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Investigation of inclusion phenomena in cyclodextrin derivatives by ultra-high resolution NMR

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Introduction of selective pulses into NMR sequences allows a tremendous increase of the apparent resolution of 2D experiments. The performances of these techniques are reported for the investigation of NMR parameters in inclusion complexes of cyclodextrin derivatives exhibiting extensive spectral overcrowding. The derived parameters are shown to be of great value in proposing a 3D structure for the complex in solution using molecular modelling.

INTRODUCTION

High resolution NMR appears to be the ultimate technique for investigating the structures and conformations of supramolecular assemblies in solution. The advantages of this spectroscopy rest upon the large amount of important information that can be derived thereof. Owing to its quantitative character and to the possibility of observing simultaneously all interacting species, NMR can provide experimental data for deriving binding characteristics (stoichiometry, association constant), as well as the possible 3D structure of the final assembly. In the field of inclusion complexes with natural cyclodextrins and derivatives, general strategies have already been published to take advantage of the versatility and flexibility of solution NMR.[1] In the case of chemically modified hosts, the situation can become more difficult owing to the complexity of the NMR spectra as induced by symmetry reduction. We propose here an efficient approach for deriving kinetic parameters as well as data dedicated to further use in molecular modelling programs. This approach is based on the use of selective excitation methods and is dedicated to afford very high resolution analysis in complicated spectra.

RESULTS AND DISCUSSION

A modified cyclodextrin (6-*S*-α-D-glucopyranosyl-6-thio-cyclomaltoheptaose) will be considered here. Its molecular structure is depicted on Figure 1. This compound and similar derivatives have been prepared to afford highly water soluble hosts in order to overcome the relatively low solubility problem of β-cyclodextrin and the corresponding inclusion complexes. These derivatives present superior properties compared with the parent cyclodextrin for the solubilization by inclusion of very sparingly soluble drugs.[2] In the present case, for the sake of simplicity, a single guest molecule (dothiepine) will be considered throughout.

Evidence for the formation of an inclusion complex can be seen from ^{1}H-NMR, as shown in Figure 1, where the spectra of the host alone and in the presence of the guest are displayed. Two comments can be made concerning these spectra: first, even in the absence of dothiepine, the NMR spectrum of the host is quite complicated and not readily prone to full assignment. This situation arises from the symmetry loss induced by the monosubstitution. This implies that all units could be virtually separated, but the extent of symmetry reduction is too weak to allow direct separation of the corresponding signals, especially of anomeric protons. Second, addition to dothiepine is shown to induce large shifts of the proton resonances of the guest as expected from the ring current effects associated with inclusion of the aromatic ring in the cavity. This corresponds to a preliminary observation but clearly cannot be used to derive a molecular structure of the complex as long as full assignment of protons is not performed.

In order to further support the formation of inclusion complexes and to show intrinsic resolution limitations associated with 2D NMR, a dipolar correlation experiment in the rotating frame (ROESY)[3] was performed. Figure 2 shows a contour plot of ROESY experiment performed at 600 MHz to show dipolar interactions (spatial proximities) between

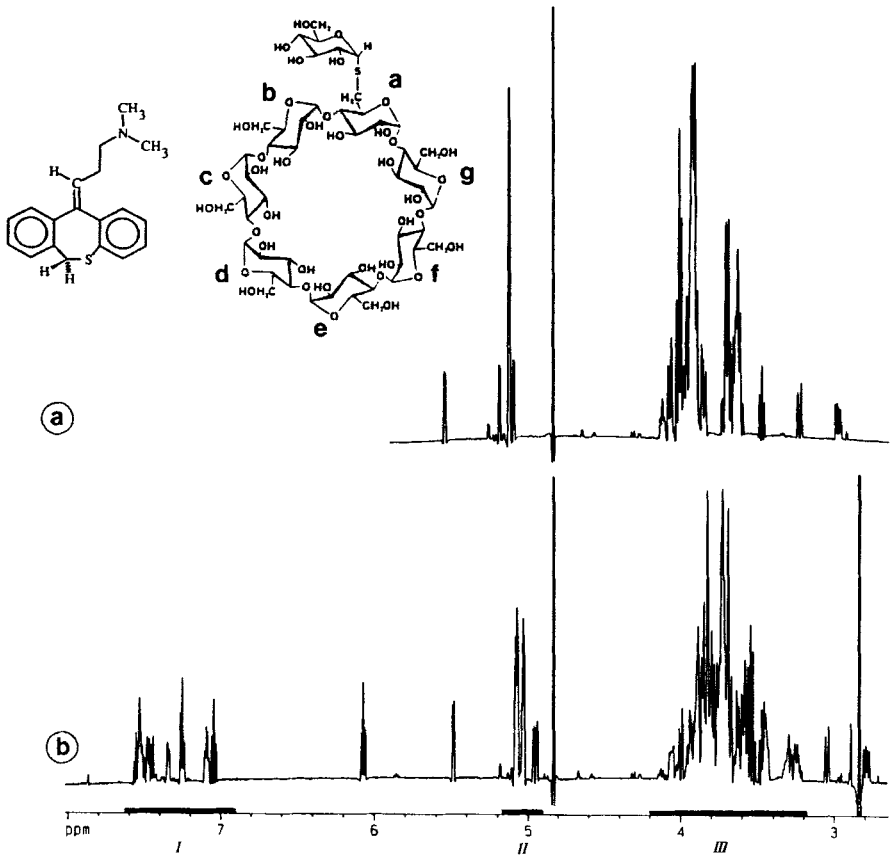

Figure 1 600 MHz ^{1}H-NMR spectra of (a) the substituted cyclodextrin alone (20 mM), and (b) an equimolar mixture (20 mM total) with dothiepine in ^2H_2O at 298 K. The three spectral domains labelled I, II and III are used for the selective experiments described later in this paper.

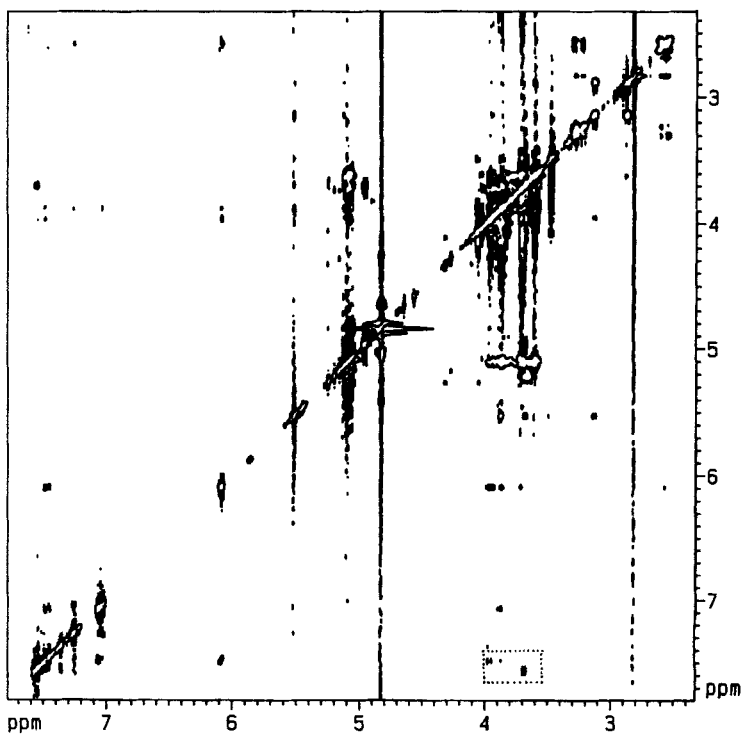

Figure 2 Complete contour plot of the ROESY experiment on a sample containing 14 mM host and 6 mM guest at 298 K and 600 MHz after F2 baseline correction. Cross-peaks showing dipolar interactions between aromatic protons of the guest and the cyclodextrin are located in the broken line box. The acquisition time was 20 h.

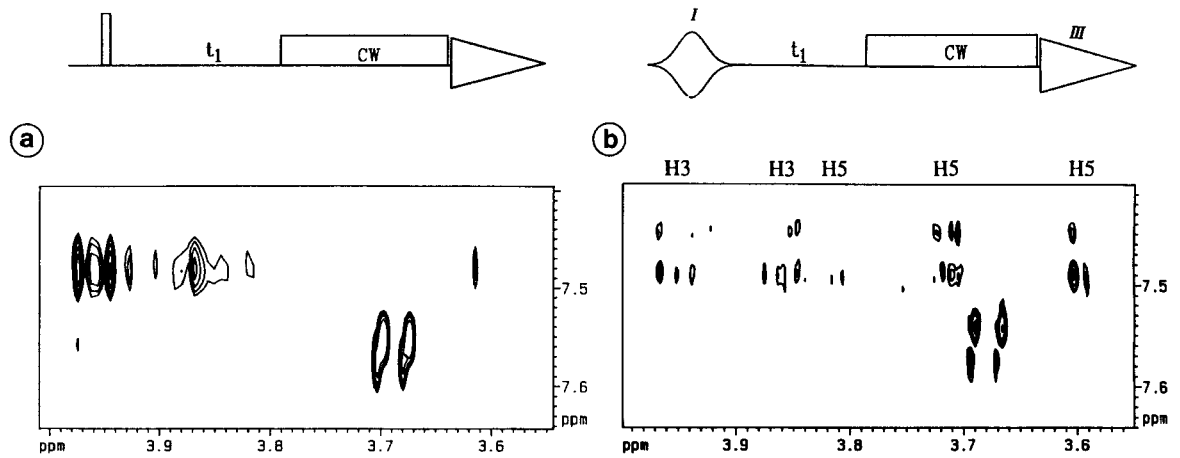

Figure 3 Comparison of the classical (hard) and semi-soft versions of ROESY experiment (same sample as in Fig 2). (a) Extension of the broken line box of Figure 2. (b) Same region in semi-soft ROESY experiment (initial excitation over the aromatic region labelled I in Fig 1, acquisition centred on the region labelled III). Total acquisition time 10 h.

protons of the host and the guest. Although this plot clearly shows these interactions as proof of the reality of inclusion, zooming-in of the region of interest (corresponding to cross-peaks between aromatic protons of the guest and protons of the cyclodextrin) reveals a lack of resolution (see Fig 3a). The intrinsic reduced resolution of 2D maps cannot be improved much further owing to storage and experiment time limitations. This situation also holds for 2D experiments dedicted to assign all signals of the cyclodextrin moiety by scalar transfer from anomeric protons. We recently proposed the use of alternative techniques to obtain ultra-high resolution of 2D maps for cyclodextrin derivatives.[4] These approaches based upon the use of selective excitation pulses (shaped pulses) can also be used in the case of inclusion complexes.

General principles of soft experiments

The basic idea for the use of selective pulses is to tailor the excitation of a spin population to the requested frequency domain without affecting the spin state of other frequencies. According to the shape, power and duration of the selective pulse, virtually any frequency domain of any size can be excited selectively. The experimental methods of achieving the requested selectivity are well documented in the literature.[5] In the present case only gaussian pulses will be considered as they are easier to implement than more complex shapes and they display the requested properties in terms of the selectivity and efficiency of the excitation. These shaped pulses (soft pulses) can replace classical hard pulses in any 1D, 2D or 3D pulse sequence leading to semi-soft or soft sequences depending upon whether one single or all hard pulses are replaced by soft equivalents. The benefits of this approach are clearly demonstrated in the semi-soft version of ROESY, as displayed in Figure 3(b). As shown before, the classical hard experiment suffers from intrinsic resolution limitations since the useful part of the full matrix only represents a small proportion of the total, most of the latter being made up of blank domains. The use of selective pulses allows collection of the useful domain only, making full use of the available storage. The semi-soft techniques are also expected to avoid spurious peaks generated by the overlap of diagonal peak tails[6] which can lead to erroneous assignments. These are clearly visible in Figure 2.

The large resolution enhancement observed by comparing Figures 3(a) and 3(b) supports the use of soft pulse NMR techniques to overcome the intrinsic limitations of classical approaches. Since the observed cross-peaks involve aromatic protons of the guest and a fairly limited number of protons of the host, it seems worth attempting a complete assignment of the latter. We wish to show in the next section that a complete analysis of the inclusion process by means of dedicated soft pulse techniques allows us to derive a large number of parameters of considerable value in proposing a 3D picture of the complex in solution.

Total assignments of the NMR spectra

It is noteworthy that cross-peaks observed in the ROESY experiment do not correspond to signals of the pure complex since fast exchange conditions are encountered because no new peaks are observed, only shifts being present. The observed chemical shifts are hence average values between the free state and the pure complex, the proportion of the complex being the weighting factor. As already shown for simpler cases,[7] a complete determination of NMR parameters in the pure complex involves a complete kinetic analysis and further processing of the derived data. The general equilibrium state can be written as:

$$m \text{ Host} + n \text{ Guest} \rightarrow \text{Complex}$$

The stoichiometry of the complex (m:n) can be derived from the continuous variation technique (Job method) as described elsewhere.[7] In the present case (data not shown), a 1:1 complex is encountered and it was shown that no other process (such as the formation of dimers of the guest) competes with the inclusion process. Under these limiting conditions, the value of any observed NMR parameter, P_{obs} (chemical shift, coupling constant, Overhauser effect, relaxation rate, etc.) can be written as:

$$P_{obs} = P_{free}\{[F]/([F] + [C])\} + P_{complex}\{[C]/([F] + [C])\}$$

P_{free} = parameter for the free species; $P_{complex}$ = parameter for the pure complex; [F] = concentration of the free species; [C] = concentration of the complex.

If any of these parameters is measured with high accuracy for several samples containing variable proportions of the host and guest, processing of the experimental data allows derivation of both the effective association constant and the value of the considered parameter in the pure complex.[8] This can be achieved by a multiparametric fitting procedure of the SIMPLEX type. It is however quite important to note that the reliability of these methods strongly depends on the accuracy of the input parameters. Similar information can be derived from signals of the guest molecules, but generally weaker effects are observed.

The first and most sensitive set of parameters to be determined is the chemical shifts of all protons of the host (and eventually of the guest) in the pure complex. Since we are dealing with inclusion processes, the most prone to experience large shift variations will be the H3 and H5 protons of the cyclodextrin derivative as they are directed towards the interior of the host cavity. Direct assignment of these protons is obviously impossible owing to the overcrowded nature of the NMR spectrum in the region of interest, 4.2 ppm to 2.8 ppm, where all non-anomeric protons are expected. Conversely, anomeric protons appear in a specific domain and they will be used as the starting point to derive all other signals by means of scalar transfers.

It has already been pointed out that magnetization transfers from the isolated anomeric protons provide a very safe stepwise assignment of all other protons in complex oligosaccharides.[9] The use of successive relayed COSYs[10] has been preferred to the more general TOCSY[11] method which provides in a single experiment the complete subspectrum of each coupled system. The large variations expected in the chemical shifts of some protons (H3 and H5) relative to the total spectral domain for non-anomeric protons, can indeed strongly affect their position in the spectrum and lead to assignment errors. The use of selective pulses is expected to afford the very high resolution required for a precise determination of chemical shifts and coupling constants.

Figure 4 represents the contour plots of semi-soft

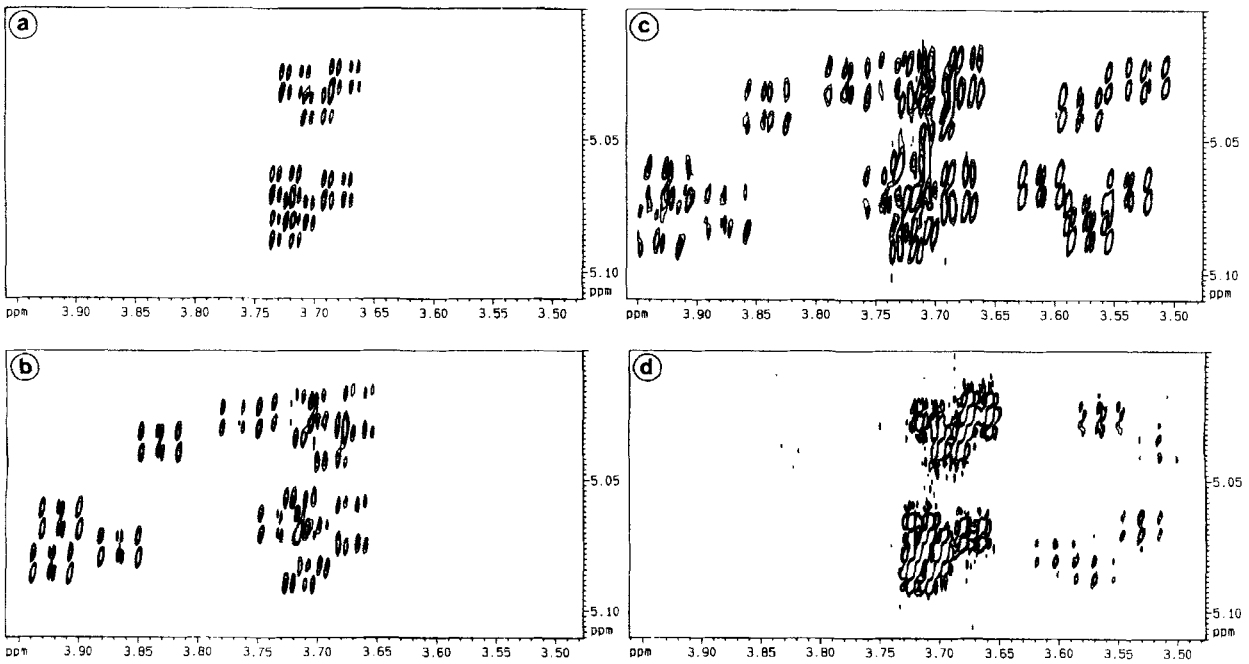

Figure 4 Selective excitation experiments for complete assignments of the 1, 2, 3 and 4 protons of the glucose units of the cyclodextrin in a 1:1 mixture. (a) Semi-soft COSY; (b) semi-soft one-step relay; (c) semi-soft two-step relay; (d) semi-soft NOESY (mixing time = 300 ms).

experiments performed on one of the mixtures. The windows used for the soft pulse (F1 dimension) and for acquisition correspond to labels II and III in Figure 1.

On the COSY map (Fig 4a) all H1–H2 cross peaks are seen. In Figure 4(b), a further relay towards H3 was performed and H2 and H3 signals appear nicely resolved. These latter protons are strongly affected by the inclusion process and show large inequivalences conversely to H2 protons which, owing to their location on the outside part of the cyclodextrin, remain virtually unaffected by inclusion. It must be borne in mind that, although all H3 protons are clearly seen, they cannot be assigned to any specific unit of the cyclodextrin. This problem can be resolved by performing a complete sequencing of the molecule. This is the purpose of the next two experiments displayed in Figures 4(c) and 4(d). In Figure 4(c), a further relay (two-step) was performed allowing an H1–H2–H3–H4 transfer and clearly shows all H4 protons. In the last plot (Figure 4d) a semi-soft NOESY experiment was performed to show dipolar interactions between H1 protons and H4 protons of the next unit. The complete sequencing of the molecule then becomes possible if a starting point can be found. In the present case, it is afforded by the specific position of H6,6′ protons of unit A which appear at high field (see Fig 2) owing to the minor deshielding effect of sulphur compared with oxygen. 1D or 2D relay experiments allow a very clear assignment of protons H4 of unit A, finally completing the sequencial analysis.

The experiments presented are dedicated to explain the general strategies used for a complete assignment of the most important signals of the host. To follow the inclusion process, they must be repeated for all considered samples under different ratios of host to guest. The most useful data is expected from the H3 protons as shown in Figure 5 where semi-soft relay experiments (as in Fig 4b) are plotted for various proportions of host to guest. Large variations of the chemical shifts of these protons are seen upon increasing the proportion of guest molecules. The observed chemical shifts can be used to derive the binding constant and the NMR characteristics of the pure complex. The importance of the variations observed does not mean that this experiment alone is sufficient. It is clear from the signals of anomeric protons, as shown in Figure 5, that the latter also experience shifts upon inclusion. Although much weaker than those observed for protons H3, they can lead to unsafe assignments from the crossing-over of H1 protons. This suggests that sequential assignments should also be performed for all samples.

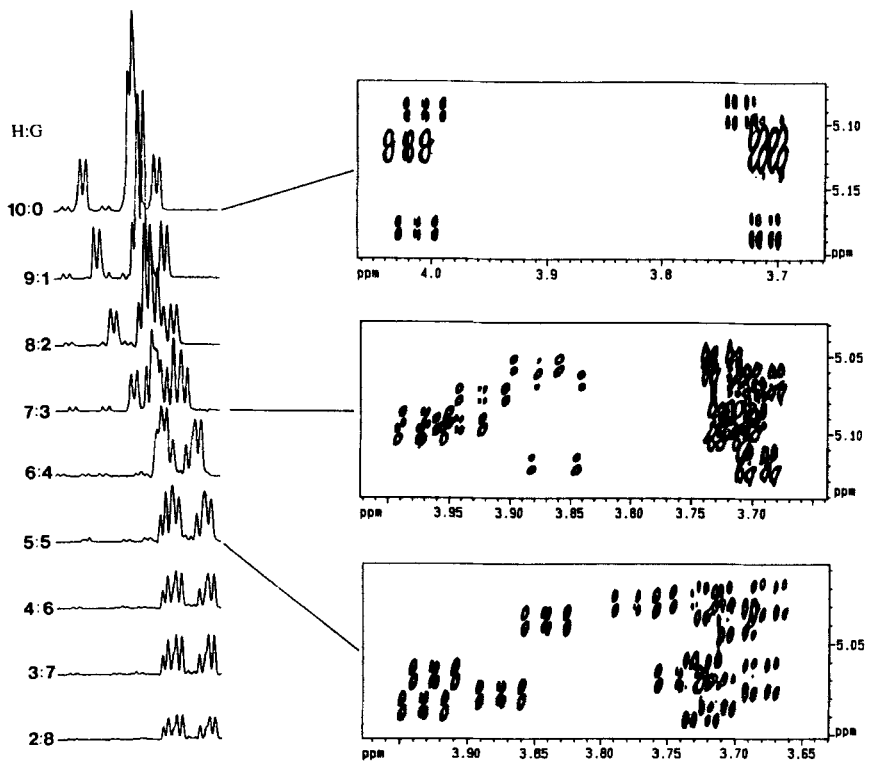

Figure 5 Left: partial 1D spectra for different host/guest (H/G) concentration ratios (20 mM total) showing the complex behaviour of anomeric protons. Right: selected semi-soft relays showing the corresponding H2 and H3 signals.

This apparently complex procedure is in reality highly simplified by the fact that all four experiments depicted in Figure 5 can be chained in a single (generally overnight) run ensuring that they are all performed under strictly identical conditions.

An example of the potential use of the derived data is shown in Figure 6 where the chemical shifts of all H3 protons are collected for various host–guest concentration ratios. The corresponding data can then be used to accurately determine both the binding constant and the observed parameter extrapolated to the pure complex. In the present case, the binding constant was found to be 1500 M^{-1}. This implies that in the 10 mM:10 mM sample, 76% of the guest is complexed. It is worth noting that this allows further extrapolation of any parameter to the pure complex by considering its value in the free host and in any mixture. Examples of this are shown in Table 1 for protons H5 which are very sensitive to the inclusion process but not easily available from anomeric protons since at least three relay steps must be performed. This experiment was therefore performed on two samples only (the free host and the 10 mM:10 mM mixture) and the parameters were directly extrapolated. This is shown in Table 1 where the pure complex values for H3 and H5 protons are reported.

Table 1 Differences (ppm) between chemical shifts in the free host and in the pure 1:1 complex with dothiepine for H3 and H5 protons. The values $\Delta\delta$H5 have been obtained by extrapolation (see text)

CD unit	*$\Delta\delta H3$*	*$\Delta\delta H5$*
b	0.12	0.18
f	0.13	0.24
e	0.18	0.31
c	0.22	0.39
a	0.31	0.15
g	0.35	0.64
d	0.36	0.91

This method, taking advantage of the high accuracy available to measure chemical shift variations, shows good reliability since the determination of the binding constant is performed using several variable parameters.

The NMR data obtained by this procedure can further be used to derive a 3D structure of the complex in solution. Obviously, the effects observed on protons H3 and H5 of the cavity are the most useful to propose an orientation of the guest in cavity. A detailed presentation of the procedure used to convert these parameters in terms of molecular conformation of the complex is beyond the scope of this paper and only a preliminary picture taking into account NOE data and chemical shift variations is presented (Fig 7).

EXPERIMENTAL

The hard and soft experiments were performed on standard BRUKER AMX500 (11.7 Tesla) and AMX600 (14 T) spectrometers equipped with HAAKE temperature regulation systems. The shaped pulse used in semi-soft experiments was a 1% truncated gaussian

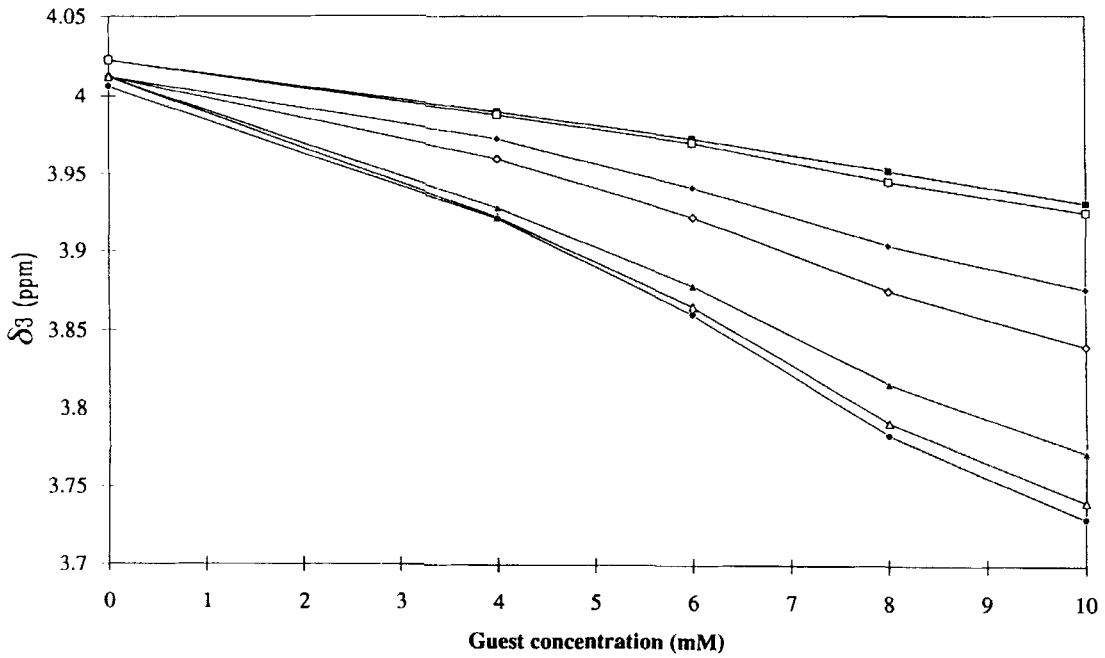

Figure 6 Chemical shift variations of H3 signals measured at different host/guest concentration ratios (20 mM total) as a function of guest concentration.

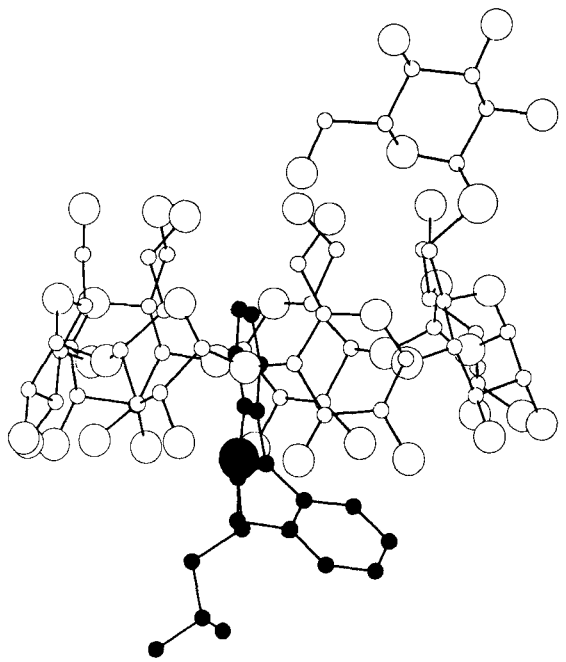

Figure 7 Molecular structure of the 1:1 complex as deduced from NMR data. (See colour plate at the back of journal.)

(defined by 2048 points) generated by the standard BRUKER Selective Excitation Unit under control of the SHAPE subroutine. Its duration was adjusted to afford a good excitation profile in a 0.3–1.0 ppm range according to experimental requirements.

Mixing times in the hard and semi-soft ROESY experiments as well as in NOESY were 300 ms. The spin-lock field strength in the former experiments was *ca.* 4 kHz. The data matrix for the hard ROESY was made of 256 free induction decays (FIDs), 2048 points each, resulting from the co-addition of 240 scans. The real resolution before zero-filling was 1.77 and 14.15 Hz/point in F2 and F1, respectively. For the semi-soft ROESY version, 128 FIDs (1024 points each, 240 scans) were collected and the real resolution as defined before was 1.17 and 5.85 Hz/point. In the case of semi-soft experiments dedicated to spectral assignment, 128 FIDs (1024 points) afforded 1.17 and 1.4 Hz/point in F2 and F1, respectively. 3D representation of the inclusion complex was performed using the QUANTA package from Molecular Simulation Inc., running on an IBM Risc 6000/530 workstation.

CONCLUSION

The use of soft excitation techniques used in 1D and 2D NMR opens up a wide range of new approaches to overcome difficulties in the NMR analysis of complex molecular systems. These difficulties may arise from spectral overcrowding (especially in 1D) and from intrinsic resolution limitations (in classical 2D). Implementation of existing sequences by incorporation of selective pulses allows these difficulties to be overcome and the extraction of accurate data from apparently hopeless situations. These procedures therefore offer the largest available flexibility in terms of selection of transfer pathways and in the sense that specific interactions can be focused on.

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