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Theoretical study of the complexation of amphotericin B with sterols

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Abstract

The aim of this present work was the study of the intermolecular complexes between amphotericin B (AmB) and either cholesterol or ergosterol. In such complexes the intermolecular interaction energy mainly proceeds from both Van der Waals and H-bonding (via water molecules) forces. Our calculations have shown that the Van der Waals forces slightly favor the AmB-ergosterol complex. Several relative positions of the sterol with regard to AmB lead to energy minima: sterol may be either in contact with the AmB polar head or repelled towards the end of the macrolide ring. It appeared that the role played by some water molecules was to maintain the sterol close to the AmB polar head.

Key words: Amphotericin; Water; Sterol; Complexation

1. Introduction

Amphotericin B (AmB) is a polyene antibiotic used to treat fungal infection. The presence of sterols in membranes is necessary for the development of the polyene antibiotic amphotericin B activity (for review, see [1,2]).

This had led to the proposal that AmB may form a complex with sterols (ergosterol in fungal cells, cholesterol in mammalian cells), the complex ergosterol-AmB being stronger, which would explain the higher activity of AmB on fungal cells as compared to that on mammalian cells [3,4]. However, up to now, there is no definite proof of such a complex with cholesterol in membranes. In contrast, with ergosterol, studies on model membrane seem to indicate the existence of a 1:1 AmB-ergosterol complex. Inside the biological membrane, the sterol is bound to phospholipid component; it may be possible that AmB has to compete for sterol: there is some evidence that ergosterol–phospholipid interaction is weaker than cholesterol–phospholipid interaction [5].

Some studies have been performed in alcohol/water mixtures [6] in order to investigate the extent of complex formation between AmB and cholesterol or ergosterol. These experiments indicate that the AmB–sterol interaction is strongly dependent on experimental conditions, especially on the nature of the solvent (water, methanol, propanol or mixture of water with an alcohol).

It has been suggested that occurrence of interaction between antibiotic and sterol is determined by some structural requirements

(a) of the sterol: presence of a 3β-OH group and of an hydrophobic side chain; these requirements are fulfilled by both cholesterol and ergosterol [7–11].

(b) of the polyene macrolide: length of polyenic chromophore, net charge of the molecule, presence or not of a free negatively charged group [12–15].

It has been suggested [13,16] that formation of sterol-antibiotic complex is due to

(a) H-bond interaction between OH group of the sterol and polar head of the antibiotic through a possible intermediate water molecule.

(b) Van der Waals interactions between the hydrophobic parts of sterols and the polyene macrolide.

Such a model needs to be borne out by the study of energetics of polyene–sterol interactions by computer modelling, since the molecular basis for the preferential binding of AmB to ergosterol as compared to cholesterol is unknown. One of our aims in this present

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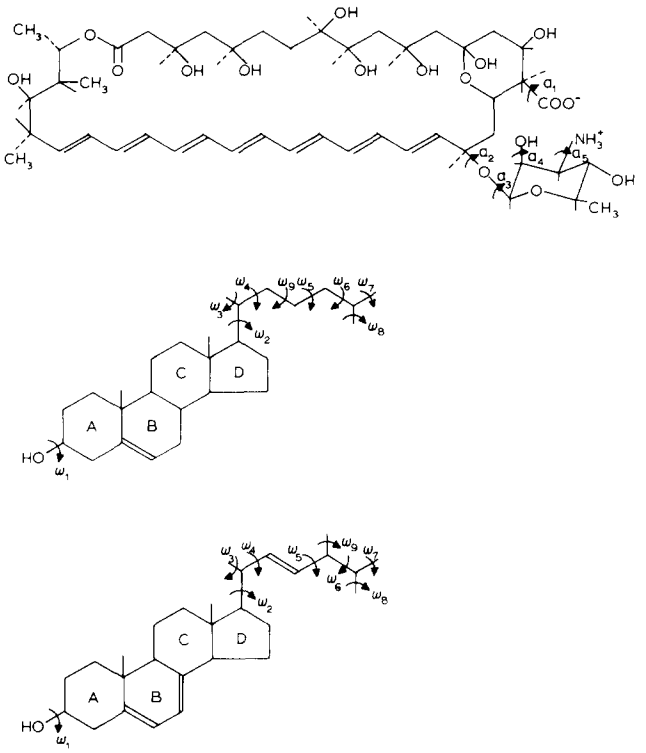

Fig. 1. AmB, cholesterol and ergosterol molecules. Degrees of freedom are indicated for the polar head of AmB and the side chains of sterol.

work was to settle the discriminating contributions of Van der Waals and H-bonded interactions in the AmB-sterol complexes.

It may be easily conceived that for such a study, a knowledge of the preferred conformations of AmB, cholesterol and ergosterol is of prime necessity. As a preliminary work [17] we performed a series of conformational investigations of the zwitterionic form of AmB; this state of ionization has been chosen because in biological media (thus at pH 7) the polar head of AmB is thought to be at the interface between membrane and water. Until now, experimental data concerning the structure of these groups of molecules are very scarce except the crystal structure determination of *N*-iodoacetyl amphotericin B [18]. Our study had dealt with both the isolated and hydrated state of AmB. We completed this previous work by dynamics calculations on isolated AmB.

Structures of cholesterol and ergosterol have been determined by the X-ray method [19–21]. A recent comparative conformational analysis of these two molecules has been carried out using molecular mechanics methods [22].

In the first part of this paper we summarize the conformational results. The second part is devoted to our results concerning AmB-sterol complexation (cholesterol and ergosterol), respectively, in the absence and presence of water molecules.

Concomitantly with us, a theoretical approach of a model of AmB-cholesterol complexes forming a channel, including eight dimers, was analyzed by molecular mechanics calculations [23]. Thus it will be interesting to compare our results concerning the intrinsic properties of the AmB-sterol dimer with the ones outcoming from the study of Khutorsky.

2. Methods

2.1. Mechanical calculations of the intra- and intermolecular energies of the complexes

Because of the complexity of the two kinds of molecules studied, AmB and sterol, it was difficult to calculate all the interaction energies in the presence of water molecules. So we adopted two strategies of calculations:

A first strategy has consisted in calculating interaction energy between sterol and AmB molecules by imposing both AmB and sterols conformations to be fixed. From a practical point of view, we used a method denoted INTER [24,25] adapted to consider the relative motions of the two molecules forming the complex.

A second strategy has consisted in taking into account eventual conformational changes of both AmB and sterol molecules forming the complex concomitantly with interactions involving some water molecules, the ones which interact strongly with the solute and are denoted as 'hydration water molecules'; method SIBFA was used [26,27].

Geometrical definitions

From a practical point of view, it must be emphasized that the geometrical parameters defining the A...B complex (i.e., the position of the second molecule with regards to the first one) are depicted differently within the two methods.

(a) In the INTER method, the cartesian coordinates of AmB and sterols are calculated using the three main inerty axes as general coordinates axis OX_i, OY_i and OZ_i (i being AmB or sterol): The OZ_i and OY_i axes are situated in the mean planes containing: (a) the macrolide ring of AmB, (b) the steroid nucleus of sterol; the OZ_i axes are directed along the long axes of the two molecules and the OX_i axes are perpendicular to these mean planes.

First the two planes containing the quasi-planar parts of the molecules (OY_iZ_i) are superimposed, the OZ_i and OX_i axes being confused. The second molecule is allowed to move along and around the coordinates axes.

So the relative positions of the two molecules are noted by the values of the displacement as ΔX, ΔY, ΔZ along the three axis OX, OY and OZ, and the

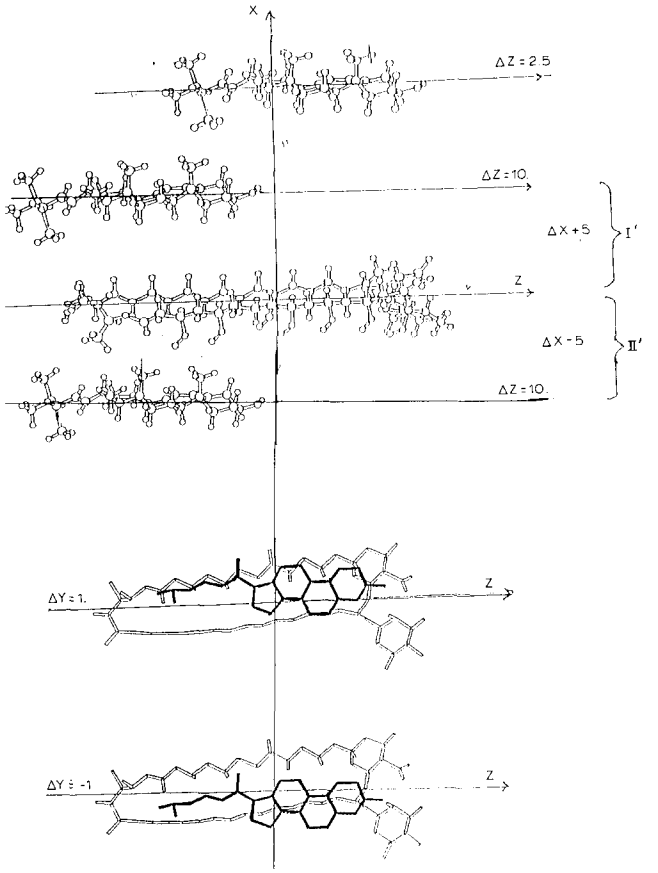

Fig. 2. Definition of the geometrical parameters for INTER calculations: (a) On the XOZ plane: motions along the OX and OZ axes (sterol being at different values of ΔX and ΔZ). Complexes I' and II' are represented. (b) On the YOZ plane: motion along OY axis (sterol is represented by black molecule).

values of the rotation angles Θ_X, Θ_Y and Θ_Z around these axes. The energy of the AmB-sterol complex is a function of six generalized coordinates.

In fact the three translation cordinates are very important, since:

(i) ΔZ defines the position of the sterol with regards to the polar head of AmB.

(ii) ΔY defines the position of the sterol with regards to the polyenic chain of the AmB macrolide ring.

(iii) ΔX defines the intermolecular distance between AmB and sterol molecules.

Among the three rotation motions, the most important one is Θ_Z angle which defines the parallelism (or not) within the two molecules forming the complex. Preliminary calculations have shown that rotations of the angles Θ_X and Θ_Y are not important. A near zero value has been obtained.

(b) In the SIBFA method, the coordinates of the whole system 'AmB + sterol' supermolecule are given in terms of internal coordinates (r_{ij}, θ_{ijk}, ϕ): r_{ij} being the distance between two atoms i, j; θ_{ijk} being the bond angle between two bonds ij and jk, and ϕ the dihedral angle between the planes containing ij and kl bonds, respectively.

Calculation methods

It has been verified that both INTER and SIBFA methods lead to results which are quite similar from both a qualitative and quantitative point of view. These two methods being previously described in detail [24–28], we will only give their main characteristic features:

Intermolecular energy. In the two methods, the interaction energy is the sum of several components, the expressions of which have been fitted in such a way to satisfactorily reproduce the results of ab-initio (SCF supermolecule or perturbation treatments) calculations on small complexes:

$$E_{\mathrm{INTER}} = E_{\mathrm{EL}} + E_{\mathrm{POL}} + E_{\mathrm{REP}} + E_{\mathrm{DISP}} + E_{\mathrm{CT}} \qquad (1)$$

(i) Use of a multicenter (atom and middle of bonds), multipolar (up to quadrupoles) expansion derived [29] from the ab-initio SCF molecular function for the calculations of *electrostatic* and *polarization* components, E_{EL} and E_{POL}. In such work, the ab-initio wave functions were calculated within an 'adapted' minimal basis [30].

(ii) Taking into account of the *charge transfer* contribution, E_{CT}, which is explicitely calculated in SIBFA and represented as a reduction of the positive repulsion component in INTER (such a representation proceeding from the exponential behaviour of the negative transfer term).

(iii) *Repulsion* contribution, E_{REP}, has a different expression within the two methods: a sum of atom-atom contributions in INTER and a sum of bond–bond, bond–lone pair and lone pair–lone pair interactions in SIBFA (such a representation accounts for the radial and directional dependence of this term).

(iv) In both these methods *dispersion* energy, E_{DISP}, is dumped in order to take into account the overestimation of the multipolar representation of this term at short distances.

We called 'hydration energy' the intermolecular energy between the AmB molecule and the water molecules.

Intramolecular energy [28]. Both intra- and intermolecular energies are calculated simultaneously in the framework of the SIBFA method. A large molecule is built out of constitutive molecular fragments separated by single bonds. In fact one calculates the variation of the conformational energy as a sum of inter-fragment interaction energies:

$$\Delta E_{\mathrm{INTRA}} = \sum_{i=1}^{N} \sum_{j=i+1}^{N} E'_{\mathrm{INTER}}(i, j) \qquad (2)$$

where N is now the number of fragments.

E'_{INTER} is calculated as a sum of the four first contributions given in Eq. (1), plus a term denoted E_{TOR} which is a transferable torsional energy contribution, calibrated for elementary rotations around single bonds.

2.2. Dynamics calculations of the AmB molecule

Energy function

Molecular mechanics and dynamics calculations were performed, using CHARMm version 21 [31]. Since the calculations were performed in vacuo, a distance-dependent dielectric was chosen to simulate the shielding effect of solvent [31,32]. The parameter set is derived from [33], while the partial atomic charges obtained from SIBFA have been modified to yield exclusively punctual charges located at the atomic positions. The Van der Waals energy terms were shifted down to yield a zero value at 9.5 Å, while the electrostatic energy function was zeroed by a sigmoid cutoff function in the interval 9.5 Å–1.5 Å.

Molecular dynamics

Newtonian equations of motion were integrated with the Verlet algorithm [34]. A 1 fs integration step was chosen and the SHAKE constraints [35,36] have been applied on the bonds involving one hydrogen atom. The procedure for MD was:

(i) a preliminary energy minimization (100 steps Adapted Base Newton-Raphson), to remove any bad contact;

(ii) a 18 ps thermalization from 0 K to 300 K in 5 K steps – a given temperature being obtained by assigning velocities from a Gaussian distribution with a variance corresponding to this temperature;

(iii) a 20 ps equilibration during which the velocities were randomly reassigned every 0.4 ps to provide an homogeneous velocity distribution;

(iv) a 30 ps equilibration during which the velocities were rescaled if the temperature falls outside a ± 10 K window, the temperature being checked every 0.4 ps (a subsequent 'productive' dynamics is performed only if no more than one rescaling occurs);

(v) a 100 ps 'productive' (i.e., neither thermalization nor equilibration) dynamics.

A time-averaged structure (over the 'productive' dynamics) was then computed

3. Results

3.1. Conformational study

AmB in an isolated state

In the previous work, all bond lengths and bond angles were fixed to the values obtained from X-ray study of *N*-iodoacetyl AmB, replacing the iodoacetyl group by a hydrogen atom. Because of the presence of a conjugated double bond system, we consider that the heptaenic macrolide ring remains rigid and therefore independent of the surrounding medium. Rinnert et al. [37] for AmB and Perun et al. [38] for erythromycin, another macrolide antibiotic, have confirmed that the conformation of the macrolactone ring in solution does not change when it is compared to crystal state. Hence we have kept this part of the molecule within the conformation obtained in the crystal, but we have been interested by the flexible polar head which is in a zwitterionic form.

The conformational energy is expressed as a function of the five variable dihedral angles $\alpha_i (i = 1,5)$ defined in Fig. 1, choosing the geometrical arrangment determined in the crystal as an initial guess (conformation A, see Table 1). We have adopted two different minimization strategies: (a) calculating different conformational energy submaps by means of systematic variations of dihedral angle values, then refining by an automatic minimization process involving the five dihedral angles simultameously; (b) performing direct minimization processes involving the five dihedral angles simultaneously. Our conformational investigation using either strategy led to nearly identical results from both

Table 1
Values of dihedral angles for the different conformations of the hydrated AmB and their energy differences

Conformations	AmB in the isolated state					ΔE_{intra}
	α_1	α_2	α_3	α_4	α_5	
A	67.7°	272.4°	142.1°	0.0°	150.0°	
C	183.8°	292.6°	92.0°	315.4°	175.2°	−34.4

Conformations	AmB in the hydrated state							
	α_1	α_2	α_3	α_4	α_5	ΔE_{intra}	ΔE_{hydra}	ΔE_{tot}
C	188.8°	288.9°	89.3°	36.9°	151.4°	−26.0	+14.3	−11.7
B	79.4°	284.8°	125.5°	7.4°	162.4°	−15.8	+4.6	−11.2

$\Delta E_{tot} = \Delta E_{intra} + \Delta E_{hydra}$. Intramolecular and hydration energies of conformation A are taken as the zeroth energies. (All values are in kcal/mol).

Table 2
rms fluctuations (Å) of the AmB and of each of its moieties (defined in the text) during a 100 ps MD simulation

AmB	Chain 1	Cycle 1	Chain 2	Cycle 2
0.60	0.59	0.58	0.53	0.52

a geometrical and energetical point of view. Furthermore an analysis of the submaps $E = f(\alpha_2, \alpha_3)$ show that only a restricted area is allowed in the α_2–α_3 conformational subspace. We obtained a folded minimum conformation, denoted C, which is stabilized with regard to the A conformation, because of an array of intramolecular H-bonds, one of them connecting the lactone ring and the sugar moiety.

As expected, the AmB is very stable during the MD simulations. Indeed a value of 1.01 Å only is observed for the rms displacement of the structure averaged over the dynamics simulations with respect to the initial structure. Fig. 3 represents the superposition of the two structures. It appears that, during the simulations, the AmB may be considered as an association of four almost rigid moieties which move relatively to each other. The moieties are the polyenic chain (chain 1), the hydroxyl chain (chain 2), the mycosamine ring (cycle 1) and the lactone ring (cycle 2). Table 2 gives the rms fluctuation (in Å) of the whole structure as well as the rms fluctuations of each of its four moieties considered as rigid bodies. All these values are in the 0.5–0.6 Å range, which confirms the lack of flexibility of the structure.

The largest fluctuations observed for the polyenic chain and the mycosamine ring are echoed in Fig. 3, which exhibits the largest structural perturbations near its junction to the hydroxyl chain. The rms fluctuations of the hydroxyl chain and of the lactone ring are lower. This is perhaps an effect of the stabilization of the hydroxyl chain by the interactions if its -OH groups.

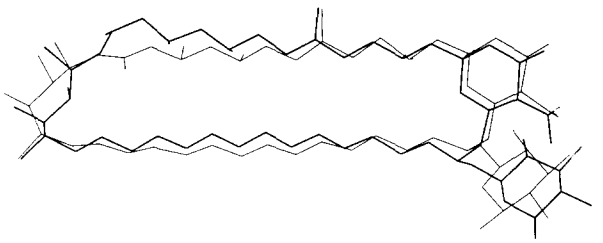

Fig. 3. Superposition of the initial AmB structure (thin line) and of the structure of the AmB averaged over the 100 ps 'productive' dynamics and energy-minimized (heavy line).

The global rms fluctuation is also higher than the corresponding values of each of the moieties (Table 2), since the former also takes into account the internal deformations. The figures indicate that the internal geometry fluctuations are negligible with respect to the rigid body movements. Fig. 4a,b represents the evolution with time of the torsional angles α_2 and α_3, which are defined around the bonds which link the cycle 1 to the rest of the AmB.

These angles fluctuate in a $\pm 15°$ range around their equilibrium value, and these fluctuations induce the rather ample rigid body movement of the mycosamine cycle (echoed in Fig. 3 and Table 2). We noted that these α_2 and α_3 values are those obtained for the close conformation of the polar head (see Table 1) and different of the RX ones.

AmB in a hydrated state

In the previous work, nine 'hydration water molecules' surrounded the polar head of AmB and a full minimization process of the sum of inter- and intramolecular energies was performed on the crystal conformation A and the folded conformation. For comparison, we kept also the AmB polar head in the fixed A conformation and optimized the hydration energy. The minimum corresponding to the folded

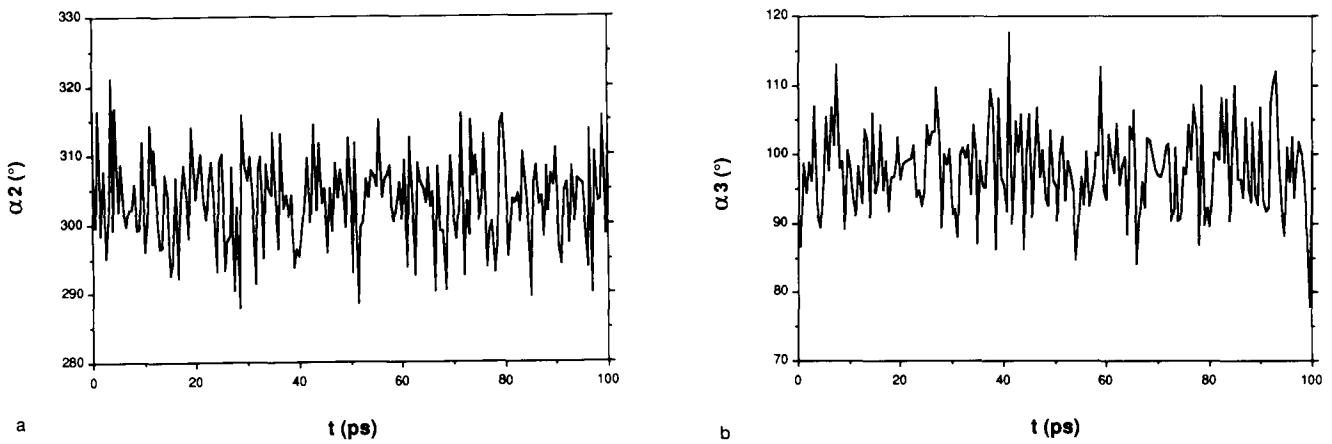

Fig. 4. (a) Evolution of the torsion angle α_2 during the 100 ps 'productive' dynamics. (b) Evolution of the torsion angle α_3 during the 100 ps 'productive' dynamic.

conformations remains relatively stable but another conformation (denoted B) was found, characterized by a geometrical structure intermediate between the folded C and the open A ones. For these most stable conformations, C and B, the 'hydration water molecules' formed particular binding arrangements, with one water molecule bridging together the NH_3^+ and CO_2^- groups. The hydration energy favors the X-ray open conformation but because of the balance of the intramolecular energy, the two B and C hydrated conformations are isoenergetic and are the most stable ones (see Table 1).

Cholesterol and ergosterol

The different X-ray studies have shown several conformers for the two sterol molecules: 8 and 2, respectively, in crystals of both anhydrous [19] and hydrated cholesterol [20], and 2 in the hydrated ergosterol crystal [21]. Cholesterol and ergosterol molecules are characterized by a steroid skeleton, which is almost the same in all independent molecules found in the crystals (see Fig. 1) and by a side chain which, because of its great flexibility, may exist within different conformations: in the anhydrous crystal of cholesterol, besides the extended all-*trans* conformations (molecules A, B, D, E, F, H [19]; ω angles being $\simeq 180°$, except for ω_8 (Table 3)), it exists a conformer with a *gauche-trans-gauche* sequence (molecules C and G [19]; ω_4 and $\omega_5 \simeq \pm 60°$ (Table 3)). In hydrated crystals of both cholesterol and ergosterol only two *gauche* conformations of the side chain may be observed.

Table 3
Values of dihedral angles for the different conformations of the cholesterol and ergosterol

Sterol	Conformations	ω_4	ω_5	ΔE_{intra}
Cholesterol	A [a]	183.8°	159.8°	0.0
	A [b]	185.8°	174.3°	−1.8
	C [b]	62.7°	259.7°	−0.7
Ergosterol	A [c]	186.3°	189.6°	0.0
	A [b]	218.0°	196.6°	−6.6

The values for the other angles are: $\omega_2 \simeq 180°$, $\omega_6 \simeq 180°$, $\omega_7 \simeq 180°$, $\omega_8 \simeq 300°$, $\omega_9 \simeq 180°$ for the cholesterol, $\omega_9 \simeq 300°$ for the ergosterol. [a] Experimental value [19]; [b] optimized value; [c] fixed value (all-*trans* conformation deduced from [21]). Energies are in kcal/mol.

Theoretical conformational analysis within the molecular mechanics method [22] taking into account all bond lengths, bond angles and dihedral angles variations have shown that:

(i) The steroid nucleus is rigid, conformational changes require a very high energy. Furthermore, following this study, rings A and D have the same conformation in both cholesterol and ergosterol while ring B, which has an additional double bond in ergosterol, is quite different for the two molecules (see Fig. 1).

(ii) Several conformations of the side chain are isoenergetic: cholesterol has a three-fold rotational barrier for the rotation of ω_4 while ergosterol has a two-fold barrier for this rotation because of the double-bond.

It was not our purpose to study the whole conformation of these two molecules, our objective was to try to

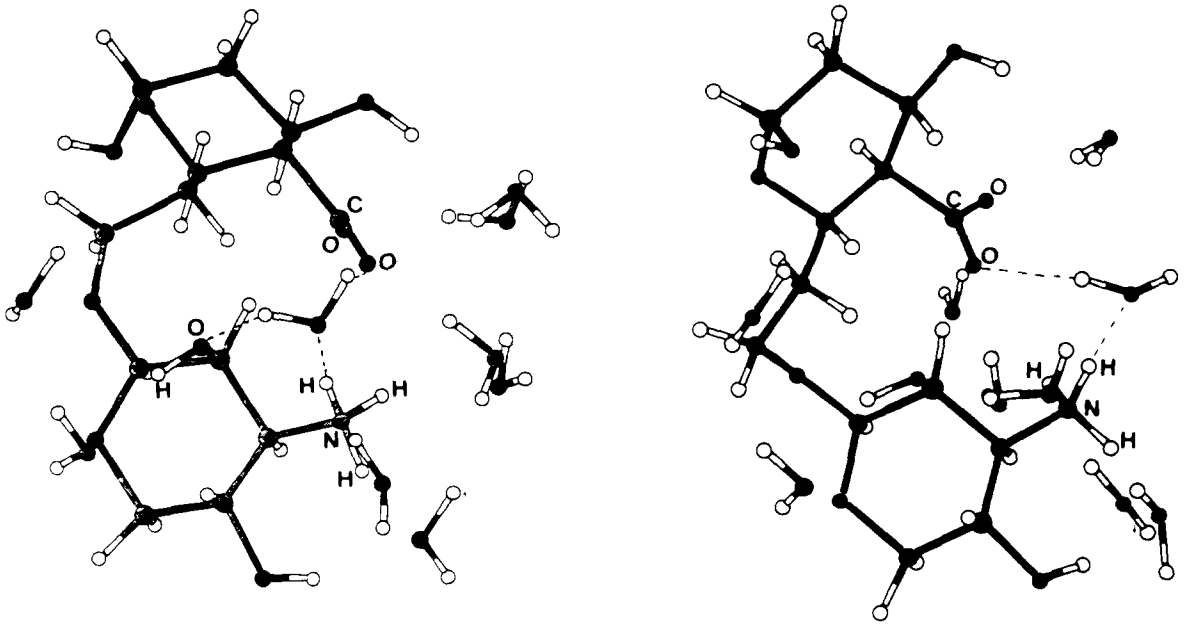

Fig. 5. Stable structures of the hydrated polar head of AmB [17] (a) Conformation C. (b) Conformation B. Intermolecular H-bonds are represented by dashed lines.

understand the different affinity of AmB for the two sterols. Thus in our study the steroid skeleton of both cholesterol and ergosterol remains fixed at the crystal values.

As a first step we selected from all the possible side chain conformers of cholesterol the ones with mainly different side chains: A (all-*trans*) and C (*gauche*) molecules in the crystal data. We optimized these conformations which remain very stable and slightly differ from the X-ray geometries.

The all-*trans* conformer of the side chain was not observed in the crystal of hydrated ergosterol, in the same way as in the crystal of hydrated cholesterol: in these crystals the water molecules play an important role in the packing (through H-bond bridges) and perhaps in the conformation of the side chain. So we did not know anything about the experimental stability of the ergosterol all-*trans* side chain. We have selected this conformation as a guessing point for our optimization calculations and it appeared that in agreement with [22], such a conformer is a little less stable that the most stable *gauche* one (Table 3).

3.2. Complexation study

Van der Waals complexes

In this present work, we are interested by the hypothesis of the eventual formation (inside the membrane) of a regular channel consisting in AmB and sterol molecules in mutual Van der Waals interaction. Our objective was to calculate a basic AmB-sterol complex in such a structure and to define the relative position and orientation of both AmB and sterol molecules.

We have noticed that, even when the sterol side chain is an all-*trans* conformation, sterol is shorter and tighter than AmB molecule, thus the two molecules cannot fully overlap. It is well known that the formation of Van der Waals complexes requires an optimal contact between the two molecules in interaction, so it can reasonably be expected (as emphasized by Khutorsky [23], De Kruijff et al. [9], Baginski et al. [22]) that the interaction between the macrolide ring of AmB and the *trans* conformation of the side chain of the sterol would be the strongest.

Calculations were carried out using method INTER, as noted above, on the complexes obtained by combinations of the *trans* conformers of the sterols (selected before) with conformation B for the polar head of AmB. Then in order to complete this study, some calculations have been performed with the sterols in a *gauche* conformation.

Beginning our work with the planes OY_iZ_i superimposed, the sterol molecule is translated along OX^+ or OX^- axis. Thus two kinds of complexes can be obtained: in complexes of type I the mycosamine sugar (which is situated out of the plane $OY_{AmB}Z_{AmB}$ with positive Z coordinate) can be approached by the sterol molecule; this is not the case with complexes II. Furthermore, considering for each of the complexes I or II a 180° rotation of the sterol molecule around the OZ axis led to two more kinds of complexes denoted I′ and II′.

In complexes I and II′, the β face of the steroid nucleus, out of which protude the two methyl groups, is in contact with AmB; the situation is the reverse with complexes I′ and II.

An insight to Fig. 2 shows that the sterol is shorter and thinner than AmB; consequently it will only partially fit into the whole AmB. Motions along the axis are illustrated in Fig. 2 and parts of sterol and AmB in interaction are pointed out:

(a) *The motion along the axis OZ* is of a great extent, comparatively to those along OX and OY axis.

(i) $\Delta Z = -10$ Å: Rings A and B of the *Steroid nucleus* of the sterol do not overlap AmB. Rings C and

Table 4
Intermolecular energies calculated within the INTER method and relative positions of the molecules of the most stable AmB-sterols complexes

Complex	AmB-cholesterol				AmB-ergosterol			
	ΔZ	ΔY	Θ_Z	E_{inter}	ΔZ	ΔY	Θ_Z	E_{inter}
I	−6.0	0.0	−20	−18.8	−6.0	−0.5	−10	−32.0 *
	0.0	−1.5	0	−19.5	−3.0	−1.0	−20	−29.0 *
I′	−7.0	1.5	200	−16.9	−5.0	−1.5	220	−27.5 *
	−2.5	−1.5	220	−23.5 *	−2.0	−2.0	230	−27.5
	1.0	2.0	210	−21.1				
II	−4.5	−1.5	10	−21.9				
	0.5	−1.5	10	−19.3				
	5.0	0.0	−50	−24.3 *	4.5	0.5	−20	−22.5
	7.5	0.0	−30	−21.2	8.0	0.5	−20	−25.5
II′	1.5	1.5	210	−23.6 *	4.0	0.5	130	−18.9
					8.5	1.0	190	−18.3

All distances are in Å, angles are in degrees and energies are in kcal/mol. Configurations noted by * are represented in Fig. 7.

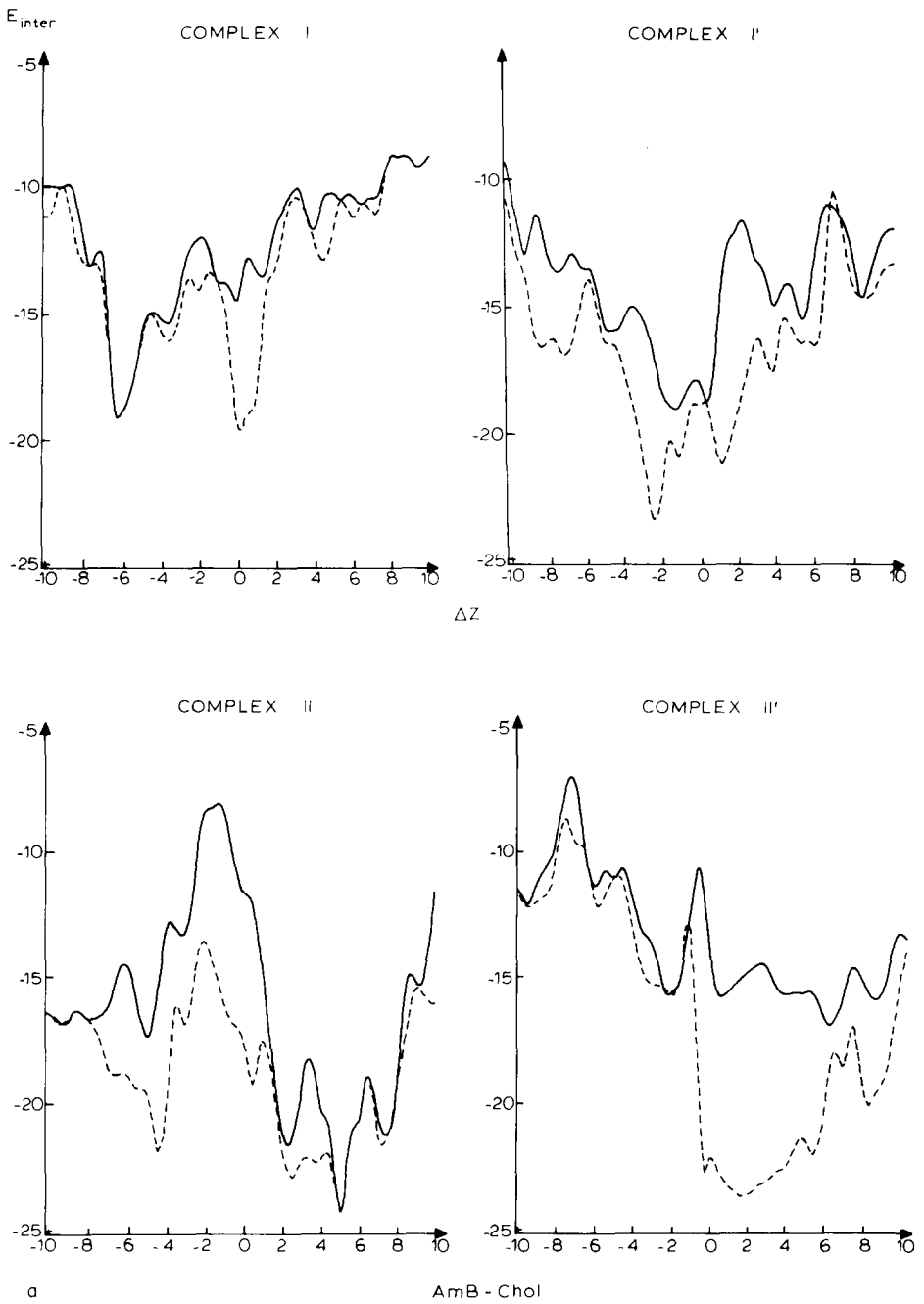

Fig. 6. Energy profiles $E_{INTER} = f(\Delta Z)$ for the four AmB-sterols complexes. (a) AmB-cholesterol. (b) AmB-ergosterol. Full line: optimized ΔX and Θ_Z, $\Delta Y = 0$; dashed line: optimized ΔX, ΔY and Θ_Z. Energy values are in kcal/mol and displacements are in Å.

D are in contact with the AmB polar head and the side chain interacts with the beginning of the macrolide ring.

(ii) $\Delta Z = -2.5$ Å: AmB and sterol interaction involves the whole sterol molecule overlapping the polar head plus a great part of the macrolide ring.

(iii) $\Delta Z = 10$ Å: the end of the side chain of sterol is out of interaction.

(b) *Motion of sterol along* OY^+ axis pushes sterol towards the aliphatic chain of the macrolide chain while motion along the OY^- shoves it towards the polyenic chain.

(c) *The motion along the axis OX* (defining the intermolecular distance between AmB and the sterol) is of a weak extent. The optimal values ΔX are found in a range of 5–6 Å.

(d) Besides the translation motions along the three main axes, we have also been interested by *the rotation around the OZ axis.*

The intermolecular interaction energy has been cal-

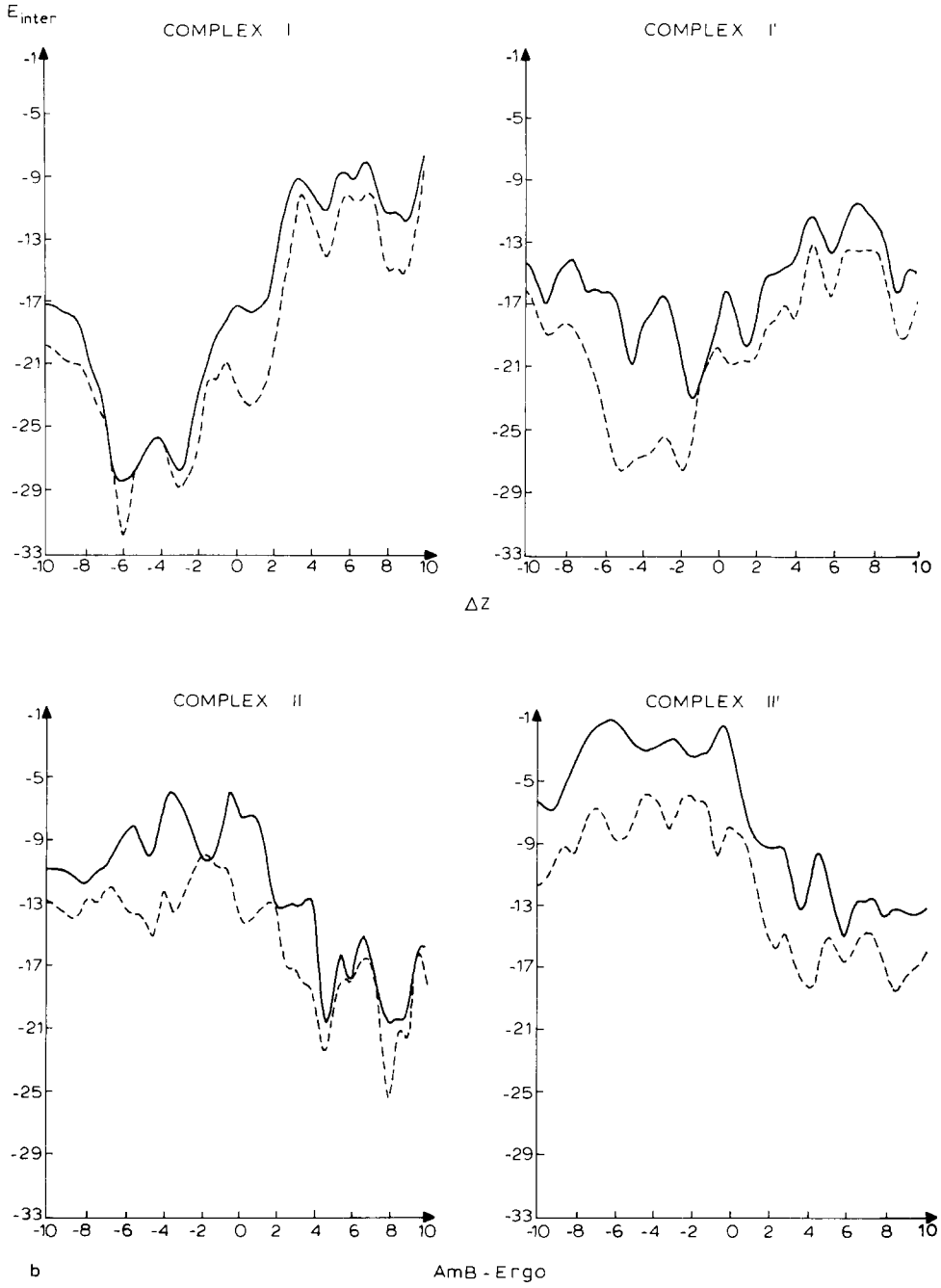

Fig. 6 (continued).

culated as a function of ΔZ first with optimization of two variables, namely Θ_Z and ΔX (curve in full line in Fig. 6) then with optimization of Θ_Z, ΔX and ΔY (dotted line in Fig. 6).

The intermolecular interaction energy and geometrical parameters defining the most stable configurations of AmB-Cholesterol (and ergosterol) complexes are listed in Table 4.

An analysis of our results has shown that optimization of ΔY does not change drastically the profile of $E_{\mathrm{INTER}} = f(\Delta Z)$. The main effect only consists in a lowering of the intermolecular motion along OY^+ (or OY^-) does not exceed 2 Å.

Rotation around the OZ axis is strongly coupled with the translation along this axis. The sterol can oscillate around this axis from $-50°$ to $+50°$ in order to minimize the repulsions between the different groups of the two molecules involved in the complex.

As illustrated by Fig. 6 the overall shape of the energy evolution $E_{\mathrm{INTER}} = f(\Delta Z)$ obtained with optimized values of the three other variables (Θ_Z, ΔX and ΔY) is quite different following the location of the

sterol with regards to AmB along the OX^+ or OX^- axis, thus for complexes of type I (or I') and II (or II').

(a) *Complexes of type I and I'*, (in both complexes involving cholesterol and ergosterol) it outcomes two main minima located at $\Delta Z = -5 \pm 1$ Å and $\Delta Z = -1.5 \pm 1$ Å (the exact value indicated in Table 4). These two energy minima are separated by an energy barrier the height of which depends on the type of complex (I or I') and on the nature of sterol (cholesterol or ergosterol).

(i) *AmB-ergosterol.* The four lowest minima lie below 27 kcal/mol, the absolute minimum ($E_{INTER} = -32.1$ kcal/mol at $\Delta Z = -6$ Å) has been obtained for complexes of type I and is separated from a second one ($E_{INTER} = -29$ kcal/mol at $\Delta Z = -3$ Å)) by a barrier of 6.2 kcal/mol. As concerns complexes of type I', we may reasonnably suppose a free motion for values of ΔZ in a range from -5 Å to $+1$ Å because these two minima located at $\Delta Z = -5$ Å and -3 Å, respectively, are isoenergetic ($E_{INTER} = -27.5$ kcal/mol) and the barrier separating them rather low: 2 kcal/mol.

(ii) *AmB-cholesterol.* The complexes of type I occur for quite similar values of ΔZ as the ones obtained with AmB-ergosterol, but they have a much higher energy ($E_{INTER} \simeq -18$ kcal/mol). In fact the two lowest minima outcome for complexes I' with $\Delta Z = -2.5$ Å ($E_{INTER} = -23.5$ kcal/mol) and $\Delta Z = 1.0$ Å ($E_{INTER} = -21.2$ kcal/mol) and they are separated by an energy barrier of 5 kcal/mol, but they are still less stable than AmB-ergosterol ones.

(b) *Complexes of type II and II'.* The energy profile $E_{INTER} = f(\Delta Z)$ calculated for AmB-ergosterol and AmB-cholesterol complexes are quite different.

(i) *AmB-ergosterol.* For values of ΔZ ranging between 0 Å and 10 Å it appears a series of deepless minima separated by low barriers, thus the motion of ergosterol may be considered a free in this range of ΔZ values. In fact the lowest minima are repelled towards values of $\Delta Z = 2.5$ Å. Table 4 and Fig. 6 clearly show that the two lowest minima occuring for complex of type II are in an energy range of -22.5 kcal/mol to -25.5 kcal/mol. They are separated by

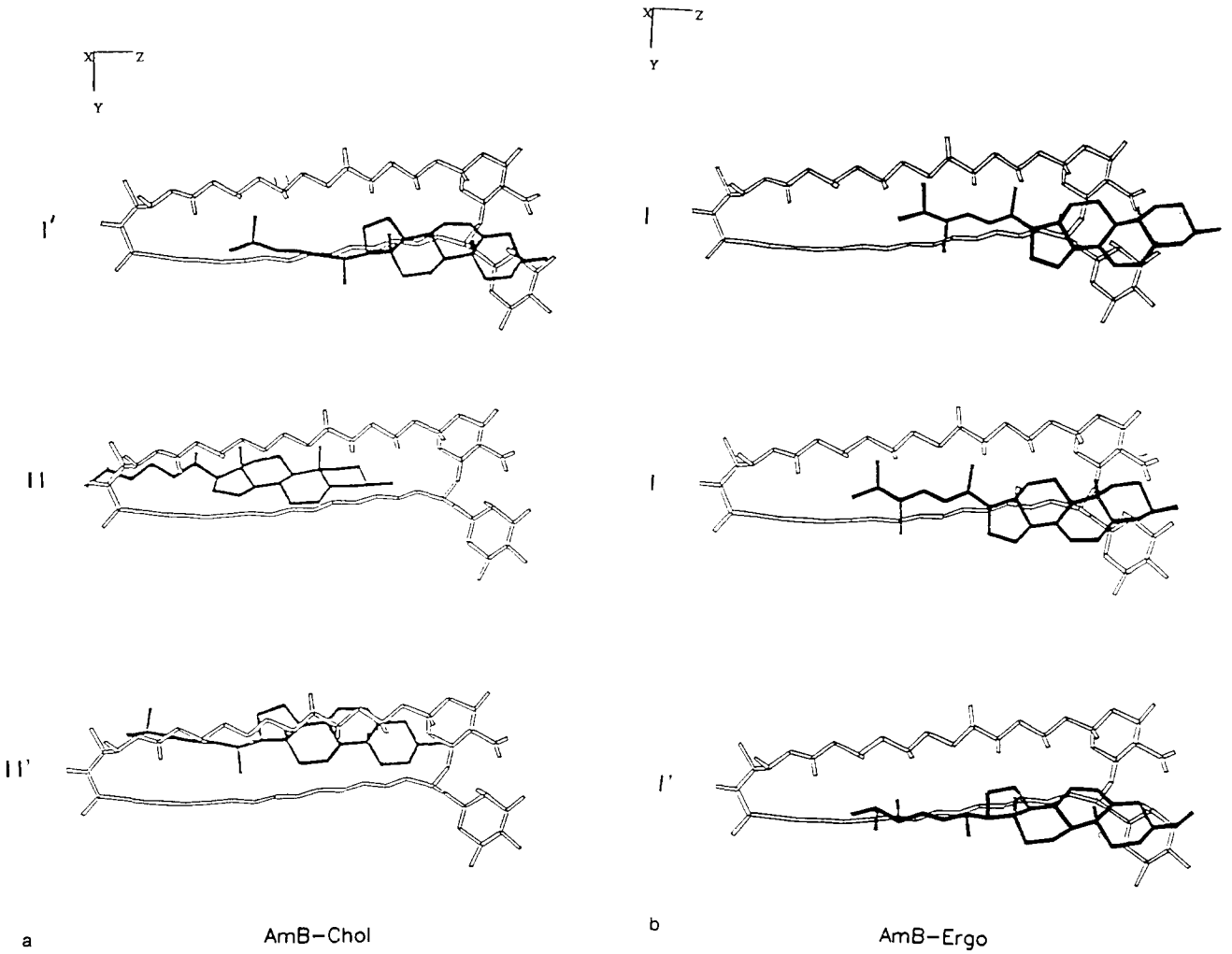

Fig. 7. Projections of different structures of some most stable AmB-sterol complexes (characterized by * in Table 2) on YOZ plane. (a) AmB-cholesterol. (b) AmB-ergosterol. Sterols are represented by dark molecules.

an energy barrier of 6 kcal/mol. Complexes of type II′ lie higher in energy (see Table 4).

(ii) *AmB-cholesterol.* As concerns complexes of type II, we get several minima, the energy of which lie between −19. kcal/mol and −25. kcal/mol for values of ΔZ ranging between −4.5 Å and +7.5 Å. The highest energies barriers separating these minima occur for $\Delta Z = -2$ Å (i.e., when the lactone ring is in contact with the ring A of cholesterol steroid nucleus). The shape of the curve $E_{INTER} = f(\Delta Z)$ obtained for complexes of type II′ is quite similar with the one we get with AmB-ergosterol (lowest minimum being repelled towards values of $\Delta Z = 0$ Å); but we now get an unique very large minimum ($E_{INTER_0} \simeq -23.0$ kcal/mol) which is spread out between −1 Å and 6 Å.

(c) *Comment on the mutual arrangment of AmB and sterols* in their most stable configurations (see Fig. 7).

After a synthetical analysis of all our results, it appears that:

(i) When sterol (cholesterol or ergosterol) approaches AmB along OX^+ axis, the lowest minima are obtained when the AmB polar head, via its mycosamine ring, is in contact with either ring B or ring A of the steroid nucleus of the sterol and the side chain is repelled towards the polyenic part of the macrolide ring. In such a mutual geometrical arrangment between AmB and the sterol, AmB interacts more strongly with ergosterol than with cholesterol. In these calculations the side chains of both cholesterol and ergosterol are in a fully elongated conformation, thus we can reasonnably think that the interaction energy difference could proceed from a conformational difference of the steroid nucleus of ergosterol with regards to cholesterol. Effectively following crystal data [21] and molecular mechanical results [22] ring B of ergosterol, because of its additional double bond is more flat than the one of cholesterol, thus its contact with AmB should be optimal.

(ii) When the sterol approaches AmB towards OX^-, excepting for one most stable AmB-cholesterol complex, the AmB polar head is excluded from the interaction with sterol which full overlaps the macrolide ring. It clearly appeared (from Fig. 7) that in such a geometrical arrangment, the sterol is repelled towards the side aliphatic chain of the macrolide ring. Furthermore, we have noticed more stable configurations (lying below −20.0 kcal/mol) for the AmB-cholesterol complex.

We have also tested some conformational effects on the stability of complexes: First considering the conformation C of the polar head of AmB, we noticed that the results are not drastically changed, as well for the most stable positions of the molecules as for the intermolecular energy; Then taking the *gauche* conformations of the side chains of the sterols we noted, as expected, that these complexes lead to energies higher (by 20–40%) than the ones displayed in Table 4.

H-bond interactions

These calculations were performed within the SIBFA method. First of all it has appeared that it does not exist any H-bond between β-OH and the polar head of AmB. So we have studied the possibility of H-bond interactions between sterol β-OH and charged groups (NH_3^+ and CO_2^-) of AmB via a water molecule forming an H-bond bridge.

(a) *Monohydrated complexes.* Our previous conformational study of the polar head of AmB in the presence of water molecules enabled us to characterize a particular arrangment for one water molecule bridging together NH_3^+ and CO_2^- groups (and eventually sugar OH group) (Fig. 5). This disposition is reminiscent of the representation proposed by Hervé et al. [15], which suggests that one water molecule can be in a favorable position to bridge by H-bonds the oxygen of the β-OH of the sterol with the charged groups of the polar head. So our first objective was to verify this hypothesis.

We have positioned the sterol in a particular way, using the internal coordinates defined in part 2. We imposed first a nearly parallelism between AmB and sterol molecules and a proximity between AmB polar head and the β-OH of the sterol. In the same way as hereabove, following the position of sterol with regards to AmB we may define complexes of type I(I′) or II(II′)

(i) *As a first step*, we performed calculations with the AmB polar head in the fixed C conformation, the cholesterol (A) and the particular water molecule hereabove cited; only intermolecular geometrical parameters (defining positions of cholesterol and water molecules with regards to AmB) and intramolecular geometrical parameters of cholesterol have been optimized. Our results have shown that the H-bonded bridged water molecule still exist but this water molecule is not close enough to the β-OH of the sterol in order to form H-bond with it ($d = 2.56$ Å). Different energy terms are displayed in Table 5 (line 1).

Table 5
Variations of intra and intermolecular energies calculated within the SIBFA method of AmB-sterol complex in presence of one water molecule

AmB	Complex	ΔE_{intra_A}	ΔE_{intra_C}	$\Delta E_{inter_{A-w}}$	$E_{inter_{A-C}}$
C [a]	II	0.0	1.5	−4.2	−20.4
C [b]	II	41.8	3.5	−14.6	−27.7
C [b]	I′	45.9	1.4	0.0	−19.8
B [b]	I′	40.2	1.6	0.2	−12.8

Energy terms are defined as: $\Delta E_{intra_A} = \Delta E_{intra}$ (hydrated complexed AmB) − ΔE_{intra} (hydrated AmB); $\Delta E_{intra_C} = \Delta E_{intra}$ (complexed sterol) − ΔE_{intra} (sterol); $\Delta E_{inter_{A-w}} = E_{inter}$ (complexed AmB-water molecules) − E_{hydra} (AmB-water molecules); $E_{inter_{A-C}} = E_{inter}$ (AmB-sterol complex). All values in kcal/mol.

[a] Optimized conformation [17]; [b] conformations in the hydrated complexes.

(ii) *As a second step*, we have varied simultaneously intermolecular and intramolecular geometrical parameters of both AmB and cholesterol. Two relative positions (II and I') of cholesterol and AmB have been considered. An analysis of our results has shown that the water molecule remains bonded to the charged (NH_3^+ and CO_2^-) groups of AmB.

(ii.a) *In complex of type II*, the distance between one hydrogen of water and the oxygen of cholesterol has decreased to 2.28 Å, so we can speak of a weak H-bond connecting the sterol with AmB via a water molecule. But such an arrangment of this water molecule with regards of both cholesterol and AmB leads to a very important loss of intramolecular energy which is moderatly counterbalanced by intermolecular energy gain. In fact we notice an opening of the polar head: the intramolecular H-bond between both CO_2^- and NH_3^+ has disappeared but AmB interacts more strongly with the water molecule. AmB-Cholesterol interaction energy is of the same magnitude as we found in stable Van der Waals complexes (see Table 4).

(ii.b) *In complex of type I'*, the particular water molecule remains H-bonded to AmB but is too far from the cholesterol (2.58 Å) in order to H-bond with it. Furthermore, the important loss of intramolecular energy is not counterbalanced by sufficiently strong intermolecular energy gain and the system AmB-cholesterol-water becomes instable. The results obtained for the complexes of AmB and cholesterol with one water molecule have been generalized for the conformation B of AmB and for the AmB-ergosterol complexes (see Table 5).

(b) *Polyhydrated complexes.* In fact, the result hereabove obtained does not eliminate the possibility of a H-bonded network relying the AmB charged groups and the sterol β-OH via one water molecule. A water molecule different from the one connecting AmB NH_3^+ and CO_2^- groups may be involved in this H-bonded bridge. Thus we have considered the polyhydrated molecule of AmB within C and B conformations. The nine hydration water molecules have been considered.

As a first alternative, we positioned the sterol molecule following geometrical arrangment leading to one stable Van der Waals complexes (following the INTER calculations).

As a second alternative, we have positioned the sterol using the internal coordinates defined hereabove. We will not display all the results that we have obtained, we will only give a digest of the most important features that have appeared for the two sterols (Tables 6 and 7).

(i) Once again we may emphasize that when the particular water molecule connecting NH_3^+ and CO_2^- AmB groups in C and B conformations remains in its initial position it does not H-bond the sterol β-OH.

(ii) In this study (in which only water molecules hydrating AmB are considered), we have not observed any water molecule forming an H-bond between NH_3^+ and β-OH of sterol, even when sterol is in position I.

(iii) When sterol is in position II, a H-bond water

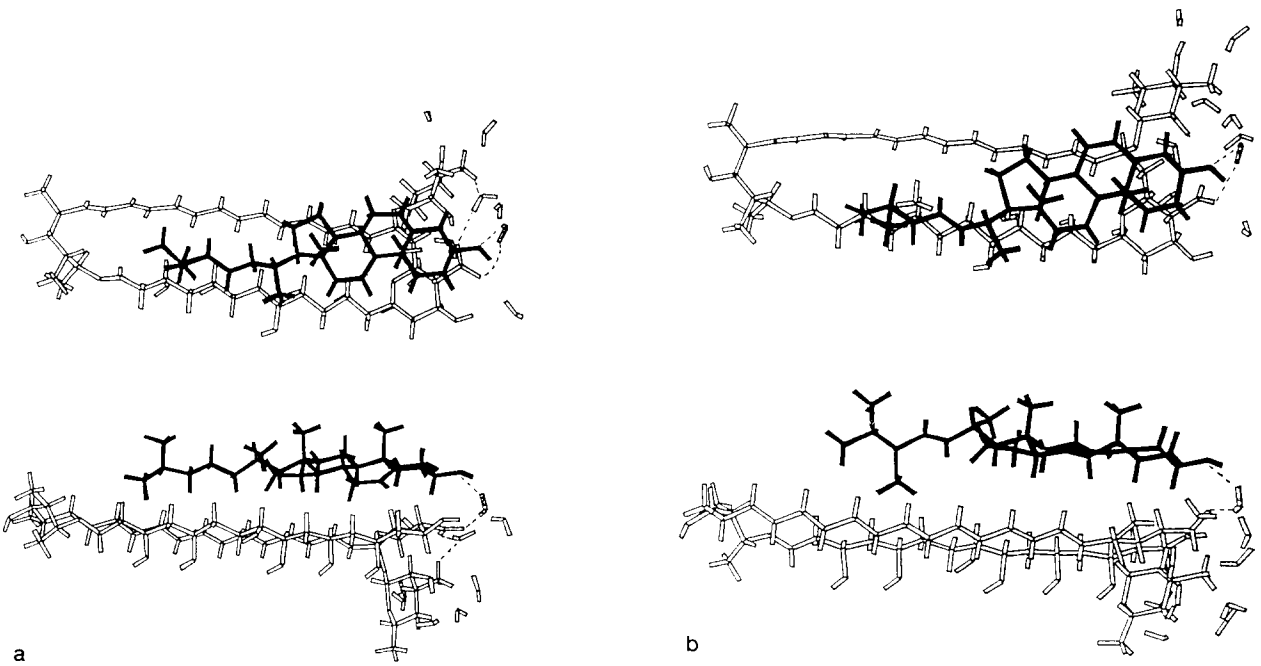

Fig. 8. Projections of different structures of some most stable hydrated AmB-sterol complexes (characterized by * in Tables 4 and 5) on *YOZ* and *XOZ* planes. (a) AmB-cholesterol. (b) AmB-ergosterol. Sterols are represented by dark molecules.

Table 6
Variations of intra and intermolecular energies calculated within the SIBFA method of AmB-cholesterol complex in presence of nine water molecules

AmB	Complex	ΔE_{intra_A}	ΔE_{intra_C}	$\Delta E_{inter_{A-w}}$	$E_{inter_{A-C}}$
C	II	7.2	3.2	9.1	−22.8
C	I′	24.5	1.9	11.3	−5.3
B	II	44.8	2.4	−6.3	−19.0 *
B	I′	41.7	1.7	11.5	−24.9

All values in kcal/mol. Configuration noted by * is represented in Fig. 8.

bridge may occur between CO_2^- group of AmB and sterol (see Fig. 8). But such an arrangment of the water molecule with regards to the AmB-sterol complex is not without any consequence as concerns both the intramolecular energy of the two molecules and the intermolecular energy of the complex. In all cases that we have studied we have observed:

(iii.a) A very weak intramolecular energy loss of the sterol molecule.

(iii.b) An AmB intramolecular energy loss depending on the conformation taken as guessing point of the optimization process. The conformation C is slightly destabilized; this effect is more important in AmB-ergosterol complex than in AmB-cholesterol complex. The conformation B is strongly destabilized in both sterols-AmB complexes ($\Delta E_{INTRA} = 43.0 \pm 4.0$ kcal/mol). We noticed an opening of the polar head (the mycosamine ring being quite perpendicular to the macrolide one).

(iii.c) An hydration energy behaviour depending again on the conformation of AmB polar head; a moderate hydration energy loss (a little more important when cholesterol is involved in the complex) occurs when AmB polar head is in a conformation quite similar to the C one. The opening of the AmB conformation B (accompanied by a loss of intramolecular energy) leads to an hydration energy gain which is more important when AmB interacts with ergosterol.

(iii.d) The AmB–sterol interaction energy remains in the ranges we have calculated for pure Van der Waals complexes.

(iii.e) As a whole the total energy balance of the hydrated complexes, taking into account the intra and intermolecular energies, is not very favourable.

Table 7
Variations of intra and intermolecular energies calculated within the SIBFA method of AmB-ergosterol complex in presence of nine water molecules

AmB	Complex	ΔE_{intra_A}	ΔE_{intra_C}	$\Delta E_{inter_{A-w}}$	$E_{inter_{A-C}}$
C	II	16.1	5.8	1.7	−18.8
C	I′	19.8	1.0	10.3	−14.4
B	II	42.4	5.9	−21.7	−24.7 *
B	I′	47.2	0.7	4.0	−21.2

All values in kcal/mol. Configuration noted by * is represented in Fig. 8.

(iv) Before ending this section, we want to emphasize that, in some cases, water molecules may exert an influence upon the relative position of sterol and AmB. Effectively, as shown in part 1 of this paragraph, sterol may interact with either both the AmB polar head and a part of the macrolide ring or with only the whole macrolide ring (see Fig. 7). After optimization of complexes involving only the whole macrolide ring in presence of water, we have noticed a displacement of the sterol molecule towards the AmB polar head; this geometrical arrangment is stabilized by a water molecule connecting CO_2^- and the β-OH groups. Such a geometrical arrangment between AmB, sterol and water molecules lead to a more or less important loss of intramolecular AmB energy because of an opening of the polar head leading to a breaking of intramolecular H-bond.

4. Discussion

This work has shown the dominant role played by Van der Waals forces in the formation of AmB-sterol complexes. As expected such an interaction requires:

(a) A full extended all-*trans* conformation of the side chain of the sterol in order to maximize its overlap with AmB. From an intramolecular point of view, *trans* conformers represent a minimum in the multidimensional conformation space of cholesterol but this is not the case with ergosterol. Nevertheless, the intramolecular energy loss due to the elongation of ergosterol side chain is strongly balanced by an intermolecular Van der Waals energy gain.

(b) A flat carboxylic skeleton: Rings A and D have the same conformation in both cholesterol and ergosterol, but because of its additional double bond, ring B of ergosterol is more flat than the one of cholesterol. This fact may explain the greater affinity of AmB for ergosterol when complexes I or I′ are formed.

It may be noticed that the energy profile $E_{INTER} = f(\Delta Z)$ presents two distinct zones with lengths of, respectively, $\simeq 10$ Å and of $\simeq 5$ Å. Within one zone, sterol can move freely along OZ axis through a continous set of relatively high energy states ($E_{INTER} \simeq -15.0$ kcal/mol), in the second one the sterol is trapped into some rather shallow low energy minima. The relative location of these two zones (which are separated by rather high energy barriers) along the OZ axis depends on the mutual geometrical arrangment of both AmB and sterol when forming complexes of type I (or I′) and II (or II′). The different minima of the complexes have been obtained for different positions of the sterol with regards to AmB polar head, polyenic or hydroxylic chains.

Because of the large distance between AmB and sterol, a direct H-bonding of the β-OH of the sterol

and a charged group of AmB is not possible, such a connection may be only formed through a possible H-bond bridge involving one water. The occurrence of such an H-bond is accompanied by a more or less important loss of the intramolecular energy of the polar head of AmB, this one being slightly balanced by the intermocular energy gain. The role played by water molecules can be a structural one, they may maintain the sterol at the interface of external aqueous medium and the inside of the membrane.

These results may be of a great interest if we go beyond the dimer, namely if we extrapolate our calculations for the channel itself for which the basic unit is a trimer constituted by the sterol incorporated between two adjacent AmB molecules. Considering an AmB-sterol dimer of type I, we may position a second AmB molecule above the sterol in such a way that both AmB molecules are localized in parallel planes. From a practical point of view, the second AmB molecule is first superimposed to the first one and then translated along OX^+ axis (see Fig. 2). The sterol molecule will form a complex of type II with it: a trimer unit I-II will be formed. Trimer unit I'-II' will be formed in a same way. The position of the sterol with regards to the two AmB molecules is obvious, since following our results the strongest interactions occur when sterol is located near the polar head in complexes I (and I') and reversely when the sterol is repelled at the end of the macrolide ring in complexes II (and II'). Then two trimers I-II (respectively, I'-II') have been considered: the sterol may be positioned in such a way to minimize the energy with the first AmB molecule (energy minimum of dimer I (or I')), or with the second AmB molecule (energy minimum of dimer II (or II')).

We can estimate roughly the intermolecular energies of these trimers as a sum of AmB-sterol intermolecular energies, $E_{\mathrm{INTER}} = E(\mathrm{I} \text{ or } (\mathrm{I}')) + E(\mathrm{II} \text{ or } (\mathrm{II}'))$ (where $E(\mathrm{I})$ is $E_{\mathrm{INTER}}(\mathrm{I})$ for the complex I), neglecting the AmB-AmB intermolecular energy. We have summarized the intermolecular energies evaluations in Table 8.

We noticed that:

(i) In both trimers I-II (or I'-II'), the two positions of the sterols, namely near the polar head or repelled towards the macrolide ring end, lead to isoenergetic dimers. Thus in trimers I-II the water molecules may limit the motions of the sterols by imposing its location near the polar head through a network of H-bonds.

(ii) The AmB-ergosterol trimers I-II are more stable than the trimers I'-II', while the two types of trimers are isoenergetic when cholesterol is involved in the complexation with AmB.

(iii) The AmB-ergosterol are more stable (by $\simeq 5$ kcal/mol) than the AmB-cholesterol ones.

It is interesting to point out that, with this rough model of trimers, we have obtained stable positions of the cholesterol repelled to the end of the AmB macrolide ring which are quite similar to the ones found by Khutorsky [23]. But following our calculations this trimer I-II represents one among the four nearly isoenergetic possible basic units of the channel. Such a difference may proceed from our intermolecular potential model which is different from the one used by Khutorsky, particularly the electrostatic component and the taking into account of the induction energy. But another explanation should be that we have only studied the intrinsical property of the trimer, which only represents a basic unit of the channel and not the channel itsel. At this stage of our work, a further extrapolation must include more parameters and constraints: the AmB–AmB interactions and the possible deformations of their polar head may be of importance as well as the constraints due to the symmetry of the channel. We may also question about the role of the water molecules at the interface between the membrane and the outside medium. Nevertheless our work has shown that at a molecular level, AmB has a greater affinity for ergosterol as well at the dimer as at the trimer level.

5. Acknowledgments

We thank Dr. M. Hervé who initiated us to this problem and Dr. J. Bolard for many helpful discussions. Molecular mechanics and dynamics calculations were performed on a Silicon Graphics 4D-35 workstation. Molecular dynamics (MD) trajectories visualiza-

Table 8
Intermolecular energies of the AmB-sterol-AmB trimers evaluated within the INTER method and relative positions of the molecules in the complexes

Trimer	AmB-cholesterol-AmB				AmB-ergosterol-AmB			
	ΔZ	$E(\mathrm{I})$	$E(\mathrm{II})$	E_{INTER}	ΔZ	$E(\mathrm{I})$	$E(\mathrm{II})$	E_{INTER}
I-II	0.5	19.1 *	−19.3	−38.4	−3.0	−29.0 *	−12.7	−41.7
I-II	5.0	−12.0	−24.3 *	−36.2	8.0	−14.6	−25.5 *	−40.1
I'-II'	−2.5	−23.5 *	−15.3	−38.8	−2.0	−27.5 *	−5.9	−33.4
I'-II'	8.0	−14.0	19.8 *	−33.7	8.5	−14.5	18.4 *	−32.9

Energy minima of AmB-sterol dimers are noted with *. All distances are in Å and energies are in kcal/mol.

tion was performed on the same workstation using the program HY-DRA (written by R. Hubbard).

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