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^{1}H and ^{13}C NMR Studies of Some Steroidal Neuromuscular Blocking Drugs: Solution Conformations and Dynamics

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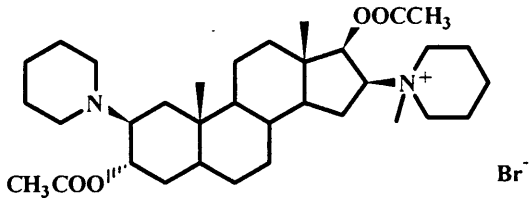
ABSTRACT: The ^{1}H and ^{13}C NMR spectra of the steroidal neuromuscular blocking drugs pancuronium bromide, vecuronium bromide, rocuronium bromide and Org. 9487 are presented. The ^{13}C NMR spectra are fully assigned. NOE data and variable-temperature studies show that there is considerable conformational freedom in both the ring A substituents and the sterically more crowded ring D substituents. The barrier to nitrogen-ring inversion in the non-quaternized piperidine ring ($\Delta G^{\neq}$) is *ca.* 10–11 kcal mol^{-1}. The quaternized ring D piperidine (or pyrollidine) rings are free to rotate with respect to the steroid. The vicinal couplings $^{13}CO–O–C–^{1}H$, *ca.* 4 Hz, suggest that the ester moieties have similar rotational freedom to simple esters. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: steroids; neuromuscular blockers; NMR; ^{1}H NMR; ^{13}C NMR; solution structure

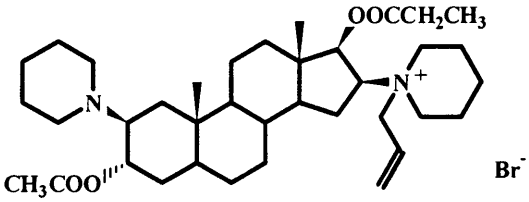
INTRODUCTION

Neuromuscular blockers are used by anaesthetists during surgery to produce complete relaxation of skeletal muscle.[1–3] Suppression of reflex laryngospasm allows rapid tracheal intubation and the reduced muscle tone considerably facilitates surgical procedures. The first muscle relaxant was tubocurarine hydrochloride.[4] Since curare, the search for non-depolarizing blockers with modified potency, faster onset times and shorter duration has led to the development of the four steroidal drugs shown in Scheme 1.[5–7]

This paper reports on an NMR study of **1–4**, including the fully assigned ^{1}H and ^{13}C NMR spectra. The data are presented in an order convenient for the discussion of their NMR properties. The historical



1
Vecuronium Bromide (1983)



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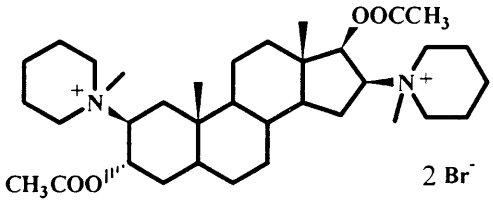
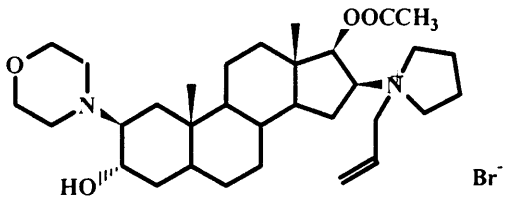
Org 9487 (1999?)



3
Pancuronium Bromide (1968)



4
Rocuronium Bromide (1994)

Scheme 1. Molecular structures of four steroidal neuromuscular blocking drugs and the year they were introduced commercially.

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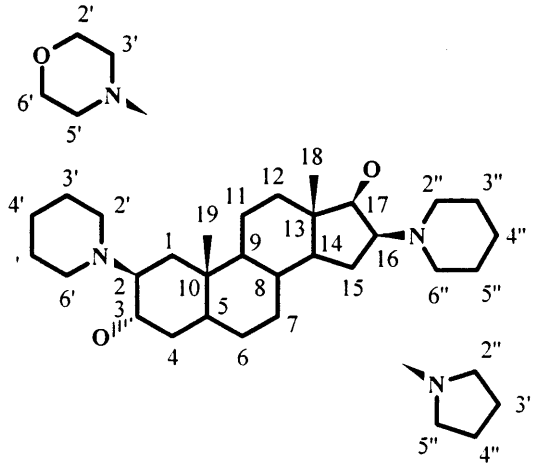

Scheme 2.

hierarchy is that **3** was discovered first; **1**, **4** and most recently **2** are the products of research driven by the search for drugs with faster onset times and shorter duration of action. Compounds **1**, **3** and **4** are frequently used drugs and are used daily in hospitals worldwide. Compound **2** has been through phase 3 clinical trials and it will be marketed in 1999.

Neuromuscular blockers act on the membrane-bound acetylcholine receptor at the neuromuscular junction.[8] Common features of **1–4** are the two alkylated nitrogen atoms (at least one of them is quaternary), separated by about 11 Å, and the acetylcholine fragment $C(O)OCH_2CH_2N^+$. Much of the discussion of the pharmacological activity of these compounds has focused on the conformational rigidity of this 'acetylcholine fragment.'[5] Hence in the following discussion of the solution conformations of these four molecules, particular attention is paid to describing the stereodynamics of the piperidine, morpholine and pyrrolidine rings which occur as ring A and ring D substituents to the androstane core.

EXPERIMENTAL

Compounds **1–4** were obtained from Organon Laboratories (Newhouse, UK). All data were obtained on a Bruker DRX 400 instrument fitted with a 5 mm inverse geometry probe incorporating self shielded *z*-gradient coils, a BGU 2 gradient unit and BGPA 10 power amplifier, and using standard software and pulse programs.[9] The ^1H data were referenced to a trace of internal TMS. The ^{13}C data were referenced to the solvent signal at 77.0 ppm ($CDCl_3$) or 39.6 ppm (DMSO-d_6).

Gradient-accelerated magnitude COSY45 experiments[10] were performed with two scans for each of 256 increments in F_1, 2048 data points in F_2 and a relaxation delay of 2.0 s. The final 512 × 512 matrix was processed with sine-bell exponential multiplication and a t_1 noise subtraction was performed.

HETCOR experiments[11] (^{13}C detected) were performed with 16 scans for each of 128 F_1 increments, 4096 points in F_2, optimized for $^2J_{CH} = 145$ Hz and a relaxation delay of 2.0 s. The final 512 × 2048 matrix was processed with 3.0 Hz exponential line broadening in F_2 and $\pi/2$ shifted sine-bell weighting in F_1.

HMQC experiments[12] for short-range ^1H–^{13}C connectivity utilized the BIRD sequence and GARP composite pulse decoupling with eight scans and 256 F_1 increments, 2048 points in F_2, optimized for $^2J_{CH} = 145$ Hz, a relaxation delay of 1.0 s and a 350 ms BIRD delay. The final 256 × 2048 matrix was processed with $\pi/2$ shifted sine-bell weighting in both dimensions.

HMBC experiments[13] for long-range ^1H–^{13}C connectivity were performed with 32 scans for each of 256 F_1 increments, 2048 points in F_2, optimized for $J_{CH} = 7.0$ Hz and a relaxation delay of 1.5 s. The final 512 × 2048 matrix was processed with $\pi/2$ shifted squared sine-bell weighting in both dimensions before t_1 noise subtraction.

Phase-sensitive (TPPI) NOESY[14] and ROESY[15] experiments were performed with 16 scans for each of 512 F_1 increments, 2048 data points in F_2, with a relaxation delay of 2.0 s and a mixing time (spin lock) as described in the text. The final 512 × 2048 matrix was processed with $\pi/2$ shifted sine-bell weighting in both dimensions and all correlations were opposite in sign to the diagonal peaks.

Long-range ^{13}C–^1H couplings were determined by a selective heteronuclear 2D *J*-resolved experiment[16] utilizing a shaped RE-BURP pulse[17] and with o2 set at the frequency of H-3β or H-17α.

In the following discussion, steroid carbons are labelled in the conventional fashion; piperidine and morpholine atoms at 2β are labelled as *N*-alkyl heterocycles with single primes and the ring D (16β) substituent atoms are labelled with double primes (Scheme 2).

RESULTS AND DISCUSSION

Analysis of the room-temperature NMR spectra of these quaternized amino steroids is complicated by the poor-quality ^1H NMR spectra that are produced. The problem is partly due to overlaps of the large numbers of protons in the spectrum (53–61 in these examples) and partly because the charged piperidine (or pyrrolidine) rings are not completely motionally averaged. The typical ^1H NMR spectra of the compounds discussed here are not fully resolved and consist of broad clusters of overlapping peaks even at moderately high magnetic fields (400 MHz). The situation can be improved by acquiring data at elevated temperatures, but the spectra are still very crowded. ^{13}C NMR gives the desired dispersion of signals and all of the carbon signals are usually resolved.

The problems of dispersion in the ^1H spectrum mean that it is not possible to follow correlations through COSY-type experiments for complete, unambiguous

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assignments. Hence the approach used for assignment was a combination of modern high-field 2D techniques and traditional chemical shift correlation analysis. The Analytical Chemistry Department at Organon Laboratories has a ^{13}C NMR database with compounds of similar structure to those considered here. The general approach to the assignment problem was to assign empirically as many carbons as possible using published ^{13}C NMR data on steroids and the Organon database of spectra for structurally similar compounds. The results of a simple ^{13}C and DEPT135 spectrum are extremely useful at this point and (with reference to the database) most carbon signals can be assigned with reasonable confidence. COSY, HETCOR, HMQC, HMBC and NOE data were then considered. The empirical assignments were rigorously checked and revised until a self-consistent complete assignment was established. With the fully assigned ^{1}H spectrum in hand, NOE experiments, VT experiments and vicinal coupling constants throw light on the solution structures.

The ^{13}C and ^{1}H chemical shift data for **1**–**4** are given in Tables 1 and 2, respectively.

Vecuronium Bromide, $C_{34}H_{57}N_2O_4Br$ (1)

An x-ray crystallographic study of the molecular structure of vecuronium bromide established that rings A, B and C of the steroid and both piperidine rings are all in chair conformations.[18] The same study also considered the ^{1}H NMR spectrum of MeOH-d_4 and $CDCl_3$ solutions and concluded from the $^{3}J_{2\alpha,\,3\beta}$ coupling that ring A remains in the chair conformation in solution.[18]

Table 1. ^{13}C chemical shift data for compounds **1–4**[a]

		1[b]	2[b]	3[c]	4[b]
	1	34.2	34.3	33.9	32.5
	2	62.9	62.9	67.7	65.2
	3	69.7	69.7	68.5	63.9
	4	30.2	30.2	34.2	37.8
	5	40.1	40.2	37.3	38.5
	6	27.3	27.3	26.3	27.8
	7	31.5	31.5	30.5	31.4
	8	33.5	33.5	33.5	33.8
	9	54.4	54.4	53.3	55.1
	10	36.0	36.0	35.9	36.0
	11	20.6	20.6	20.5	20.8
	12	37.6	37.6	37.1	34.0
	13	45.1	45.3	44.5	45.4
	14	46.3	46.6	45.3	47.0
	15	26.9	27.4	26.0	28.4
	16	69.9[e]	68.5[e]	68.8[e]	70.8[e]
	17	78.6	78.8	77.6	78.1
	18	13.3	13.4	12.6	13.6
	19	13.1	13.1	16.7	15.9
2β	2′, 6′	51.9 (2)	51.9 (2)	61.3, 59.6	67.3 (2)
	3′, 5′	26.3 (2)	26.3 (2)	20, 20, 20[f]	49.5 (2)
	4′	24.7	24.7		
	N^+CH_3			43.2	
3α	CO	170.2	170.2	169.0	
	CH_3	21.4	21.4	21.2	
17β	CO	168.4	172.2	168.8	168.6
	CH_2		28.0		
	CH_3	21.1	9.1	20.8	21.1
16β	$N^+C_xH_y$[d]	62.0, 60.4[e]	58, 58[e]	61.3, 60.1[e]	63.7, 62.6[e]
		20.6, 20.4, 20.3	20.7, 20.1, 20.1	20, 20, 20[f]	24.4, 24.1
	N^+CH_3	45.9		45.5	
	N^+allyl CH_2		58.0		65.3
	=CH		124.8		126.1
	$=CH_2$		128.8		128.5

[a] A number in parentheses following an assignment indicates the number of magnetically equivalent carbon atoms.
[b] In $CDCl_3$ at 52 °C.
[c] In DMSO-d_6 at 52 °C.
[d] Where $x = 5$, $y = 10$ for **1**, **2** and **3** and $x = 4$, $y = 8$ for **4**.
[e] These peaks are not visible in the conventional ^{13}C spectrum at 52 °C. Data from HMQC experiment or ^{13}C NMR at higher temperatures (*ca.* 80 °C).
[f] Six peaks at 20.4, 20.4, 19.5, 19.4, 19.3 and 19.2 ppm are assigned to C-3′, C-4′, C-5′, C-3″, C-4″ and C-5″.

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Table 2. ^{1}H chemical shift data for compounds **1–4**[a]

		1[b]	2[b]	3[c]	4[b]
	1α	1.22	1.21	1.94	1.45
	1β	1.87	1.86	1.62	1.45
	2α	2.32	2.31	4.28	2.54
	3β	5.26	5.26	5.20	3.89
	4α	1.34	1.34	1.43	1.45
	4β	1.77	1.77	2.04	1.78
	5α	1.51	1.50	1.66	1.58
	6α	1.26	1.27	1.40	1.39
	6β	1.26	1.27	1.12	1.19
	7α	1.06	1.06	0.91	1.03
	7β	1.70	1.77	1.68	1.70
	8β	1.54	1.55	1.43	1.47
	9α	0.82	0.81	0.97	0.82
	11α	[e]	1.59	1.65	1.57
	11β	1.24	1.31	1.25	1.31
	12α	1.38	1.36	1.31	1.47
	12β	1.73	1.71	1.68	1.78
	14α	1.24	1.22	1.13	1.20
	15α	2.18	2.21	1.95	2.28
	15β	1.74	2.04	1.85	1.81
	16α	4.80	4.37	4.42	4.64
	17α	5.32	5.29	5.16	5.24
	18	0.82 (3)	0.84 (3)	0.79 (3)	0.83 (3)
	19	1.00 (3)	1.00 (3)	0.89 (3)	0.89 (3)
2β	2′, 6′	2.46 (4)	2.45 (4)	3.5 (4)	3.68 (4)
	3′, 5′	1.51 (4)	1.52 (4)	1.8 (4)	2.46, 2.61 (4)
	4′	1.39 (2)	1.39 (2)	1.6 (2)	
	N^+CH_3			2.96 (3)	
3α	CH_3	2.05 (3)	2.05 (3)	2.09 (3)	
17β	CH_2		2.49 (2)		
	CH_3	2.19 (3)	1.21 (3)	2.19 (3)	2.23 (3)
16β	$N^+C_xH_y$[d]	3.88–3.70 (4)	3.87–3.55 (4)	3.5 (4)	3.9 (4),
		2.0 (4)	2.1 (4), 1.9, 1.75	1.8 (4), 1.6 (2)	2.28 (4)
	N^+allyl CH_2		4.51, 4.17		4.36, 4.18
	$=CH$		6.22		6.22
	$=CH_2$		5.73 (2)		5.72 (2)
	N^+CH_3	3.39 (3)		3.15 (3)	

[a] A number in parentheses following an assignment indicates the number of magnetically equivalent protons.
[b] In $CDCl_3$ at 52 °C.
[c] In DMSO-d_6 at 52 °C.
[d] Where $x = 5$, $y = 10$ for **1**, **2** and **3** and $x = 4$, $y = 8$ for **4**.
[e] H-11α is not located in the HETCOR spectrum.

The ^{1}H NMR spectrum of **1** in $CDCl_3$ at 52 °C [Fig. 1(a)] consists of three fully resolved peaks around 5 ppm assigned as H-17α, H-3β and H-16α, signals from the seven remaining protons on carbons α to the quaternary nitrogen around 3.3–3.9 ppm and signals from the remaining 47 protons between 0.8 and 2.5 ppm. The 52 °C $^{13}C\{^1H\}$ NMR spectrum of **1** consists of only 29 lines. The signals from C-2′/C-6′ and C-3′/C-5′ are coincident and three of the carbons α to N^+ (C-16, C-2″ and C-6″) are invisible because of severe line broadening.

In broad terms, any discussion of the solution structure of **1** has to address steroid ring conformations and the conformations (and if possible the dynamics) of the acetate and piperidine groups (the acetylcholine fragments). The former problem is trivial but the latter is a more complex issue. Fortunately, there is a considerable body of data relating to the stereochemistry and dynamics of *N*-alkylpiperidines, including excellent reviews by Rubiralta *et al.*[19] and Delpuech.[20]

It is clear that ring A of the steroid is in a chair conformation with a *trans* amino-ol ester configuration [H-2α and H-3β are both equatorial, Fig. 1(a)]. The 2β-piperidine protons occur as three signals with integrals 4:4:2, indicating that an exchange averaging is occurring in this part of the molecule. It is well established that nitrogen inversion occurs at a similar rate to ring reversal in saturated six-membered heterocycles, and that the coupled nitrogen-ring inversion process allows an *N*-alkyl group (i.e. the steroid) to remain equatorial.[19,20] Does the 1,3-transannular interaction with 19-CH_3 restrict the rotation of the piperidine about the C-2—N bond? The rapid averaging of 2β-

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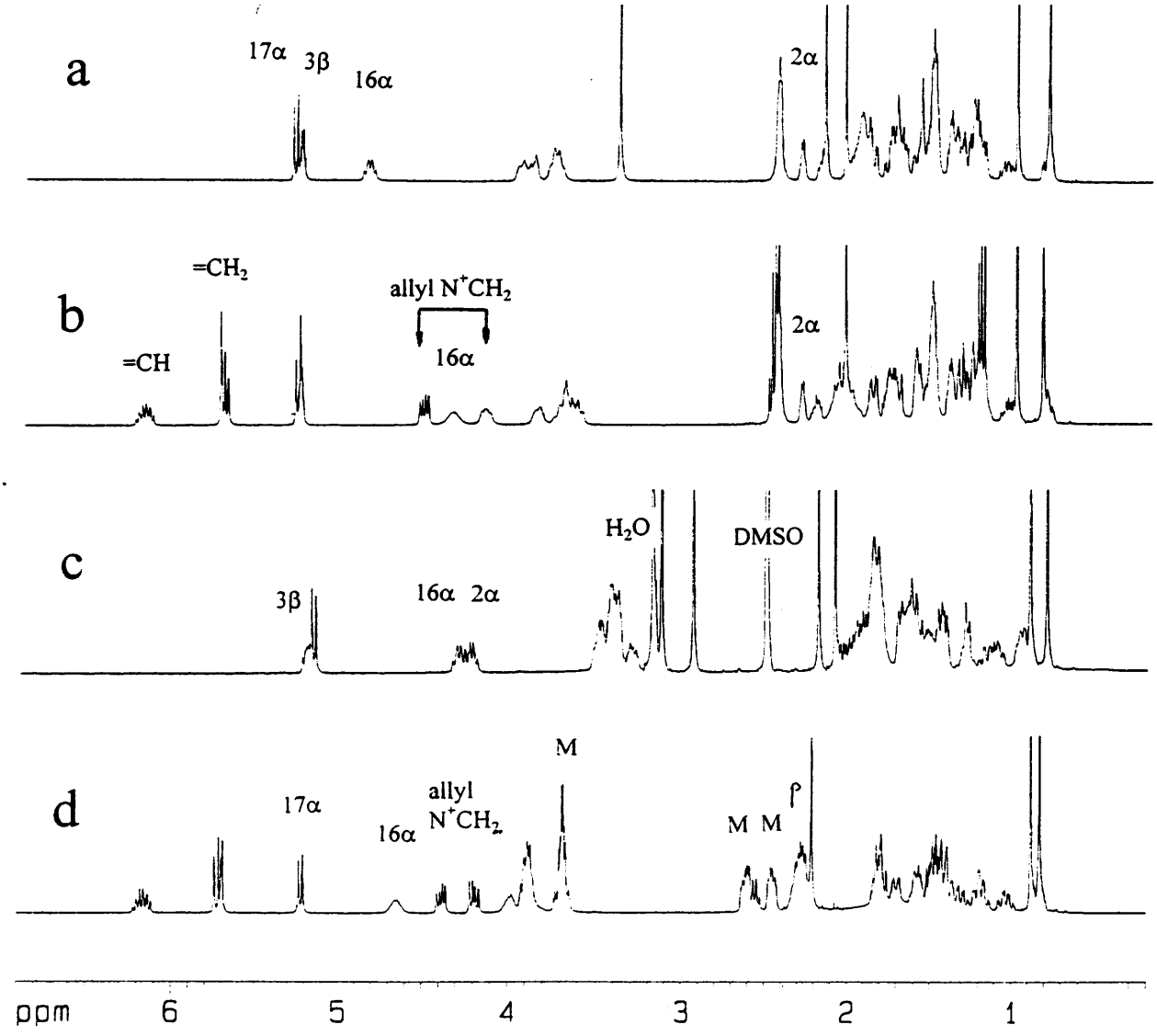

Figure 1. 400 MHz ^{1}H NMR spectra of (a) **1** in $CDCl_3$, (b) **2** in $CDCl_3$, (c) **3** in DMSO-d_6 and (d) **4** in $CDCl_3$. All data are from 10 mg ml^{-1} solutions at 52 °C. The data are scaled to the height of the 18- and 19-methyl group signals and are clipped at approximately 40% of the height of those signals.

piperidine signals negates any attempt to probe the orientation of the piperidine moiety by NOE experiments, but the fully collapsed piperidine ^{1}H signals point to efficient averaging about the piperidine N—C-4 symmetry plane rather than a fixed piperidine–steroid geometry. The same conclusion may be drawn from the ^{13}C spectrum (Table 1), where only three piperidine signals are seen. Note that the presence of the steroid makes C-2′ and C-6′ diastereotopic and hence magnetically non-equivalent regardless of C—N bond rotations. This intrinsic shift difference was not detected (<0.01 ppm). Presumably coupled nitrogen inversion and C—N bond rotations bypass steric clashes during rotation about C-2—N, and therefore allow the piperidine ring to rotate freely. The asymmetric environment also implies that the two chair conformations of the piperidine ring do not have the same free energies.

Investigations of the side-chain conformational equilibria in *N*-alkylpiperidines have shown that although specific staggered conformations can be indirectly detected, the alkyl group rotates freely even at −148 °C.[21]

The ^{1}H NMR spectra of **1** were recorded in $CDCl_3$ and MeOH-d_4 and in $CDCl_3$–toluene-d_8 and $CDCl_3$–MeOH-d_4 binary mixtures at a range of temperatures until the solutions froze. These experiments showed that the 2β-piperidine signals at 2.5 ppm (integral 4), which are sharp in warm solutions, start to broaden at room temperature, continue to broaden as the temperature is lowered and become undetectable (coalesce) around −35 to −50 °C (depending on which solvent is used), and reappear again as two peaks around 3.1 ppm (integral 2) around −65 °C. If we assume that the two peaks at 3.1 ppm are H-2′$_{eq}$ and H-6′$_{eq}$, and that the fully averaged peak at 2.5 ppm is equidistant between the equatorial and axial peaks (i.e. the corresponding axial peaks are at 1.9 ppm; a chemical shift difference of approximately 1 ppm is expected

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between the geminal protons at the α-position of an alkylpiperidine[22]), we can calculate $\Delta G^{\neq}$ for the coupled nitrogen-ring inversion of this piperidine to be *ca.* 9.8–10.5 kcal mol^{-1} (1 kcal = 4.184 kJ).

When we turn to ring D, it is instructive to consider **1** as an *N*-alkyl-*N*-methylpiperidinium salt where the steroid is the alkyl group. The ^{1}H and ^{13}C NMR spectroscopy and the conformational equlibria of quaternary piperidine salts have been thoroughly documented.[19,20] In this system, ring reversal can occur, but nitrogen inversion is prohibited, hence an equilibrium between two chair conformations is expected. The lowest energy conformer should be the one with the bulky steroid group in an equatorial position. Rotation of the piperidine moiety about the C-16—N^{+} bond is also possible.

One of the most characteristic features of the ^{1}H NMR spectra of **1** (and of **2**, **3** and **4**) is that the signals between 3 and 5 ppm (protons β to the charged nitrogen) are broad at room temperature and they sharpen on warming (Fig. 2). Cooling produces further broadening until a maximum around −40 °C, and then the signals start to sharpen again. Unfortunately, the spectra never become sharp enough for confident assignments to be made before the solutions freeze (*ca.* −100 °C).

It might be expected that the 10 protons in the quaternized piperidine ring would be magnetically non-equivalent and indeed the spectrum of the protons on C-2″ and C-6″ are observed to be distinct (Fig. 2). Note also that in the ^{13}C NMR spectrum (Table 1), C-2″ and C-6″ are also non-equivalent. The solvent system was adjusted to achieve the best resolution of the piperidine C-2″ and C-6″ protons and an optimum was found with a mixture of 30% $CDCl_3$ and 70% toluene-d_8 at 50 °C [Fig. 2(c)]. Under these conditions, the spectrum has the appearance of two resolved (downfield) equatorial protons and two overlapped (upfield) axial protons.

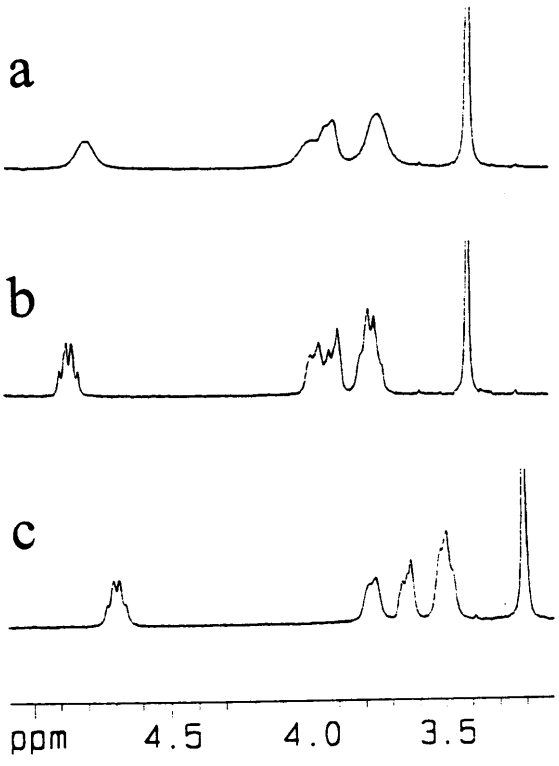

Figure 2. Expansion of the 400 MHz ^{1}H NMR spectrum of **1** (10 mg ml^{-1}) showing the signals from protons β to N^{+}. (a) $CDCl_3$ at 22 °C; (b) $CDCl_3$ at 52 °C; (c) $CDCl_3$–toluene-d_8 (30 : 70) at 52 °C.

The piperidine ring is locked into a chair conformation by the quaternary nitrogen, so only ring inversion and C-17—N^{+} bond rotation are realistic candidates for the dynamic process (ring D of the steroid should be pseudo-rotating at these temperatures and the 17α-OAc group is discounted for now). How do we rationalize the apparent paradox that a dynamic process involving piperidine protons (most likely interconversion of the two chair conformations) is slow at room temperature, yet at elevated temperatures the sharper spectrum has the appearance of a single conformation (i.e. with resolved 'equatorial' and 'axial' protons on C-2″ and C-6″)? We propose that the high-temperature data correspond to fast exchange between two chair conformations with the equilibrium shifted to favour one conformer. Hence the shifts and the couplings observed for the rapidly exchanging protons are weighted and retain some of the characteristics of equatorial and axial protons.

The experimental NOESY cross peak intensities (Table 3) support the above notion of a shifted equilibrium and allow an estimate of the equilibrium constant. NOEs from $N^{+}CH_3$ to the 'equatorial' protons on C-2″ and C-6″ are of equal intensity (0.8% each), and weak NOEs to the 'axial' protons are also present (0.5% total). The NOE to the 'axial' protons could be accounted for if the equilibrium mixture contained approximately 30% of the equatorial $N^{+}CH_3$ conformer and 70% of the axial $N^{+}CH_3$ conformer. [It is assumed that an equatorial NCH_3 would give rise to equal enhancements to both equatorial and axial protons on C-2″ and C-6″, and an axial NCH_3 would produce zero enhancement for the axial protons on C-2″ and C-6″. If we further assume that the NOE process $CH_{3(ax)} \rightarrow H_{(eq)}$ is the same as $CH_{3(eq)} \rightarrow H_{(ax)}$, we can infer that the observed 0.8% enhancement of an equatorial proton is made up of a contribution of 0.25% from $CH_{3(eq)}$ and 0.55% from $CH_{3(ax)}$.] NOE transfers from $N^{+}CH_3$ across the piperidine ring are also consistent with a high proportion of axial $N^{+}CH_3$. NOEs were also observed from $N^{+}CH_3$ to 18-CH_3, H-15β and H-16α, suggesting that the piperidine ring is able to rotate about the C-16—N^{+} bond and adopt conformations other than those found in the crystal structure.[18] NOEs from H-16α to H-2″$_{ax}$ and H-6″$_{ax}$ are of equal intensity and are stronger than those from H-16α to H-2″$_{eq}$ and H-6″$_{eq}$. This result supports the notion of a predominantly axial methyl group and also implies some rotational freedom in the quaternary piperidine moiety.

The ^{13}C chemical shift of the $N^{+}CH_3$ group has been used for configuration assignment and as an indicator

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Table 3. ^1H–^1H NOE enhancements (%)[a] observed around ring D in **1**, in $CDCl_3$–toluene-d_8 (30:70) solution at 47 °C[b], and in $CDCl_3$ solution at 47 °C[c]

	N^+CH_3		H-16α	
	$CDCl_3$–toluene-d_8	$CDCl_3$	$CDCl_3$–toluene-d_8	$CDCl_3$
H-14α			2.6	4.3
H-15α			2.7	4.2
H-15β	0.7	1.3		
H-16α	0.7	0.7		
H-17α			7.6	13.2
18-CH_3	0.6	1.2		
H-2″$_{eq}$ + H-16″$_{eq}$	1.6		1.0	
		3.2		8.9
H-2″$_{ax}$ + H-6″$_{ax}$	0.5		3.7	
H-3″, H-4″, H-5″	1.6	2.4	4.6	7.3

[a] Cross peak intensities were obtained by integrating a row from the 2D NOESY spectrum and normalizing the diagonal peak to −100%.
[b] Mixing time 500 ms.
[c] Mixing time 600 ms.

of the methyl group equatorial–axial equilibrium in quaternary piperidine salts.[23,24] Generally (in conformationally locked molecules) an axial N^+CH_3 group occurs upfield of an equatorial N^+CH_3 group,[23,24] but exceptions to this trend do occur.[25] Hence we will refrain from attaching significance to the chemical shifts of the N^+CH_3 groups in these molecules.

The x-ray crystal structure of **1** has the methyl group in equatorial position of the piperidine ring and the steroid in the axial position[18] (i.e. the reverse of the observed solution structure and the reverse of what is expected from simple steric arguments).[19] Presumably crystal packing forces are responsible for the inversion.

Each acetyl group contains three rotatable bonds. Rotation of the terminal CH_3 group is not of interest. The acyl–oxygen bond is expected to exist in a planar *trans* conformation (the other planar *cis* conformation is usually higher energy in esters). The alkyl–oxygen bond may have more conformational freedom and is of interest to this study. The orientation of the OAc groups was addressed by considering the heteronuclear vicinal couplings $^3J_{H\text{-}3,C=O}$ and $^3J_{H\text{-}17,C=O}$ (Table 4). The magnitude of $^3J_{CH}$ is generally a factor of about 0.6 smaller than $^3J_{HH}$ and the Karplus-type curve relating $^3J_{CH}$ to the dihedral angle typically has a minimum near

Table 4. Vicinal ^{13}C–^1H couplings (Hz) for compounds **1–4**

	^{13}CO–O–C–^1H	
	H-3β	H-17α
1[a]	3.9	4.3
2[a]	3.7	4.2
3[b]	3.4	4.1
4[a]		4.2

[a] In $CDCl_3$ at 52 °C.
[b] In DMSO-d_6 at 52 °C.

zero and a maximum around 6–8 Hz.[26] The averaged CO–O–C–H couplings observed in simple esters are around 3–4 Hz.[27] Hence, although we note that the observed CO–O–C–H couplings in **1** may of course correspond to any one of four possible conformations of the ester, because the couplings closely match those observed in small molecules it seems likely that the CH_3CO group is no more constrained in **1** than it is in, for instance, ethyl acetate.

Are these findings of freedom of movement in the ring D substituents consistent with the physical reality of the molecule? Examination of any molecular model of **1** reveals that the acetyl group cannot rotate freely without clashes with the piperidine atoms. A mechanism which includes geared rotation of the acetyl group and piperidine group does, however, seem to allow the freedoms which are experimentally observed.

Org 9487, $C_{37}H_{61}N_2O_4Br$ (2)

Org 9487 is the latest neuromuscular blocker to be developed. This non-depolarizing muscle relaxant with rapid onset and short duration will be marketed shortly as an alternative to succinylcholine. There are no previously published structural data.

The ^1H NMR spectrum of **2** is shown in Fig. 1(b). Many of the features that have been explained in **1** are reproduced in **2**. Ring A is in the expected chair conformation, and at 52 °C the 2β-piperidine ring rotates freely and converts between two energetically non-equivalent chair conformations (the four protons on C-2′ and C-6′ of the 2β-piperidine fragment are a single peak, integral 4 at 2.45 ppm). At −60 °C in mixed (2:1) $CDCl_3$ and MeOH-d_4 these four protons are magnetically non-equivalent. This is shown most clearly in Fig. 3, which shows the correlation between H-2′$_{eq}$ and H-6′$_{eq}$ at 2.99 and 3.13 ppm with the signals from H-2′$_{ax}$ and H-6′$_{ax}$ at 2.08 and 1.94 ppm. Using a mean coalescence

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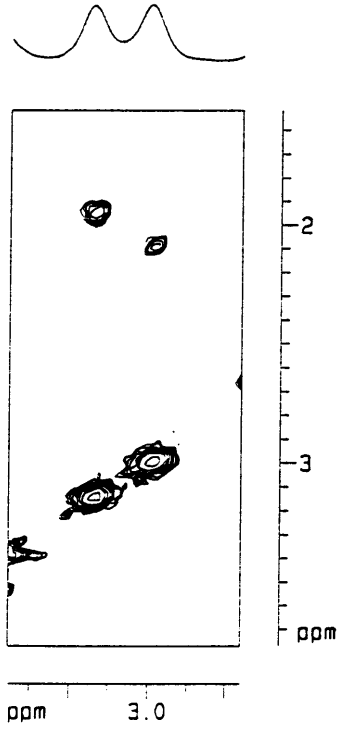

Figure 3. Part of a 400 MHz ^{1}H COSY spectrum of **2** in $CDCl_3$–MeOH-d_4 (2:1) at −75 °C showing the correlations between the decoalesced axial and equatorial protons on C-2′ and C-6′ of the 2β-piperidine. Nitrogen-ring inversion is frozen out.

temperature and the averaged chemical shifts of the C-2′ and C-6′ protons, $\Delta G^{\neq}$ is calculated to be 11.0 kcal mol^{-1}.

Signals from the 16β-piperidine and allyl fragments also broadened, coalesced and then sharpened again as the temperature was lowered. At −45 °C most of the low-field signals (6.3–3.3 ppm) were sharp, producing a complex spectrum with overlapping signals from more than one species. At −60 °C the olefinic protons of the allyl fragment were resolved as major (83%) and minor (17%) sets and a 7 Hz splitting was apparent in the 18-CH_3 signal. Assignment difficulties due to inadequate resolution, a variety of different coalescense temperatures and the number of bonds that may be freezing restrict the conclusions which can be drawn.

NOEs (at 57 °C) between the quaternary *N*-allylpiperidine moiety and the steroid were very much weaker than those observed in **1**. Possibly replacing N^+CH_3 with N^+allyl defocuses the spin–spin interaction around several peaks and introduces new relaxation pathways that overcome any useful steroid–quaternary piperidine NOE interactions. Weak NOEs (*ca.* 0.1–0.2%) were observed between the 18-CH_3 and all of the C-2″ and C-6″ hydrogens and N^+allyl hydrogens in both NOESY and ROESY experiments (mixing time/spin lock, 300 ms). This result is consistent with multiple conformations about the C-16—N^+ bond. The ROESY data also showed many correlations between the allyl hydrogens and the piperidine. Similar NOEs were observed from each of the magnetically non-equivalent geminal protons at 4.51 and 4.17 ppm, implying free rotation about the allyl N^+—C bond.

Pancuronium bromide, $C_{35}H_{60}N_2O_4Br_2$ (3)

The x-ray crystal structure of **3** shows that ring A adopts a twist-boat conformation, that both piperidine rings are chair (the 2β-N^+CH_3 is axial, the 16β-N^+CH_3 is equatorial) and both of the N^+CH_3 groups are located on the β side of the steroid.[28] It was suggested that the pharmacological activity of **3** is due in part to a 'rigidity' of the acetylcholine fragments (i.e. the ring A and ring D *trans* amino-ol esters).[28,29] In a limited ^{1}H NMR study of **3** in MeOH-d_4, a large $^3J_{2\alpha,3\beta}$ and a single NOE (5%) from 19-CH_3 to H-3β (but no NOEs from 19-CH_3 to the 3β-*N*-methylpiperidine group) were taken as evidence of a twist-boat conformation of ring A in solution.[18]

Pancuronium bromide appears to dissolve in $CDCl_3$ after a few drops of MeOH-d_4 have been added; however the presence of oily striations at 50 °C (30 mg ml^{-1}) suggest that the mixture may not be a true solution. No such problems were observed with MeOH-d_4 or DMSO-d_6 in which **3** is readily soluble. Preliminary studies established that the ^{13}C NMR spectrum and the ^{1}H lineshapes are essentially independent of solvent. In the discussion which follows, data were selected from the most appropriate solvent system, usually DMSO-d_6.

Figure 1(c) shows the ^{1}H NMR spectrum of **3** in DMSO-d_6. H-2α and H-3β are clearly pseudo-axial, as required for a ring A twist-boat conformation. It is important to note that the ring A twist-boat conformation in **3** is observed in all solvents and is not simply a result of choosing DMSO-d_6 as the solvent.

Nitrogen inversion can no longer occur in this bis-quaternary compound, hence questions of stereochemistry are reduced to determining the proportions of axial versus equatorial N^+CH_3 groups, the orientation of *N*-methylpiperidine groups with respect to the steroid and the orientation of the acetyl groups. ROESY data (spin lock, 300 ms) established the following: at ring A of the steroid, there was an interaction between 19-CH_3 and H-3β (2.5%), but no observable correlation from 19-CH_3 to the piperidine ring protons, which are remote in the twist-boat conformation of ring A. These observations echo the previous NOESY study in MeOH-d_4.[18] Correlations were detected from the 2β-N^+CH_3 group to both α and β protons on ring A of the steroid (H-1α, 1.2%; H-1β, 2.2%; H-2α, 1.6%; H-3β, 2.8%). The significantly stronger response to the β protons suggests that the 2β-*N*-methylpiperidine group is free to rotate, but that it spends more time with the N^+CH_3 group above the β face of the steroid, as is found in the crystal structure.[28] Correlations to the signals from protons on C-2′ and C-6′ were not fully resolved, so analysis of the conformational preference of the 2β-N^+CH_3 group (as for **1**) was not possible.

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ROESY correlations around ring D were similar to those observed from **1** (16β-N^+CH_3 to H-16α, 1.1%; and 16β-N^+CH_3 to 18-CH_3, 2.1%) except that, again, in DMSO-d_6 discrete 'axial' or 'equatorial' signals were not observable.

The long-range ^1H–^{13}C couplings (Table 4) are consistent with some freedom in the acetyl groups.

Rocuronium bromide, $C_{32}H_{53}N_2O_4Br$ (4)

The ring D environment of **4** is similar to that in the preceding three steroids, but the 3α-ol is a significant change over the 3α-acetates and has a large effect on the ring A stereochemistry of **4**. Intramolecular hydrogen bonding within the *trans* amino-ol configuration stabilizes a twist-boat conformation of ring A. The solvent dependence and the energetics of the chair–twist-boat equilibrium in ring A have been fully investigated in the model compound 3α-hydroxy-2β-(4-morpholinyl)-5α(*H*)-androstan-17-one.[30,31] The key point to arise from this earlier work is that in solution there is *always* an equilibrium between chair and twist-boat conformations of ring A which is fast on the NMR time-scale. Non-polar solvents favour the twist-boat conformation (86% twist-boat in $CDCl_3$) and polar aprotic solvents favour the chair conformation (88% chair in DMSO-d_6).[31] The equilibrium is slightly temperature dependent and shifts to favour the chair conformation at lower temperatures.

The 400 MHz ^1H NMR spectrum of **4** is shown in Fig. 1(d). The ring A and ring B ^1H and ^{13}C chemical shifts are in full agreement with those reported for 3α-hydroxy-2β-(4-morpholinyl)-5α(*H*)-androstan-17-one.[30]

As before, we will concentrate on the conformational freedom of the morpholine and *N*-allylpyrrolidine groups, so steroid–heterocycle NOEs are important. The six membered morpholine ring is expected to be subjected to rapid flips between two chair conformations via coupled nitrogen-ring inversion as already described for piperidine.[20] The ^1H NMR spectrum of **4** contains two multiplets (integrals 2 + 2) at 2.46 and 2.61 ppm for the morpholine $N(CH_2)_2$ protons. This is due to diastereotopic C-3′ and C-5′ protons and fast exchange between the axial and equatorial sites (but note that in the ^{13}C spectrum C-3′ and C-5′ are not resolved). The ROESY spectrum of **4** showed equally intense correlations from the (second order) H-1α/1β frequency and from H-3β to the pair of peaks at 2.46 and 2.61 ppm [Fig. 4(a)], indicating fast rotation of the morpholine ring about the C-2—N bond. This implies that the intramolecular hydrogen bond in ring A must exchange at least as fast as the ring rotation.

At ring D we are interested in rotation of the pyrrolidine ring about C-16—N^+, rotation of the allyl moiety about N^+—CH_2 and one rotation within the allyl moiety. Although two multiplets at 3.9 and 2.28 ppm (integrals 4 + 4) corresponding to pyrrolidine are seen in the high-temperature ^1H spectrum of **4** (which look like collapsed multiplets), four non-equivalent pyrrolidine carbons are seen in the high-temperature ^{13}C

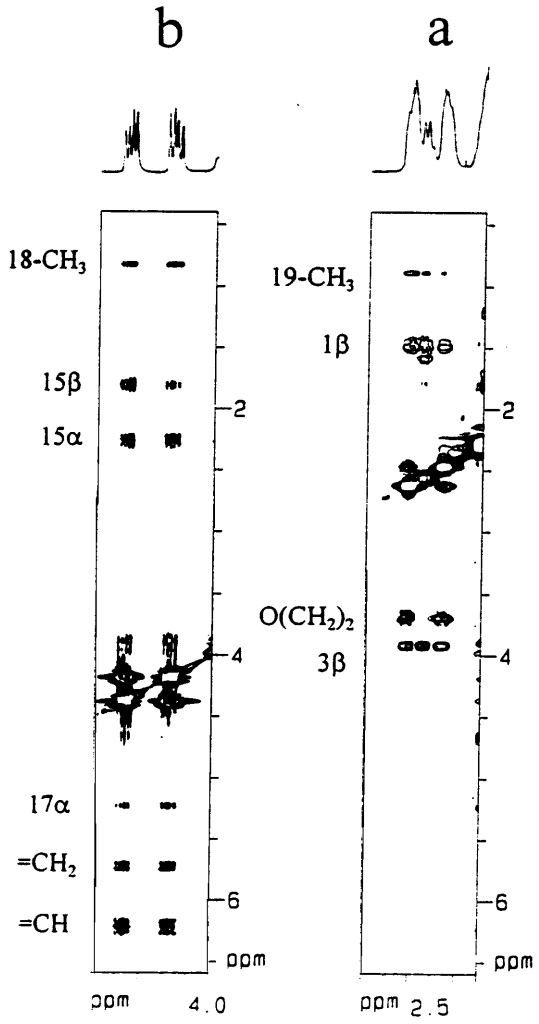

Figure 4. Parts of the 400 MHz ROESY spectrum (mixing time 300 ms) of **4** in $CDCl_3$ at 52 °C showing (a) correlations to the morpholine $N(CH_2)_2$ protons and (b) correlations to the allyl N^+CH_2 protons. Correlations from H-2α are also visible along the centre column of (a).

NMR spectrum. It seems most likely that the ^1H spectrum is deceptively simple and individual ^1H resonances are unresolved at 400 MHz. The ROESY data [Fig. 4(b)] showed several strong correlations between the steroid (18-CH_3, H-15α and H-15β) and the allyl (N^+CH_2) protons, and intra-allyl NOEs between N^+CH_2 and $CH{=}CH_2$ which are only consistent with rapid reorientations of the entire pendant allyl moiety. The relationship between the pyrrolidine ring and the steroid was not well defined by the ROESY data. Only one unambiguous steroid–heterocycle NOE was observed (a weak correlation between 18-CH_3 and the four unresolved C-2″ and C-5″ protons at 3.9 ppm). If the *N*-allylpyrrolidine moiety was freely rotating, an NOE might also be expected between 18-CH_3 and the C-3″ and C-4″ protons. The evidence, then, is for a rotating *N*-allylpyrrolidine group which (because of the missing NOE) spends more time with the allyl group above the β face of the steroid than under the α face.

Variable-temperature ^1H NMR experiments were performed in $CDCl_3$ (86% ring A twist-boat) over the

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range +52 to −65 °C and in MeOH-d_4 (corresponding to 23% ring A twist-boat)[31] over the range +52 to −85 °C. The $CDCl_3$ spectra first broadened as the temperature was decreased, consistent with slowing of morpholine and pyrrolidine motions, and then started to become sharper again around −50 °C. At −65 °C the ring A ^{1}H signals were not significantly different from those at +52 °C; however, the 17β-OAc and 18-CH_3 signals were split and all ring D ^{1}H signals were doubled. This was particularly clear in the COSY spectrum, which locates otherwise unresolved H-14 and H-15 signals (Fig. 5). Similar results were obtained in MeOH-d_4, except that in this solvent the lowest temperature data are best explained as a 1:1:2 mixture of three different species (perhaps the same species are present in $CDCl_3$, but they are not adequately resolved). In addition, in MeOH-d_4 it was possible to observe the H-2α signal change from a quartet (J = 4.5 Hz) at +52 °C to a narrower signal with unresolved couplings at −63 °C, corresponding to a shift in the ring A equilibrium towards more of the chair conformation.

It is interesting to compare these findings about the dynamics of the 2β-morpholine in **4** (fast exchange between two chair conformations and free rotation about C-2—N), with recent findings about the dynamics of the 2-ethylmorpholine moiety in 2β-[4-(2-ethylmorpholinyl)]-3α-hydroxy-5α(*H*)-pregnane-11,20-dione (**5**).[32] The ring A environments of these two molecules are very similar, yet in **5** (in both $CDCl_3$ and in DMSO-d_6) the morpholine ring exists in a single chair conformation with equatorial *N*-steroid and equatorial 2′-ethyl groups and no rotation about the C-2—N bond. Are these findings consistent? Presumably in **5**, the 2-alkyl group acts as a conformational lock on the morpholine, preventing ring inversion, and this together

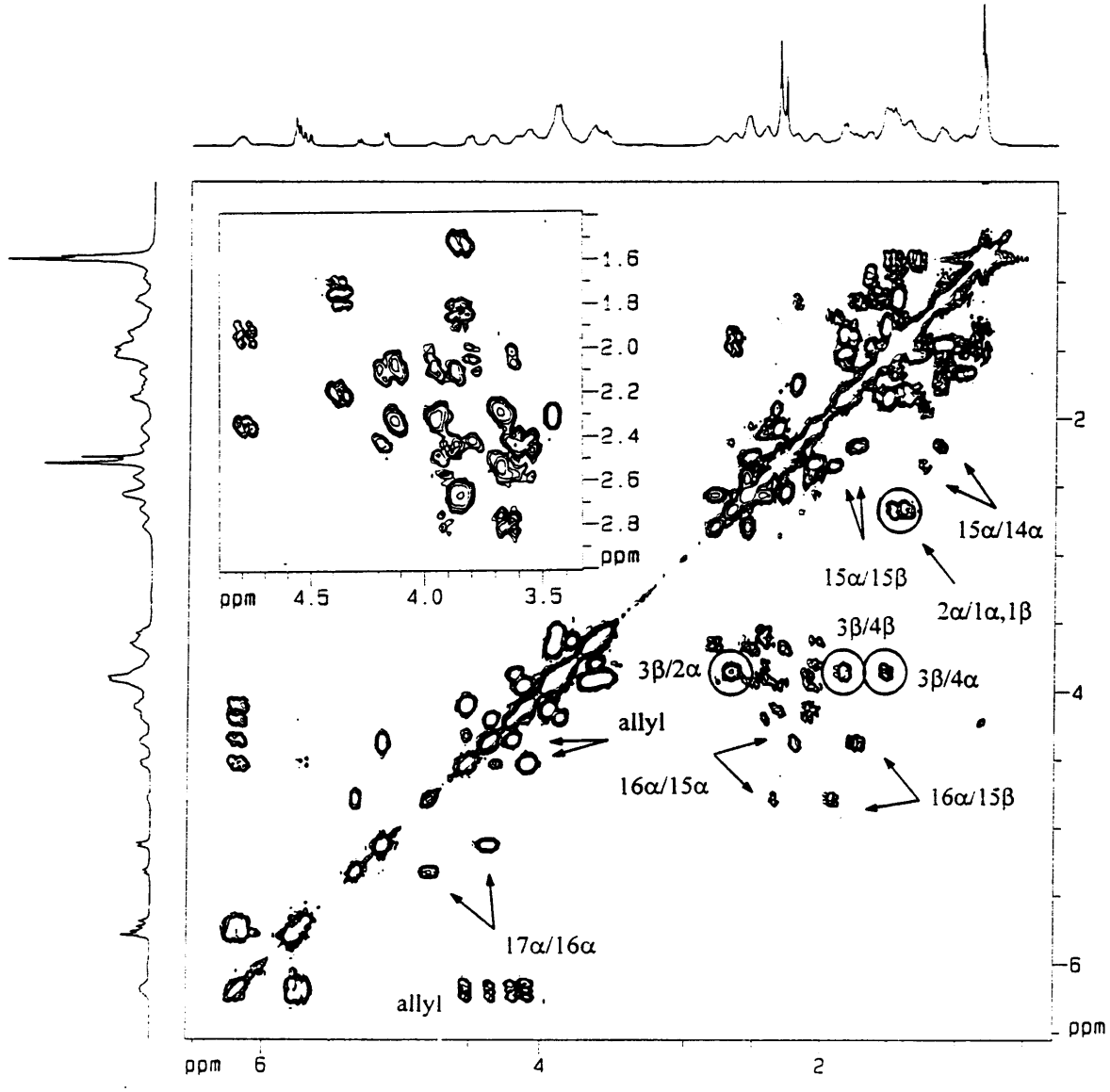

Figure 5. 400 MHz ^{1}H COSY spectrum of **4** in $CDCl_3$ at −65 °C showing single ring A correlations (circled) and a doubling of ring D resonances and correlations. The cluster of unassigned off-diagonal peaks around 2–4 ppm represent intra-pyrrolidine correlations.

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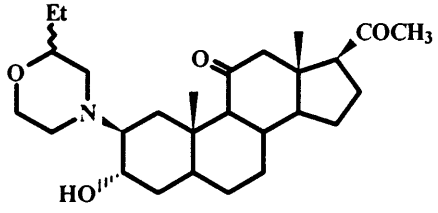

5

with the intramolecular hydrogen bond, prevents rotation of the morpholine moiety. *Both* restraints are necessary.

This supports the notion that rotation of the morpholine group in **4** (or piperidine as described for **1** and **2**) is facilitated by nitrogen-ring inversion to bypass the steric pressure of the axial 19-CH_3 group. Indeed, ring inversion is essential for the morpholine ring to pass the axial CH_3 group.

CONCLUSION

The acetylcholine fragments of steroidal neuromuscular blockers are not conformationally rigid. The rates of some conformation changes are slow on the NMR timescale at room temperature and this results in broad ^1H and ^{13}C NMR spectra.

Vecuronium bromide (**1**) exists in solution with the steroid ring A in a chair conformation, the axial 2β-piperidine moiety is present in two chair conformations which are rapidly averaged by the coupled nitrogen-ring inversion process and is freely rotating about the C-2—N bond. The 3α-OAc moiety is also mobile. The 16β-*N*-methylpiperidine moiety is in two rapidly converting chair conformations. In the major conformer (70%), the steroid occupies an equatorial position of the tetrahedral nitrogen and the CH_3 group is axial. The whole quaternary moiety is able to rotate about the C-16—N^+ bond. The 17α-OAc moiety is also unrestrained.

The same conclusions apply to Org 9487 (**2**), except that the proportions of axial and equatorial N^+allyl are undetermined.

Pancuronium bromide (**3**) exists in solution with ring A of the steroid in a twist-boat conformation. The twist-boat conformation of ring A is due solely to steric pressure on the β face. Both the 2β-*N*-methylpiperidine and the 16β-*N*-methylpiperidine are in chair conformations with a dynamic equilibrium existing between axial and equatorial N^+CH_3 groups. Both of the *N*-methylpiperidines and both of the acetyl groups are unrestrained.

Rocuronium bromide (**4**) exists in solution with a dynamic equilibrium between chair and twist-boat conformations of ring A. The 2β-morpholine group is free to rotate and is rapidly inverting between two chair conformations. The 16β-*N*-allylpyrrolidine group rotates about C-16—N^+ and the 17β-acetyl group is not constrained. The intramolecular hydrogen bond is an essential contribution to the stability of the twist-boat conformation, hence the substitution of 3α-OH by 3α-OAc blocks any possible hydrogen bond in **1**, **2** and **3**.

Rotation of the 2β-piperidine (or morpholine) moiety in **1**, **2** and **4** is correlated with the nitrogen-ring inversion process for six membered rings.

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