The conformation of the A-ring fragment of neuromuscular blocking agents structurally related to pancuronium. X-Ray structure determinations, molecular mechanics calculations and molecular dynamics simulations

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Org 9616 [1-{(2β,3α,5α,16β,17α)-3-acetoxy-17-(1-oxobutoxy)-2-(piperidin-1-yl)androstan-16-yl}-1-methylpiperidinium bromide] is a non-depolarizing neuromuscular blocking agent, structurally related to pancuronium. The reported crystal structures of the free base and the HBr salt show that the monoquaternary aminosteroid changes from a vecuronium-like conformation with the A-ring substituents in an axial orientation to a pancuronium-like conformation with the A-ring substituents in an equatorial orientation upon protonation. The conformations observed in the X-ray structures of Org 9616 and several other neuromuscular blockers, suggest a different conformational behaviour of the piperidino group attached to the A-ring of the steroid skeleton in pancuronium and protonated vecuronium. The conformational flexibilities of the A-ring and substituents in pancuronium, vecuronium and protonated vecuronium are more closely investigated by means of molecular mechanics calculations and molecular dynamics simulations. A model explaining the difference in selectivity between pancuronium and vecuronium is based on the conformational behaviour of this part of the molecule. The relevance of the presented model for the 3β-methyl analogues of pancuronium and vecuronium and the 2β-trimethylammonium analogue of pancuronium is discussed.

The aminosteroids pancuronium **1** and vecuronium **2** both show neuromuscular blocking activity, *i.e.* antagonism with respect to the nicotine receptors situated at the neuromuscular junction. Pancuronium is a bisquaternary compound, whereas vecuronium is a monoquaternary compound with a tertiary nitrogen atom in the piperidino substituent at position 2 of the steroid skeleton. Due to additional muscarinic antagonism, pancuronium displays cardiac side-effects, such as an increased heart rate (tachycardia) and hypertension. These types of side-effects were first observed for gallamine, another neuromuscular blocking agent, and are also known for a number of other blockers, like fazadinium, alcuronium and chandonium.[1] The cardiac side-effects of these substances are caused by a number of processes, one of the most important of which is the blockade of the actions of acetylcholine released from the cardiac vagus. Other processes include the blocking of the controlling influence of the vagus on noradrenergic output. Affinity for muscarinic receptors on dopaminergic interneurones is also observed.

The affinity for these muscurinic receptors is much lower in vecuronium than in pancuronium. In contrast to pancuronium, vecuronium can therefore in practice be considered as a selective neuromuscular blocker. Other selective neuromuscular blockers include the non-steroidal tubocurarine and atracurium. The pharmacology for the neuromuscular function is reviewed by Bowman[1a] and Ramsey.[1b] The neuromuscular and vagal blocking potencies of pancuronium and vecuronium are summarized in Table 1.

In their crystal structures pancuronium[2] and vecuronium[3] show different conformational features. In pancuronium, the 2-piperidinyl and 3-acetoxy A-ring substituents are both in equatorial orientations, whereas vecuronium displays these groups in axial orientations. These conformations will be referred to throughout this paper as the equatorial and axial conformations, respectively.

Table 1 Neuromuscular blocking potencies of the discussed compounds (measured on the tibialis and soleus muscles) and the vagolytic potencies, all measured in the cat. Tabulated ED_{50} values are given in μg kg^{-1}

Compound	ED_{50} tib	ED_{50} sol	ED_{50} vagus
Pancuronium (**1**)	27	18	50
Vecuronium (**3**)[a]	33	26	2749
Org 9616 (**5**)[a]	118	156	2020
Org 7677 (**6**)	10	8	450
Org 9269 (**7**)	127	143	697
Org 9255 (**8**)[a]	170	215	1780

[a] Vecuronium, Org 9616 and Org 9255 are thought to interact with the receptor in protonated form.

Vecuronium is thought to be active in the protonated form (**3**) but its crystal structure is still unknown, due to the lack of suitable crystals. NMR experiments and molecular mechanics calculations suggest that protonated vecuronium adopts an equatorial conformation.[3]

Here we present the crystal structures of a monoquaternary aminosteroid, structurally related to vecuronium, as a free base (Org 9616, **4**) and in protonated form (Org 9616HBr, **5**). Since the A-ring fragment of Org 9616 chemically corresponds to that of vecuronium, an axial conformation is anticipated for Org 9616. The protonated form, Org 9616HBr, is expected to display an equatorial conformation.

Results and discussion

The crystal structure of Org 9616

The molecular structure and adopted atom numbering scheme of Org 9616 [1-{(2β,3α,5α,16β,17α)-3-acetoxy-17-(1-oxobu-

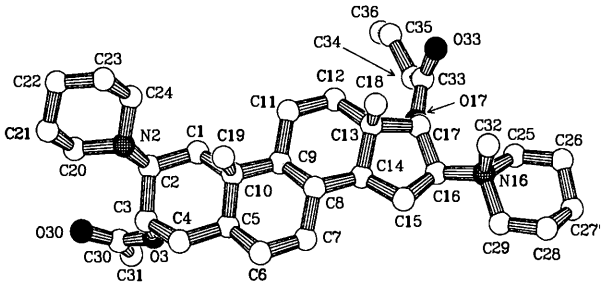
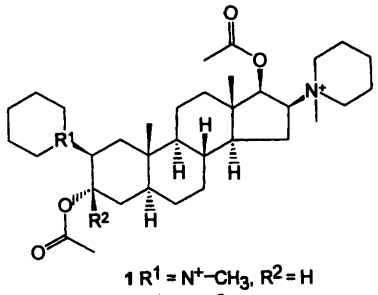
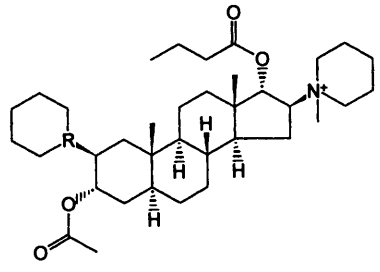


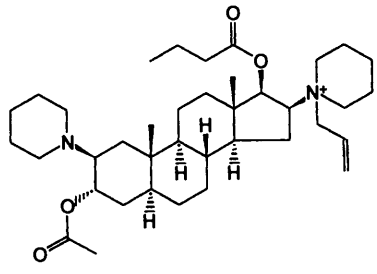
Fig. 1 Molecular conformation of Org 9616 in the crystal structure. Hydrogen atoms have been omitted for clarity.



1 $R^1 = N^+-CH_3$, $R^2 = H$
2 $R^1 = N$, $R^2 = H$
3 $R^1 = N^+-H$, $R^2 = H$
7 $R^1 = N^+-CH_3$, $R^2 = CH_3$
8 $R^1 = N^+-H$, $R^2 = CH_3$



4 R = N
5 $R = N^+-H$



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toxy)-2-(piperidin-1-yl)androstan-16-yl}-1-methylpiperidinium bromide, **4**] are presented in Fig. 1.

As anticipated, Org 9616 has adopted an axial conformation, which is also observed for vecuronium. There is, however, a small difference in the overall conformation of the A-ring side. The orientation of the acetoxy group with respect to the steroid skeleton is not equal in the two molecules. This orientation is characterized by the torsion angle C(2)–C(3)–O(3)–C(30), which amounts to −87.8(17)° for Org 9616 and to −142(3)° and −155(2)° for the independent molecules observed in the crystal structure of vecuronium bromide. The A ring itself has adopted a chair conformation, with all relevant asymmetry parameters[4] less than 9°. The B and C rings also have adopted a chair conformation.

Table 2 Relative steric energies (kcal mol^{-1}) of the conformations of some neuromuscular blocking agents. A–F refer to the minima equivalent to those displayed for pancuronium in Fig. 3. The extension .H indicates a protonated molecule

Compound	A	B	C	D	E	F
Pancuronium (**1**)	0.0	0.4	0.7	5.9	6.5	6.7
Vecuronium (**2**)	4.5	3.8	2.7	0.0	8.6	7.8
Vecuronium.H (**3**)	3.0	2.1	0.0	3.1	7.5	5.8
Org 9269 (**4**)	0.0	0.4	0.8	6.7	6.9	7.4
Org 9255.H (**5**)[a]	0.0	2.8	0.8	3.2	7.6	2.9
Org 7677 (**6**)[b]	0.0	0.0	0.0	5.8	5.8	5.8

[a] An extra minimum is observed for Org 9255.H, located between the minima C and F, with a relative steric energy of 1.8 kcal mol^{-1}, corresponding to the alternative twist-boat conformation (see text). [b] Due to the presence of three-fold rotation symmetry, the equatorial minima A, B and C display the same steric energy, as do the axial minima D, E and F.

The conformation of the D ring fragment of Org 9616 is different from that observed in vecuronium. The D ring itself has adopted a 14α envelope conformation, slightly distorted towards a 14α,15β half chair, as was also the case in vecuronium. The 17-ester function is located at the β position in vecuronium, and at the α position in Org 9616. A combination of packing effects and steric hindrance between the ester group and the 16β piperidino group causes the latter to adopt the alternative chair conformation to that observed in vecuronium. Molecular mechanics calculations show a large degree of flexibility for this part of the molecule. For vecuronium, the two conformations described above and two others, which differ in the orientation of this piperidino group around the C–N bond, all lie within an energy range of 0.5 kcal mol^{-1} (ref. 5); (1 cal = 4.184 J).

Analysis of the crystal packing is of interest because it can give an insight into possible intermolecular interaction geometries, which are of great importance in the ligand–receptor interaction. The crystal packing of Org 9616 includes a water molecule at typical hydrogen bonding distance with respect to the bromide anion. The positions of the hydrogen atoms of the water molecule could not be directly determined, but were introduced from geometrical considerations (*vide infra*). As in vecuronium, the bromine ions cluster around the positive charge of the quaternary nitrogen. In Org 9616 three bromine ions lie at distances between 4.35 and 4.76 Å from the quaternary nitrogen atom. Interestingly, no anion is located opposite to the C(16)–N(16) bond. At this position the partially negatively charged O(33) $[-x, -\frac{1}{2}+y, 1-z]$ is located, at a distance of 4.780(14) Å of the quaternary nitrogen atom; the angle C(16)–N(16)···O(33) amounts to 153.0(6)°. No bromine ions are located within 6 Å of N(2).

The crystal structure of Org 9616HBr

The molecular structure of Org 9616HBr observed in the crystal is presented in Fig. 2, together with the adopted atom numbering scheme. Selected geometrical parameters are included in Table 2.

The D-ring has adopted a 14α envelope conformation, again slightly distorted towards a 14α,15β half-chair conformation. The D-ring fragment has taken approximately the same conformation as is observed for Org 9616. The main difference is the orientation of the 17 ester group. The torsion angle O(17)–C(33)–C(34)–C(35) amounts to −73.8(8)° in Org 9616HBr and to 141(1)° in Org 9616. This difference is probably caused by packing influences, and is indicative of the flexibility of the 17α substituent. Both B and C-rings have taken a chair conformation. The A ring has taken an intermediate boat–twist–boat conformation {the asymmetry parameters[4] with the lowest values are $\Delta C_s[C(2)] = 26.5(4)°$, $\Delta C_s[C(3)–C(4)] = 19.3(5)°$, $\Delta C_2[C(1)] = 19.0(4)°$ and $\Delta C_2[C(2)–C(3)] = 23.3(5)°$} in order to orientate the 2 and 3 substituents in equatorial

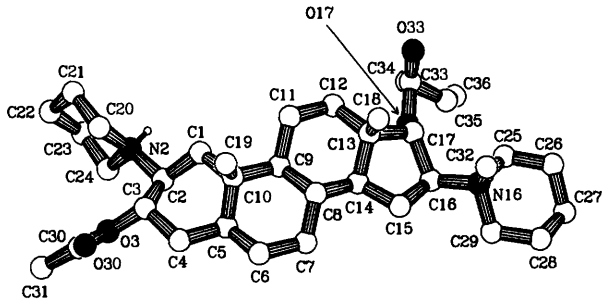


Fig. 2 Molecular conformation of Org 9616HBr in the crystal structure. All hydrogen atoms except the amino hydrogen of N(2) have been omitted for clarity.

positions. The observed conformation of Org 9616HBr resembles that of pancuronium bromide in the crystal structure, which also displays an equatorial conformation for the A-ring fragment. Whereas the orientation of the 3α-acetoxy group is similar, the orientation of the piperidino-ring around the C(2)–N(2) bond differs significantly between the two molecules. The torsion angle C(3)–C(2)–N(2)–C(24) amounts to −88.9(4)° in Org 9616HBr, while a value of 180° is found for the corresponding torsion angle in pancuronium.

The crystal packing of Org 9616HBr involves a water molecule, as is also the case in pancuronium, Org 7617 (**6**)[5] and Org 9616. No discrete water molecules were observed in the crystal structure of vecuronium bromide, although large disordered solvent areas were observed in this structure. The water molecule in Org 9616HBr links the two independent bromine ions Br(1) $[\bar{x}, \frac{1}{2}+y, \frac{1}{2}-z]$ and Br(2) with hydrogen bonds; O(40)···Br(1) = 3.237(4) Å, O(40)–H(402)···B(1) = 165(4)° and O(40)···Br(2) = 3.265(5) Å, O(40)–H(401)···Br(2) = 164(3)°. The angle Br(2)···O(40)···Br(1) $[\bar{x}, \frac{1}{2}+y, \frac{1}{2}-z]$ amounts to 99.57(11)°. The protonated tertiary nitrogen is hydrogen bonded to Br(2), with N(2)···Br(2) = 3.379(3) Å, N(2)–H(24)···BR(2) = 172.1(3)°. There are two other bromine anions located at a distance of 4.228(3) and 4.967(3) Å from N(2), with H(24)–N(2)···Br angles of 82.4(3)° and 131.9(3)°, respectively. Three anions surround the quaternary nitrogen attached to the D-ring, with Br···N distances 4.254(4)–5.096(3) Å. No anion is located on the extension of the C(16)–N(16) vector. This was also the case in Org 9616. The lack of bromine ions in this particular direction may be a consequence of a less favourable interaction energy with the quaternary nitrogen atom.

Molecular mechanics calculations on vecuronium and pancuronium

The crystal structures of Org 9616 (**4**) and Org 9616HBr (**5**) demonstrate the changes of conformation which a monoquaternary aminosteroid may undergo upon protonation, as already suggested by NMR experiments and molecular mechanics calculations on vecuronium.[3] A new feature arising from the crystal structure determinations is the observed flexibility of the A ring substituents. The 3-acetoxy group in Org 9616 shows an alternative conformation to the one observed in vecuronium. Molecular mechanics calculations indicate an energy difference between these two conformations of less than 0.5 kcal mol^{-1} for both molecules.

As described above, the 2-piperidino ring has changed its orientation with respect to the steroid skeleton in the transition from the axial to the equatorial conformation. In order to obtain a more detailed insight into the accesssible conformations of the A ring fragment, extensive molecular mechanics calculations were performed by means of the method of Prudent Ascent (*vide infra*).[9] The calculations were performed on pancuronium and vecuronium, since these two neuromuscular blocking agents are actually used in clinical practice. The results obtained for vecuronium can be expected to apply to Org 9616 as well, since these molecules have the same configuration in the A ring fragment.

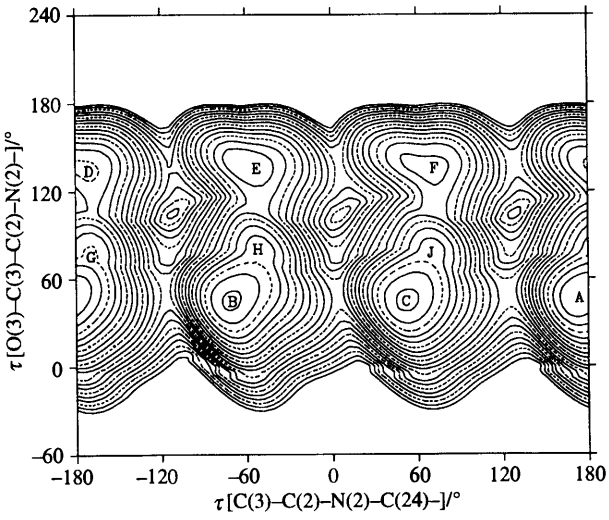


Fig. 3 Steric energy of pancuronium as a function of the torsion angles C–C–N–C and O–C–C–N (see text). The contour lines are drawn at an interval of 1.0 kcal mol^{-1}. Dashed contours are drawn at an interval of 3.0 kcal mol^{-1}; with a longer dash for the 3.0 kcal mol^{-1} level. The capitals refer to minima and other points of interest, see text.

The steric energy is calculated as a function of the overall conformation of the A-ring fragment and the orientation of the 3β-piperidino group. The overall conformation is described by means of the O(3)–C(3)–C(2)–N(2) torsion angle. The atom numbering used for pancuronium and vecuronium refers to the numbering scheme adopted for Org 9616 and its HBr salt, rather than the numbering schemes adopted in the original papers describing the crystal structures of vecuronium and pancuronium. The orientation of the piperidino ring is described with the torsion angle C(3)–C(2)–N(2)–C(24), where C(24) is the methylene carbon atom in the piperidino ring which is located in the anti position with respect to C(3) if the unique direction, *i.e.* the direction of the N-methyl group, the amine hydrogen or the amine lone pair, is located at the β side of the steroid molecule.

The energy function calculated for pancuronium is presented in Fig. 3 in the form of a contour map. Six minima, indicated A–F, can clearly be discerned. The three minima A–C, display an overall equatorial conformation, in which the C(3)–C(2)–N(2)–C(24) torsion angle has adopted values of 170°, −70° and 50°, respectively. The corresponding orientations of the piperidino group will be referred to as anti, −gauche and +gauche. These three equatorial minima display approximately equal steric energy (see Table 2). The minima D–F represent axial conformations. Due to steric hindrance between the piperidino group and the C(19) methyl group, the O(3)–C(3)–C(2)–N(2) torsion angle cannot reach a value of 170°, as is observed in the axial conformation of vecuronium, but instead adopts a value of approximately 140°. Also in the axial conformations, the piperidino group can take three orientations, with approximately equal steric energy, corresponding to an anti, −gauche and +gauche orientation of the piperidino group. The equatorial conformations are approximately 6 kcal mol^{-1} lower in energy than the axial conformations.

The conformation of the A ring is a slightly distorted chair in the axial conformations (D, E and F). In the equatorial conformations (A, B and C) the A ring adopts a twist–boat conformation, with a two-fold rotation axis running through C(1) and C(4). In the energy map, all three equatorial conformations display a small recess around O(3)–C(3)–C(2)–N(2) = 80°; these recesses are marked G, H and J in Fig. 3. At these points the molecule displays an alternative twist–boat conformation, characterized by a two-fold rotation axis running through C(3) and C(10). Careful inspection of the contour map in Fig. 3 reveals a

Table 3 Crystal data and details of the structure determination of Org 9616 (**4**) and Org 9616HBr (**5**)

Crystal data	**4**	**5**
Formula	$[C_{36}H_{61}N_2O_4]^+ \cdot Br^- \cdot H_2O$	$[C_{36}H_{62}N_2O_4]^{2+} \cdot 2Br^- \cdot H_2O$
Formula mass	683.80	764.72
Crystal system	Monoclinic	Orthorhombic
Space group	$P2_1$ (No. 4)	$P2_12_12_1$ (No. 19)
a, *b*, *c*/Å	8.5561(18), 11.484(2), 19.0334(19)	11.7638(12), 16.177(2), 20.086(2)
β/°	94.794(12)	—
V/Å^3	1863.7(5)	3822.4(7)
Z	2	4
D_x/g cm^{-3}	1.219	1.329
F(000) (electrons)	736	1616
μ/cm^{-1}	11.3	30.2
Data collection		
T/K	Ambient	Ambient
Radiation (Å)	Mo-Kα, Zr filter (0.710 73)	Cu-Kα, Ni filter (1.541 84)
θ_{min}, θ_{max}/°	1.08, 27.50	2.20, 70.00
Scan type	$\omega/2\theta$	$\omega/2\theta$
Scan/°	1.20 + 0.35 tan θ	0.45 + 0.14 tan θ
Hor., vert. aperture/mm	3.00, 5.00	3.00, 4.00
Reference reflections	$\bar{1}\,\bar{1}\,4$; $1\,\bar{1}\,\bar{4}$	$3\,3\,0$; $\bar{3}\,\bar{2}\,\bar{3}$
Dataset (*hkl* range)	0: 9; −14: 0; −24; 24	−14: 14; 0: 19; −20: 0
Total, unique data	4479, 4178 (R_{int} = 0.057)	7763, 7256 (R_{int} = 0.041)
Observed data [$I > 2.5\,\sigma(I)$]	1644	5476
Refinement		
Nref, Npar	1644, 401	5476, 414
R, *wR*, *S*	0.060, 0.058, 1.21	0.044, 0.044, 1.39
Weighting scheme	$1/[\sigma^2(F) + 0.000\,143F^2]$	$1/\sigma^2(F)$
$(\Delta/\sigma)_{max}$, $(\Delta/\sigma)_{av}$	0.31, 0.017	0.63, 0.035
Max, min resid dens/e A^{-3}	0.44, −0.28	0.48, −0.46

small band, with τ[O(3)–C(3)–C(2)–N(2)] between approximately 75° and 110°, marked by a point of inflection in the contour lines. This band corresponds to the described alternative twist–boat conformation, which apparently forms an intermediate conformation between the low-energy twist–boat, found at low O(3)–C(3)–C(2)–N(2) torsion angles, and the high-energy chair conformation, observed at high O(3)–C(3)–C(2)–N(2) torsion angles.

The orientation of the acetoxy group, as indicated by the torsion angle C(30)–O(3)–C(3)–C(2), varies from −gauche in the axial minima to anti in the equatorial minima. Intermediate values are observed in between. The C(30)–O(3)–C(3)–C(2) torsion angle adopts values of approximately 120° at O(3)–C(3)–C(2)–N(2) values of approximately 0°, as is indicated by a ripple in the contour map. Additional calculations show this alternative value to be only slightly higher in energy for all equatorial configurations, except for those at low O(3)–C(3)–C(2)–N(2) torsion angles. It is illustrative of the efficacy of the method of Prudent Ascent that such small details emerge.

Calculation of the steric energy of vecuronium as a function of the torsion angles C(3)–C(2)–N(2)–C(24) and O(3)–C(3)–C(2)–N(2) yields a map reasonably similar to the one observed for pancuronium. The major differences are found in the relative steric energies of the minima, resulting in a change of preferred conformation from equatorial to axial, and in the position of the minimum equivalent to D in Fig. 3. For vecuronium, this minimum is located at τ[O(3)–C(3)–C(2)–N(2)] = 170° because there is virtually no steric hindrance between the lone pair direction of N(2) and the C(19) methyl group. Minima E and F are again located at τ[O(3)–C(3)–C(2)–N(2)] = 140°, due to the steric hindrance between a methylene group of the piperidino ring and C(19). Since vecuronium as a free base is of less interest than its protonated form, we hereby suffice with the inclusion of the relative steric energies of the minima in Table 2.

Fig. 4 shows the steric energy as a function of the discussed torsion angles for protonated vecuronium. This map shows essentially the same features as the pancuronium map presented in Fig. 3. The relative steric energies of the minima are summarized in Table 2. As in unprotonated vecuronium, the minimum marked D is shifted to a higher O(3)–C(3)–C(2)–N(2) torsion angle than observed for pancuronium, although not as high as in unprotonated vecuronium. The location of the minimum is in accord with the increased steric hindrance between the nitrogen substituents and the C(19) methyl group of vecuronium, protonated vecuronium and pancuronium. The equatorial conformations A, B and C all display a lower steric energy than the axial conformations D, E and F, although the energy difference between the A and D minima is hardly significant. The conformation of the A-ring in the various minima of protonated vecuronium is equal to the one observed in the related pancuronium minima. As in pancuronium, an alternative twist–boat conformation forms an intermediate zone between the axial and equatorial conformations. Unlike pancuronium,

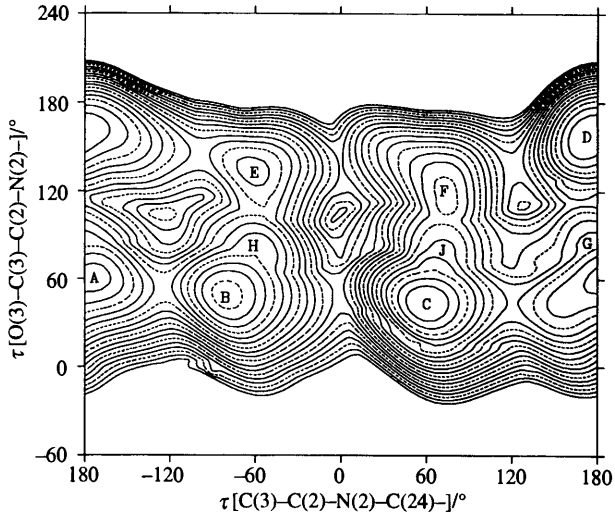


Fig. 4 Steric energy of protonated vecuronium as a function of the torsion angles C–C–N–C and O–C–C–N (see text). The contour lines are drawn as in Fig. 3. The capitals refer to minima and other points of interest, see text.

where all equatorial conformations display approximately equal energy, there is a clear preference for the orientation of the piperidino group in protonated vecuronium. The equatorial +gauche minimum represents the global minimum.

The only important conformational difference between pancuronium and protonated vecuronium observed so far, is the preference of protonated vecuronium for this +gauche orientation of the A ring piperidino group, whereas pancuronium lacks an orientational preference of this group. Since the orientation of the relatively bulky piperidino group can be an important factor in the interaction of the antagonist molecules with the muscarinic receptor, we suggest that the different vagolytic activities of these two molecules are a consequence of this particular conformational feature.

As was suggested earlier,[3] pancuronium and protonated vecuronium are thought to interact with the neuromuscular receptor in the overall equatorial conformation. As an addition to this model, we now suggest that pancuronium might be a less selective blocker due to its multiple minima behaviour. One of the corresponding conformations, not being preferred by protonated vecuronium, might be compatible with the muscarinic receptor.

Another factor governing the difference in vagolytic activity may be found in the different interaction geometries of a protonated amino function and a quaternary ammonium group with a negatively charged receptor site.[6] Examples of these different interaction geometries can be found in the crystal structures of some neuromuscular blocking agents. The quaternary nitrogen atom of the piperidino group attached to the A-ring of pancuronium is surrounded by three bromine anions, located at distances of 4.34–4.77 Å from the central nitrogen atom. Each of these ions is located more or less at the centre of a face of the carbon tetrahedron enclosing the central nitrogen. The face shielded by the presence of the 2α-acetoxy group displays no contact with a bromine ion. Interestingly, a partially negatively charged Cl atom of the solvent molecule CH_2Cl_2 is in contact with this tetrahedron face, albeit not at the centre. In the case of a protonated amine group, like in the structure of Org 9616HBr described here, a single bromine anion is able to approach the central nitrogen atom as close as 3.2 Å in order to form a linear hydrogen bond. The ion is therefore located at a tetrahedron vertex, rather than at the centre of a tetrahedron face.

Molecular dynamics calculations on pancuronium and vecuronium

The hypothesis explaining the difference in selectivity of vecuronium and pancuronium, described above, is based on the assumption that protonated vecuronium adopts a subset of the conformations of pancuronium. Molecular mechanics calculations show that this is true for the molecules *in vacuum*. In order to obtain information concerning the conformational preferences of these molecules in solution, molecular dynamics simulations were performed.

The stability in solution of the six conformations of pancuronium and protonated vecuronium as suggested by molecular mechanics, was investigated by a series of molecular dynamics simulations. In each simulation a blocker molecule was immersed in a box with water molecules and appropriate counter ions. All six conformations of pancuronium marked A–F in Fig. 3, proved to be stable during 25 ps of simulation time, preceded by a 5 ps equilibration period. In the case of protonated vecuronium, however, only four of the six conformations proved to be stable. The equatorial anti (A) and the axial −gauche (E) conformations could not be simulated for a longer time. For conformation A six different starting configurations were chosen, differing in the configuration of solvent molecules and showing small differences in the starting conformation of the solute, including the alternative conformation of the 3-acetoxy group, described earlier. During these simulations a transition to another conformation, mostly equatorial +gauche (C), was observed within 5 ps, often within the first ps of simulation. During the remaining simulation period, no transition back to conformation A occurred. Six different solvent starting configurations for E also showed transition to another conformation, mostly D, within 5 ps. When compared to the in vacuum calculations (Fig. 4), it is somewhat surprising that the conformations A and E turn out to be the least stable and not for instance conformation F. This illustrates once more that the conformational energies calculated in vacuum are not directly transferable to the situation in solution.

In order to obtain more detailed knowledge of the possible conformation in solution, the potential of mean force (*vide infra*) was calculated as a function of the C(3)–C(2)–N(2)–C(24) angle of protonated vecuronium. During simulation an overall equatorial conformation was imposed. The result is presented in Fig. 5. As is evident from Fig. 5, the equatorial anti minimum, marked as A in the molecular mechanics calculations, has completely disappeared. No clear preference for either the +gauche or the −gauche orientation of the piperidino group is found.

With regard to our hypothesis concerning the absence of muscarinic antagonism in vecuronium, we suggest that the conformation demanded by the muscarinic receptors should involve the anti orientation of the piperidino group, since this is not stable in solution for protonated vecuronium, but it can be adopted by pancuronium.

3β-Methyl-substituted analogues of pancuronium and vecuronium

In order to investigate the validity of our hypothesis for other molecules, the steric energy map as a function of the described torsion angles was calculated for Org 9269 (**7**) and Org 9255HBr (**8**), which are the 3β-methyl analogues of pancuronium and protonated vecuronium, respectively. The energy maps have virtually the same appearance as those calculated for pancuronium, vecuronium and protonated vecuronium. Both energy maps contained three axial and three equatorial minima, representing the anti, −gauche and +gauche conformations of the piperidino group of Org 9255HBr. The relative steric energy values of the calculated minima are listed in Table 2. The A-ring takes the same twist–boat conformation in the equatorial conformations as in vecuronium and pancuronium; apparently the extra substituent does not influence its conformation. As with the other molecules discussed so far, an alternative twist-boat conformation, the existence of which is revealed in the energy map by the presence of small recesses associated with the equatorial minima, forms the intermediate between the equatorial twist–boat and the axial chair conformations of the A-ring. At the +gauche orientation of the piperidino group, the intermediate twist–boat conformation forms a separate minimum

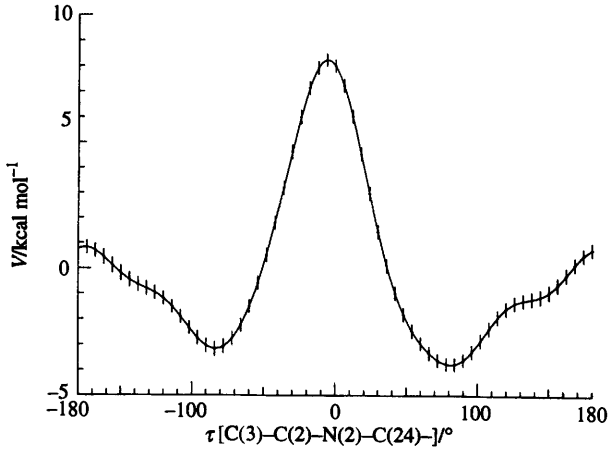


Fig. 5 Potential of mean force as a function of C(3)–C(2)–N(2)–C(24) in protonated vecuronium. The overall conformation of the molecule is equatorial. Vertical bars give the estimated standard deviation, calculated according to Straatsma *et al.*[7]

with a relative energy of 1.8 kcal mol^{-1} rather than a recess associated with the equatorial minimum. A ripple at low values for the O(3)–C(3)–C(2)–N(2) torsion angle indicates a change in the orientation of the 3-acetoxy substituent.

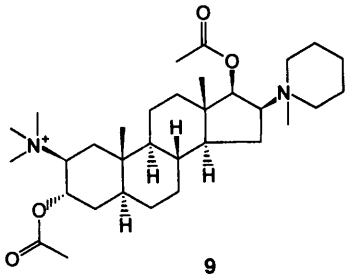
Molecular dynamics simulations were applied to determine the stability in solution of the minima found with molecular mechanics simulations. The same procedure as described for protonated vecuronium was followed. All six conformations of Org 9269 proved to be stable in solution. Org 9255HBr appeared to be stable in the same four conformations as protonated vecuronium, the other conformations could not be simulated for longer than 5 ps.

The ED_{50} values of Org 9255HBr and Org 9269 are included in Table 1. According to molecular mechanics, Org 9269 shows no clear conformational preference for any of the three equatorial conformations A, B and C, and can be regarded as a non-selective blocker. Org 9255HBr is a selective blocker and shows preference for the equatorial anti conformation, marked A in Table 2. Molecular dynamics simulations indicate, however, that this minimum is not stable in solution. It is clear that Org 9255HBr has a pronounced conformational preference, both in vacuum and in solution. Compared to pancuronium, Org 9269 is a relatively weak muscarinic antagonist; this cannot be fully explained within the current model.

Summarizing, we suggest that the muscarinic receptor probably requires an equatorial anti conformation in order to establish a favourable interaction geometry. Both Org 9255HBr and protonated vecuronium are in high energy states in this conformation. This may be related to their property not to display cardiac side-effects. Pancuronium and Org 9269 can adopt all three equatorial conformations and therefore are able to associate with the cardiac muscarinic receptors. The differences between the molecular mechanics and molecular dynamics results make it clear that the solvent effects should not be neglected. The molecular mechanics energies cannot therefore be used to correlate with potency. However, molecular mechanics remains an interesting tool to determine potential minima. Although the relative potencies cannot be explained in detail, a division into non-selective and selective neuromuscular blockers can be made on the basis of the conformational preference of the orientation of the 2β-piperidino group.

Org 7677, the 2-trimethylammonium analogue of pancuronium

The 2-trimethylammonium analogue of pancuronium (Org 7677, **9**), will display three equal equatorial minima, due to the three-fold rotation symmetry of the onium group. The steric energy map of Org 7677 was therefore calculated in the region $-10° < \tau[C(3)–C(2)–N(2)–C(24)] < 140°$, where C(24) is chosen at random. The overlapping area, *i.e.* the area expanding beyond a 120° segment, proved to be reproduced perfectly. In the unique area, one equatorial and one axial minimum was located. The same A-ring conformations as observed for all other compounds were also found for Org 7677, including the alternative twist-boat conformation present between the equatorial and axial conformations. Since there is no conformational preference, we expect a nonselective compound. The ED_{50} values in Table 1 show a certain amount of selectivity for Org 7677. However, this is mainly due to the high neuromuscular blocking potency, rather than low vagolytic activity, as displayed by vecuronium. Compared to pancuronium, Org 7677 is a less potent muscarinic antagonist, which might be caused by the fact that the trimethylammonium group is too small to stabilize the interaction with the muscarinic receptor. It is likely that a larger lipophilic group is needed to provide a better stabilization.



9

Experimental

Molecular mechanics calculations

The calculations described in this paper were performed with MMP2(1985).[8] Instead of standard bond dipole moments, an atomic point charge model was used to describe the electrostatic interactions. The actual atomic point charges were the same as those applied in the molecular dynamics simulations (*vide infra*). No lone pairs were added to the ether oxygen atoms, while a ghost lone pair had to be introduced at the tertiary nitrogen of vecuronium in order to prevent amine inversion while crossing high energy barriers.

The two-dimensional steric energy maps were calculated by means of the method of Prudent Ascent.[9] This method does not rely on a fixed order of computation of the points in the map; instead the order of computation is determined from all previous results. Furthermore, a recalculation of points is considered if a conformational change is detected in the unconstrained parts of the molecule.

In the Prudent Ascent calculations described in this work, a grid spacing of 4° was used for the O(3)–C(3)–C(2)–N(2) torsion angle at a spacing of 6° for the C(3)–C(2)–N(2)–C(24) torsion angle. The starting conformation of pancuronium was that observed in the crystal structure.[2] Hydrogen atoms were added with a modelling program. The starting configuration of protonated vecuronium was constructed from the crystal structure conformation of Org 9616HBr by means of a modelling program.

The energy map of vecuronium was calculated three times under different conditions. Besides a calculation in which the grid spacing described above was used, another calculation with a larger grid spacing of 5° and 10° for the O(3)–C(3)–C(2)–N(2) and C(3)–C(2)–N(2)–C(24) torsion angles was performed. A map with a higher, *i.e.* a less demanding, minimization convergence criterion was also calculated. This can be validated by the consideration that most lattice points are calculated a number of times, especially the low energy points, which are of the greatest interest. Both calculations showed a significant decrease in computational time, however, neither method was able to reproduce the energy map in a satisfactory way. Although all the major features were present, there were a number of discrepancies concerning the small conformational details like the conformation of the 3-acetoxy group and alternative twist–boat conformation of the A-ring. The above mentioned values for grid spacing and the default convergence criterion were therefore adopted for all other calculations.

Molecular dynamics simulations

Molecular dynamics simulations were carried out using the GROMOS forcefield and program package.[10] The bond lengths were constrained to their natural values *via* the SHAKE procedure in order to reduce computation time.[11] Another measure to reduce computation time is the introduction of a cut-off radius beyond which the non-bonded interactions are neglected. The electrostatic energy of the system is calculated as the interaction of charge groups. In the simulations of protonated vecuronium and pancuronium described here, the protonated amino and quaternary ammonium groups were each included in a charge group with a total charge of +1. The Cl^- counter ions each formed a separate charge group. All other atoms were included in neutral charge groups. Although it is in principle incorrect to use non-neutral charge groups, simulations of crystal structures show that this approximation, combined with

a cut-off radius of 11 Å rather than the usually applied value of 8 Å, are able to produce reasonable results.[12] The atomic point charges of the non-neutral charge groups are, as far as possible, based on standard GROMOS charges for neutral charge groups. For the charges of the quaternary ammonium group we have chosen the simplest possible delocalization scheme, *i.e.* the nitrogen and its four carbon substituents carry a charge of +0.2 e each. Molecular mechanics test calculations of these compounds showed this distribution to render virtually the same results as charge distributions based on semi-empirical quantum chemical calculations with the AM1, MNDO, MINDO/3 and PM3 methods.[13]

A new atom type had to be introduced to represent the quaternary carbon atom. Parameters defining the covalent geometry around this atom type were copied from the other aliphatic carbon types or based upon ideal tetrahedral geometry. Van der Waals parameters were equated to the values used for other bare carbon atoms.

Starting coordinates were obtained by immersing a molecular mechanics conformation in a box with water molecules and appropriate counter ions. The positions of the solvent molecules were optimized by an energy minimization run. Starting velocities were taken from a Maxwellian distribution for 300 K. The system was loosely coupled to a temperature and pressure bath in order to maintain a temperature of 300 K and a pressure of 1 atm with coupling times of 0.1 and 0.5 ps, respectively. A time-step of 2 fs was employed and the list of interacting charge group pairs was updated every five time-steps. In order to reduce computation time a truncated molecule was simulated, *i.e.* the *C* and *D* rings with their substituents were deleted from the molecule, since only the *A*-ring fragment of the discussed molecules is of interest. Molecular mechanics calculations on complete molecules and truncated molecules showed virtually the same results.

In order to calculate the potential of mean force as a function of a torsion angle, a simulation is carried out with the normal forcefield, to which an extra harmonic potential with a large force constant is added. This harmonic potential constrains the torsion angle of interest at the specified value. It can be shown[14] that the force necessary to keep the torsion angle at the desired value is equal to the force constant of the applied harmonic potential multiplied by the averaged value of the deviation of the torsion angle from the specified value. This force is calculated for a number of torsion angle values and a function is fitted to the observed values. Analytical integration of this function yields the potential of mean force. It is convenient to specify the force function as a Fourier series.

For the calculations on protonated vecuronium, an extra harmonic potential with a force constant of 100 kcal mol^{-1} was added to the force field in order to restrain the torsion angle C(3)–C(2)–N(2)–C(24). The mean force was calculated at the points $\tau = 12° + n \times 24°$ ($n = 0,15$). Each point was simulated during 40 ps, preceded by a equilibration period of 5 ps. For most points this period proved to be long enough to produce a stable average. At some points, however, long period fluctuations were observed. In order to get a more precise determination, much longer simulation periods (approximately 10^2 ps) are needed. A six-term Fourier series was fitted through the calculated forces by means of a weighted singular value decomposition algorithm.[15]

X-Ray crystallography

Experimental details of the structure determination of Org 9616 (**4**) and Org 9616HBr (**5**) are presented in Table 3. Intensity data were collected on an Enraf-Nonius CAD-4 diffractometer.

Crystals of Org 9616, suitable for X-ray structure determination were grown by vapour diffusion of diethyl ether in a solution of 5 mg Org 9616 in 200 μl methylene chloride. The structure was determined by means of automated Patterson methods. Full-matrix least-squares refinement on *F* included positional and anisotropic thermal parameters of the non-hydrogen atoms and two overall isotropic thermal parameters for the hydrogen atoms with values of 0.143(16) and 0.067(6) $Å^2$ for the hydrogen atoms in the ester methyl groups and the piperidinyl ring attached to the *A*-ring, and all other hydrogen atoms, respectively. The hydrogen atoms of the water molecule included in the structure of Org 9616 could not be located on a difference Fourier map, nor on a difference Fourier map with weights depending exponentially on $\sin\theta/\lambda$. The distance of the oxygen atom of the water molecule to the bromine ion clearly suggests the presence of a hydrogen bond. The only other short contact of the oxygen atom to a potential hydrogen bond acceptor is to O(33), however, the distance of 3.0 Å is rather long for a hydrogen bond. The water molecule with hydrogen atoms was included in the refinement as a rigid group, free to rotate around the oxygen atom. A significant lowering of the *R*-factor was obtained with the rigid group orientated in such a way that both hydrogen atoms formed a hydrogen bond with the bromine ion.

Crystals of Org 9616HBr were grown by vapour diffusion of pentane in a solution of 5 mg Org 9616 in 400 μl methylene chloride. The crystal of Org 9616HBr used in the diffraction experiment turned out to consist of two fragments, with intensity ratio of 10.8:1. The smaller fragment was rotated over 11.8° around the vector in reciprocal space (−3.0 14.3 7.9). Data were collected for the larger fragment. The structure was solved by means of automated Patterson methods. Full-matrix least-squares on *F* included anisotropic thermal parameters of the non-hydrogen atoms and two overall isotropic thermal parameters for the hydrogen atoms with values of 0.31(2) and 0.071(2) $Å^2$ for the hydrogen atoms in the 17α-oxobutoxy group and other hydrogen atoms, respectively.

The structures of both compounds were solved by SHELXS86.[16] Least-squares refinement was carried out with SHELX76[17] and absorption effects were corrected for with DIFABS.[18] Absolute configurations were derived in accordance with the well known configuration of the androstane steroid skeleton. Refinement of the alternative chirality lead to significantly higher *R*-values (0.066 and 0.053 for Org 9616 and Org 9616HBr, respectively). Scattering factors were taken from Cromer and Mann;[19] anomalous-dispersion corrections from Cromer and Liberman.[20] Geometrical calculations were performed by PLATON.[21]

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Atomic coordinates, bond lengths and angles, and thermal parameters have been deposited at the Cambridge Crystallographic Data Centre (CCDC). For details of the deposition scheme, see 'Instructions for Authors', *J. Chem. Soc., Perkin Trans. 2*, 1996, Issue 1. Any request to the CCDC for this material should quote the full literature citation and the reference number 188/22.

References

1 (*a*) W. C. Bowman, *Pharmacology of Neuromuscular Function*, John Wright & Sons, Bristol, 1980, pp. 103–105; (*b*) F. M. Ramsay, *Anesthesiol. Clin. North Am.*, 1993, **11**, 219.
2 D. S. Savage, A. F. Cameron, C. Hannaway and I. R. Mackay, *J. Chem. Soc. B*, 1971, 410.
3 H. Kooijman, V. J. van Geerstein, P. van der Sluis, J. A. Kanters, J. Kroon, C. W. Funke and J. Kelder, *J. Chem. Soc., Perkin Trans. 2*, 1991, 1581.
4 W. L. Duax and D. A. Norton, *Atlas of Steroid Structure*, vol. I, IFI/Plenum Data Company, New York, 1975, pp. 16–22.
5 H. Kooijman, J. A. Kanters, J. Kroon and J. Kelder, unpublished results.

6 H. Kooijman, J. A. Kanters, J. Kroon and J. Kelder, *Acta Crystallogr., Sect. B.* 1993, **49**, 356.
7 T. P. Straatsma, H. J. C. Berendsen and A. J. Stam, *Mol. Phys.*, 1986, **57**, 89.
8 N. L. Allinger, MMP2(85), QCPE, Indiana University.
9 R. W. W. Hooft, J. A. Kanters and J. Kroon, *J. Comput. Chem.*, 1991, **12**, 943.
10 W. F. van Gunsteren, GROMOS, Groningen molecular simulation computer program package, University of Groningen, The Netherlands, 1986.
11 W. F. van Gunsteren and H. J. C. Berendsen, *Mol. Phys.*, 1977, **34**, 1311.
12 H. Kooijman, B. P. van Eijck and J. Kroon, *J. Mol. Struct.*, 1991, **268**, 283.
13 J. P. Stewart, MOPAC 5.0, QCPE program 455, Indiana University, 1989.
14 E. Paci, G. Ciccotti, M. Ferrario and R. Capral, *Chem. Phys. Lett.*, 1991, **176**, 581.
15 W. H. Press, B. P. Flannery, S. A. Teukolskly and W. T. Vetterling, *Numerical Recipes*, CUP, Cambridge, 1988, pp. 515–519.
16 G. M. Sheldrick, SHELXS86, Program for crystal structure determination, University of Göttingen, Germany, 1986.
17 G. M. Sheldrick, SHELX76, Program for crystal structure determination, University of Cambridge, England, 1976.
18 N. Walker and D. Stuart, *Acta Crystallogr., Sect. A*, 1983, , **39**, 158.
19 D. T. Cromer and J. B. Mann, *Acta Crystallogr., Sect. A*, 1968, **24**, 321.
20 D. T. Cromer and D. Liberman, *J. Chem. Phys.*, 1970, **53**, 1891.
21 A. L. Spek, *Acta Crystallogr., Sect. A.*, 1990, **46**, C34.

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