 MHz: (a) aromatic region, CR and (b) anomeric region (H1).

interactions can be rationalised by the notion that the wide γCD is able to slide effortlessly along the molecule's backbone, and in effect, what we perceive originates from two 1:1 complexes in fast exchange between them. Regarding the minor complex ab, the CR peaks assigned as *H2′a*, *H2′b*, i.e. located in the biphenyl part of CR, interact with the primary side protons of the a-type γCD ring H5a, H6a, whereas *H2a*, *H2b* and possibly *H5a, H5b* of the naphthyl portion interact with the internal H3a, H3b of two different host cavities.The conclusion is that the minor complex is also a pseudorotaxane. There was no detectable interaction between CR peaks that could reveal any information on the arrangement of CR molecules, which is not surprising, given the weakness of the minor peaks and the unfavourable temperature factor (323 K) for the development of roe. We, therefore, arbitrarily consider the two guests to be π−π-stacked.

Variable Temperature Studies

The ^{1}H NMR spectra, examined in the range of 288–343 K in D_2O, showed that an increase in the temperature resulted in a decrease in the "major" complex c, an increase in the free γCD, and an unexpected increase in the "minor" ab complex (Fig. 8). The formation of the major 1:1 complex is therefore exothermic, and that of the minor complex is endothermic. Heating causes slippage of CR off the cavity, hence the observed increase in the free γCD, and also promotes formation of the 2:2 adduct at the expense of the 1:1 species, so the second process comes as a consequence of the first.

The data so far suggest a scheme that involves a slow threading of CR inside γCD to form a 1:1 complex (in fast exchange between conformational isomers) which then in a further slow step self-associates to produce the 2:2 adduct. Dimerisation of 1:1 complex to 2:2 species has been reported before for cyclodextrin complexes [28,29]. A three-step pathway from 1:1 to 1:2 and then to 2:2 complexes, such as that reported for Methyl Orange in γCD [25], is not justified by our data. The proposed mechanistic model then comprises two steps, and the equations for the calculations of K_{11} and K_{22} at the various temperatures (Table I) are shown in the Materials and Methods section. It should be noted that K_{22} characterises the second step only. As noted previously, there was a sudden increase in the amount of the minor complex at 343 K, and indeed, we observe that from 333 to 343 K, the value of K_{22} doubles. We also observe some additional very minor peaks at the high temperatures that can be attributed to aggregated complexes [26]. These peaks could not be characterised further. A plot of ln K vs. $1/T$ (Fig. 9) provided the thermodynamic parameters shown in Table II.

TABLE I Association constants at various temperatures

T (K)	K_{11} (M^{-1})	K_{22} (M^{-1})
288	36,400 ± 700	7 ± 2
293	40,100 ± 800	10 ± 3
298	38,000 ± 1100	13 ± 3
303	35,900 ± 1100	17 ± 4
313	29,800 ± 1200	28 ± 5
323	24,200 ± 1200	44 ± 7
333	14,800 ± 900	76 ± 9
343	8000 ± 600	141 ± 14

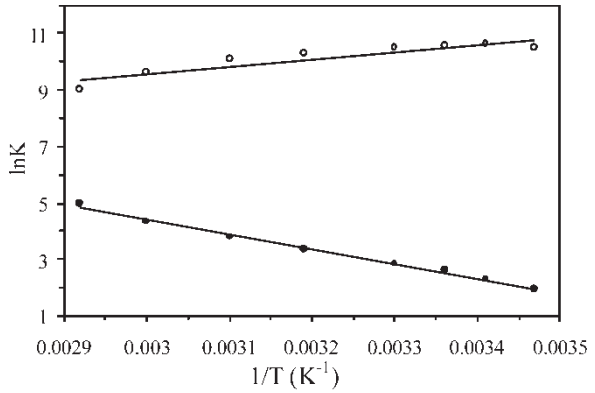

FIGURE 9 Plots of ln K vs. $1/T$ for (○) 1:1 complex and (•) 2:2 complex.

Microcalorimetric Studies in Aqueous Solution

Isothermal titration calorimetry (ITC) [30] was also employed to study the thermodynamics of the interaction of γCD with CR. Titration experiments have been carried out at 298 K and 333 K involving a total of 38 5 μL injections of 3 mM CR into a 0.3 mM solution of γCD. Typical experimental titration curves at the two temperatures are presented in Figs. 10a and 11a, respectively. The corresponding enthalpies of the reaction vs. the molar ratio CR/γCD are shown in Figs. 10b and 11b. A straightforward deduction is that the interaction of γCD with CR is certainly a more complicated process than a simple 1:1 binding. Both titration curves exhibit a maximum at molar ratio 1.2 and 1.0 for 298 and 333 K, respectively. Indeed, in agreement with the NMR data, the enthalpy curves appear to comprise at least one major exothermic process and a minor endothermic process. At high temperatures, the latter appears to be enhanced with respect to the exothermic major. Any attempt to fit the data with theoretical models involving two different binding sites, or two sequential binding sites, has failed unless the stoichiometry of the minor endothermic interaction assumes non-physical values.

In our attempt to analyse and understand the calorimetric data, we have applied the following procedure. Based upon the NMR results, an exothermic, single-site, 1:1 binding was assumed to fit the low molar ratio data, since this is the major complexation process at these ratios. This way, for 298 K, isothermal binding curves produced best fits for molar ratios <1 (Fig. 10b), leading to a binding

TABLE II Thermodynamic parameters of the 1:1 and 2:2 complexes γCD/CR (298 K)

Stoichiometry	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 (J/mol·K)
1:1 (NMR)	−26.1 ± 0.1	−21.7 ± 1.0	14.5 ± 0.7
1:1 (ITC)	−29.7 ± 0.2	−21.7 ± 0.9	26.8 ± 0.4
2:2 (NMR)	−6.36 ± 0.58	43.3 ± 8.7	166 ± 33

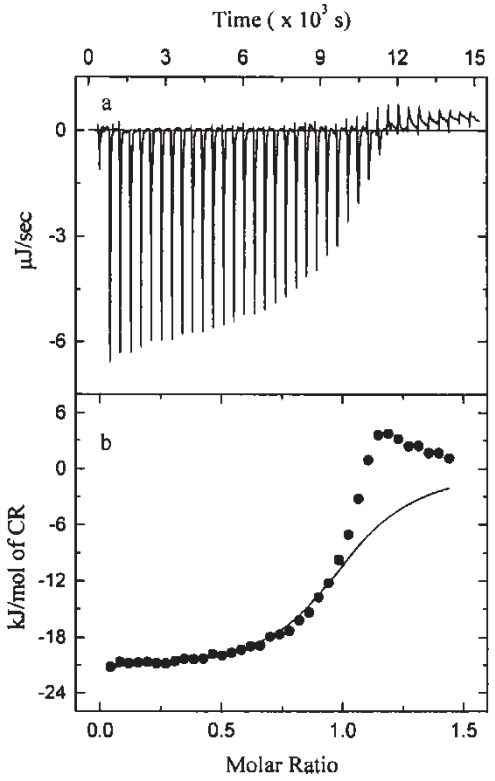

FIGURE 10 (a) Raw ITC data (heat flow vs. time) for the titration of 5 μL aliquots of CR (3 mM, pH 7) in the reaction cell filled with γCD (0.3 mM, pH 7) at 298 K. (b) Reaction enthalpy data (obtained by integrating the area under the trace of the upper curve) vs. the molar ratio of CR/γCD. The solid line represents the best non-linear, least-squares, 1:1-binding fit for the low-molar-ratio range of the data (<1).

constant K_{11} value of $1.3 \pm 0.3 \times 10^5\,M^{-1}$ and an enthalpy change ΔH of -21.7 ± 0.9 kJ/mol of injected CR. While ΔH is identical to the NMR result for the 1:1 exothermic complex, the calorimetric K_{11} value is about three times larger. Considering the calorimetric results for K_{11} in comparison with the present NMR study as well as with previous circular dichroism measurements [18], a dependence of K_{11} upon the concentration is revealed. This K_{11} dependence, while the corresponding values for ΔH and stoichiometry are constant, is indicative of the fact that the binding process may be complicated by "dimerisation" or aggregation of the complexed species. Such behaviour has been observed for protein-ligand interactions as well [31]. Of all the K_{11} values reported, those measured by NMR are certainly the most accurate since, with this spectroscopic method, the two complexation processes appear at separate frequencies, thus enabling the direct measurement of the K for each of the processes distinctly.

For the high-temperature experiment, the situation is qualitatively no different than what is already described for the low-molar-ratio data. Reasonably good fits for 1:1 binding isotherms could only be

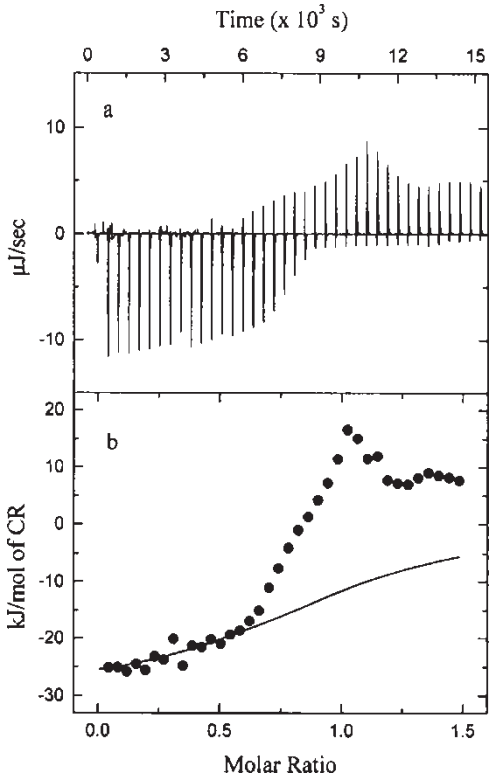

FIGURE 11 (a) Raw ITC data (heat flow vs. time) for the titration of 5 μL aliquots of CR (3 mM, pH 7) in the reaction cell filled with γCD (0.3 mM, pH 7) at 333 K. (b) Reaction enthalpy data vs. the molar ratio of CR/γCD. The solid line represents the best non-linear, least-squares, 1:1-binding fit for the low-molar-ratio range (<0.5).

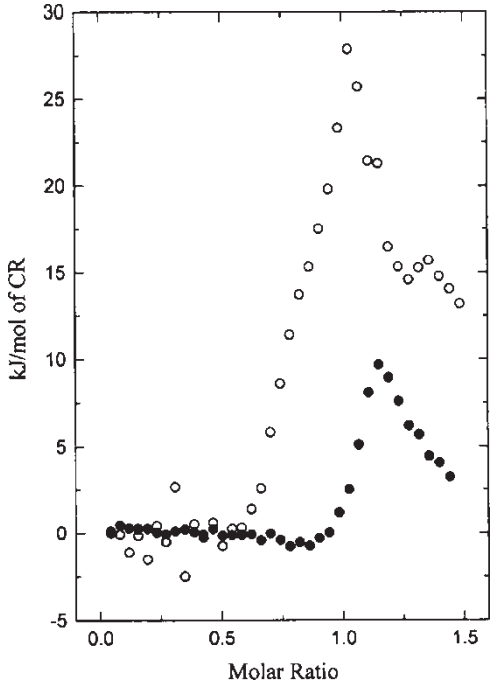

FIGURE 12 Experimental data after subtracting the best-fit, 1:1 binding curves described in the text for (●) 298 K and (○) 333 K.

produced for a much narrower range of the molar ratio (less than 0.5). The fits lead to values of $K_{11} = 8.0 \pm 1.0 \times 10^4\,M^{-1}$, almost half of the result for room temperature and $\Delta H = -31.3 \pm 1.1$ kJ/mol, considerably larger than at 298 K. Regardless of the temperature, the 1:1 binding isotherms for molar ratios larger than 1 will always fail to fit the ITC data, which exhibit a maximum followed by a pronounced decreasing trend for ΔH. Subtracting the 1:1, best-fit curves from the experimental data, one is left with a revealing situation presented in Fig. 12. ΔH exhibits an abrupt increase in $\Delta(\Delta H) = 8.5$ kJ/mol of CR at a molar ratio of $\sim$ 1.2 for 298 K and $\Delta(\Delta H) \sim 25$ kJ/mol of CR at a molar ratio 1.0 for 333 K. Once again, this behaviour can be interpreted in terms of the NMR results, and thus an endothermic "dimerisation" of 1:1 complexes into 2:2 adducts can be assumed. In agreement with NMR, the abrupt enhancement of the 2:2 complexation is observed for molar ratios in the neighbourhood of 1.

Finally, at a high molar ratio, an additional exothermic process is taking place. This process's occurrence at low and high temperatures as well, at an excess of CR in the solution, may be attributed to aggregation between the 2:2 complexes as well as the CR molecules. These various aggregation effects have been depicted by the NMR spectra of the present study and have also been seen in other γCD complexes recently reported [26]. The nature and structure of the aggregates are very difficult to deduce, since, especially for 333 K, several pronounced aggregation processes appear to take place at molar ratios >0.8.

Equilibria and Thermodynamics of the Process

The present studies provide a full description of the γCD/CR system in solution, characterised by the equilibria and structures shown in Fig. 13. The structures proposed for the 2:2 species are based on the existence of two different γCD rings around two—probably π−π-stacked—CR molecules, with the NMR data insufficient to differentiate further. The first step (1:1 complexation) is thus exothermic, as commonly observed in cyclodextrin complexes [32,33], characterised by an uncommonly large association constant. Here, the contribution of the hydrophobic interactions to the stability of the complex originates from the insertion of the biphenyl part of CR inside the γCD cavity. The rest of the guest molecule is allowed to interact with the water molecules and to solvate the sulphonate groups. We therefore have a thermodynamically very favoured, but kinetically unstable, pseudorotaxane. The second step (2:2 formation) is endothermic, which is rather

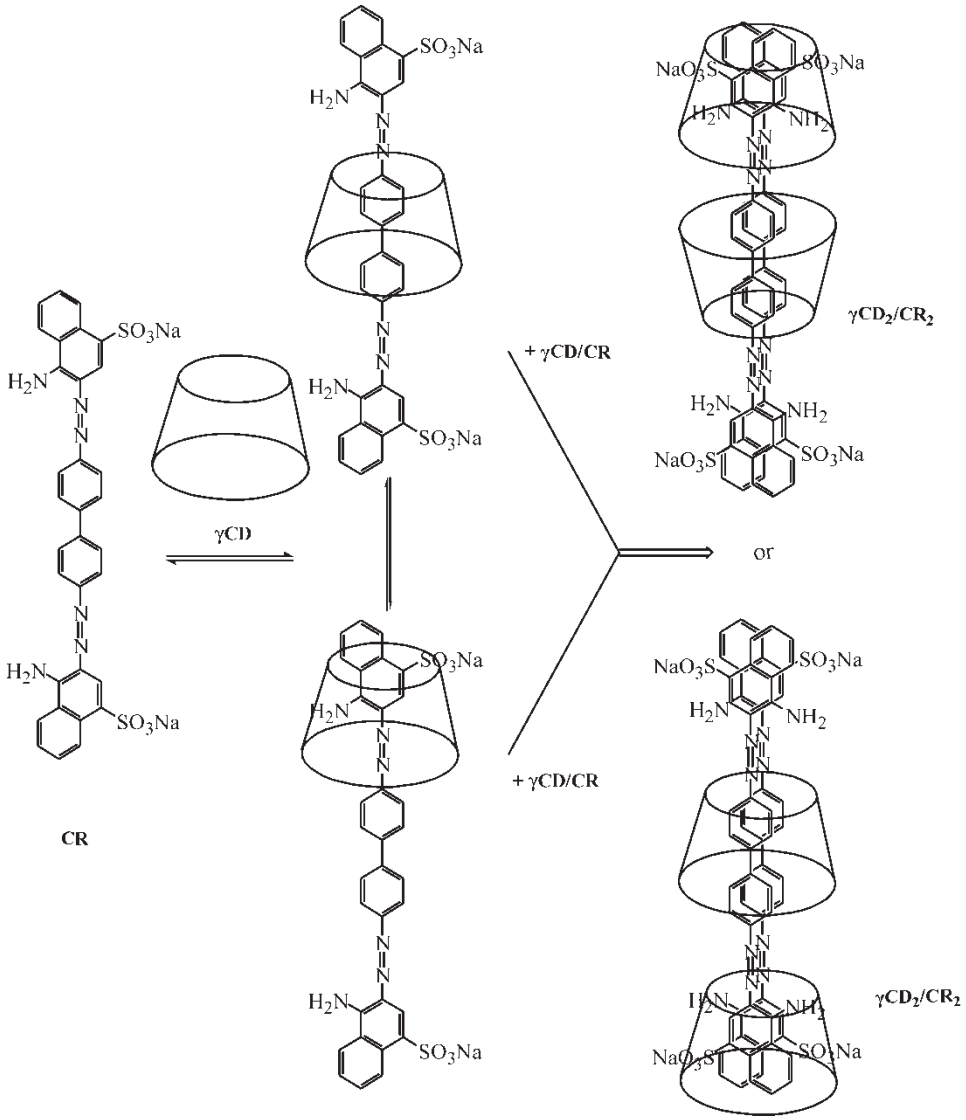

FIGURE 13 Proposed equilibria and structures of species in D_2O in the system CR/γCD.

unusual. One plausible cause could be the reorganisation that the γCD ring has to undergo to be positioned on to the naphthyl part of CR and enable the accommodation of the second complex, thus establishing many extra contact points in the compact 2:2 structure. This process is also characterised by sizeable positive entropy, which could be the result of release of solvation water molecules from the sulphonate groups as a consequence of dimerisation of the complex.

CONCLUSIONS

We have studied the solution properties of the system of γCD with CR with NMR spectroscopy and ITC. We have found that a major, thermodynamically very favoured 1:1 pseudorotaxane forms via an exothermic process. Dimerisation of this species in an endothermic process results in a 2:2 complex. In the major 1:1 γCD/CR complex, the sulphonate ends appear to be as free as in the CR molecule itself, while the wide γCD ring is not expected to restrict the biphenyl part considerably. Since CR interacts through its sulphonate groups with arginine residues and the biphenyl spacer (hydrophobic portion) is known to possess two conformations [34], we expect that in a solution of γCD/CR, the guest will behave as the free molecule, with the additional feature of not forming large aggregates even at high concentrations, up to 60–70 mM. In addition, there are indications from both NMR and ITC that the complexes themselves aggregate. One further important finding is that with NMR, we observe and quantify each of the two complexation processes separately (K_{11}, K_{22}) and independently. With ITC, however, we measure the sum of the heat changes involved, so the association

constants are concentration-dependent due to the dimerisation and further aggregation processes involved.

MATERIALS AND METHODS

Commercial (purity 90%) Congo Red (CR) was recrystallised from H_2O/EtOH 1:3 v/v. The γCD used was purchased from either Jansen or Cyclolab, and the deuterated solvents were products of Aldrich. Buffered aqueous solutions were prepared using Na_2HPO_4 and KH_2PO_4 in D_2O to maintain a pH 7 and were used throughout the experiments.

The NMR spectra were recorded on a Bruker Avance DRX 500 MHz. Two-dimensional spectra were acquired using the standard sequences in the spectrometer's library. Titrations (molar ratio method) were carried out by adding solid CR to a 30 mM solution of γCD in buffered D_2O. For the continuous variation plots, solutions of 5 mM in each γCD and CR were mixed to create 10 solutions with proportions from 100% to 10% for γCD and 0% to 90% CR, respectively. The ^{1}H NMR spectra were carried out using a relaxation delay of 1 s and processed using a small LB factor. Integrations in the anomeric proton region were done after careful baseline correction.

The calculations were based on the following scheme:

$$\gamma CD + CR \overset{K_{11}}{\rightleftharpoons} (\gamma CD/CR) \quad K_{11} = [(\gamma CD/CR)]/[\gamma CD][CR]$$

$$2(\gamma CD/CR) \overset{K_{22}}{\rightleftharpoons} (\gamma CD_2/CR_2) \quad K_{22} = [(\gamma CD_2/CR_2)]/[(\gamma CD/CR)]^2$$

We define the integrals as $I_{total} = I_f + I_{ab} + I_c$, where *f* denotes free γCD, ab the minor complex, and c the major complex.

Mass-balance gives:

$$[\gamma CD]_0 = [\gamma CD] + [(\gamma CD/CR)] + 2[(\gamma CD_2/CR_2)]$$

and

$$[CR]_0 = [CR] + [(\gamma CD/CR)] + 2[(\gamma CD_2/CR_2)]$$

Then, $I_f/I_{total} = [\gamma CD]/[\gamma CD]_0$, $I_c/I_{total} = [(\gamma CD/CR)]/[\gamma CD]_0$, $I_{ab}/I_{total} = 2[(\gamma CD_2/CR_2)]/[\gamma CD]_0$.

Substitution of the integral values to the above equations provides the numbers of K_{11} and K_{22} at each temperature used for the plot of Fig. 9.

The ITC measurements have been carried out using an MCS-ITC unit by MicroCal Inc., Northampton, MA. The reaction cell with a 1.4 mL volume was filled with 0.3 mM buffered γCD solution (pH 7, phosphate). A 250 μL titration syringe was used for experiments at 298 K and 333 K. Thirty-eight-injection experiments of 5 μL each were programmed and executed automatically for a 3 mM buffered solution of CR (pH 7, phosphate). The duration of each injection was 3.14 s, and a time delay of 400 s was set between injections. The injection-stirrer syringe was set at a rotation speed of 200 rpm throughout the experiment. Control experiments have also been carried out to measure the dilution heat of CR. Identical to the titration experiments, the control experiments involved the injection of a 3 mM buffered CR solution into the titration cell that contained only buffer. The calorimetric data were analysed using the ORIGIN® software by MicroCal.

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References

[1] Wenz, G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 803–822.
[2] *Chem. Rev.* **1998**, *98*, issue dedicated to cyclodextrins.
[3] *Cyclodextrins and their Industrial Uses*, Duchêne D., Ed.; Editions de Santé: Paris, 1987.
[4] Szejtli, J. *Cyclodextrin Technology*; Kluwer Academic: Dordrecht, 1988.
[5] Tokihiro, K.; Irie, T.; Uekama, K. *Chem. Pharm. Bull.* **1997**, *45*, 525–531.
[6] Aachmann, F. L.; Larsen, K. L.; Otzen, D. E.; Wimmer, R. *11th Int. Cyclodextrin Symp.*, 2002, 5–8 May, Reykjavik, Iceland.
[7] Pinotsis, N.; Leonidas, D. D.; Zographos, S. E.; Oikonomakos, N. G.; Mavridis, I. M. 31st International School of Crystallography. *Strength from Weakness: Structural Consequences of Weak Interactions in Molecules, Supermolecules and Crystals*; Erice: Italy, 2001; 23 May–3 June.
[8] Pigorsch, E.; Elhaddaoui, A.; Turell, S. *J. Mol. Struct.* **1995**, *348*, 61–64.
[9] Edwards, R. A.; Woody, R. W. *J. Phys. Chem.* **1983**, *87*, 1329–1337.
[10] Balzarini, J.; Mitsuya, H.; De Clercq, E.; Broder, S. *Int J. Cancer* **1986**, *37*, 451–457.
[11] Brinkworth, R. I.; Fairlie, D. P. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 624–630.
[12] Lyon, R. P.; Atkins, W. M. *J. Am. Chem. Soc.* **2001**, *123*, 4408–4413.
[13] Ladewig, P. *Nature* **1945**, *156*, 81–82.
[14] Westermark, G. T.; Johnson, K. H.; Westermark, P. *Meth. Enzymol.* **1999**, *309*, 3–25.
[15] Klunk, W. E.; Pettegrew, J. W.; Abraham, D. J. *J. Histochem. Cytochem.* **1989**, *37*, 1273–1281.
[16] Caughey, B.; Ernst, D.; Race, R. E. *J. Virol.* **1993**, *67*, 6270–6272.
[17] Hiromichi, T.; Umemura, J.; Takenaka, T. *J. Phys. Chem.* **1982**, *86*, 4660–4664.
[18] Hirai, H.; Toshima, N.; Uenoyama, S. *Bull. Chem. Soc. Jpn.* **1985**, *58*, 1156–1164.
[19] Hamada, K.; Take, S.; Iijima, T.; Amiya, S. *J. Chem. Soc., Faraday Trans.* **1986**, *82*, 3141–3148.
[20] Neumann, B.; Huber, K.; Pollmann, P. *Phys. Chem. Chem. Phys.* **2000**, *2*, 3687–3696.
[21] El-Sabbagh, I. A.; Moussa, E. A.; El-Fass, M. M.; Tourky, A. S.; El-Mariah; Afaf, A. R. *Egypt. J. Chem.* **1994**, *37*, 259–272.
[22] Neumann, B.; Pollmann, P. *Phys. Chem. Chem. Phys.* **2001**, *3*, 4508–4514.
[23] Pigorsch, E.; Elhaddaoui, A.; Turell, S. *Spectrochim. Acta* **1994**, *50*, 2145–2152.
[24] Steullet, V.; Dixon, D. W. *J. Chem. Soc., Perkin Trans. 2* **1999**, 1547–1558.
[25] Clarke, R. J.; Coates, J. H.; Lincoln, S. F. *Carbohydr. Res.* **1984**, *127*, 181–191.

[26] Suzuki, M.; Tsutsui, M.; Ohmori, H. *Carbohydr. Res.* **1994**, *261*, 223–230.
[27] Giastas, P.; Yannakopoulou, K.; Mavridis, I. M. *Acta Crystallogr. B* **2003**, *B59*, 287–299.
[28] Hamai, S. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 2721–2729.
[29] Herkstroeter, W. G.; Martic, P. A.; Farid, S. *J. Chem. Soc., Perkin Trans. 2* **1984**, 1453–1457.
[30] Jelesarov, I.; Bosshard, H. R. *J. Mol. Recognit.* **1999**, *12*, 3–18.
[31] Wiesman, T.; Williston, S.; Brandts, T. S.; Lin, L.-N. *Anal. Biochem.* **1989**, *179*, 131–137.
[32] Clarke, R. J.; Coates, J. H.; Lincoln, S. F. *Adv. Carbohydr. Chem. Biochem.* **1988**, *46*, 205–249.
[33] Inoue, Y.; Reharsky, M. V. *Chem. Rev.* **1998**, *98*, 1875–1917.
[34] Ojala, W. H.; Ojala, C. R.; Gleason, W. B. *Antiviral Chem. Chemother.* **1995**, *6*, 25–33.