Journal of Computer-Aided Molecular Design, **14:** 689–703, 2000.
KLUWER/ESCOM

Conformational properties of amphotericin B amide derivatives – impact on selective toxicity

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Received 14 October 1999; Accepted 14 April 2000

Key words: Amphotericin B, amide derivatives of Amphotericin B, cholesterol, conformational analysis, drug design, ergosterol, membrane ion channel, potential energy surface, semiempirical quantum chemistry methods

Summary

Even though it is highly toxic, Amphotericin B (AmB), an amphipathic polyene macrolide antibiotic, is used in the treatment of severe systemic fungal infections as a life-saving drug. To examine the influence of conformational factors on selective toxicity of these compounds, we have investigated the conformational properties of five AmB amide derivatives. It was found that the extended conformation with torsional angles (ϕ,ψ)=(290°,180°) is a common minimum of the potential energy surfaces (PES) of unsubstituted AmB and its amide derivatives. The extended conformation of the studied compounds allows for the formation of an intermolecular hydrogen bond network between adjacent antibiotic molecules in the open channel configuration. Therefore, the extended conformation is expected to be the dominant conformer in an open AmB (or its amide derivatives) membrane channel. The derivative compounds for calculations were chosen according to their selective toxicity compared to AmB and they had a wide range of selective toxicity. Except for two AmB derivatives, the PES maps of the derivatives reveal that the molecules can coexist in more than one conformer. Taking into account the cumulative conclusions drawn from the earlier MD simulation studies of AmB membrane channel, the results of the potential energy surface maps, and the physical considerations of the molecular structures, we hypothesize a new model of structure-selective toxicity of AmB derivatives. In this proposed model the presence of the extended conformation as the only well defined global conformer for AmB derivatives is taken as the indicator of their higher selective toxicity. This model successfully explains our results. To further test our model, we also investigated an AmB derivative whose selective toxicity has not been experimentally measured before. Our prediction for the selective toxicity of this compound can be tested in experiments to validate or invalidate the proposed model.

Introduction

Amphotericin B (AmB) is an amphipathic polyene macrolide antibiotic (Figure 1) used in the treatment of severe systemic fungal infections [1–3]. Even though it has serious side effects [3–5], it has been in clinical use for nearly 40 years due to the lack of a better alternative. It has maintained a good track record and new fungal strains resistant to the drug are very infrequent [6]. The accepted molecular mechanism of AmB's anti-fungal action is that it forms membrane ion channels which cause the loss of the cell contents, consequently leading to cell death. The molecular structure of the channels has not been determined. However, the membrane sterols are believed to play a role in the channel formation or at least in maintaining the channel in the open form more efficiently [7–11]. How the channel is actually formed is not yet known. It has been speculated that the polar head groups of AmB help in attaching to the membrane. In one mechanism, the AmB molecules insert themselves into the

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membrane individually. Then, as a result of the lateral diffusion, a certain number of AmB molecules group together to form a cluster which later takes the form of an ion channel. In another suggested mechanism, the AmB molecules group together on the membrane surface. To reduce the exposure of their hydrophobic parts to the solvent, the AmB molecules prefer to imbed themselves in the membrane and form an ion channel.

The antibiotic is quite lethal, both for mammalian and fungal cells, i.e., it is quite toxic, with only a slightly enhanced toxicity for fungal cells. Noting that fungal and mammalian cell membranes differ mainly in their sterol content (fungal and mammalian cell membranes contain ergosterol and cholesterol, respectively), the observed toxicity difference has been interpreted as being due to somewhat higher affinity of AmB towards ergosterol-containing cell membranes [12, 13]. To increase the selectivity of AmB toward ergosterol-containing membranes, experimental efforts have been focused on the synthesis of new AmB derivatives that would prefer to form ion channels in the fungal cell membranes, thus lowering its toxicity for the host tissues. For this purpose various AmB derivatives were synthesized and their membrane activities were examined [9, 14–20].

Although the three-dimensional structure of the membrane channel formed by the antibiotic AmB has yet to be determined, the crystal X-ray diffraction study of AmB N-iodoacetyl derivative [21], the NMR study of AmB methoxycarbonyl-methyl amide [22], and the CD and NMR studies of AmB aggregates in solution [23] have revealed the molecular structure of AmB (Figure 1). The most important property defining the overall molecular conformation of AmB is the mutual orientation of the two rigid fragments of the antibiotic, its macrolide ring and the mycosamine sugar moiety [24]. The mycosamine residue is β-glycosidically linked to the macrolide ring and can rotate around the $C_{19}O_{41}$ and $O_{41}C_{42}$ bonds (Figure 1). Therefore, AmB can be regarded as a semi-rigid molecule formed by two rigid fragments. The mutual positions of these two fragments play an important role in determining the AmB-AmB and the AmB-sterol interactions within an ion membrane channel complex [24, 25]. The ϕ ($C_{18}C_{19}O_{41}C_{42}$) and ψ ($C_{19}O_{41}C_{42}C_{43}$) dihedral angles (Figure 1) have been determined respectively as 267.6° and 142.0° in the crystal X-ray diffraction study of N-iodoacetyl AmB [21]. A semiempirical quantum chemistry study of unsubstituted AmB in its neutral form has determined the best values for ϕ and ψ as 275° and 183°, respectively [24].

Under physiological conditions Amphotericin B acquires a zwitterionic form where its amino and carboxyl groups are charged positively and negatively, respectively. Depending on the relative orientation of the macrolide ring and the AmB sugar moiety, these polar groups of an AmB molecule can either form hydrogen bonds with each other (intra-molecular hydrogen bonds) or with the polar groups of other AmB molecules (inter-molecular hydrogen bonds) in a membrane channel complex. These conformations can respectively be classified as the *folded* and the *extended* forms (Figure 2). In the extended conformation, the amino group of the sugar moiety points outwards which allows for the formation of an intermolecular hydrogen bond with the carboxyl group of the adjacent AmB molecule in the channel. In the folded conformation, the orientation of the sugar moiety makes it possible for the amino group to form an intramolecular hydrogen bond with the carboxyl group of the same AmB molecule. In particular, the conformational behavior of the substituents at the AmB carboxyl site can alter the hydrogen bond formation patterns of AmB and its derivatives. Note that AmB's polar groups can also form hydrogen bonds with other molecules present in the channel-membrane supramolecular system, i.e. sterols and phospholipids. The collection of all these hydrogen bonds in turn determines the stability of the AmB membrane channel and can help to explain its structure-function relationship [25]. For this reason, this study focuses on investigating the possible hydrogen bond formation patterns of AmB and its derivatives.

Since the conformation of AmB is determined mainly by the relative positioning of two rigid fragments, this study investigates the conformational preferences of AmB derivatives around the $C_{19}O_{41}$ and $O_{41}C_{42}$ bonds which link these fragments (Figure 1). Among the large number of AmB derivatives synthesized and studied earlier, an amide series was observed to constitute a group of AmB modification products with varied selective toxicity [15, 16]. Thus, this amide series forms a compound group suitable for theoretical studies on structure-selective toxicity relationships.

The conformational properties of five AmB amide derivatives (Figure 3) were studied: AmB 2-hydroxyethyl amide (S1), AmB 3-dimethylaminopropyl amide (S2), AmB *n*-decyl amide (S3), AmB cyclohexyl amide (S4), and AmB 2-methoxyethyl amide

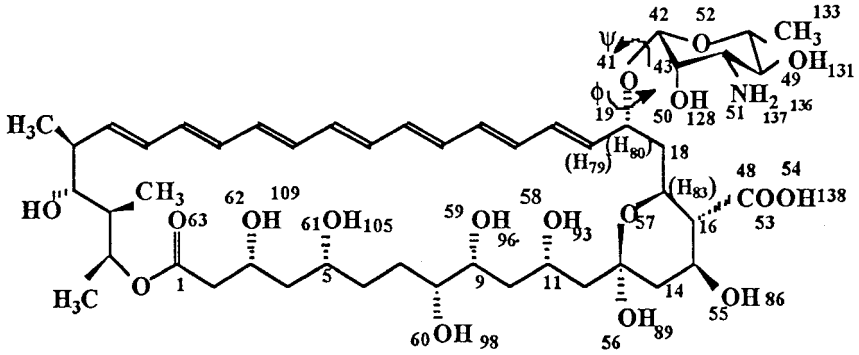

Figure 1. Unsubstituted Amphotericin B molecule.

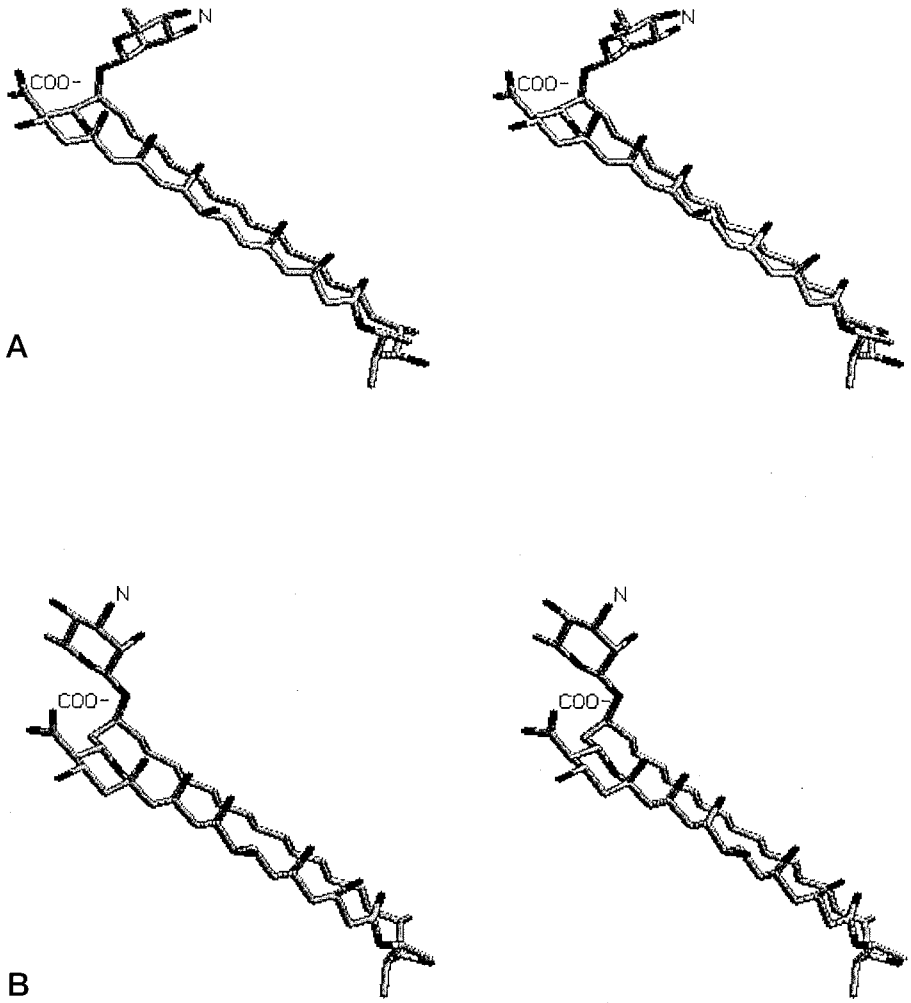

Figure 2. Stereo pictures of the (A) extended and (B) folded conformations of Amphotericin B.

(S5). While the selective toxicity of compound S4 is similar to that of AmB, S3 has a lower, and S1 and S2 have higher selectivity toxicity compared to AmB. The fifth derivative, AmB 2-methoxyethyl amide (S5), has not been synthesized yet but was included to make a prediction which can serve as a case study to test our hypothesis experimentally.

In the following sections we first outline the approach used to investigate the conformational properties of the AmB derivatives. Then our results are presented and discussed with a particular emphasis on the structure-selective toxicity relationship of the examined AmB derivatives. It was found, with the exception of AmB cyclohexyl amide (S4), that the lowest energy conformers prefer the extended conformation. In other words, amide substituted AmB derivatives are expected to have a dominant tendency toward forming intermolecular hydrogen bonds when they form ion channel complexes. It was also observed that the differences in the conformational space potential energy maps could give clues about the AmB-sterol interactions in the channel. Based on our results for the AmB derivatives' ability to form different intermolecular hydrogen bond networks, we postulate a new explanation for the observed differences in the selective toxicity of the amide AmB derivatives. Our hypothesis could be a valuable aid in the rational design of a less toxic antibiotic. In such an approach, theoretical conformational analysis can suggest lead candidates that would be worth to synthesize and test for their side effects. The conformational analysis is rather simple and does not require an unreasonable amount of computational resources. The selected compounds can also be further included in more sophisticated atomic level molecular dynamics studies of the AmB membrane channels.

Methods

Since our ultimate aim is to identify an antifungal antibiotic which has higher toxicity towards fungal cells compared to mammalian ones, we have chosen the amide AmB derivatives for the subject of this study. They have been measured to have varying selective toxicity. A total of five AmB derivatives obtained by introducing various amide groups at the carboxyl site of AmB were studied (Figure 3). Four of the derivatives (S1-S4) have been synthesized and their toxicity has been measured [15, 16]. Of the studied derivatives, S1 and S2 exhibit higher selective toxicity than AmB (i.e., their effectiveness towards fungal cells is much higher than for mammalian cells) and S3 exhibits a smaller selective toxicity than AmB [15,16]. The selective toxicity of the derivative S4 is only slightly higher than that of the unsubstituted AmB. Additionally, a hypothetical S5 AmB derivative was also included in the study to make a prediction that would make it possible to test the structure-selective toxicity relationship proposed in this report.

Due to the inflexibility of the conjugated double bonds, the macrolide ring of AmB can be treated as a rigid group to simplify the calculations. This helps to keep the computational expenses at a feasible level. This approximation has been shown not to cause any significant errors in previous conformational studies of AmB or AmB derivatives [26–28]. Therefore, we have employed this simplification and assumed that the sugar moiety and the substituents at the carboxyl group are the only flexible regions in our calculations. The macrolide ring was kept fixed and all calculations were done in vacuum. An earlier conformational analysis investigation of the unsubstituted AmB showed that the zwitterionic form of AmB has much higher energies in vacuum so in our calculations the neutral form of AmB was used [24]. The three-dimensional molecular structure of unsubstituted AmB was used as the starting geometry in our calculations. This structure was obtained from Baginski et al. [24], and the substituents were added using the standard parameters as initial values for the bond lengths and angles.

Our investigation of the conformational preferences of AmB derivatives was aimed at obtaining the potential energy surface (PES) maps defined using the torsion angles $\phi(C_{18}C_{19}O_{41}C_{42})$ and ψ $(C_{19}O_{41}C_{42}C_{43})$ as free parameters (Figure 1). The PES maps of an unsubstituted AmB and of AmB derivatives having the substituents shown in Figure 3 were generated by performing a conformational search for the angles ϕ and ψ. Our calculations involved several steps. First, to eliminate the orientations with steric hindrances due to the somewhat arbitrary addition of the substituents and to find the possible low energy regions, the sugar moiety was kept frozen and a conformer search analysis was performed for the substituents. In this first step, the conformation preferences of the carboxylate site substituents were determined using molecular mechanics. This preference search was done using the Spartan program (version 4.0) [29] and the Sybyl force field as implemented in the program [30] was employed to define the interaction potentials.

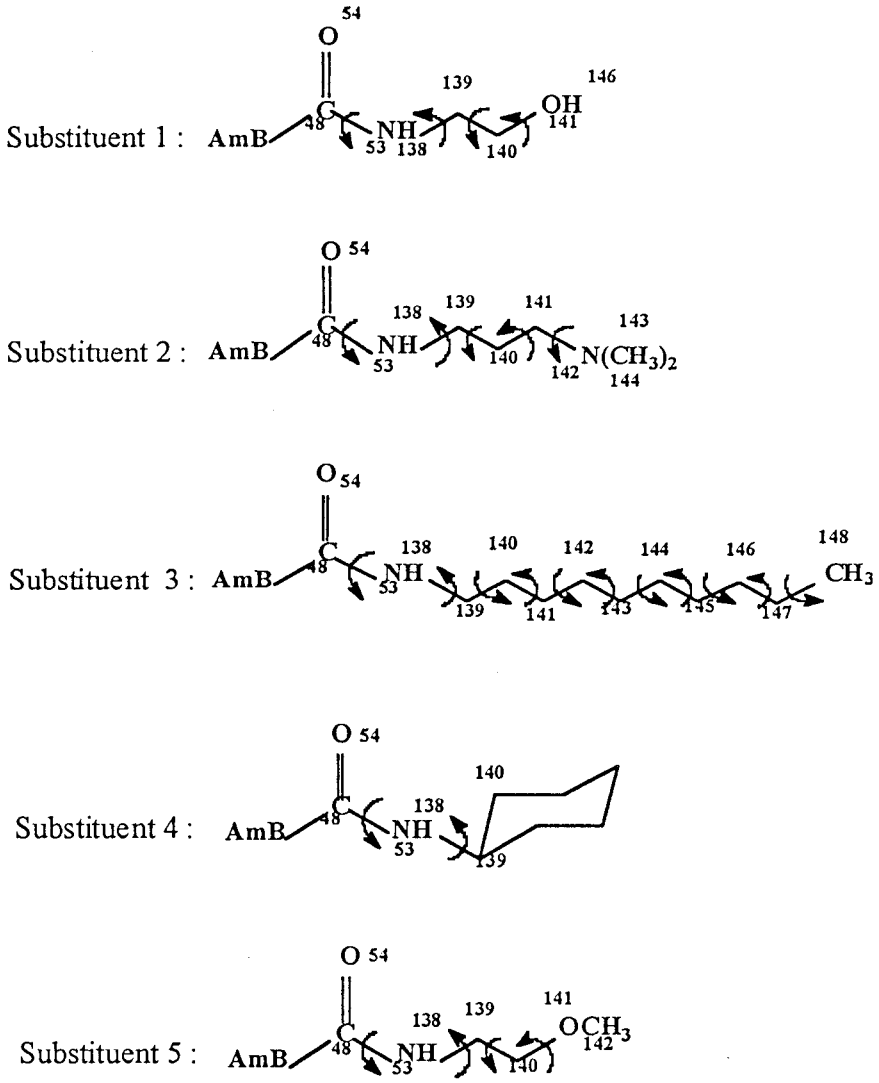

Figure 3. The structure of the investigated amide substituents.

The conformer search results can be expressed as functions of the dihedral angles α_i which are defined in Figure 3. For each torsion angle three possibilities, $\alpha_i = -60.0°$, 60.0°, or 180.0°, were considered, i.e. each torsion angle was allowed to be in one of the *gauche*$^-$, *gauche*$^+$, and *trans* conformations. The torsion angles used in the conformer search are listed in Table 3. For each substituent, 10 conformations with the lowest energies were chosen as the seed structures to be used in the more detailed and accurate calculations of the second step.

In the second step, 10 low energy conformations obtained in the first step were further optimized with the semi-empirical AM1 method [31]. This part of the calculations allowed determination of the best possible ring structure of the amino sugar group in the vicinity of the substituent. In this second step, and in the remainder of our calculations, the MOPAC program (version 7.0) [32] was used. Ten structures chosen in the first step were allowed to relax in such a way that the macrolide ring was again frozen, but, in addition to the substituent, the sugar moiety was also left free during the energy minimization. This procedure was repeated for every derivative.

In the third step, the lowest energy structure found in the second stage was used to compute the potential energy surfaces (PES) around the $\phi(C_{18}C_{19}O_{41}C_{42})$ and $\psi(C_{19}O_{41}C_{42}C_{43})$ angles (Figure 1). As discussed

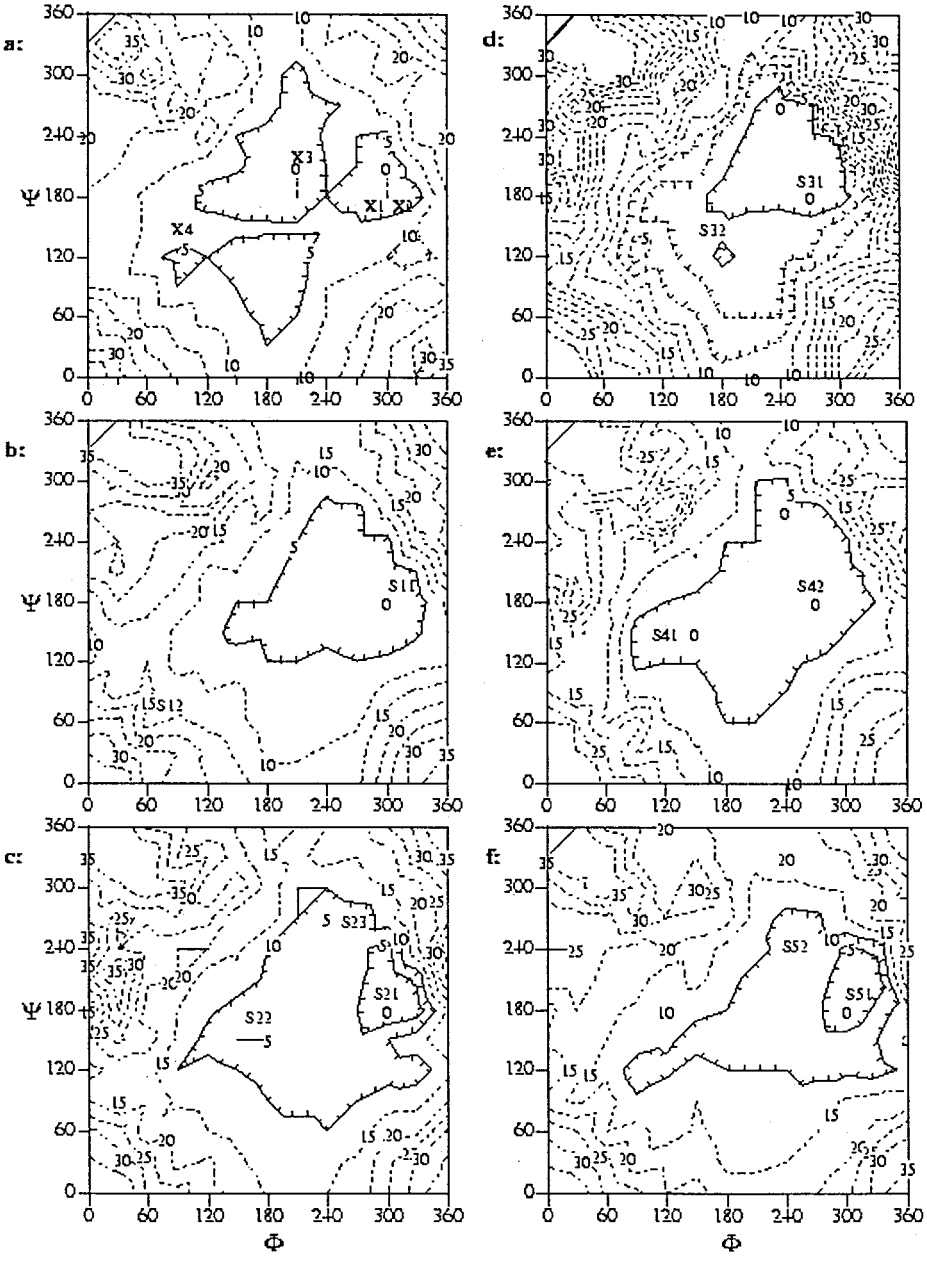

Figure 4. The PES map of investigated compounds. As explained in the text, X*n* and S*nm* respectively represent the location of the *n*th minima position for the unsubstituted AmB and its S*n* amide derivative. The ϕ/ψ angles range from 0° to 360°. (a) Unsubstituted AmB; (b) 2-hydroxyethyl amide substituted AmB; (c) 3-dimethylaminopropyl amide substituted AmB; (d) n-decyl amide substituted AmB; (e) cyclohexyl amide substituted AmB; and (f) 2-methoxyethyl amide substituted AmB.

in the Introduction section, ϕ and ψ are the torsion angles most important in defining the overall structure of AmB and its derivatives. Therefore, our investigation concentrated on these angles. PES maps were generated on a grid by varying the dihedral angles by 30° increments. For each (ϕ,ψ), the structure of the molecule was optimized using the semi-empirical AM1 method [31]. During optimization the macrolide ring was kept frozen and the best geometry for the sugar moiety and for the substituent at the carboxyl group was determined. The constant energy contour maps reported in Figure 4 were drawn by interpolated fitting to the values calculated at $12 \times 12 = 144$ discrete (ϕ,ψ) points.

As usual, PES maps contain several local minima. Since the maps were generated using a grid, the fourth step involved the accurate determination of the conformers corresponding to local energy minima on the (ϕ,ψ) map. The (ϕ, ψ) angles that correspond to regions of the lowest energy were located in the PES maps. The determined distinct minimum energy conformers appearing in the PES maps were further optimized. In this step, the macrolide ring and the sugar moiety were frozen and the rest of the molecule was allowed to relax to the optimal (ϕ,ψ) point. The conformations found in this step corresponding to minima are marked in Figures 4a–f as X*m* for the unsubstituted AmB, and as S*nm* for AmB derivatives where '*m*' denotes the minima and '*n*' denotes the substituent. Various structural properties and the values of energy for PES minima corresponding to appropriate conformers are tabulated in Tables 1 and 2.

Results and discussion

The membrane activity of AmB and its derivatives is due to ionic channel formation induced by the antibiotic in the membrane. According to the well-established sterol hypothesis, sterol molecules present in phospholipid membranes participate in the AmB channel formation or they are at least responsible for the channel's stability in its open state [33, 34]. Even though it is not clear whether AmB channels formed in sterol-containing and sterol-absent membranes have the same molecular properties, it should be noted that the formation of AmB channels in the absence of sterols has also been observed [35, 36]. It has been shown in Reference 36 that the existence of sterols is not necessary in the channel formation but sterols affect the channel conductance and stability by modifying the membrane structure.

Formation and stability of a supramolecular ion channel is governed by the various complex intermolecular interactions between different components of the system, namely by the (i) AmB-AmB, (ii) AmB-sterol, (iii) AmB-phospholipid, and (iv) sterol-phospholipid interactions. Of this list, it is believed that only the first two types of interactions are responsible for the channel's stability. Since AmB is an amphipathic molecule, and since sterols (cholesterol or ergosterol) are mainly hydrophobic molecules with one hydroxyl group, the interactions between an AmB and another AmB or a sterol can be of two types, hydrophobic and polar. The governing polar interactions are due to the hydrogen bonds formed between the polar head groups of AmBs and, undoubtedly, the hydroxyl groups of sterols also participate in hydrogen bond formation. As confirmed by experiments, these polar intermolecular interactions are essential for the membrane activity of AmB and its derivatives [10].

In addition to the effect of the sterols [10, 37, 38], the following modifications of AmB have also been observed to effect the selective toxicity: (i) annulment of AmB's free carboxyl group (AmB amides and esters) [9, 10], and (ii) appropriate substitution at the AmB amino group by *N*-alkylation [17] or aminoacylation [39]. These modifications can have an effect by changing the hydrogen bond network that can be formed between the antibiotic molecules or between these molecules and sterols in the channel. It has also been observed that in order to exhibit membrane activity (i.e., to be able to form a channel), AmB or its derivative must have an amino group which can be protonated [9, 10, 13, 40]. As for the sterol, the presence of the hydroxyl group is indispensable for the antibiotic membrane activity [41].

The selectivity difference of AmB and its derivatives towards cholesterol- and ergosterol-containing membranes can be regarded as being due to the different affinity of the antibiotic to ergosterol and cholesterol. Thus, a more favorable AmB-ergosterol (compared to AmB-cholesterol) interaction in the channel might be responsible for the higher stability of AmB-ergosterol channel and, as a consequence, can help to explain the higher toxicity of the antibiotic against fungal cells. A close look at the molecular structures shows that structural differences between ergosterol and cholesterol can give rise to different short range and hydrophobic interactions of these sterols with AmB. Compared to cholesterol, ergosterol has two

additional carbon-carbon double bonds and one more methyl group. Therefore, it can be expected that the flexibility and the bulkiness of ergosterol would be different than that of cholesterol. Additionally, ergosterol's additional double bonds can be regarded as polar spots that can interact electrostatically with the polyene fragment of the AmB and thus enhance the AmB-ergosterol affinity [42–44]. Therefore, both the hydrophobic and the electrostatic interactions and ability to form different networks of hydrogen bonds can be important for AmB's selective toxicity.

It has been shown using semi-empirical quantum chemistry methods in vacuum [24, 26, 27] and classical Molecular Dynamics (MD) studies [25, 45, 46] that polar functional head groups of AmB can form *intra*- and *inter*-molecular hydrogen bonds. When AmB has a *folded* structure (Figure 2), an intramolecular hydrogen bond can be formed between its amino sugar moiety and its carboxyl group. In this conformation the sugar ring is oriented in such a way that the amino group points towards the carboxyl group. In the *extended* structure (Figure 2), the sugar ring is rotated in such a way that the amino group points away from the carboxyl group. When appropriately oriented (as in a membrane channel configuration), the amino group can form an intermolecular hydrogen bond with the carboxyl group of the adjacent AmB molecule. It can be expected that intermolecular hydrogen bonds are important for keeping the *open*, i.e. transmitting, form of the ion channel intact [25, 47]. It has been observed in the MD simulation studies that there is a network (which has the shape of a ring) of intermolecular hydrogen bonds between the amino and the carboxyl groups of the pore forming AmB molecules. This hydrogen bond network adds structural stability to the pore and prolongs the open state of the channel.

All these previous results show that hydrogen bond patterns can give clues to the stability and selectivity of AmB membrane channels. Therefore, our analysis in this report has concentrated mainly on the hydrogen bonding characteristics. In addition, the distances between key groups were analyzed to determine possible close contacts.

Figures 4a–f present the potential energy surfaces (PES) in terms of ϕ and ψ torsion angles. The PES of unsubstituted AmB and of its studied derivatives have common minima in the ranges [155°:225°] for ψ and [135°:305°] for ϕ. AmB molecules having ϕ and ψ angles in these ranges can form intermolecular hydrogen bonds with the adjacent AmB molecules in a membrane ion channel. These minima regions are quite spread out and include the absolute minima (Table 1). Even though the reported PES maps are similar in appearance, there are several differences that can be important and should be noticed.

Table 1. ϕ/ψ angles and relative energies of unsubstituted and substituted AmB molecules before and after optimization processes as described in the text

	$[\phi,\psi]_{before}$ (°)	$[\phi,\psi]_{after}$ (°)	ΔE_{before} (kcal/mol)	ΔE_{after} (kcal/mol)
X1a	214, 183	275, 183	±0	±0
X1b	305, 183	271, 185	±0	±0
X2	184, 123	306, 186	+1.0	+0.7
X3	165, 244	219, 224	+6.0	+1.0
X4	123, 94	75, 138	+4.0	+3.4
S11a	290, 184	290, 184	±0	±0
S11b	260, 247	290, 186	+1.1	±0
S12	50, 64	52, 64	+12.8	+12.8
S21a	292,215	292,190	±0	±0
S21b	82,305	295,195	+36.0	+2.8
S22	172,155	170,183	+4.7	+5.3
S23	262,245	277,247	+4.9	+5.6
S31	270, 210	270, 208	±0	±0
S32	30, 330	170, 155	+38.9	+1.2
S41	52, 305	137, 155	+38.1	±0
S42	263, 185	264, 185	±0	+1.4
S51a	291, 190	291, 190	±0	±0
S51b	80, 129	291, 190	+2.3	±0
S52	170, 310	253, 268	+15.9	+2.0

Figure 4a shows the PES map of an unsubstituted AmB in its neutral form. There are four distinct minima (X1,X2,X3,X4) (Table 1). Three of these, which are also the three lowest energy minima, have dihedral angles in the ranges [185°:225°] and [220°:305°] for ψ and ϕ, respectively. The fourth minimum X4 with ϕ=75° and ψ=138° has a relative energy of +3.4 kcal/mol. Since there are several minima with similar energies it can be stated that the AmB molecule is flexible around the glycosidic bond. This flexible nature of the sugar moiety with regard to the macrolide ring has been observed before in molecular simulation studies [48].

In its three lowest energy conformers X1, X2, and X3, the AmB has the extended conformation. The differences between these three structures are the rotations around ϕ and ψ. These rotations mainly change the polar interactions between oxygen O_{52} (Figure 1)

and the macrolide ring atoms and result in energy differences of about 1 kcal/mol. The X4 conformer has the folded structure where the amino group of the sugar moiety turns toward the carboxyl group and can have favorable polar interactions with it. There are also favorable contacts between $O_{53}\ldots H_{133}$ and $O_{52}\ldots H_{83}$ with distances of 2.518 Å and 2.149 Å, respectively. However, because of the closeness of the groups, there is a steric hindrance between the sugar moiety and the heptaenic part of AmB. This hindrance increases the energy of this configuration. The hydrogen bonds between $H_{128}\ldots O_{41}$, $H_{93}\ldots O_{59}$, $H_{105}\ldots O_{62}$, $H_{109}\ldots O_{63}$, and $H_{89}\ldots O_{58}$ were observed in all the conformers X1, X2, X3, and X4, in accord with the earlier studies [24, 25].

The PES map of AmB 2-hydroxyethyl amide (S1) is primarily composed of a single minimum region (Figure 4b). In this global minimum, ϕ is equal to 275°, a 15° shift relative to the lowest energy conformer of unsubstituted AmB. Due to this 15° rotation around the $C_{19}O_{41}$ moiety, the distance between O_{52} and H_{80} decreases from 2.35 Å to 2.29 Å and has a stabilizing effect. The rotation around the $C_{16}C_{48}$ bond from 29.0° (unsubstituted AmB) to −94.0° (AmB 2-hydroxyethyl amide) results in O_{53} and H_{126} becoming closer. Their interaction also contributes to the stability of this conformation. The ψ angle is equal to 180° and the molecule is in the extended conformation, i.e. it can form intermolecular hydrogen bonds in a channel. There is a local minimum (Table 1) at (ϕ,ψ)=(52°, 64°). In this conformation, the mycosamine sugar ring is folded towards the carboxyl group. However, the energy of this conformer is +12.8 kcal/mol higher than the energy of the global minimum. Because of this large energy difference, the probability of observing this conformation would be extremely small, and therefore, this conformation can be omitted from the discussion.

Although there are three distinct minima in the PES map (Figure 4c) of AmB 3-dimethylaminopropyl amide (S2), the energy of the absolute minimum is much lower than the other minima. The lowest energy conformer S21 has values of 292° and 190° for ϕ and ψ, respectively. This is very close to the lowest energy conformer of the derivative S1. The energies of the other two conformers, S22 with (ϕ,ψ)=(170°, 183°) and S23 with (ϕ,ψ)=(277°, 247°), are 5.3 kcal/mol and 5.6 kcal/mol higher than the lowest energy conformer S21, respectively. Based on the corresponding Boltzmann factors, it can be safely stated that these two conformers would not be very populated. The analysis of intramolecular distances listed in Table 2 shows that conformer S21 is extended and conformers S22 and S23 are more folded. Even though the (ϕ,ψ) values for S21 and S22 conformers differ only slightly, there is a 5.3 kcal/mol energy difference between these two configurations. This reveals the steepness of the absolute minimum well. Experiments show that AmB 2-hydroxyethyl amide (S1) and AmB 3-dimethylaminopropyl (S2) derivatives exhibit much higher selective toxicity than unsubstituted AmB [15, 16]. As will be proposed below, the lack of another minimum in the PES map may be an indicator of their observed higher selective toxicity.

The AmB n-decyl amide (S3) has a PES map with two minima regions (Figure 4d). The position of the lowest energy conformer (ϕ,ψ)=(270°, 208°) is close to the global minima of the unsubstituted AmB and its S1 and S2 derivatives. Because of the extended conformation and the bulkiness of the substituent, there is no possibility of forming an intramolecular hydrogen bond between the amino group of the mycosamine moiety and the alkyl substituted carboxyl group. The energy of the local minimum S32 with (ϕ,ψ)=(170°, 155°) is +1.2 kcal/mol higher than the lowest energy conformer S31. Because of this small energy difference (less than 2 *kT* at room temperature) it is highly likely that the higher energy conformer S32 would also be reasonably populated.

The fourth AmB derivative, S4, is an AmB cyclohexyl amide – equatorially substituted. The PES of this molecule has two distinct minima regions (Figure 4e). These regions are very similar to the S31 and S32 regions of the S3 derivative with the difference that the locations of the global and higher energy minima are reversed. The angles for the lowest energy conformer S41 are (ϕ,ψ)=(137°, 155°) and in this conformation, the sugar ring is close to the macrolide ring. As a consequence there are weak but stabilizing interactions between $O_{52}\ldots H_{79}$, $O_{41}\ldots H_{83}$, and $O_{52}\ldots H_{83}$ with distances of 2.45 Å, 2.51 Å, and 2.65 Å, respectively. Even though it is slightly folded, the sugar moiety is still quite extended and the amino sugar group cannot form intramolecular hydrogen bonds. The local minimum conformer S42 has a relative energy of +1.4 kcal/mol and its ϕ and ψ angles are very close to the global minimum of the unsubstituted AmB.

According to the experimental results, selective toxicity (ST) of the studied compounds has the order S2 > S1 > S4 > AmB > S3 with ST values of 117.0: 93.1: 64.5: 45.0: 4.4, respectively [15, 16]. The selec-

Table 2. Distances (Å) and dihedral values (degrees) of unsubstituted and substituted AmB molecules

Dihedrals (°) and distances (Å)	X1a,b	X2	X3	X4	S11a,b	S12	S21a	S21b	S22	S23	S31	S32	S41	S42	S51a,b	S52
$C_{15}C_{16}C_{48}N(O)_{53}$	29.0	28	24	23	−94	−95	−94	−96	54.4	−93	60	58	61	57	−92	−94
$C_{15}C_{16}C_{48}O_{54}$	−148.5	−149	−152	−150	86	85	87	85	−123.4	85	−119	−122	−120	−121	89	86
$H_{128}\ldots O_{41}$	2.39	2.40	2.43	2.39	2.39	2.56	2.39	2.39	2.39	2.43	2.39	2.43	2.39	2.39	2.39	2.49
$N_{51}\ldots O_{54}$	7.29	7.83	8.38	8.75	5.45	5.73	5.51	5.64	8.76	6.92	8.30	9.02	9.15	7.76	5.56	7.72
$N_{51}\ldots N(O)_{53}$	6.50	8.42	8.83	8.75	7.57	7.73	7.62	7.73	7.26	8.77	6.79	7.56	7.45	6.22	7.69	9.53
$N_{51}\ldots H_{138}$	7.05	8.36	9.02	8.95	8.52	8.22	8.58	8.69	6.72	9.59	6.43	7.12	6.90	5.86	8.64	10.37
$H_{136}\ldots O_{41}$	3.40	2.55	2.55	2.55	2.54	2.54	2.63	2.54	2.49	2.55	3.79	2.55	3.05	3.77	2.55	2.53
$H_{137}\ldots N(O)_{53}$	7.16	7.53	8.68	8.84	5.84	6.08	7.68	7.87	7.07	9.20	8.70	8.22	9.02	8.30	5.93	8.23
$H_{83}\ldots O_{41}$	2.45	2.40	2.38	2.78	2.48	2.92	2.48	2.49	2.39	2.44	2.43	2.42	2.51	2.43	2.48	2.41
$H_{79}\ldots O_{52}$	4.41	2.50	3.73	3.85	4.60	4.59	4.61	4.62	2.64	4.56	4.74	2.38	2.44	4.32	4.61	4.55
$H_{83}\ldots O_{52}$	2.28	3.40	2.21	4.07	2.35	4.11	2.32	2.31	3.27	2.25	2.15	3.60	4.01	2.29	2.32	2.21
$H_{133}\ldots O_{54}$	7.67	9.05	8.61	2.52	5.77	5.43	5.56	5.27	9.44	3.94	6.13	9.09	7.85	7.54	5.59	4.36
$H_{83}\ldots O_{52}$	4.54	4.08	4.68	2.15	4.27	3.63	4.20	4.10	4.15	3.79	4.37	3.63	2.68	461	4.22	4.01
$H_{93}\ldots O_{59}$	2.06	2.05	2.06	2.06	2.06	2.06	2.06	2.06	2.05	2.06	2.06	2.05	2.06	2.06	2.06	2.06
$H_{105}\ldots O_{62}$	2.25	2.26	2.26	2.25	2.26	2.26	2.26	2.26	2.25	2.25	2.25	2.25	2.26	2.25	2.26	2.26
$H_{109}\ldots O_{63}$	2.25	2.25	2.23	2.24	2.25	2.23	2.24	2.24	2.25	2.24	2.24	2.24	2.25	2.25	2.25	2.25
$H_{89}\ldots O_{57}$	2.35	2.35	2.35	2.35	2.35	2.35	2.35	2.35	2.35	2.35	2.35	2.35	2.35	2.35	2.35	2.35

Table 3. Dihedral angles that are taken into consideration during the conformer search analysis of substituents

2-Hydroxyethyl amide substituted AmB	$C_{16}C_{48}N_{53}C_{139}$, $C_{48}N_{53}C_{139}C_{140}$, $N_{53}C_{139}C_{140}O_{141}$
3-Dimethylaminopropyl amide substituted AmB	$C_{16}C_{48}N_{53}C_{139}$, $C_{48}N_{53}C_{139}C_{140}$, $N_{53}C_{139}C_{140}C_{141}$, $C_{139}C_{140}C_{141}N_{142}$, $C_{140}C_{141}N_{142}C_{143}$
n-Decyl amide substituted AmB	$C_{16}C_{48}N_{53}C_{139}$, $C_{48}N_{53}C_{139}C_{140}$, $N_{53}C_{139}C_{140}C_{141}$, $C_{139}C_{140}C_{141}C_{142}$, $C_{140}C_{141}C_{142}C_{143}$, $C_{141}C_{142}C_{143}C_{144}$, $C_{142}C_{143}C_{144}C_{145}$, $C_{143}C_{144}C_{145}C_{146}$, $C_{144}C_{145}C_{146}C_{147}$, $C_{145}C_{146}C_{147}C_{148}$
Cyclohexyl amide substituted AmB	$C_{16}C_{48}N_{53}C_{139}$, $C_{48}N_{53}C_{139}C_{140}$
2-Methoxyethyl amide substituted AmB	$C_{16}C_{48}N_{53}C_{139}$, $C_{48}N_{53}C_{139}C_{140}$, $N_{53}C_{139}C_{140}O_{141}$, $C_{139}C_{140}O_{141}C_{142}$

tive toxicity has been defined as the ratio of IC_{50} and EH_{50} ($ST=EH_{50}/IC_{50}$). The IC_{50} and EH_{50} factors, respectively, are the concentration of compound tested which in standard conditions inhibits the growth of *Saccharomyces cerevisiae* by 50% and the concentration of compound causing 50% heamolysis of human erythrocytes [15, 16].

The results of our PES study allow us to propose a new way of estimating the relative selective toxicity of AmB derivatives. This proposal is an extension of the arguments given by Herve et al. [10] and Meddeb et al. [26]. As stated in Reference 10, the different affinity of ergosterol and cholesterol towards AmB is observed when the antibiotic and the sterol molecules interact mainly through hydrophobic interactions at short ranges. When there are polar interactions (very likely mediated by the water molecules) between the hydroxyl group of sterol and the antibiotic's amino and carboxyl groups, the sterol and the antibiotic form a binary complex (triplex if a water is involved). Due to the strength of these polar interactions, the AmB-sterol structural configuration is quite hindered and the relative molecular positions do not fluctuate considerably. Therefore, since they are not close enough, the sterol and the antibiotic do not sample each other's hydrophobic surface. This type of interaction cannot distinguish between sterol types (cholesterol versus ergosterol), and hence, there is poor selectivity. In contrast, when there are no strong polar interactions (as in the case of a blocked carboxyl group [10]) to form a rigid antibiotic-water-sterol complex, the sterol can approach the antibiotic and short-range hydrophobic interactions would start to dominate. This model of AmB-sterol interactions was used to rationalize a structure-activity relationship for the observed selective toxicity differences between AmB derivatives. Although this model is successful, it has the shortcoming that it is based only on an AmB-sterol binary complex and does not take into account the constraints imposed on the system when a membrane ion channel is formed.

In addition to the findings of the present report, our extension of the selectivity model presented above also uses the results of the previous molecular modeling studies of the AmB-sterol membrane channel [25, 46, 47] and takes into account the molecular properties of AmB and sterols [24, 26, 27, 37, 43, 49]. We propose that the interactions between the sterol and the antibiotic molecules would be mainly hydrophobic in character only when the structures of antibiotic molecules satisfy certain conditions. We start by noting that

in aqueous biomolecular systems, the polar groups exposed to the solvent form as many salt bridges or hydrogen bonds as possible, either among themselves or with the surrounding water molecules. We also note that Herve et al.'s [10] argument for the selective toxicity requires that the polar interactions between AmB's ionizable groups and sterols should be very weak. Such a situation can be achieved if the polar head groups of AmB (or its derivatives) have *saturated* interactions in the channel. Here the word saturated is used to describe the configurations in which the groups that can participate in electrostatic interactions (like hydrogen bonds or salt bridges) establish these interactions to the fullest extent, i.e., there are no unpaired polar groups. When the polar head groups of the antibiotic have saturated interactions, they would not need to interact electrostatically with the sterols. Also, because of the strength of the polar interactions, the head groups of the antibiotic can be expected to be quite immobile. As a result, sterols would be able to approach the antibiotic molecules. This would allow for hydrophobic interactions, which is essential for the selective toxicity.

At reasonably high temperatures, the molecules of a condensed biomolecular system do not stay in a rigid configuration. Therefore, explanations based on the analysis of a (semi-) rigid configuration have to be supported with further discussions whether the described configuration is reasonable and whether the system will be in that state almost all the time. In statistical terms, the mechanism described above would be valid if and only if it is shown that the discussed rigid configuration is observed almost one hundred percent of the time. The information that certain structures will be populated comes from the conformational search studies (i.e., the PES maps). If the PES map of a molecule contains only one accessible minimum (like in the case of the S1 and S2 derivatives of AmB), then the molecule can be expected to have a structure corresponding to the minimum energy conformation. Lacking other favorable conformations to transform to, the molecule would not change its conformation. In contrast, if there are other thermodynamically accessible states (i.e., if there are other states with slightly higher relative energies, and if the energy barriers between the PES minima do not prohibit conformation transformations within the relevant time scale), then the molecules can change conformations between those states. Such switches between the accessible states would require that the groups involved in the conformation change have enough space around them so that their movements are not limited due to steric effects. This is analogous to stating that (freely) rotating groups have their own space in a condensed medium. For AmB and its derivatives this translates to the condition where AmB's moving groups would not let the sterols come close enough to interact through hydrophobic forces. In contrast, if the AmB channel structure is rigid, the sterol molecules are not blocked from approaching the AmB molecules. In such cases, the sterols can interact with AmB molecules through hydrophobic forces. If there is a good match between the interacting hydrophobic surfaces of the sterol and the antibiotic (as in ergosterol), the favorable short range interactions contribute to the channel stability and increase the affinity of the antibiotic. One must also point out that the sterols do not have to interact with the antibiotics. There are other natural components of the membranes (such as phospholipids) and sterols can prefer to interact with them to form stable configurations. In general, there would be a competition between sterol-AmB and sterol-phospholipid interactions.

Our results for the PES maps of unsubstituted AmB and AmB derivatives show that, except for AmB 3-dimethylaminopropyl amide (S2) and AmB 2-hydroxyethyl amide (S1), the PES maps of the molecules contain more than one thermodynamically accessible minimum. In our analysis the states which have energies up to 2.5 kcal/mol (about 4 kT at room temperature) above the global minimum are counted as accessible states. One of the minima for each of these molecules is in the region $(\phi,\psi) \approx (270^\circ, 180^\circ)$ which corresponds to the extended conformation of the antibiotic, i.e., the amino group of the sugar moiety can form intermolecular hydrogen bonds with the free or substituted carboxyl group of the adjacent antibiotic molecule. The formation of such a hydrogen bond network has been observed in the MD simulations investigating the dynamical behavior of an AmB channel in the membrane consisting of cholesterol and phospholipid molecules [25]. The same MD simulations also showed that this hydrogen bond network would align the amino groups in such a way that they would not strongly interact with the sterols. The amino groups of AmBs can also interact with the phosphate group of the phospholipids which further blocks them from interacting electrostatically with the sterols. Even though hydrogen bonds between AmB and cholesterol molecules can be formed, they are very infrequent [25]. These all point to the fact that the amino group of the sugar moiety has *saturated* electro-

static interactions when the antibiotic molecules form a membrane channel.

Satisfying this condition of saturated amino group interactions in the channel, the selective toxicity of an AmB derivative would then depend on the possible proximity of antibiotic and sterol molecules and on the lack of conformation transformations within the polar head region of the antibiotic molecules. As discussed above, the possibility of conformational transformations of the antibiotic molecules can be estimated from the PES maps. Our results, Figures 4a–e, reveal that only the S1 and S2 derivatives are expected to exist in a single conformation, i.e., they would not go through conformational changes. This would allow for the close approach of the sterols to the antibiotic which, as discussed above, would enhance the strength and the selectivity of the hydrophobic interactions between AmB and sterol. The possibility of short range encounters also explains the high selective toxicity of S1 and S2 AmB derivatives. In contrast, unsubstituted AmB, and the S3 and S4 AmB derivatives can exist in more than one conformation (Figures 4a, 4d, and 4e) and are expected to undergo conformation transformations. As explained above, conformation changes would limit their close range interactions with sterols and, as a result, the selective toxicity of these compounds would be smaller than that of the S1 or S2 derivatives. A closer look at the structures of the molecules can explain the experimental observation that the selective toxicity of the S3 derivative is smaller than those of the rest. This derivative has a bulky hydrocarbon chain which, being hydrophobic, would try to bury itself into the membrane to avoid being exposed to the aqueous solution. Therefore, the expected average distance between the antibiotic and the sterols would be larger. Large separations would effectively diminish the hydrophobic interactions between sterol and the S3 derivative and the antibiotic selective toxicity would be lower than for other derivatives.

To test the consistency of our extended model of selective AmB-sterol interactions, we investigated an additional AmB derivative and made predictions for its selective toxicity. Since our work is purely theoretical, we chose an AmB derivative which has not been studied before. This choice allowed us to make a prediction that can be tested experimentally. Such a test would validate or invalidate our explanation for the selective toxicity of AmB's amide derivatives. The AmB 2-methoxyethyl amide (S5) was chosen for this part of the work. This derivative S5, AmB 2-methoxyethyl amide, differs from the first derivative S1, AmB 2-hydroxyethyl amide, only by the replacement of the –OH group with $–OCH_3$ (Figure 3). The PES map of S5 (Figure 4f) has two distinct minima. The global minimum conformation $(\phi,\psi)=(291°, 190°)$ is nearly identical to that of the S1 derivative (Table 1). In this conformation, the sugar ring is extended and the amino group can form intermolecular hydrogen bonds in a membrane channel. The main distinction between the PES maps of the S5 and S1 derivatives is that there is a second and reasonably accessible conformation for the S5. This local minimum conformation has a relative energy of $+2.0$ kcal/mol compared to the global minimum and this state can be considered as being thermodynamically accessible. In this respect, we expect that the selective toxicity of S5 would be smaller than that of S1. Judging from the size of the substituent and the slightly higher energy of the local minimum, we predict the selective toxicity of S5 to be slightly higher than that of the S4 derivative (ST=64.5) or that of unsubstituted AmB (ST=45.0).

Conclusions

Amphotericin B amide derivatives form a good test group for theoretical studies of the molecular nature of the structure-selective toxicity relationship of the modified antibiotic. The conformational properties of AmB itself and five AmB amides with differentiated selective toxicity were studied and the impact of conformational preferences to the selective toxicity of the examined molecules was investigated.

It was found that the AmB derivatives with improved selective toxicity have a preference to exist in the extended conformation of the antibiotic. This conformation allows for the formation of intermolecular hydrogen bonds between amino and substituted carboxyl groups of adjacent antibiotic molecules. Moreover, it was speculated that the preference for the extended conformation and the existence of only one well-defined minimum point to a membrane ion channel structure in which the polar head groups of the antibiotic have saturated electrostatic interactions. A network of such saturated electrostatic interactions is believed to increase the stability of a membrane ion channel, and thus, increase the lifetime of its open form [25]. Saturated electrostatic interactions also immobilize the antibiotic molecules and allow for close range hydrophobic interactions between the antibiotic and sterol molecules. Such hydrophobic interactions are believed to introduce selective toxicity. In con-

trast, the derivatives with low selective toxicity have the ability to acquire a folded conformation and have more flexible polar head groups. The folded conformation makes the formation of intramolecular hydrogen bonds possible. In consequence, the stability of the channel is reduced and the close contact between the antibiotic and sterol is impeded.

The results of our conformational analysis correlate well with experimental data for the selective toxicity of AmB amides. We introduced a new model to explain the structure-selective toxicity relationship of AmB amide derivatives. This new explanation was designed by building on earlier models [10, 26]. In this new model, a derivative with high selective toxicity should have the following properties: (i) The extended conformation of the antibiotic molecule should be an accessible conformer. This allows for the formation of intermolecular hydrogen bonds and for the polar head groups to have saturated electrostatic interactions. (ii) The extended conformation should be the *only* thermally accessible configuration. Unavailability of other conformers helps to immobilize the antibiotic's polar head group. Sterols with planar ring groups can approach the inflexible antibiotic molecules and such close range hydrophobic interactions make it possible for the antibiotic to distinguish between sterols. Different hydrophobic affinity of ergosterol and cholesterol for the antibiotic introduces the desired selective toxicity. (iii) The substituent at the carboxyl site should be polar enough so that it would not try to immerse itself into the hydrophobic region of the membrane. Such an effect practically diminishes the interaction between the antibiotic and the sterols and reduces the selective toxicity.

In this report, we investigated five amide derivatives of AmB. Obviously, the conclusions based only on a limited number of compounds could be misleading. However, the fact that the selected compounds had a wide selective toxicity range and the chemical structures of the substitutent groups were quite different makes us believe that the introduced model can be a good starting point in the rational design of new AmB amide derivatives without serious side effects. Our model makes it possible to predict whether an amide derivative compound will have a high selective toxicity prior to its synthesis. In other words, theoretical conformational analysis as outlined above will serve as the first step for this design. Moreover, the physical properties of derivatives initially selected with this simple approach can be studied in more detail using more complicated theoretical studies such as the molecular simulations of the membrane ion channels formed by the selected AmB derivatives. Though it requires demonstration through examples, it can be expected that the proposed model can be widely applicable to other types of AmB derivatives having polar groups capable of forming both inter- and intra-molecular hydrogen bonds.

Acknowledgements

A.S. performed the calculations reported in this manuscript and this study forms a part of her Ph.D. thesis. The computations were performed using the computing resources of Bogaziçi and Koç Universities. This study was funded in part by a grant from the State Committee of Scientific Research, Warsaw (Poland) – grant number 3T09A07014. We would like to thank Jaroslav Koca for his help in preparing the figures.

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