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# An NMR study of cyclodextrin complexes of the steroidal neuromuscular blocker drug Rocuronium Bromide

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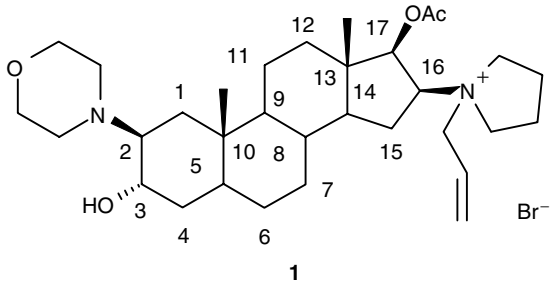
**The interaction of Rocuronium Bromide, and a model steroid Org 7402, with three cyclodextrins (β-cyclodextrin, γ-cyclodextrin and Org 25969) was studied by solution state NMR experiments. Stoichiometries and binding constants were determined from $^1$H chemical shift titrations. All of the systems formed 1 : 1 complexes. Most of the complexes were in fast exchange with unbound species on the NMR time scale, but the most tightly bound complex (Rocuronium Bromide–Org 25969) was in the slow exchange regime. The geometry of the complexes was inferred from $^1$H and $^{13}$C NMR shift changes upon complexation and from intramolecular NOE correlations. Rocuronium Bromide forms a weak complex with β-cyclodextrin ($K_a = 3.3 \pm 0.5 \times 10^3$ M$^{-1}$) and no clear picture of the structure of the complex emerges. The complexes with γ-cyclodextrin ($K_a = 1.8 \pm 0.2 \times 10^4$ M$^{-1}$) and Org 25969 ($K_a > 10^5$ M$^{-1}$) are true inclusion complexes with the steroid located inside the central void of the cyclodextrin. Copyright © 2002 John Wiley & Sons, Ltd.**

**KEYWORDS:** NMR; $^1$H NMR; $^{13}$C NMR; host–guest chemistry; steroids; cyclodextrins; anaesthetics

## INTRODUCTION

Neuromuscular blocking drugs are used by anaesthetists during surgical procedures to ease tracheal intubation and to improve muscle tone.[1] Many different drugs with different durations of action are available.[2] Rocuronium Bromide (**1**) is typical of the highly successful amino steroids class of neuromuscular blockers that are in general use worldwide.[3]




**1**

However, it remains a problem that sometimes patients are still under the effects of the neuromuscular block (and are therefore paralysed) after the surgical procedures are finished. Current practice is to administer an inhibitor of acetylcholinesterase (e.g. Neostigmine) to reverse the action of the blocking drug. It would be desirable for anaesthetists to have an alternative course of action. One such line that has been followed at these laboratories was the development of neuromuscular block reversal agents by means of sequestering the blocking drug.[4] In the course of this research cyclodextrins were considered as prospective reversal agents because of their known ability to sequester drugs by forming host–guest complexes.[5]

Cyclodextrins are cyclic oligosaccharides composed of a ring of α-1,4 linked D-glucosyl residues, and comprising six (α-cyclodextrin), seven (β-cyclodextrin (**2**)) or eight (γ-cyclodextrin (**3**)) units. Tertiary structures resemble a hollow, truncated cone with primary hydroxyl groups crowning the narrow rim and secondary hydroxyl groups crowning the wider rim. The low polarity central void is able to encapsulate smaller molecules and is responsible for the great interest in cyclodextrins in host–guest chemistry.[6,7]

There are many reports of steroid–β-cyclodextrin complex formation,[5] particularly with regard to drug delivery[8] and molecular recognition,[9–12] and binding constants for 1 : 1 complexes are typically in the range $10^4$ to $10^5$ M$^{-1}$.[9,11] Thus there is little doubt that steroids can at least partially penetrate the core of β-cyclodextrin.[13–16] However, there are not always clear pictures of the nature of the complexes. A demonstrated interaction of the steroid with the internal cyclodextrin protons H-3 or H-5 is usually taken as proof of internalization. There is a larger literature describing the binding of bile salts to cyclodextrins, but the evidence shows that for this class of compounds it is almost always the alkyl chain (C-20 to C24) that enters the cyclodextrin

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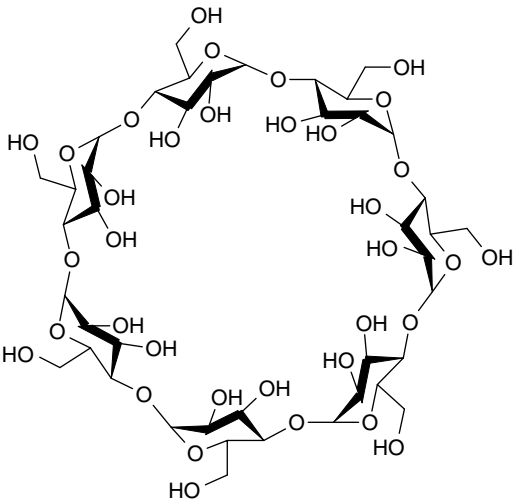

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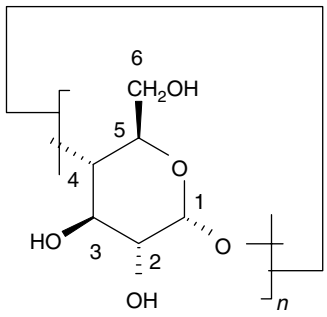
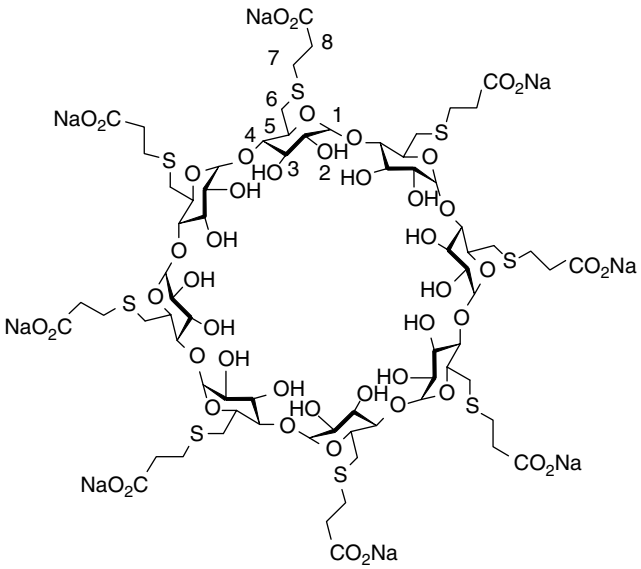


**3** ($n = 8$)



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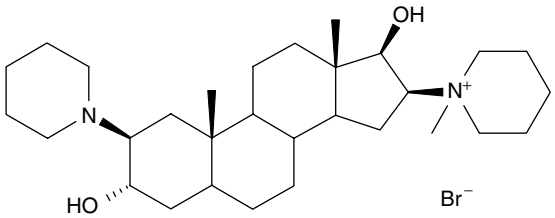

**5**

cavity.[17–21] Recent ROESY studies of ursodeoxycholic acid with $\beta$-cyclodextrin[22] and sodium cholate and sodium deoxycholate with $\beta$-cyclodextrin[23] are producing more detailed pictures. Again, the aliphatic side chain plays the major role in binding. Some reports implicate that ring A of (non-bile) steroids is involved in formation of the complex.[13,16,23,24] Less work has been published on the interactions of steroids with the larger $\gamma$-cyclodextrin.

A modified $\gamma$-cyclodextrin, Org 25969 (**4**), was developed as a potent and rapid reversal agent for the neuromuscular blocking drug Rocuronium Bromide.[4] A natural question arises as to what the structure of the cyclodextrin–steroid complex is.

The principal aim of the study was to elucidate the structure of the Rocuronium Bromide–Org 25969 complex (**1:4**). This was a challenging problem, and we found it convenient to study also some simpler systems, and so we also report on the properties of the complexes of Rocuronium Bromide with the naturally occurring $\beta$-cyclodextrin and $\gamma$-cyclodextrin, and on a model system, the Org 7402–Org 25969 complex (**5:4**). This communication reports on the stoichiometry and formation constants of the steroid–cyclodextrin complexes **1:2**, **1:3** and **1:4**. Evidence is presented for internalization of the steroid in complexes with the larger $\gamma$-cyclodextrin **3** and the $\gamma$-cyclodextrin derivative **4**.

## EXPERIMENTAL

$\beta$-Cyclodextrin [85,608-8] was purchased from Aldrich; $\gamma$-cyclodextrin [17465-86-0] was purchased from Fluka; Rocuronium Bromide, Org 7402 and Org 25969 were from Organon Laboratories Ltd. All materials were dried at 70 °C for 48 h before use. Solutions for binding studies were prepared by mixing and dilution of stock solutions in 0.2 M phosphate-buffered $D_2O$. For instance, a series of solutions of cyclodextrin was made to cover the concentration range 0.2 to 6 mM (each 400 µl). To each solution was then added 400 µl of 0.820 mM Rocuronium Bromide solution. Solutions for Job plots were prepared by mixing appropriate quantities of 1 mM Rocuronium Bromide with 1 mM Org 25969 (all in 0.2 M phosphate-buffered $D_2O$ at pH 7.5, pH 4.3 or pH 9.1) so that a range of different solution compositions was sampled, but the total molarity remained 1 mM. Equimolar solutions were prepared for the structural studies and for the purposes of tabulating bound shifts. Concentrations are given in the text. All NMR experiments were performed on a Bruker DRX 400 spectrometer fitted with an inverse geometry 5 mm probe and under VT control. 1D $^1$H NMR spectra for binding studies and Job plots were acquired with a digital resolution of 0.0002 ppm/point and using a small amount of MeOH as an internal chemical shift reference. ROESY experiments were performed in the phase-sensitive mode and with presaturation of the water signal, 16 scans, sw = 6.4 ppm, 256 $t_1$ increments, mixing time 400 ms. $^{13}$C chemical shifts were recorded from the HSQC spectra. HSQC data were obtained with eight scans, $sw_H = 6.3$ ppm,

$sw_C$ = 180 ppm, and 256 $t_1$ increments. Analysis of the NMR binding data was performed within the environment of an Excel spreadsheet. The spreadsheet was configured to solve the quadratic equation that relates specific species concentrations to the known total concentrations present and the association constant for formation of a 1:1 complex.[25] The embedded solver tool was used to minimize the difference between the calculated curve and the experimental data. This spreadsheet is available from the authors. The following discussion contains frequent references to steroid and cyclodextrin protons. To clarify any confusion about which protons are being discussed, cyclodextrin protons are always referred to as H-3, H-2, etc., with no subscripts, whereas notations for steroid protons bear an additional $\alpha$ or $\beta$ identification.

## RESULTS AND DISCUSSION

### Stoichiometry and binding constants

Quantitative descriptors of binding are stoichiometry and binding constants. NMR is routinely used to measure these parameters for host–guest complexes. The terms of reference for such studies are either fast exchange or slow exchange. In the slow exchange regime, all components of a mixture (steroid, cyclodextrin and steroid–cyclodextrin complex) give rise to their own discrete signals in the NMR spectrum. If spectral dispersion is sufficient for these discrete signals to be resolved, analysis of the data is simple. The spectrum is assigned and information about stoichiometry and binding comes directly from integrating the spectrum.

In the fast exchange regime, the observed nuclear spin appears as though it were in two sites at the same time, and the observed NMR frequency is the mole fraction weighted average between the frequencies in the native form and the complexed form of the observed molecule. For example, if the observed proton is located on the host (cyclodextrin) molecule.

$$\delta_{obs} = X_H\delta_H + X_{HG}\delta_{HG} \quad (1)$$

where $\delta_{obs}$, $\delta_H$ and $\delta_{HG}$ are the chemical shifts of the observed nucleus in the experiment, in the host molecule and in the complex respectively, and $X_H$ and $X_{HG}$ are the mole fractions of host molecule distributed between the two sites. In this case the analysis is more complicated than the slow exchange case because the chemical shift in the complex cannot be observed. This problem may be overcome by obtaining information on chemical shift over a range of different solution compositions—an NMR titration. Once the stoichiometry of the complex is established, a binding equation can be written, which can be combined with Eqn (1) to produce non-linear expressions relating the observed chemical shift to $K_a$ and $\delta_{HG}$. The values $K_a$ and $\delta_{HG}$ are determined by minimizing the difference between the experimentally observed data and the calculated curve.

In this study, fast exchange was observed in complexes **1:2** and **1:3**, and slow exchange was observed in **1:4**.

*Rocuronium Bromide complexes with β-cyclodextrin and γ-cyclodextrin (**1:2** and **1:3**)*

For the determination of stoichiometry and binding constants we simply need to identify protons that are sensitive to complex formation. In the present case this was generally the cyclodextrin H-3 proton and the steroid axial methyl groups (18-$CH_3$ and 19-$CH_3$) or the steroid H-9$\alpha$ proton. In this section we are only concerned with markers of complex formation, and not with structure. A good marker will be a peak that is easy to measure, for instance a sharp singlet, and/or is a peak that shifts significantly upon complexation.

The $^1$H and $^{13}$C NMR spectra of **1** have been fully assigned.[26] The stoichiometry of the complexes **1:2** and **1:3** were determined to be 1:1 by Job's method (data not shown).[27] Results of binding assays are summarized in Table 1.

Binding curves observed from the system Rocuronium Bromide and $\beta$-cyclodextrin at room temperature are shown in Fig. 1. The change in chemical shift of the 19-$CH_3$ peak of **1** as a function of the concentration of **2** is shown in Fig. 1(A). The 19-$CH_3$ group is a good marker because it is a sharp singlet that can be seen in a simple 1D spectrum and it shifts significantly (>0.2 ppm downfield) in the presence of **2**. In the form of binding curve shown here, the concentration of **1** is kept constant and the concentration of **2** is varied. The solution composition is expressed on the $x$-axis as a mole fraction of **2**, i.e. [**2**]/([**1**] + [**2**]). Throughout this range the spectra appeared as a two-component mixture of Rocuronium Bromide and cyclodextrin. This confirms that the complexed and non-complexed components are in fast exchange on the NMR time scale.

The solid line is the least squares fitted curve for a 1:1 complex with $K_a$ = 3500 $M^{-1}$. Figure 1(B) shows the experimental data and the corresponding calculated curve from the proton (H-5) on the cyclodextrin. The chemical shift range experienced by this proton is considerably less than that seen on the steroid axial methyl group, but it is enough of a shift to calculate $K_a$ reliably. The value obtained (2600 $M^{-1}$) is in satisfactory agreement with the value obtained from observations on the steroid, and averaging all of the available

**Table 1.** Binding constants of Rocuronium Bromide with cyclodextrins[a]

| Observed proton[b] | β-Cyclodextrin **2** | γ-Cyclodextrin **3** | Org 25969 **4** |
|---|---|---|---|
| H-9α | | 20 400 | >60 000 |
| 19-$CH_3$ | 3 500 | | |
| H-3 | 3 900 | 17 000 | |
| H-5 | 2 600 | 17 800 | |

[a] From curve fitting the NMR titration data. The $\delta_H$, $\delta_G$ or $\delta_{HG}$ parameters that accompany this fitting are as follows. **1:2**, 19-$CH_3$ $\delta_G$ = 0.87, $\delta_{HG}$ = 1.10 ppm; H-3 $\delta_H$ = 3.94, $\delta_{HG}$ = 3.89 ppm. **1:3**, H-3 $\delta_H$ = 3.91, $\delta_{HG}$ = 3.81 ppm; H-5 $\delta_H$ = 3.83, $\delta_{HG}$ = 3.64 ppm; H-9α $\delta_G$ = 0.78, $\delta_{HG}$ = 0.63 ppm. **1:4**, H-9α $\delta_G$ = 0.81, $\delta_{HG}$ = 0.68 ppm.

[b] H-9α and 19-$CH_3$ are located on the steroid, and H-3 and H-5 are the 'internal' protons in cyclodextrins.

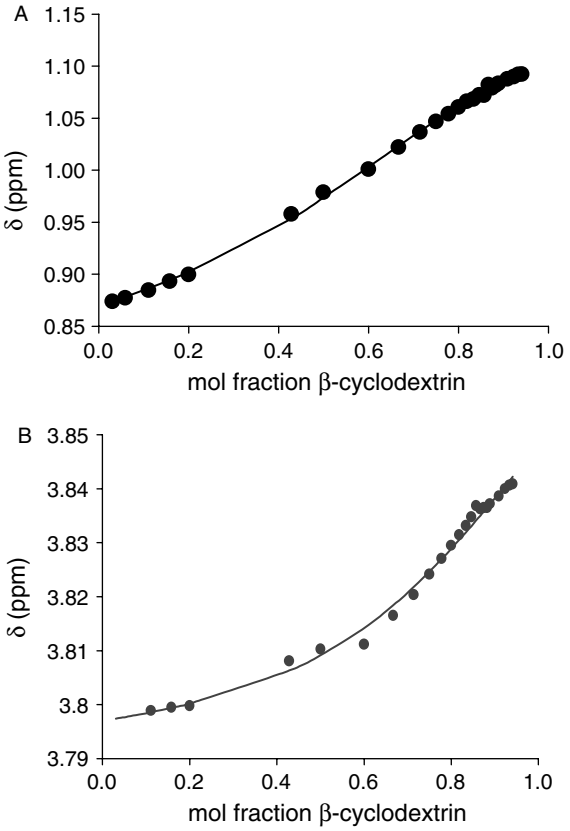


**Figure 1.** $^1$H NMR chemical shift changes observed during the titration of Rocuronium Bromide with $\beta$-cyclodextrin. (A) The behaviour of the steroid 19-$CH_3$ proton signal. (B) The behaviour of the cyclodextrin H-5 signal. The points are the experimental data, [**1**] = 0.41 mM, [**2**] ranging from 0.013 to 6.6 mM at 30 °C, and the solid line is the calculated curve using parameters from Table 1.

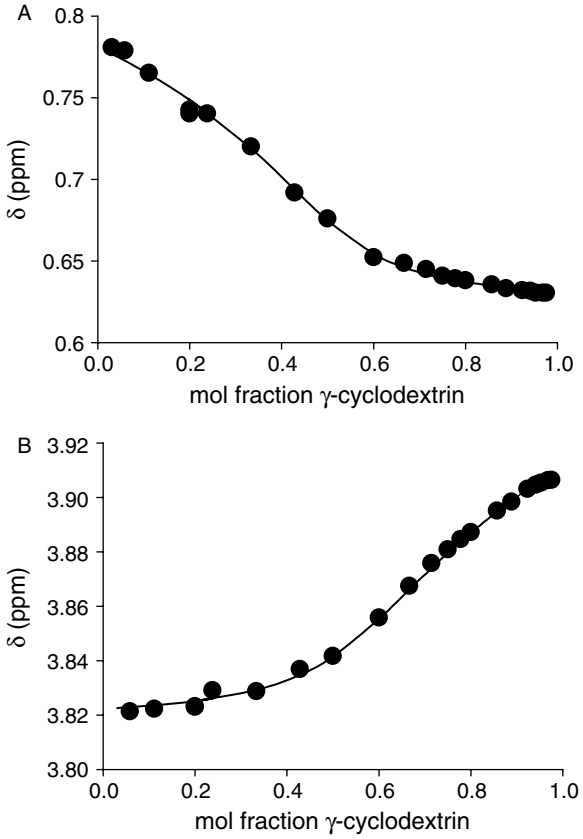


**Figure 2.** $^1$H NMR titration curves for Rocuronium Bromide with $\gamma$-cyclodextrin. (A) The behaviour of the steroid H-9$\alpha$ proton signal. (B) The behaviour of the cyclodextrin H-3 signal. The points are the experimental data, [**1**] = 0.41 mM and [**3**] ranging from 0.013 to 8.2 mM at 30 °C, and the solid line is the least squares fitted calculated curve using parameters from Table 1.

data gives $K_a = 3.3 \pm 0.5 \times 10^3$ M$^{-1}$ for formation of a 1:1 complex. Similar curves were obtained from the titration of Rocuronium Bromide with $\gamma$-cyclodextrin (Fig. 2). Again, we see (Table 1) a good correlation between the $K_a$ derived from a steroid proton shift and that derived from a cyclodextrin proton, and $K_a = 1.8 \pm 0.2 \times 10^4$ M$^{-1}$. With $\gamma$-cyclodextrin, H-9$\alpha$ of the steroid also proved to be very sensitive to the presence of the cyclodextrin, and so this was used as the marker. In plots such as Figs 1 and 2 the difference between the chemical shift of the observed proton in the complex ($\delta_{HG}$) and the non-complexed molecule ($\delta_H$ or $\delta_G$) defines the total chemical shift range of the *y*-axis (often referred to as $\Delta\delta_{max}$) and the value of $K_a$ determines the degree of curvature. Hence, compared with the flatter curves of Fig. 1, the S-shaped curves observed in Fig. 2 graphically illustrate the stronger affinity of $\gamma$-cyclodextrin over $\beta$-cyclodextrin for Rocuronium Bromide.

The non-linear relationship between $\delta_{obs}$ and $K_a$ necessitates the curve fitting approach discussed above. An alternative treatment, dating back to early spectroscopic studies of complexation, is to make a numerical approximation that results in a linear relationship between $\delta_{obs}$ and $K_a$. If the not observed species (e.g. the host) is present in large excess, then the concentration of free host can be assumed to be equal to the starting concentration $[H]_0$ and linear graphical solutions ensue.[28] One such linearization that has been widely used in studies of host–guest complexation is the Benesi–Hildebrand method.[29] The data from both of the binding experiments were also suitable for treatment by the Benesi–Hildebrand method ($[H]_0 \gg [G]_0$). This data treatment was applied and very similar results were obtained: $K_a = 3100$ M$^{-1}$ for **1:2** from the data of 19-$CH_3$; $K_a = 11\,000$ M$^{-1}$ for **1:3** from the data of H-9$\alpha$ .

### *The complex of Rocuronium Bromide with Org 25969 (**1:4**)*

Org 25969 has eight pendant mercapto-propionate groups ringing the primary face of the cyclodextrin torus. The studies with the **1:4** system were performed at several different conditions of pH to probe the importance of ion interactions. The studies were also performed at a range of temperatures in order to explore the fast exchange and slow exchange NMR time scales.

The room temperature $^1$H NMR spectra of Rocuronium Bromide–Org 25969 mixtures were more complicated than mixtures of **1** with $\beta$-cyclodextrin or $\gamma$-cyclodextrin. This system was in slow exchange on the NMR time scale, and so both free **1**, free **4**, and the **1:4** complex contributed to the spectrum. A portion of the 400 MHz $^1$H spectrum of a mixture

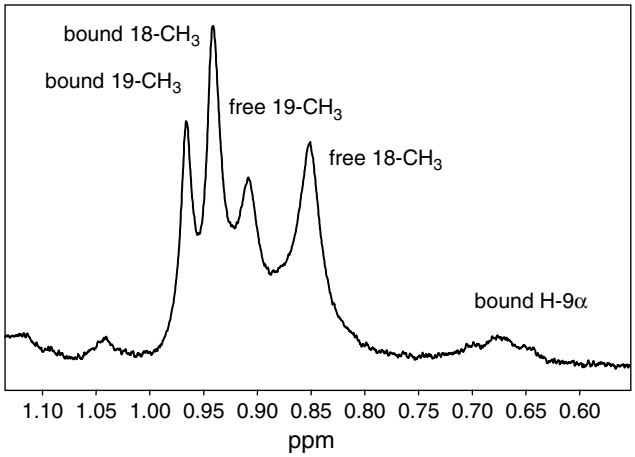


**Figure 3.** Part of the 400 MHz $^1$H NMR spectrum of a mixture of Rocuronium Bromide (0.5 mM) and Org 25969 (0.3 mM) in $D_2O$ at pH 7.5, 30 °C, showing peaks for both free and bound Rocuronium Bromide.

of **1** and **4** (5 : 3 mole ratio) is shown in Fig. 3. This spectrum displays the doubling of signals that results from observing the steroid axial methyl signals (18-$CH_3$ and 19-$CH_3$) in two different environments. In this solution the steroid is present in higher concentration than the cyclodextrin, and the integrated peak ratios (bound versus free) are consistent with very strong binding and formation of a 1 : 1 complex (i.e. the amount of complex formed is limited by the amount of cyclodextrin present, [**1 : 4**] = [**4**]). Also visible in this portion of the spectrum is the signal from H-9$\alpha$ in the complex. This peak is shifted from 0.81 ppm in the uncomplexed steroid.

For a system in slow exchange on the NMR time scale, the concentrations of constituents can be measured directly from signal intensities. Hence, the stoichiometry of the complex is determined in a straightforward fashion from the binding isotherm, which is expected to be in the form of two straight lines intersecting at the stoichiometry of the complex. Figure 4 shows the pH 7.5 data plotted to show the proportion of steroid bound in the complex (determined from the peak height ratios of spectra such as Fig. 3) versus the mole fraction of Org 25969. The correlation coefficient $R^2$ for the least squares fitted straight line is 0.982, and the line intersects the 100% bound axis at a stoichiometry of 0.47. The binding constant for the **1 : 4** complex is too high to measure from this data. In order to measure $K_a$, there must be a measurable quantity of uncomplexed **1** or **4** present at sub-stoichiometric ratios. If we assume that we should be able to detect at least 0.1 mM of the uncomplexed species, the lower limit of $K_a$ is $10^4$ M$^{-1}$.

As the temperature was increased, the signals due to non-complexed and complexed species coalesced and then sharpened. At elevated temperatures the system was completely in the fast exchange regime, and data could be treated by the methods detailed above for $\beta$-cyclodextrin and $\gamma$-cyclodextrin complexes. Figure 5 shows the result of a Job plot analysis for the **1 : 4** system at pH 9.1 and 70 °C. In the Job plot a physical parameter (the $^1$H chemical shift) is measured at different ratios of [**1**]/[**4**], while keeping the total concentration [**1**] + [**4**] constant. This data is a confirmation of the 1 : 1 stoichiometry observed in slow exchange conditions at 30 °C (Fig. 4). An almost identical plot was obtained at pH 4.3 and 60 °C , indicating that the stoichiometry of the complex is independent of pH.

Attempts to extract a value for $K_a$ from the higher-temperature fast-exchange data failed for the same reason (sensitivity) as outlined above, but consideration of the fast exchange data allowed us to revise the lower limit to $K_a > 6 \times 10^4$ M$^{-1}$. At 30 °C (slow exchange regime) there was a clear trend for the spectra to become sharper as pH increased. This indicates that the exchange (on/off rate) for complex formation is faster at higher pH, which in turn suggests that the formation constant $K_a$ decreases as pH increases. Microcalorimetry experiments indicate that the complex **1 : 4** has an association constant of *ca* $1 \times 10^7$ M$^{-1}$.[30]

## Geometry of the complexes

The previous section dealt with the thermodynamic descriptors of binding. We turn now to structural issues and ask questions about the geometry of the steroid–cyclodextrin complex. The cyclodextrin molecule is analogous to a short pipe with one opening (the side of the secondary hydroxy groups) larger than the other. The depth of the cavity is 7.9 to 8.0 Å, and the diameter varies between 6.0 and 6.5 Å for

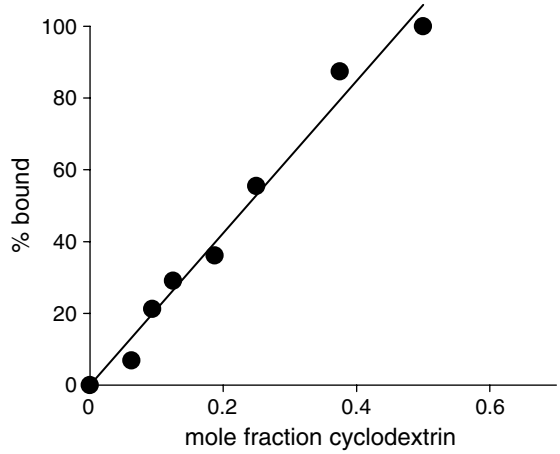


**Figure 4.** Binding isotherm for the slow exchange system, Rocuronium Bromide with Org 25969 at pH 7.5, 30 °C.

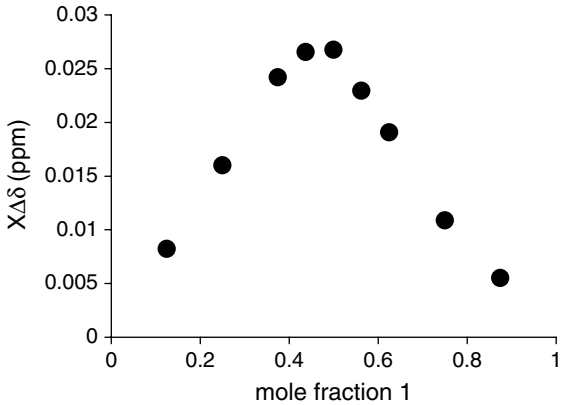


**Figure 5.** Job plot for the fast exchange system, Rocuronium Bromide and Org 25969 at pH 9.1, 70 °C. The total concentration [**1**] + [**3**] = 1.0 mM.

$\beta$-cyclodextrin and between 7.5 and 8.3 Å for $\gamma$-cyclodextrin. It is well established that steroids are able to enter the cavity in $\beta$-cyclodextrin.

One source of structural information is the chemical shift changes relative to the unbound forms observed upon formation of the complex. This parameter is referred to as the complexation-induced shift (CIS, or $\Delta\delta$). Such information from the steroid is likely to be useful to distinguish between postulated binding models. A consideration of cyclodextrin chemical shift changes is expected to be even more profitable, and indeed it is well established that changes in the chemical shifts of cyclodextrin H-3 and H-5 protons are indicative of internalization in guest–cyclodextrin complexes. Observation of $\Delta\delta$H-3 > $\Delta\delta$H-5 is taken to indicate guest binding from the secondary side.

A second NMR probe of the structure of the complex is the detection of through-space dipole–dipole interactions (NOE and/or ROESY) between the steroid and the cyclodextrin. Again, NOESY contacts between the guest and the cyclodextrin protons H-3 and H-5 are hallmarks of internalization. As with the CIS method, stronger NOEs from H-3 compared with H-5 are taken to indicate entry of the guest molecule from the secondary side. With only a few exceptions, guests generally produce stronger NOEs and larger $\Delta\delta$ effects at H-3 than H-5. ROESY experiments are generally preferred to NOESY because of the short correlation times of these small molecules.

### *Mapping the complexation-induced chemical shift changes*

Table 2 summarizes the observed CIS values for Rocuronium Bromide in the three cyclodextrin complexes. For clarity, only the largest shifts are displayed. There is a wealth of structure information in this data. The objective of this part of the analysis is to look for patterns in the magnitude of these shifts that might indicate possible complex geometries. For instance, a preference for binding to the face of the steroid instead of the edge of the steroid would be signalled by larger shifts of axial protons over equatorial protons. Larger shifts of $\alpha$ protons compared with $\beta$ protons would indicate stronger binding to one face of the steroid compared with the other.

### *Rocuronium Bromide–β-cyclodextrin (**1:2**)*

There are 11 entries in Table 2 for the complex with $\beta$-cyclodextrin (**1:2**). We have noted already that the largest shift occurs for the axial 19-$CH_3$ signal (+0.19 ppm). Nine other protons have shifted by >0.1 ppm, and with only one exception (H-15$\alpha$) they are located on rings A, B and C of the steroid. Seven of these strongly shifted protons occupy axial positions, and only three are equatorial. This may be suggestive of a face-on interaction of the steroid, rather than an edge-on binding. The protons with large CIS are equally distributed over both sides of the steroid (five on the $\alpha$ face, and five on the $\beta$ face). CIS effects in the $^{13}$C spectrum were significant, but small (typically *ca* ±0.3 ppm, and up to +1.7 ppm) and without an apparent pattern. The CIS in the $^1$H spectrum of the $\beta$-cyclodextrin are listed in Table 3. The protons that are most sensitive to the presence of the steroid are H-3 and H-5, which are located inside the torus-shaped cyclodextrin (Fig. 6). The shifts that are observed on other protons are insignificant. This suggests that significant internalization of the steroid occurs.

These results are inconclusive and even contradictory. They are suggestive that, in the complex **1:2**, Rocuronium Bromide is incorporated into the cyclodextrin cavity with

**Table 2.** Compiled $^1$H chemical shift perturbations[a] and NOE data[b] observed in Rocuronium Bromide upon binding to cyclodextrins

| | $\beta$-Cyclodextrin (**2**) | $\gamma$-Cyclodextrin (**3**) | Org 25969 (**4**) |
|---|---|---|---|
| 1$\alpha$ | −0.10 | | |
| 1$\beta$ | | | |
| 2$\alpha$ | | | |
| 3$\beta$ | −0.12 | | |
| 4$\alpha$ | | | −0.12 |
| 4$\beta$ | | −0.07 | |
| 5$\alpha$ | −0.16 | | |
| 6$\alpha$ | | −0.09 | −0.13 |
| 6$\beta$ | −0.14 | | |
| 7$\alpha$ | −0.10 | +0.07 | +0.10 |
| 7$\beta$ | −0.15 | | −0.10 |
| 8$\beta$ | | | |
| 9$\alpha$ | | +0.14 | +0.14 |
| 11$\alpha$ | −0.12 | +0.07 | |
| 11$\beta$ | | +0.08 | |
| 12$\alpha$ | −0.15 | +0.10 | |
| 12$\beta$ | | | +0.14 |
| 14$\alpha$ | | | |
| 15$\alpha$ | −0.10 | | −0.16 |
| 15$\beta$ | | | |
| 16$\alpha$ | | | |
| 17$\alpha$ | | | −0.31 |
| 18-$CH_3$ | | | −0.10 |
| 19-$CH_3$ | −0.19 | | |
| 2$\beta$-Morpholine | +0.13 | −0.12[c]<br>+0.07[d] | |
| 16$\beta$-Pyrollidine/allyl | | −0.09 | −0.15<br>−0.10 |
| 17$\beta$-$CH_3CO$ | | | |

[a] Chemical shift change, ($\Delta\delta = \delta_{free} - \delta_{bound}$). The threshold value was set to ±0.10 ppm for $\beta$-cyclodextrin and Org 25969 and ±0.07 ppm for $\gamma$-cyclodextrin. Data from equimolar mixtures (6.1 mM of each component) at 30 °C.

[b] The threshold value for NOEs was arbitarily set so that only the strongest eight to ten NOEs appear in the table (grey 15% applied to cell). Data from equimolar mixtures (33 mM of each component) at 30 °C.

[c] $OCH_2$.

[d] $NCH_2$.

**Table 3.** $^{1}$H chemical shift changes observed in cyclodextrins upon forming a 1:1 complex with Rocuronium Bromide

| | $\beta$-Cyclodextrin (**2**) | $\gamma$-Cyclodextrin (**3**) | Org 25969 (**4**) |
|---|---|---|---|
| H-1 | −0.01 | +0.01 | +0.04 |
| H-2 | −0.02 | −0.03 | −0.07 |
| H-3 | +0.05 | +0.10 | +0.19 |
| H-4 | −0.02 | −0.02 | +0.07 |
| H-5 | +0.08 | +0.18 | +0.19 |
| H-6a | +0.01 | +0.02 | +0.07 |
| H-6b | | | −0.25 |
| H-7 | | | −0.06 |
| H-8 | | | −0.01 |

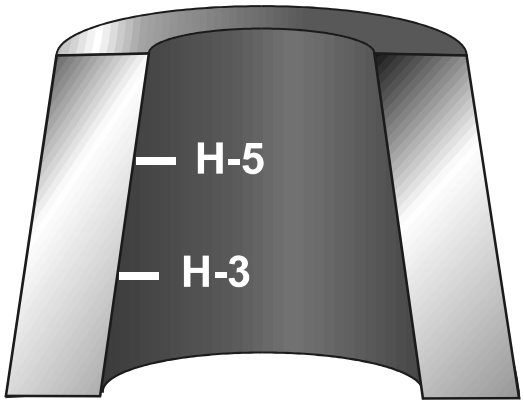


**Figure 6.** Sketch of the internal proton positions in cyclodextrins.

no single preferred conformation. However, the 'face-on' model suggested by the steroid CIS values is not compatible with the inclusion model suggested by the cyclodextrin CIS values.

*Rocuronium Bromide–$\gamma$-cyclodextrin (**1:3**)*

The CIS values observed in the **1:3** system were weaker, and so the threshold value for inclusion in Table 2 was lowered. Smaller CIS effects in the $\gamma$-cyclodextrin system compared with the $\beta$-cyclodextrin system may indicate that the $\Delta\delta_{max}$ values are less in this system, in turn suggesting a looser fit in the larger $\gamma$-cyclodextrin ring. All of the significant shifts were again seen from protons on steroid rings A, B and C. Of the seven entries for framework protons, there is no preference for axial (four) to equatorial sites (three). Neither is there any argument to support preferential binding to the $\alpha$ or $\beta$ face of the steroid. The data from observations on **3** again show much stronger CISs for H-3 and H-5 than for any other cyclodextrin protons. These results strongly suggest that the steroid is included into the centre void of $\gamma$-cyclodextrin. A model where ring B of the steroid was centred in the cyclodextrin torus, but with no preferred orientation, would explain most of these observations.

*Rocuronium Bromide–Org 25969 (**1:4**)*

There are 11 entries in Table 2 for CISs > 0.1 ppm, and the largest shift is observed for H-17$\alpha$. There is a nearly equal distribution of strongly shifted protons around rings B, C and D, but only one entry from ring A. There are almost equal representations from protons on the $\alpha$ face of the steroid (six) and from the $\beta$ face (five), and there is a nearly equal distribution of entries from axial (three) and equatorial steroid protons (four). As noted previously for the other two systems, the $^{13}$C CIS values for the **1:4** system were small and there was no discernable pattern when they were mapped onto the steroid skeleton. Hence the $^{1}$H and the $^{13}$C steroid CIS values indicate no preference for edge binding, face binding, or for any particular face of the steroid. The CIS values from the $\gamma$-cyclodextrin (Table 3) suggest incorporation of the steroid into the cavity. The observed shifts for the internal protons H-3 and H-5 are two times greater than those observed for analogous $\beta$-cyclodextrin protons and are an order of magnitude greater than those observed for any other $\gamma$-cyclodextrin protons.

Taken together, the CIS from both **1** and **4** suggest that the steroid is incorporated in the cyclodextrin and that it rotates freely inside the cavity. This model is also consistent with the large $K_a$ measured for the **1:4** complex.

*Mapping the NOE contacts*

More direct information on the complex geometry came from nuclear Overhauser experiments. The rotational correlation times of the complexes studied here are such that NOEs are very weak, and so the 2D ROESY experiment was used to detect steroid–cyclodextrin interactions. The results of the ROESY experiments are summarized in Table 2, where the shading indicates the presence of a correlation between a steroid proton and H-3 or H-5 of cyclodextrin.

*Rocuronium Bromide–$\beta$-cyclodextrin (**1:2**)*

For the complex **1:2**, the interpretation of ROESY data was straightforward. Only correlations between steroid and H-3 and H-5 on cyclodextrin were observed (there were no correlations to any other cyclodextrin protons). The distribution of NOE contacts follows roughly the general pattern of large CISs, so that correlations are noted to rings A, B and C, and there are none to ring D. However, for the rings A, B and C, there is no correlation between the observed NOEs and the larger CISs. Only half of the previously discussed CISs have NOE boxes shaded, and half of the noted NOE contacts do not correlate with a large CIS. Like the distribution of CISs, the NOE contacts are equally distributed over the $\alpha$ face and the $\beta$ face of the steroid, and are equally distributed between axial and equatorial protons. The general conclusion from the consideration of both the CIS values and the NOE data is that rings A, B and C of Rocuronium Bromide are buried within the cavity of $\beta$-cyclodextrin. A likely model would be for the cyclodextrin to form an annular belt around ring B of the steroid, leaving the ring A substituents projecting into the solvent space and ring D with its bulky substituents plugging the other side. It is implicit in such a model that ring A of the steroid passes through the torus first. This is possible because ring A of Rocuronium Bromide is an equilibrium mixture of chair and twist-boat conformations (see below).

*Rocuronium Bromide–γ-cyclodextrin (**1:3**)*

Analysis of the ROESY data for the complex **1:3** was hindered by the poor dispersion of the γ-cyclodextrin signals. In the 1 : 1 mixtures the cyclodextrin H-3 signal overlaps with H-6, and H-5 overlaps with H-2. Hence it was impossible to distinguish a binding mode from this data. In addition, weak correlations were observed to almost every proton in the steroid (Table 2). But, taken together with the CIS data, and especially with the noted strong CIS of H-3 and H-5 of the cyclodextrin, these results are strongly suggestive of internalization of the steroid into the cyclodextrin.

*Rocuronium Bromide–Org 25969 (**1:4**)*

Attempts to observe NOE contacts between Rocuronium Bromide and Org 25969 were greatly hindered by the inadequate dispersion at 400 MHz, and the broad lines that characterize this system. The situation was particularly difficult because signals from the morpholine and pyrrolidine signals of **1** overlap with the signals from **4**. This made it virtually impossible to distinguish intramolecular NOEs from intermolecular NOEs. Accordingly, the remainder of this communication discusses the simpler model systems **5:2**, **5:3** and **5:4**.

## NOESY studies of the model steroid Org 7402 (5)

Several benefits were obtained by choosing Org 7402 (**5**) as a model system for Rocuronium Bromide. Changing the morpholine and pyrrolidine rings to piperidine completely solved the problem of overlaps between steroid $^1$H signals and essential cyclodextrin signals. Replacement of the 17β-OAc by 17β-OH made a more stable system (slow hydrolysis of the 17β-OAc group was noted in the aqueous solutions of **1**). The structural changes between **5** and **1** are considered to be minor with respect to the size and shape of the steroid, and so we assume that they have no impact on the gross morphology of the complex. Org 7402 is a metabolite of a similar steroidal neuromuscular blocker—Vecuronium Bromide.[31]

*Org 7402–β-cyclodextrin (**5:2**)*

Both species showed changes in their chemical shifts when mixed together, showing that some interaction was occurring, although no clear pattern emerged from these changes. A section from the ROESY spectrum of an equimolar mixture of **5** and **2** in $D_2O$ is shown in Fig. 7. As can be seen, several intermolecular ROE cross-peaks appear. In the following discussion, stronger NOE contacts are indicated by bold type. Protons **H-3β** (not visible in Fig. 7), H-4α, H-4β, **H-5α**, **H-6α**, **H-7α** and/or **H-14α**, H-11α, H-11β, H-12β, H-15α, H-17α, 18-$CH_3$ and **19-$CH_3$** are close to H-3 of the cyclodextrin. There are weak interactions from H-3β, H-6α, H-7α and/or H-14α, H-12β, H-12α, H-11α, H-15α and 19-$CH_3$ to H-5 of β-cyclodextrin, whereas H-7β, and 18-$CH_3$ show medium interactions. The 18-$CH_3$ also shows a correlation to H-6. This result would appear to indicate that the steroid can sit on the secondary face of the cyclodextrin with only small sections at both ends of the skeleton entering the cavity. No correlations were observed between the piperidine rings in the 2β and 16β positions and the cyclodextrin, and there were only spurious peaks to the H-1, H-2 and H-4 signals of the cyclodextrin to the steroid, showing there was no binding to the outside of the cyclodextrin. There was no evidence of any binding to the primary face.

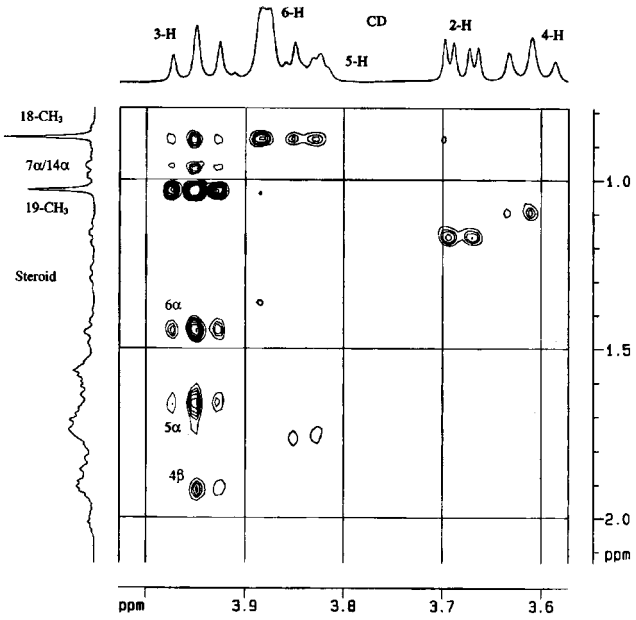


**Figure 7.** A section of the ROESY spectrum of Org 7042 (6 mM) and β-cyclodextrin (6 mM) in pH 7.6 buffered $D_2O$ showing strong correlations between the steroid and the H-3 β-cyclodextrin proton, and weaker correlations to H-5.

*Org7402–γ-cyclodextrin (**5:3**)*

As with the previous sample, both species showed changes in their chemical shifts when mixed together. This was very apparent for 3-H and 5-H of the cyclodextrin. A section of the ROESY spectrum is shown in Fig. 8. It is evident that there

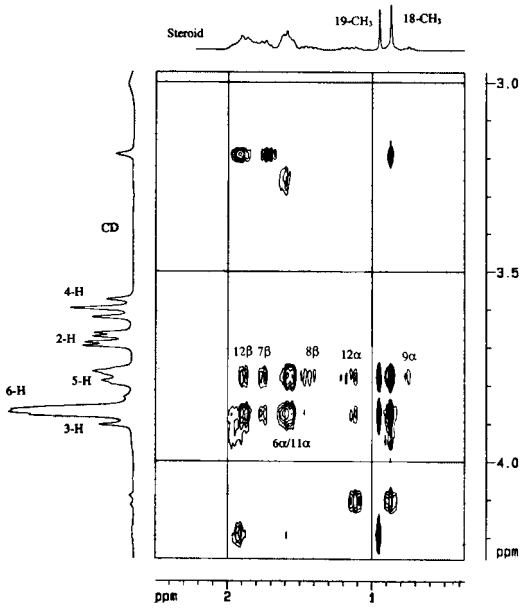


**Figure 8.** A section of the ROESY spectrum of Org 7042 (6 mM) and γ-cyclodextrin (6 mM) showing clear correlations between the steroid protons and the cyclodextrin protons H-3 and H-5.

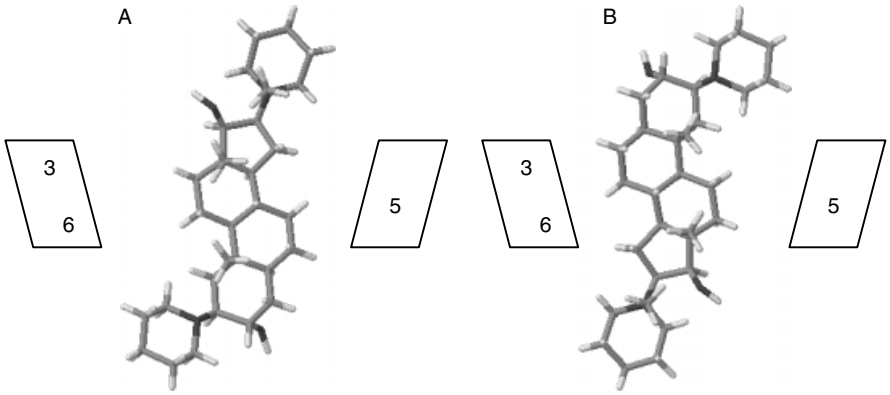


**Figure 9.** Model of the 1 : 1 complex formed between Org 7042 and $\gamma$-cyclodextrin.

are many more correlations from H-5 of the cyclodextrin to steroid signals, compared with the $\beta$-cyclodextrin mixture. Unfortunately, the signals of H-3 and H-6 overlap, making any assignments difficult, although most steroid signals appear to correlate to both H-3 and H-6. The steroid protons showing correlations to H-3 and/or H-6 are **H-6**$\alpha$ and/or **H-11**$\alpha$, H-7$\beta$, H-8$\beta$, H-9$\alpha$(?), **H-12**$\beta$, H-14$\alpha$ and/or H-7$\alpha$, **18-CH$_3$** and **19-CH$_3$**. The steroid protons showing correlations to H-5 are H-6$\beta$, **H-6**$\alpha$ and/or **H-11**$\alpha$, **H-7**$\beta$, H-8$\beta$, H-9$\alpha$, H-11$\beta$, H-12$\alpha$, H-12$\beta$, **18-CH$_3$** and **19-CH$_3$**.

There are strong interactions between the protons in the cyclodextrin cavity and the protons in the central rings (B and C) of the steroid. This result would seem to indicate that the main skeleton of the steroid lies within the cavity of the cyclodextrin in two ways, as sketched in Fig. 9.

### *Org 7402–Org 25969 (**5 : 4**)*

This mixture was much more difficult to study. The signals at room temperature were extremely broad, presumably because of the smaller $K_a$ compared with **1 : 4**. The sample had to be heated to 343 K to make the signals reasonably sharp. The piperidine $CH_2$ signals overlapped with the cyclodextrin signals, and the signals of H-3 and H-5 of the cyclodextrin overlapped. This made the separation of intramolecular and intermolecular correlations impossible, so no conclusions on the structure of the steroid–cyclodextrin complex could be made.

## CONCLUSIONS

It is difficult to imagine that non-core binding would be responsible for the impressive association constant between Rocuronium Bromide and Org 25969. Although individual experiments seem sometimes to give conflicting results, when taken as a whole the balance of the evidence suggests strongly that Rocuronium Bromide is completely encapsulated in the central void of Org 25969. The hallmarks of inclusion into a cyclodextrin (CIS to the H-3 and H-5 protons of the cyclodextrin, and intermolecular ROESY correlations between guest protons and H-3 and H-5) are clearly demonstrated.

Does physical reality (the size and shapes of these molecules) permit this conclusion? Ring D of Rocuronium Bromide is bulky, with large substituents projecting above the plane of the molecule. Ring A of Rocuronium Bromide is known to exist in a dynamic equilibrium between chair and twist-boat conformations, with both conformers significantly populated and in fast exchange at room temperature.[32,33] In the ring A chair conformation the 2$\beta$-morpholine and the 3$\alpha$-ol are trans axial, and would present a substantial steric constraint to passage of the steroid into the cyclodextrin. In the ring A twist-boat conformation the 2$\beta$-morpholine and the 3$\alpha$-ol are equatorial and project along the long axis of the steroid. This arrangement would certainly allow the steroid to thread through the centre of the cyclodextrin.

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