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MOLECULAR MODELLING IN THE RATIONAL DESIGN OF SOME ANTI-TUMOR AND ANTIFUNGAL AGENTS

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Abstract: In this paper we present our approaches and results concerning application of molecular modelling techniques in the design of new chemotherapeutic agents for the control of eukariotic systems, comprising compounds for the treatment of systemic fungal infections and tumor deseases. In the case of anti-tumor agents we focused our attention on molecular properties of natural and synthetic anthraquinones. In the area of antifungal compounds we adopted two approaches. In one of them we examine molecular nature of undesirable properties of polyene macrolide antifungal antibiotic — amphotericin B using molecular modelling techniques. Another approach was aimed at the development of selective inactivator of glucosamine synthase, a novel target for antifungal compounds. In this problem we have used computational chemistry methods to identify structural features responsible for the selective inactivation of the target enzyme.

Keywords: molecular modelling, molecular mechanics, molecular dynamics, semi-empirical methods, antifungal agents, anti-tumor agents

1. Introduction

Our scientific program, carried out in the Department of Pharmaceutical Technology & Biochemistry in Technical University of Gdansk, is being focused on the development of theoretical basis for the rational design of chemotherapeutic agents for the control of eukariotic systems. We are particularily interested in the two the most important groups of eukariotic pathogens: fungal micro-organisms and cancer cells.

Design of new chemotherapeutic agents, active against eukariotic pathogens, is a difficult task due to the high biochemical and molecular similarity between pathogens and cells of the treated patient. Molecular differences between constituents of these two types of cells, with which active agents interact, are so small that toxic effect is one of the significant limitation in their clinical use.

In such situation a rational design seems to be the most promising way to obtain new, effective and non-toxic drugs. Such a design has to be based on the knowledge of biological properties of active agents at a molecular level as well as of cell processes which we would like to control by those agents. A computational

chemistry and a molecular modelling may be very effective in providing sources of this knowledge. Consequently, a few years ago, our scientific program has been extended to include these theoretical branches of chemistry.

In this paper we would like to present our approaches and results concerning application of molecular modelling techniques in the design of new chemotherapeutic agents active against systemic fungal infections and tumor deseases.

In the case of anti-tumor chemotherapeutic agents we focus our attention on natural and synthetic anthraquinones. This group of compounds include the most important anti-tumor drugs, like doxorubicin and its congeners or mitoxantron. However, these drugs exert also essential side-effects. Recognition of molecular factors responsible for these effects is one of our main goals.

In the area of agents active in systemic fungal infections we adopted the two approaches. In one of them we studied polyene macrolide antifungal antibiotic – – amphotericin B. This antibiotic exhibits very promising biological properties, but has also two significant disadvantages: a low water solubility and a high animal toxicity. We have used molecular modelling techniques to examine molecular nature of these two undesirable properties.

Another approach was aimed at the development of a selective inactivator of glucosamine syntase, an enzyme playing important role in the synthesis of constituents of fungal and bacterial cell walls. In this problem we have used computational chemistry approach to find structural features responsible for the selective inactivation of the target enzyme.

2. Anti-Tumor Anthraquinones

Anthraquinone anti-tumor antibiotics (anthracyclines), such as Daunorubicine and Adriamycine (Figure 1) belong to the most important anti-tumor chemotherapeutics. They are particularly useful against leukaemia which can't be treated by a surgical intervention or by a radiotherapy.

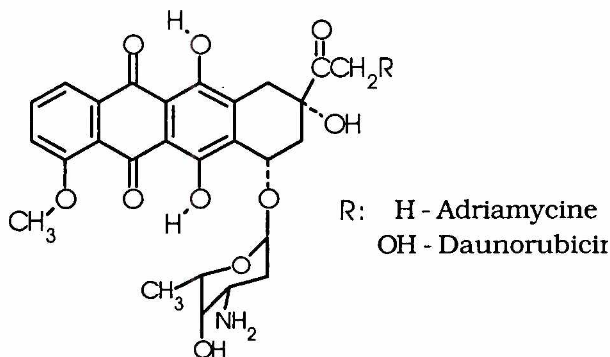


Figure 1. *Chemical structures of two main anthraquinone antitumor antibiotics (anthracyclines).*

However, their use is significantly limited by their toxicity toward heart (cardiotoxicity). It has been shown that cardiotoxicity of the anthracyclines results

from their peroxidating activity due to the overproduction of active oxygen species (AOS). The overproduction of AOS is induced not only by the anthracyclines but also by some other quinone-moiety containing compounds.

2.1. Mechanism of Anthraquinone Mediated Generation of AOS

During last decades many authors have searched for anthraquinone anti-tumor compounds with a low peroxidating activity (low cardiotoxicity). This problem has been also studied in our group. A typical approach to solve this problem was based on the hypothesis that the generation of AOS depends on a flavine enzyme mediated one-electron reduction of an anthraquinone system to a semiquinone anion-radical [1]. Non-enzymatic reoxidation of these radicals by a molecular oxygen generates AOS (Figure 2). As a result of this hypothesis the new anti-tumor compounds have been screened among anthraquinones with a high redox potential. However, results of such screening have not been satisfactory. Moreover, a few derivatives of the anthracyclines have been found which exhibited low cardiotoxicity in spite of the

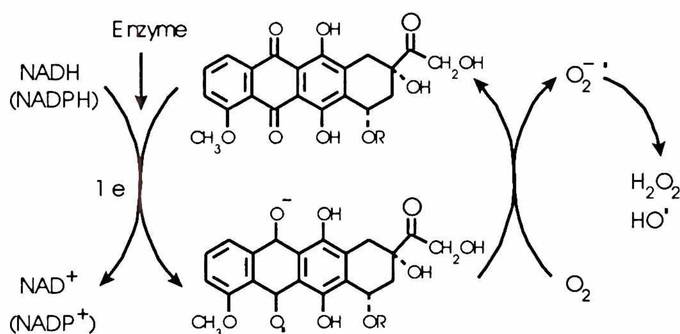


Figure 2. *A commonly accepted mechanism of anthraquinone mediated generation of AOS.*

same redox potential as that of the native antibiotics.

In such a situation we developed a new hypothesis, concerning the molecular mechanism, by which anthraquinones mediate the generation of AOS. During our studies, we observed two new phenomena which allowed us to put forward such a hypothesis. The molecular modelling was successfully used in these studies. The hypothesis explains peroxidative properties of all known anthraquinone agents.

2.2. Complex with a Singlet Oxygen

At the end of eighties, jointly with Chemistry Institute of University of Gdansk, we carried out quantum-chemistry calculations relating to mechanism of one-electron reduction of the anthraquinone system of the anthracycline antibiotics. These calculations allowed us to propose an alternative mechanism of the generation of AOS mediated by the antibiotics (Figure 3).

Semi-empirical calculations (CNDO/2) have shown [2] that some anthraquinone systems are able to form a stable complex with an oxygen molecule

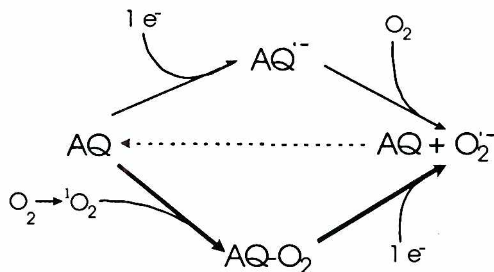


Figure 3. Two alternative paths on which anthraquinones (AQ) may generate active oxygen species ($O_2^{\cdot-}$). The path with singlet oxygen ($^1O_2$) has been proposed by us on the basis of the quantum-chemistry calculations [2].

in a singlet state (Figure 4). The redox potential of the complex is significantly lower than that for the free anthraquinone. It is clear from the calculations that an electron binds to the oxygen part of the complex, with its simultaneous dissociation to a super-oxide anion-radical and a free, unchanged anthraquinone. Thus, on this path the semi-quinone anion-radicals are not formed as an intermediate.

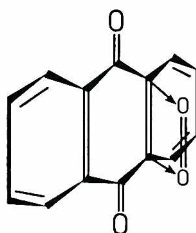


Figure 4. A proposed structure of anthraquinone-singlet oxygen complex [2].

As a consequence, the one-electron transfer occurs rather by the reduction of the complex (bottom path in Figure 3) than by the reduction of the anthraquinone system (upper path in Figure 3).

The path of one-electron transfer, suggested by the quantum-chemistry calculations, was experimentally confirmed by an electrochemical reduction of solutions of the anthracycline antibiotics in aerobic conditions.

Further calculations revealed that the complex with the singlet oxygen is formed when the anthraquinone moiety is characterised by:

— assymmetric distribution of an electron density

— high electron density in the quinone system

The anthraquinone antibiotics such as daunorubicine or adriamycine fulfil these conditions (Figure 5). Based on these rules we can eliminate some anthraquinone systems, at the design step, without the need of chemical synthesis and biochemical evaluations.

More detailed calculations indicated that the complex is formed when a dipole moment may be induced in the singlet oxygen molecule [3]. Such induction occurs effectively only when a component of a dipole moment of the anthraquinone,

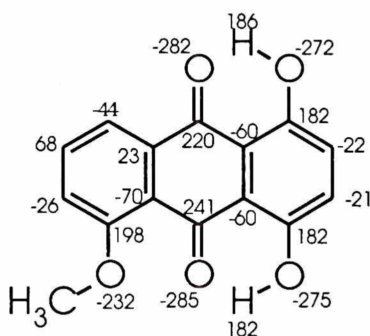


*Figure 5. A partial charge distribution (as 1000*e) in model compound related to π-electron system of daunorubicine and adriamycine [2].*

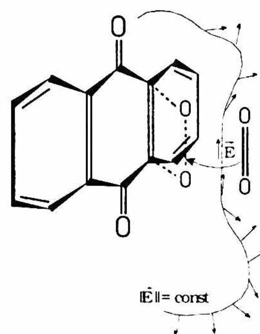


Figure 6. An orienting effect of an anthraquinone electrostatic field on the oxygen molecule during complex formation [3].

parallel to the quinone axis, has sufficiently high strength, and an electrostatic field of the anthraquinone molecule could orient inducing dipole of the singlet oxygen parallel to the quinone system (Figure 6).

2.3. Substrate Properties

The anthraquinone compounds, which do not fulfil above mentioned conditions, do not form complex with the singlet oxygen. However, such compounds can also generate AOS by direct one-electron reduction to semi-quinone anion-radicals (Figure 3). This path of the AOS generation is not so effective like reduction of the complex with singlet oxygen, but it occurs. Further diminishing of a productivity of this path, or even its elimination, needed more information about the factors which determine yield of the path. We found that the enzymatic catalysis is such an important factor. Without the enzymatic catalysis the electron transfer does not occur, even for species with a relatively low redox potential, such as complexes with the singlet oxygen. This observation strongly suggests that some anthraquinones, and/or their complexes with oxygen should be substrates for oxydoreductases. We carefully studied this problem for one of such enzyme: the NADH dehydrogenase. This enzyme plays main role in the anthraquinone mediated generation of the AOS.

We have shown experimentally that anthraquinones, which exhibit peroxidating activity, are substrates for this enzyme and exhibit affinity to the same binding site as does ubiquinone (Figure 7) — natural substrate for the enzyme [4]. This fact allowed us to extend our hypothesis [5], and to state that not only a redox potential but also substrate properties of the anthraquinones are factors determining their ability to generate the AOS. According to this complete hypothesis, an anthraquinone compound would stimulate generation of the AOS if it is a good substrate for the enzyme and is bound to the enzyme active site. On the basis of the structure — activity relationships, observed for derivatives of the anthracycline antibiotics as well as for some model compounds, a number of structural criteria which must be fulfilled by anthraquinone compound to be a good substrate for

NADH dehydrogenase, has been identified in our Laboratory [5]. We have also shown that the decrease of a peroxidative activity of some daunorubicine derivatives is a result of their low affinity to the dehydrogenase.

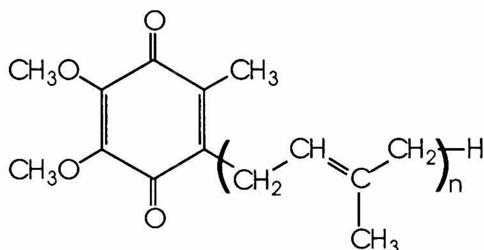


Figure 7. *A chemical structure of an ubiquinone — a natural substrate for the NADH dehydrogenase.*

However, one of the synthetic analogues of the Daunorubicine — Mitoxantrone (Figure 8) — seemed to be an exception to these rules. Mitoxantrone fulfils all identified structural requirements to be a good substrate for NADH dehydrogenase, but its ability to generate the AOS is extremally low. This does not result from the chemical structure of its anthraquinone moiety because 1,4-dihydroxy-5,8-diaminoanthra-quinone (Mitoxantrone without side chains) is a good mediator of one-electron transfer.

This problem is interesting from theoretical point of view and very important from practical one. To solve it we analysed a molecular shape of Mitoxantrone and its close analogue Ametantrone (Figure 8) by the molecular dynamics simulations. The simulations were performed by the programs from GROMOS 87 package in a hydrophobic environment, which is probably present in an enzyme active site [6].

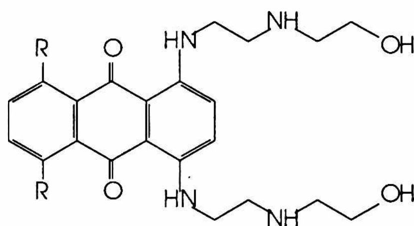


Figure 8. *Chemical structures of synthetic anti-tumor anthracenediones.*

The simulation results indicated that side chains of the studied molecules may exist in a few different conformational states (Figure 9). This result was not surprising as we expected such a situation. However, the limited number of these conformational states was not to be expected. The results suggested that functional groups, present in the side chains, should take part in some interactions which limit the number of the

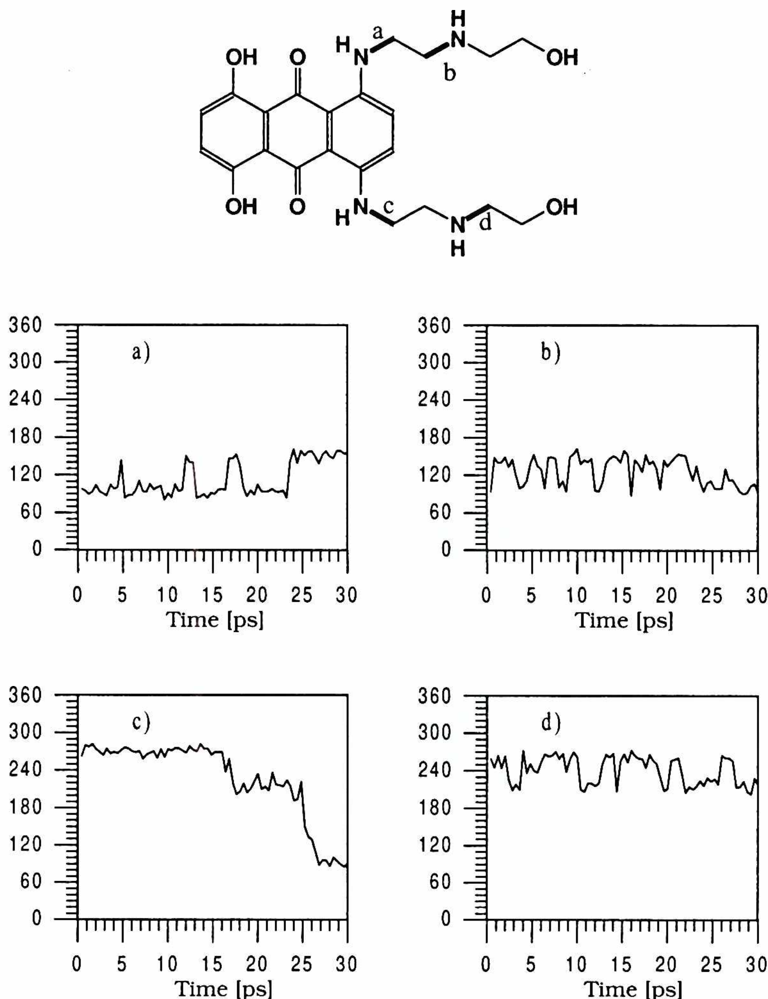


Figure 9. *Time dependence of torsion angles deciding of conformational state of Mitoxantrone side chains [6].*

side chains conformational states. The chemical nature of the side chains functional groups strongly suggests that the interactions may have the character of a hydrogen bonding.

Detailed analysis of hydrogen bonds, observed during simulations, revealed that in such molecules there exist two types of interactions which determine the overall shape of the molecule. These hydrogen bonds are formed between:

i) aliphatic hydroxyl groups from the two side chains. The bonds were observed during more than 80% of the simulation period.

ii) aliphatic hydroxyl and/or amino groups from the side chains and oxygen atoms from the quinone system. The stability of these bonds varied from 15 to 95% of the simulation period.

The both types of interactions may diminish similarity of the studied compounds to the ubiquinone. The two side chains of the molecule have to be simultaneously on the same side of a mean plane of the anthraquinone system to form the hydrogen bond between their terminal hydroxyl groups. Formation of such a bond not only stabilises the side chains conformation but also changes the overall shape of the molecule (Figure 10). The isolated anthraquinone moiety is nearly planar and has a thickness of about 0.3 nm, whereas drug molecule, in which this hydrogen bond exists, has a thickness of about 0.8 nm. Taking into account that ubiquinone, the natural substrate of the enzyme, is a planar molecule with a thickness of 0.3 nm, we could suppose that molecule, with a shape resulted from our simulations, should not fit to the enzyme active site.

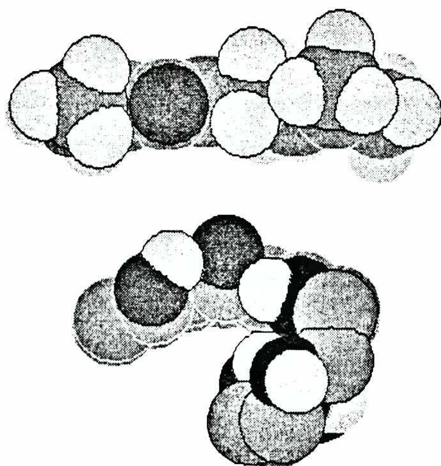


Figure 10. *A comparison of molecular shapes of ubiquinone (top) and Mitoxantrone (bottom) [6].*

Also a formation of hydrogen bonds between the side chains and the quinone oxygen atom, second type of observed intramolecular interactions, may significantly decrease susceptibility of the quinone system to the enzymatic reduction (Figure 11). Such interactions may form significant steric hindrance in an axis of the quinone system.

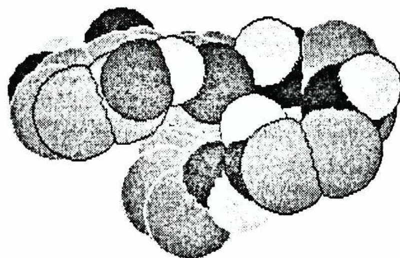


Figure 11. *The hydrogen bond between terminal hydroxy group and quinone oxygen forms steric hindrance [6].*

2.4. Interactions with DNA

The knowledge about molecular mechanisms responsible for the main side effects of the anthraquinone anti-tumor drugs is the goal of molecular modelling studies presented above. Such knowledge is very important during design of new drugs. However, one must still remember about the molecular mechanisms of a main mode of action of these drugs: their anti-tumor activity. There is a well--founded assumption that structural requirements for strong interaction with DNA are different than those for the enzymatic reduction. Thus, effective design of new drugs with high anti-tumor activity and low cardiotoxicity is possible but needs information about the both mechanisms.

Despite the efforts of intensive chemical and biological research, the molecular mechanism responsible for the anti-tumor activity of anthraquinone derivatives has not been fully clarified. The overall picture of the data available strongly suggests that the ability to bind to DNA is a necessary though not a sufficient condition for the drug activity. It is widely accepted that an intercalation of the drug molecule between base-pairs of DNA is a first and very important step of that interactions.

To determine factors responsible for the stability of the intercalation complex one needs information about its structure. There are known structures of complexes between some anthracycline antibiotics and DNA fragments of different length [7, 8]. The structures of the complexes have two common features:

i) a long axis of the anthraquinone system is nearly perpendicular to the direction of the hydrogen bonds in both neighbouring base-pairs

ii) the sugar moiety of the drugs is located in the minor groove of the DNA double-helix.

For the synthetic anthracenediones, Ametantrone (AMT) and Mitoxantrone (MIT), such information is not available. We have been carrying out since '94, in cooperation with the group from Department of Chemical Sciences of University of Camerino (Italy), molecular modelling studies on the structure of an intercalation complex of 12 base-pairs DNA fragment with anthracenediones and their synthetic analogs. The molecular dynamics approach has been used to simulate a behaviour of the complex [9]. During the simulations a complex is placed in a periodic boundary box filled with counter-ions and about 1 700 molecules of water.

A rationally selected set of different starting orientations of the AMT molecule in the DNA intercalation cavity was established. Energies of different types of drug-DNA interactions were monitored during the dynamics simulations for each starting geometry. We showed [9] that the best levels of the interactions are observed when both side chains are located in the minor groove of DNA (Figure 12).

Further dynamics simulations of AMT as well as MIT revealed that side chains of the two drugs fit tightly to the helical shape of the minor groove. The fitness is so good that the functional groups of the chains could interact not only with phosphates and a sugar moiety but also could form hydrogen bonds with the base-pairs.

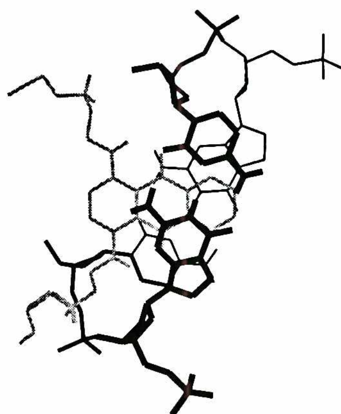


Figure 12. The best starting geometry of the AMT-dodecamer intercalation complex obtained in pre-simulation step [9]. The AMT molecule is shown in green. The major groove is on the right. For clarity the intercalator and two neighbouring base-pairs are presented only.

Overall structures of the intercalation complexes formed by the two drugs are very similar but some subtle differences of the drug orientation in the cavity, as well as of conformation of the side chains, were observed (Figure 13). In the MIT-DNA

AMETANTRONE MITOXANTRONE

Figure 13. Composite of 40 snapshot geometries of the anthracenedione — dodecamer complexes obtained during main simulations [9]. The phosphorus atoms in each snapshot have been fitted to their positions in the first snapshot. The sodium cations and water molecules are omitted for clarity.

complex the anthracenedione part of the intercalator molecule is parallel to the neighbouring base-pairs and the side chains are helicaly twisted. Such orientation of the intercalator molecule results in the best interactions with DNA. However, in the AMT-DNA complex the anthracenedione part is significantly inclined (of about 13) in regard to the neighbouring base-pairs. As a result of the inclination the side chains might fit to the minor groove without helical twisting, but intercalation with DNA is not so strong as in the case of MIT complex.

These differences may explain well known difference in the affinity of the two compounds to DNA.

Currently, we try to use similar approach to other synthetic anti-tumor anthraquinones and their analogs.

2.5. Conclusions

Our research program on a rational design of synthetic anthracenediones with anti-tumor activity and devoid of peroxidating properties (cardiotoxicity) is of multidisciplinary character. The molecular modelling is an essential and very helpful component of this program. In this revue paper the contribution of this approach to our general program has been outlined.

The molecular modelling methods allowed us to conclude as follows:

i) electron affinity of anthraquinones is markedly increased when assymmetric substitution of π electron moiety of the molecule, causing the asymmetric distribution of electron density and thus creating the dipole, leads to the formation of the anthraquinone-singlet oxygen complex, better electrons acceptor than the anthraquinone itself. This finding constitutes an essential part of our novel hypothesis of the molecular mechanism of the mediation of one electron transfer by anthraquinones.

ii) the presence of side chains in the anthracenedione molecules, bearing particular functional groups and moieties, e.g. mitoxantrone (MIT), may lead to the formation of a network of hydrogen bonds stabilising the particular conformation of the molecule. Such interactions may form significant steric hindrance in the axis of the quinone system, thus causing the perturbation in the contact of the drug with the NADH dehydrogenase catalysing the electron transfer.

The recognition of molecular basis for the rational design of non-peroxidating anthracenediones should be paralleled by the identification of structural factors allowing for the preservation of the affinity of a drug to DNA, indispensable for its anti-tumor activity.

Our calculations have shown that the presence of hydroxyl groups, attached to the anthraquinone moiety of the MIT molecule, does not result in additional interactions between the intercalator and the neighbouring base pairs. These groups, however, are essential for the increased affinity of the drug to DNA, because they force the intercalator molecule to more parallel orientation inside the intercalation cavity.

3. Amphotericin B

An antifungal antibiotic Amphotericin B (AMB) is one of the main chemotherapeutic agents clinically used in the treatment of systemic fungal infections. AMB, Figure 14, induces lethal membrane permeability changes of cells containing sterols in their membranes.

An increase of membrane permeability for monovalent ions, as well as for small polar organic compounds up to glucose, is observed as a result of an AMB action. A molecular mechanism, by which AMB induces the permeability changes, is still not clearly understood. A hypothesis suggested more than 20 years ago by DeKruijff and Demel [11] is widely accepted. According to these authors 8 molecules of AMB together with 8 molecules of membrane sterols (Figure 15) form a cylindrical complex which penetrate the cell membrane. In a central part of the complex a hydrophilic channel (aqueous pore) is formed. A movement of water, monovalent ions, and small polar organic molecules is possible through this channel due to concentration gradients.

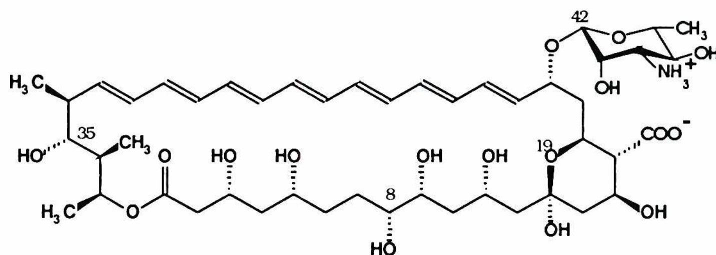


Figure 14. Chemical structure of AMB determined by X-ray analysis [10].

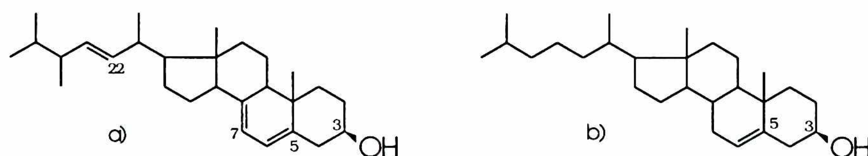


Figure 15. Chemical structures of the main membrane sterols: a) ergosterol in fungal cells, and b) cholesterol in mammalian cells.

An outside part of the complex, containing an apolar part of AMB molecules as well as sterol molecules, is highly lipophilic and exhibits high affinity to the hydrophobic part of a cell membrane.

A permeabilizing channel is formed in a cell membrane only when sterol molecules are present in the membrane. The fungal as well as mammalian cell membranes contain sterols, thus AMB induces the permeability in the two types of cells. Ergosterol (Figure 15a) is the main sterol of fungal cell membranes, whereas cholesterol (Figure 15b) is the main sterol component in mammalian cell membranes. This difference in membrane composition is the basis of the selective toxicity of AMB. However, the differential interaction of the native antibiotic with the pathogen

and host cells is relatively small and toxicity of AMB in regard to the host cells is the important limitation of AMB clinical use.

The extremely low water solubility of the AMB is an additional limitation of its use in the treatment of systemic fungal infections. An intensive search for new AMB derivatives with improved solubility and decreased toxicity has been carrying out in our Laboratory for a number of years. We have used a rational approach based on recognition of the molecular nature of physicochemical and biological properties of AMB. Molecular modelling techniques have played an important role in this work.

3.1. Single Molecule of AMB

Up to now there are no experimental data concerning the conformation of the AMB molecule. Experimental data are available only for AMB derivatives:

i) X-ray data for the N-iodoacetyl derivative [10]

ii) 2D NMR data for the metoxycarbonylmethylamide derivative [12]

In two cases very similar results were obtained (Figure 16). The macrolide ring forms nearly planar rectangle with an approximate size 1.9 nm × 0.55 nm. Nearly all hydroxyl groups, attached to the macrolide ring, exist in a pseudo-axial position on the same side of the ring. The aminosugar piranosyl ring is located out of a mean plain of the macrolide ring. This similarity may suggest that also native antibiotic has similar conformation. However, the molecule of AMB contains in physiological conditions two charged functional groups (Figure 14):

i) a carboxylate anion attached to the macrolide ring

ii) a protonated amino group of the aminosugar.

The presence of the charged functional groups may significantly influence the conformation of the native antibiotic molecule. In the two derivatives, which conformation was experimentally determined, only one charge was present.

In such a situation we started our molecular modelling study from a conformational analysis of AMB and a rationally selected set of its derivatives.

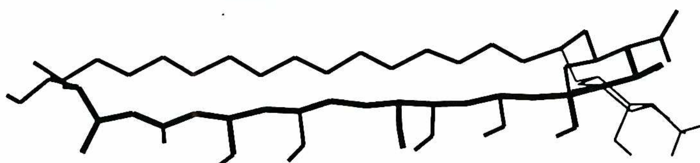


Figure 16. *3D-Structure of AMB suggested on the base of X-ray [10] and 2D NMR [12] studies.*

3.1.1. Conformational analysis

Results of earlier calculations suggest that the macrolide as well as the aminosugar rings are relatively rigid [13, 14]. We focused our attention on the orientation of the aminosugar ring in relation to the macrolide one. This orientation

determines an overall shape of the molecule and space distribution of the main functional groups.

Molecular mechanics (MM2P parametrization) and semiempirical (AM1) calculations of neutral and zwitterionic forms of AMB in vacuum revealed that aminosugar moiety may exist in several low-energy conformations [15]. Only one of them (for the zwitterionic and the neutral forms of the antibiotic) was observed experimentally.

3.1.2. Distribution of electrostatic potential

We performed calculations of the molecular electrostatic potential (MEP) for the zwitterionic form of AMB in vacuum and in condition mimicking water as well as water/membrane environment [16]. The MEP distribution around a AMB molecule is very complex, but qualitatively the same in all environments studied.

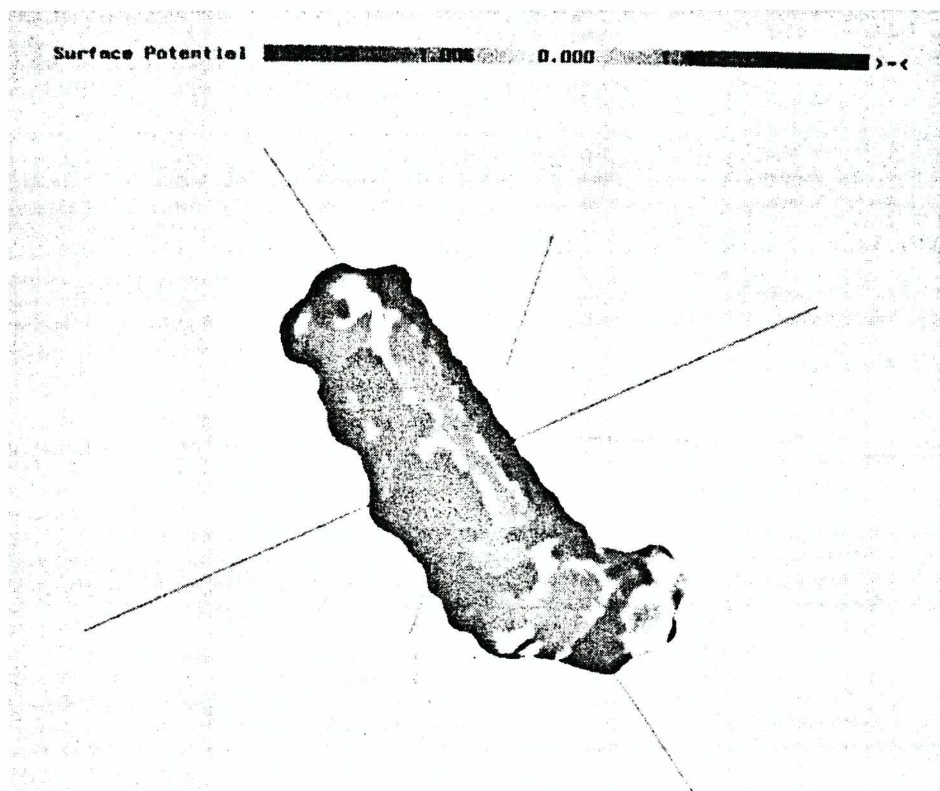


Figure 17. *The distribution of electrostatic potential on the molecular surface of AMB for water/membrane environment [16].*

The polar head of the molecule has a positive area of MEP next to the protonated amino group and a negative one close to the carboxylic anion. Negative areas correspond also to the hydroxyl groups. The MEP distribution brings also completely new information about the system of conjugated double bonds. For years this system was regarded as a purely hydrophobic one. However, we found that

there is a large negative area along this part of the molecule. It means that the hydrophobic/hydrophilic pattern of the AMB molecule is much more complicated than the one expected. It also means that the system of conjugated double bonds may interact with other molecules by electrostatic interactions.

3.1.3. Dynamics of the AMB molecule

AMB reveals its biological activity in a water environment. It looks also that water molecule(s) contributes in the AMB/sterol complex. Thus, analysis of interactions between AMB and water molecules is necessary to understand molecular properties of the antibiotic studied.

The analysis was done by means of molecular dynamics simulations (GROMOS87 package). The AMB molecule in zwitterionic form was immersed in rectangular periodic boundary filled by 354 water molecules [17]. For comparison, parallel simulations were also done in vacuum. During detailed analysis of obtained dynamic trajectories we detected several important features revealed by the system.

Mean structures obtained from vacuum and water simulations were compared on a basis of their dihedral angles. The structure of the aminosugar ring was nearly identical in both simulations and similar to the crystal one.

It is interesting to note that the dynamically averaged orientations of the aminosugar moiety, in relation to the macrolide ring in vacuum as well as in water environment, were very similar to the crystal one. However, significant differences were observed in a dynamic behaviour of two dihedrals determining the aminosugar orientation. In vacuum simulation a high variability of the dihedrals was observed. This variability was not the result of transitions between different conformers. It rather looks that an almost free and continuous change of the dihedral could occur in the interval of more than 100. In water simulation the twisting of these dihedrals was more restricted. We found that around the polar head of the AMB molecule there is dynamic network of hydrogen bonds linking protonated amino group of the aminosugar with the carboxylic anion. Several water molecules participate in the network (Figure 18). The presence of this network significantly decreases the mobility of the aminosugar moiety.

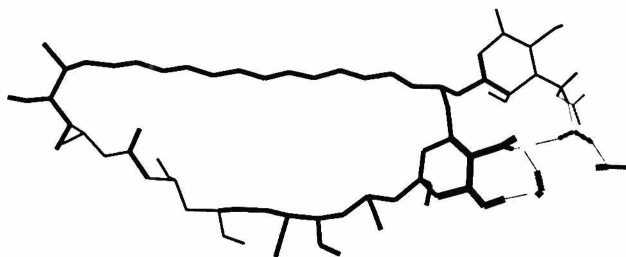


Figure 18. *Representative hydrogen bonds network linking protonated amino group and carboxylate anion (water molecules in grey) [17]. Water molecules which do not contribute to the network are omitted for clarity.*

The macrolide ring was more flexible and sensitive to the type of environment than the sugar ring. Values of several dihedral angles in both simulations were significantly different from the respective values in the crystal structure (Figure 19). While the general shape of the molecule was rather conserved during the simulations, the macrolide ring was able to change dynamically the conformation of its parts. Such flexibility of the macrolide ring, together with a variability of the orientation of the aminosugar moiety, may be important when the AMB molecule takes part in the formation of supramolecular complexes (aggregates, pores). They allow the molecule to create better-packed, better-fitted and more stable structures.

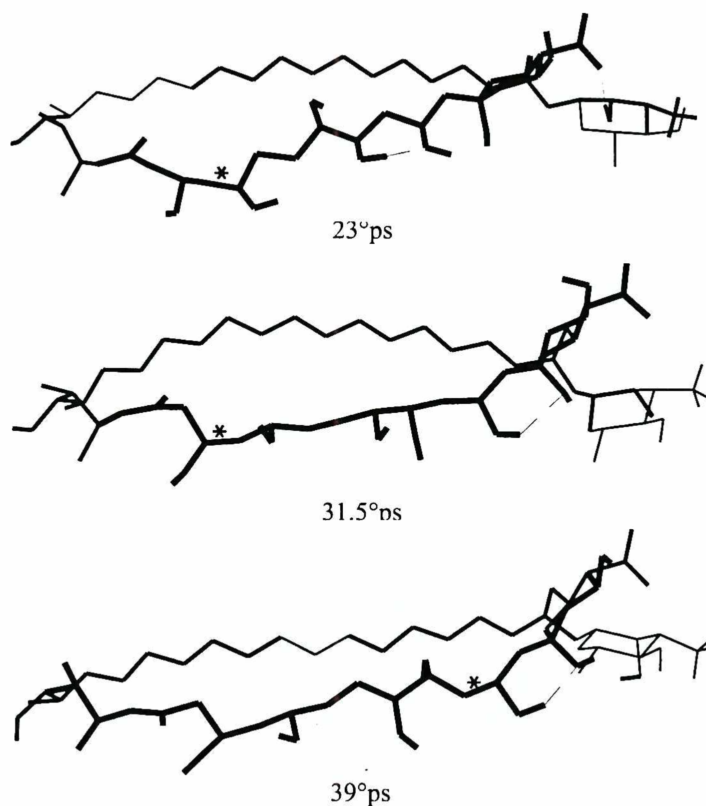


__Figure 19.__ Representative conformers of the macrolide ring during simulation in water [17]. The bonds in which the dihedral angle differs from the crystal conformation is marked by an asterisk.

3.2. Dimer of AMB

The results presented in previous subsection indicate that the AMB molecule is highly amphiphilic. It is also amphoteric. These molecular characteristics provoke AMB to form aggregates in aqueous media which decrease its solubility. The poor water solubility is one of the important factors limiting an application of AMB in the therapy of systemic fungal infections. However, the main disadvantage of AMB is its high animal toxicity. Recent studies show that toxic effects of AMB depend on its

aggregation state [18, 19]. Thus, the examination of aggregation might be of importance for the design of a modified antibiotic with better therapeutic properties.

On the basis of experimental data we have proposed a multi-step model of AMB self-association [20]. In this model we take into consideration the fact that aqueous solutions of AMB and some of its derivatives contain many different species with a molecular weight in range of one thousand (monomer) to a few million (colloid type micelles). Dimerisation of monomers to more hydrophilic dimers should be the first step in the aggregation. The formed dimers may subsequently interact to continue diminishing of the hydrophobicity by the formation of hydrophilic, colloid-type micelles.

There is no experimental evidence of a structure of the dimers or other aggregates. A few hypotheses concerning 3D arrangement of the aggregates were proposed. All of them were based on the analysis or modelling of spectroscopic properties of the aggregates [21-23]. However, the aggregation of amphiphilic and amphoteric molecules in an aqueous medium is a very complicated process, in which hydrophobic and polar substituents play their parts with a delicate balance between the attractive and the repulsive forces. In our opinion molecular dynamics simulation could be one of the approaches which may solve this problem. In addition, our results obtained by this technique for single AMB molecule [17] strongly suggest that the dynamic behaviour of the molecule could be an important factor determining the structure of the aggregate. Thus, analysis of interactions between the two AMB molecules and an appropriate number of water molecules was done by means of the molecular dynamics simulations (GROMOS87 package). The AMB molecules in zwitterionic form were immersed in a rectangular periodic boundary fulfilled by water molecules.

We started our simulations [24] with the three carefully selected initial geometries. After initial step of calculation the geometry with the highest dimerisation energy was selected for the main simulation. Results obtained during the main simulation show that AMB molecules forming the dimer are placed in such a manner that (Figure 20):

— they create a "head-to-tail" system, but the mean value of the angle between long axis of the chromophores differs significantly from 180°

— the mean planes of the macrolide ring of both AMB molecules are near parallel with the distance of about 0.5 nm

— the polyene chromophores overlap as much as possible to decrease hydrophobic interactions

We also found that several important elements of asymmetry exist in the dimer architecture. They concern a relative orientation of the aminosugar as well as conformations of the macrolide rings. The asymmetry observed in the dimer internal structure corroborate our earlier observation [20], that dimer is responsible for extremely high intensity of the CD spectra of samples containing aggregated forms of AMB.

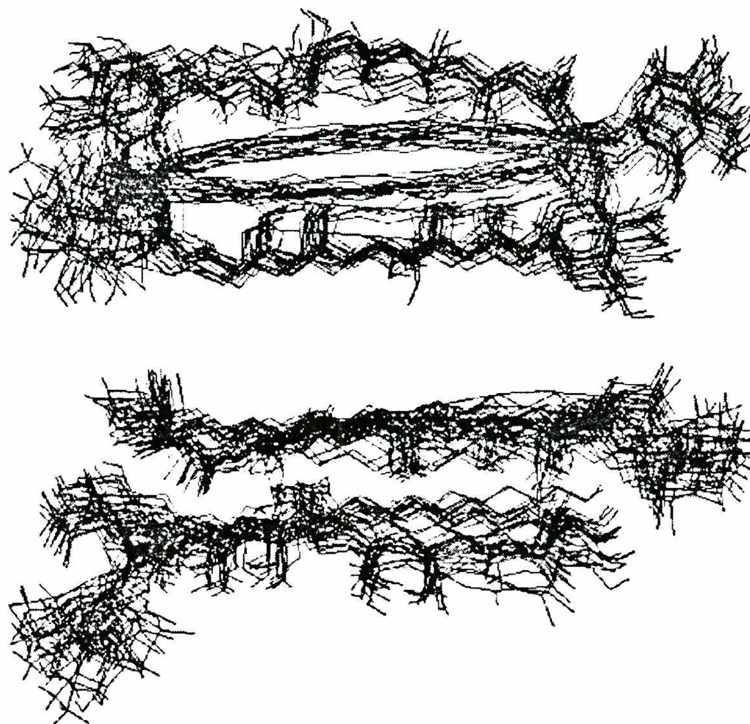


Figure 20. Orthogonal views of 21 snapshots taken from the main dynamic simulation every 1.5 ps [24].

A detailed analysis of intermolecular interactions indicate that dimerization driving-forces originate not only from hydrophobic interactions but also from attractive electrostatic interactions between AMB molecules. The electrostatic interactions, directly or mediated by water molecules, stabilise the dimer structure and are responsible for its specific asymmetric architecture.

A near antiparallel arrangement of AMB molecules arises not only from interactions between rigid chromophores but also from stabilising effects of hydrogen bond networks which exist between the polar terminals of antibiotic molecules. The key fragment of one such network is formed by the terminal 35-OH" group and aminosugar oxygen O42' (Figure 21). Intramolecular hydrogen bonds between 43-OH' and the carboxyl group of AMB', as well as between carboxyl group and 15-OH' supported by hydrogen bonds with water molecules, complete this network. On the opposite side of the dimer, water-mediated interactions between 35-OH' and the carboxyl group of the AMB" form the second network. Geometries of these networks determine, in our opinion, the stability and the architecture of the dimer.

3.3. Conformation of Sterols

It has been generally accepted that selective toxicity of AMB is based on a difference in the affinity of the drug for ergosterol and cholesterol. However, high

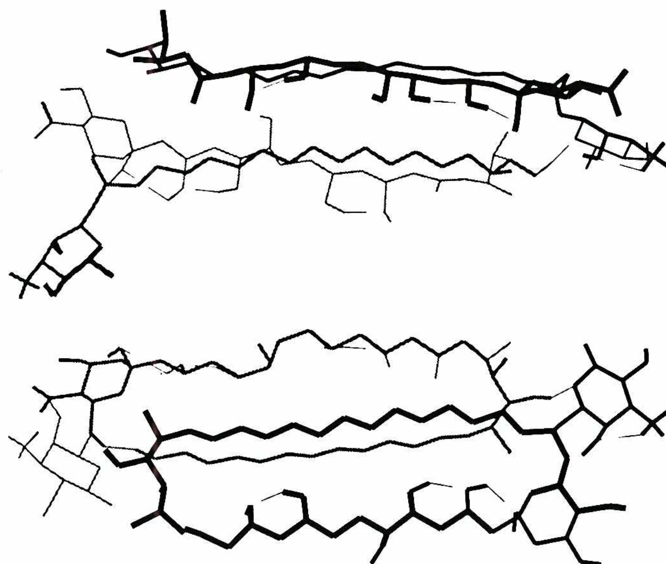


Figure 21. Orthogonal views of snapshot representative for interactions between AMB' (black) and AMB" (grey) [24]. Dashed lines represent hydrogen bonds.

similarity of the structures of both sterols (Figure 15) results in high toxicity of AMB. In addition, the molecular basis for the preferential binding of AMB to ergosterol, compared with cholesterol, is still unknown. In such situation we decided to identify molecular properties which differentiate the two sterols. In our opinion, two such properties could play the most important role: the shape of a molecule, and distribution of the molecular electrostatic potential (MEP).

The importance of the shape of a sterol molecule, particularly a conformation of the side-chain, has been pointed out as the main structural feature which may determine differential affinity of AMB. It was postulated that the planarity of the sterol molecule is crucial for high affinity to AMB. We used the molecular mechanics calculations to estimate the most probable orientation of the sterol side-chains in relation to the ring system [25]. The flexible side-chain of cholesterol may exist in three, practically isoenergetic, conformations. Only in one of them the coplanarity of the side-chain and the ring system occurs. In case of the more rigid side-chain of ergosterol two low energy conformations have been found. The conformations slightly differ in their energy level. The conformation with a lower energy is practically planar, the side-chain in the second one is bent out from the mean plane of the ring system. However, the difference in their energy is in a range of thermal movement at the physiological temperature.

We also used the molecular dynamics simulations to determine a preference in the conformational state of different sterols [26]. These simulations also exhibited that the two sterols may exist in physiological conditions in the planar as well as bent conformations. Only small, quantitative difference between occupancy of both types of conformations were observed. In our opinion the difference is insufficient to

explain the observed difference in affinity of AMB to the both sterols.

We also carried out a comparative analysis of the distribution of MEP for both sterols by the semiempirical CNDO/2D method for a planar conformer of each sterol [27]. The results obtained revealed the existance of subtle but qualitative differences between ergosterol and cholesterol in the distribution of MEP around the side-chain of the sterols. The number of MEP minima in this area is higher for ergosterol than for cholesterol. The most important difference results from the existance of a double bond in the ergosterol side-chain. In spite of fact that the depth of the minima is similar for both molecules and not lower than about -4.0 kcal/mol an electrostatic „envelope", described by the MEP distribution, is richer and more negative in the case of ergosterol.

These results were recently confirmed by calculations of electrostatic potential on molecular surface in physiological environment using the Poisson-Boltzmann method [16]. We found that the both sterols studied have quite different pattern of an electrostatic potential of the side-chains (Figure 22).

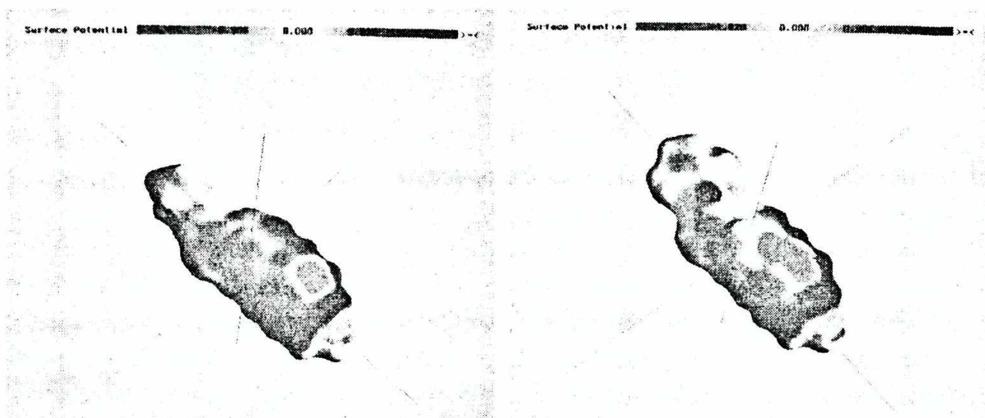


Figure 22. *The distribution of an electrostatic potential on the molecular surface of cholesterol (left) and ergosterol (right) for water/membrane environment [16].*

3.4. AMB-Sterol Complex

According to the suggestion of DeKruijjf and Demel [11] the water channel responsible for AMB inducing changes in the membrane permeability, consists of 8 molecules of AMB and 8 molecules of the sterol. However, the authors did not indicate functional groups nor a type of interactions responsible for the architecture and stability of the channel.

During last ten years we have developed the two conceptual models of the AMB-sterol complex. In this endeavour we focused our attention on interactions between a polar head of the antibiotic molecule and a hydroxyl group of the sterol [28, 29]. The both models concerned the primary complex consisting of 1 molecule of AMB and 1 molecule of the sterol. Interactions responsible for assembling the primary complexes into the channel were not described in these models.

The first reasonable molecular model of the channel was proposed by Khutorsky

[30] at the beginning of '90. The model was built from rigid molecules of AMB and cholesterol with a strict 8-fold symmetry. The AMB molecules play the most important role in this model. The function of cholesterol molecules is limited to a role of filling. The conclusion, that electrostatic interactions between ammonium cation of one AMB molecule and carboxylate anion of the neighbouring one are mainly responsible for the stability of the channel, was the most important result of this simplified calculations. Taking into consideration results of our molecular dynamics simulations of AMB [17, 24] and sterol molecules [26] such rigid model of the complex appeared to be insufficient. In addition, the Khutorsky's model can not be directly used to explain stability of the channel formed with some derivatives of AMB and do not explain differential affinity of AMB to ergosterol and cholesterol.

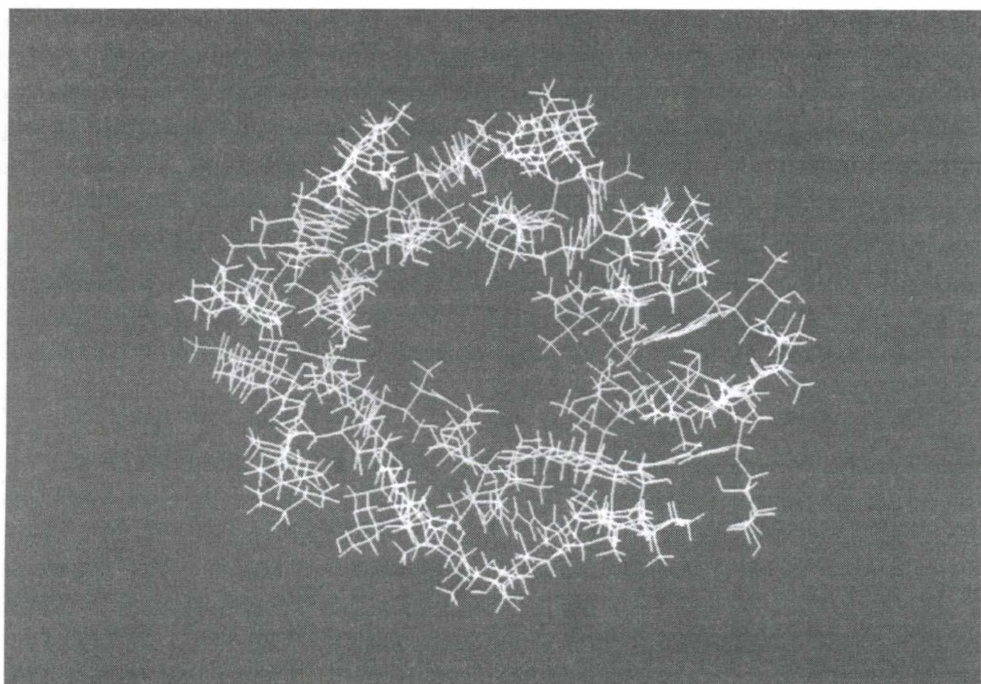


Figure 23. *A typical snapshot of the AMB-cholesterol channel showing nonsymmetric structure of the pore [32]. Magenta, AMB molecules; cyan, cholesterol molecules.*

In such situation we have decided to create dynamic models of the primary complex as well as of the whole channel. We started dynamics simulations of the primary complex from system consisting of one molecule of AMB and one molecule of the sterol (cholesterol or ergosterol) in water environment [31]. During simulations of such binary complexes we obtained a number of different complex architectures depending on starting geometries. The complexes were relatively unstable and during long simulations (>100 ps) exhibit a tendency to disintegration. No stable interactions were observed during the simulations of such complexes.

Primary complexes containing two AMB molecules separated by the sterol molecule exhibit totally different dynamics behaviour. Characteristic, stable

interactions between AMB molecules result in a well characterised architecture of the complex. The results obtained suggested that the electrostatic interaction between an ammonium cation of one antibiotic molecule and a carboxyl anion of the other one decided of the complex stability. We also identified some intermolecular hydrogen bonds which increased the complex stability and decided of its architecture. One of such characteristic interaction occurs between the O15 hydroxyl group attached to the macrolide ring and the O43 hydroxyl group from aminosugar moiety. Other very important hydrogen bond is formed between the O8 hydroxyl group and several hydroxyl groups from other AMB molecule. This system of intramolecular hydrogen bonds was observed during 40 to 90% of the simulation period.

It is interesting to note that sterol molecule interacts directly with antibiotic molecules only by the Van der Waals interaction. The sterol hydroxyl group does not form a stable direct hydrogen bond with the polar groups of AMB molecules. However, we identified indirect hydrogen bonds in which water molecules bridge interacting molecules.

The dynamics of the multimolecular complex of 8 AMB molecules and 8 cholesterol molecules (channel) were simulated in water and in lipid bilayer [32]. Obtained results were very similar. During analysis of the dynamics trajectories we focused our attention on interactions responsible for the stability of the complex. We found that the complex is stabilised by several types of interactions, mainly between neighbouring molecules of AMB.

Our simulations in a membrane environment confirmed the conclusion of Khutorsky as well as our earlier results from primary complex simulations [31], that strong attractive electrostatic interactions between charged functional groups of neighbouring AMB molecules are the major forces stabilising the complex. These interactions form a closed ring at the channel entrance. The ring is formed by seven of the eight AMB molecules (on average). It is very stable and exists throughout almost the entire simulation period. On average, one AMB molecule does not take part in this ring and instead interacts with a nearby phospholipid molecule. Strong electrostatic interaction of this AMB with a phospholipid molecule takes place through the positively charged amino group of AMB and the negatively charged phosphate group of the lipid. The trimethylammonium group of the same phospholipid molecule interacts electrostaticaly with the carboxyl group of the next AMB molecule. Thus, the phospholipid molecule can act as an electrostatic bridge in the AMB/lipid/AMB system.

An additional chains of hydrogen bonds is formed between the O15 hydroxyl group of one AMB molecule and the O43 hydroxyl group in the aminosugar moiety of its neighbouring AMB molecule. These chains have dynamic nature but an average of 5 or 6 such hydrogen bonds are observed.

A next dynamic chain of hydrogen bonds is observed in the central part of the channel. It was found that the out-of-plane O8 hydroxyl groups form intermolecular

hydrogen bonds with either O9 or O5 hydroxyl groups of the neighbouring AMB molecules. The involvement of O8 hydroxyl groups in the intermolecular hydrogen bond chain is due to their favourable orientation, which is opposite the other hydroxyl groups in the polyhydroxyl chain (Figure 14). The formation of the intermolecular chain of hydrogen bonds is supported by mutual shift of the adjacent AMB molecules along the pore axis. In addition, we found that the formation of this bond correlates with the conformation change in the C6-C7 bond. On the basis of our earlier simulations [17, 24] we expected that such conformational changes of the lacton ring could occur. It is noteworthy that we detected the same intermolecular interaction during simulations of the primary AMB/sterol/AMB complex in water [31].

3.5. Conclusions

The major problem with otherwise valuable antifungal drug AMB is its poor selective toxicity (high animal toxicity). Our molecular modelling studies have essentially contributed to understanding of the molecular effects responsible for this undesirable property.

Molecular mechanics and dynamics as well as semiempirical quantum chemical methods have lead us to a recognition of the molecular properties of antibiotic and its membrane targets. It was evidenced that the AMB molecule is not rigid and can acquire various geometries depending on the environment and the presence of interacting molecules. Conformational changes are due to flexibility of the polyoxygenated side of the macrolide ring as well as to the rotation of the amino sugar moiety.

The analysis of MEP's of the antibiotic and sterols molecules essentially contributed to the recognition of the interactions responsible for the formation and stability of the primary AMB-sterol complexes. This information allowed us, on the molecular basis, to put forward the hypothesis of the differential affinity of the antibiotic to ergosterol and cholesterol (Figure 24).

The main forces binding the both complex components are the Van der Waals interactions, which are similar for both sterols. However, for the stability of the complex the orienting forces are essential. To properly orient the complex constituents, the presence of at least two attach points located in proper distance, is indispensable. One of them is the hydrogen bonds network generated by the protonated amino group of the aminosugar moiety. The OH group of sterols participates in this network. The second attach point, stabilising the complex, is formed by the interaction between the high electron density region of the ergosterol side chain and the appropriate area (chromophore) of the antibiotic molecule. In case of cholesterol this second attach point is lacking.

The above findings contributed essentially to our multidisciplinary research program which succeeded in the rational design of modified AMB with a markedly improved selective toxicity [33].

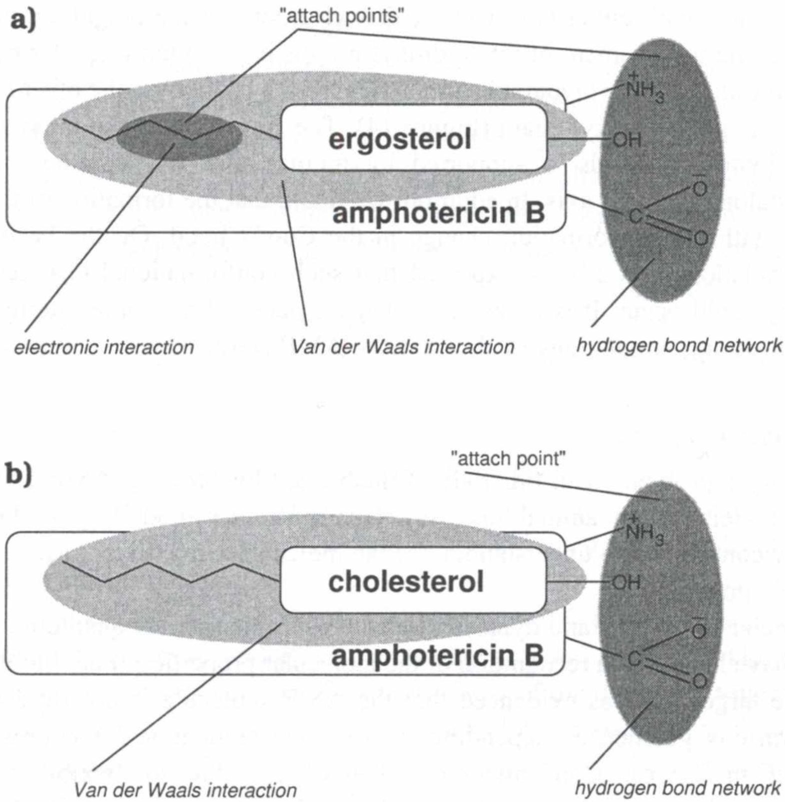


Figure 24. The conceptual models of the AMB-ergosterol (a) and AMB-cholesterol (b) complexes.

4. Inactivators of Glucosamine-6-Phosphate Synthase

A great biochemical similarity between host and pathogen cells is the main problem in the design of chemotherapeutic agents for the treatment of systemic fungal infections. The presence, in fungal cells, of a cell wall is one of few qualitative differences observed between both types of cells. Thus, selective inhibitors or inactivators of enzymes of cell wall biosynthesis might be potentially very promising drugs for the treatment of systemic fungal infections.

Results of recent studies indicate that glucosamino-6-phosphate synthase (EC.2.6.1.16) is one of the most important enzyme in the microbial cell wall biosynthesis. This enzyme catalyses one of the early steps in the biosynthesis of aminosugar containing macromolecules: glycoproteins, mannoproteins, chitin, peptidoglican, and lipopolisaccharides. The inactivation of this enzyme in microorganisms results in the inhibition of the cell wall formation and, in effect, in fast cell death. In mammalians, in contrary, inhibition of this enzyme has not so dramatic consequences due to a longer life cycle and a slow turnover of a glycoprotein pool.

The glucosamino-6-phosphate synthase (GlcN-6-P synthase) catalyses the transfer of amino group from glutamine to fructoso-6-phosphate with simultaneous

conversion of the sugar to glucosamino-6-phosphate (Figure 25).

Up to now a few antibiotics, which mode of action is based on the inhibition or inactivation of this enzyme, have been found. Active fragments of these antibiotics are glutamine analogs which interact with the active center of the GlcN-6-P synthase. First of them, discovered in our Laboratory in '50, was tetaine. Some synthetic inhibitors and inactivators of this enzyme are also known.

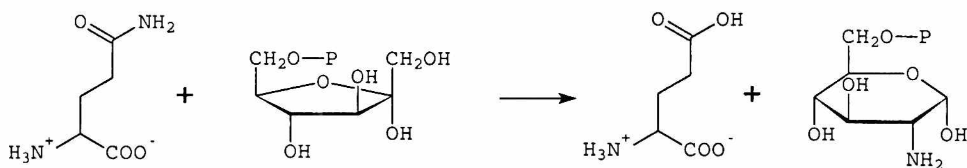


Figure 25. The reaction catalysed by GlcN-6-P synthase.

4.1. Mechanism of Enzymatic Reaction

Earlier studies performed in our Laboratory have shown that the nucleophilic attack of a cysteine -SH group on the γ-carbonyl carbon of glutamine is the first step of this enzymatic reaction. As a results a thioester is formed and the glutamine amino group is transferred to Fru-6-P [34]. All known inhibitors and inactivators of a GlcN-6-P synthase affect this step. We have performed theoretical studies of this reaction by calculating the model reaction because 3D structure of the enzyme is yet unknown. Methanethiol was used as a model of cysteine moiety and acetamide as a model of glutamine.

At the beginning we model the nucleophilic substitution on the carbonyl carbon of the acetamide by means of the semi-empirical (MNDO) and in same part *ab initio* methods [35]. Our calculations have shown that this reaction is possible only when a sulfhydryl group, but not a sulphide anion, is the nucleophilic reactant. A transition complex is formed when a sulfhydryl group takes part in the reaction (Figure 26). This complex decomposes to the methyl thioester of acetic acid and ammonia. In a real enzymatic reaction ammonia is not released but interacts with Fru-6-P. The thioester hydrolyses reproducing cysteine and liberating glutamic acid.

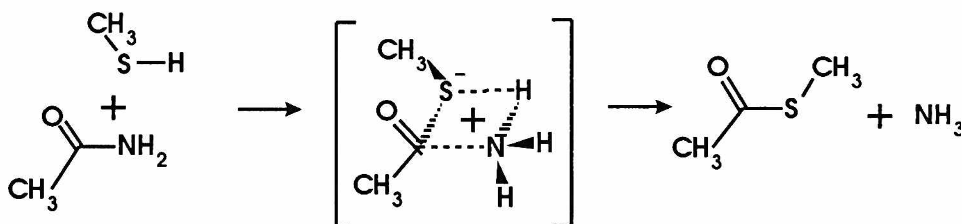


Figure 26. The pathway of reaction between metanethiol and acetamide as a model of the first step of the reaction catalysed by GlcN-6-P synthase [35].

A careful analysis of the model reaction pathway as well as the transition complex structure provide very important information about the reaction studied. The sulphur atom approaches the carbonyl carbon simultaneously with partial decomposition of the S – H bond and formation of the partial H – N bond. This step is connected with an energy barrier of about 50 kcal/mol. The charge distribution in a transition complex is also very characteristic. The negative charge is practically localised on the sulphur atom, whereas the positive charge is delocalised between a carbonyl carbon, nitrogen, and sulfhydryl hydrogen. It is noteworthy that the C = O bond posseses in part a double-bond characteristics. Thus, the reaction may be treated rather as cycloaddition than classical nucleophilic substitution.

Taking into account that the intermediate complex is characterised by a large charge distribution, one may expect that in case of the real enzymatic reaction highly polar inside of the active center should stabilise this state and thus decrease an energy barrier of its formation.

We also studied the pathway of direct transfer of the ammonia from the intermediate state to Fru-6-P [36]. The molecular mechanics calculations indicate that such possibility exists in simple model system with intermolecular stabilising interactions (Figure 27) as well as in more sophisticated systems, taking into consideration possible conformations of the enzyme peptide chain near a cysteine residue.

More detailed modelling of the reaction was not possible without information of the 3D structure of the synthase or its active center. However, even such partial information allowed us to begin modelling of enzyme inactivation.

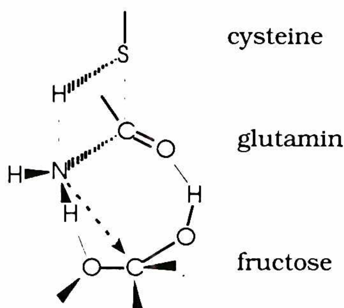


Figure 27. Proposed structure of GlcN-6-P synthase/substrates complex [36]. An arrow indicates postulated transfer of ammonia; dashed lines symbolise hydrogen bonds stabilising the complex but dotted lines bonds of the intermediate state.

4.2. Modelling of Enzyme Inactivation

The antibiotic tetaine, discovered in our Laboratory, was the first antifungal agent which mode of action is based on the inhibition of GlcN-6-P synthase. This fact has promoted a broad biochemical, enzymological, and chemical studies on inhibitors and inactivators of the enzyme. Results obtained in our Laboratory [37] indicated that in fungi the tetaine is hydrolysed by intracellular peptidases. The

C-terminal amino acid of tetain, anticapsin (Figure 31), is a very potent inactivator of the enzyme studied. However, the production of tetaine is very difficult both by fermentation and by a chemical synthesis. Thus, new, more stable and easier to synthesise, inactivators of GlcN-6-P synthase have been looked for.

In '72, Molloya et al. published information about next antibiotic, A 19009, which acts by inhibition of a GlcN-6-P synthase. Also, in this case only part of the antibiotic: ω-amide of N^3-fumaroyl-L-2,3-diaminopropanoic acid (FCDP, Figure 31), acts as an enzyme inactivator. In our Laboratory a series of fumaroyldiamino-propanoic acid derivatives have been designed and synthesised. These compounds appear to be potent and highly specific inactivators of the enzyme studied. Among them the N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP, Figure 31) exhibits the highest potency. This compound was the first our object of modelling the inactivation reaction.

Experimental works done in Badet laboratory [38] have evidenced that the product of the Michael's reaction (1 on Figure 28) is a first step of the reaction between FMDP and cysteine in water solution. This compound, in reaction conditions, undergo cyclisation in a pH dependent manner. A derivative of thiazynon (2) is the main product of cyclisation at pH higher than 6, whereas in acidic solution, in addition, a derivative of succinimide (3) was also found. The succinimide derivative (3) isomerises to a compound (2) in alkaline solution (Figure 28). The structure of obtained products, as well as kinetic study, allowed Badet and co-workers to suggest the two alternative mechanisms of compound (2) formation:

i) one step in which the nucleophilic substitution of a cysteine amino group on a α-carbonyl group of the FMDP fumaroyl moiety occurs simultaneously with a transfer of diaminopropionic moiety on a ω-carbonyl group

ii) two steps in which a succinimide derivative (3) is an intermediate product undergoing intramolecular cyclisation in the reaction conditions.

In our theoretical work in this field we tried to determine which of the mechanisms proposed by Badet is more probable from energetic point of view [39]. We calculated heats of formation in vacuum for the two cyclic products by the semi-empirical PM3 method. The obtained results showed that the succinimide derivative (3) is less thermodynamically stable and its presence in reaction mixture may result from kinetic reasons (to a slow isomerisation in acidic condition). However, the compounds studied contain a number of polar or ionisable groups and the results obtained in vacuum should be treated as a first approximation. Thus, in our further works we will take into consideration a possible role of the polar environment.

FMDP is a strong Michael reagent for sulfhydryl compounds. In consequence, this compounds, as well as its peptides, are inactivated by the serum constituents before they reach fungal cells. Thus, we have looked for the analogs of FMDP with decreased affinity to sulfhydryl groups but with a high affinity to the enzyme. The derivatives of N^3-*trans*-epoxysuccinamoylo-L-2,3-diaminopropanoic acid appear to be such compounds. It is noteworthy, that different structure-activity relationships

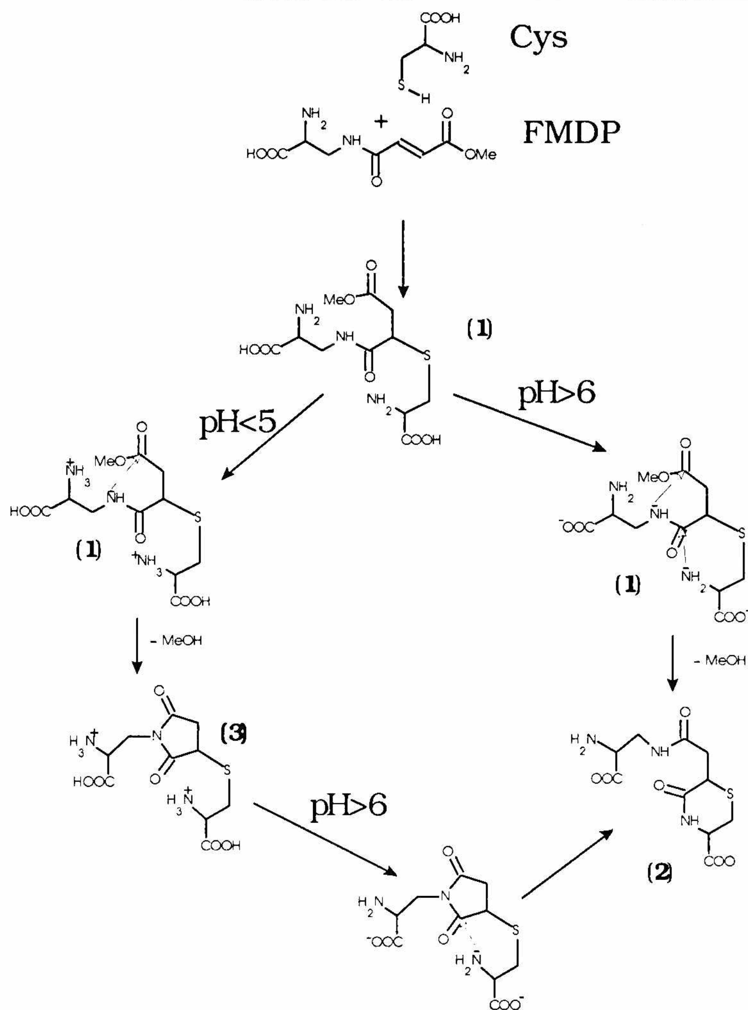


Figure 28. *Suggested mechanisms of the reaction between FMDP and cysteine in water solution [38].*

are observed for the two families of compounds. An unsubstituted ω-amide of N^3-*trans*-epoxysuccinamoylo-L-2,3-diaminopropanoic acid (EADP, Figure 28) is the most active member of this family, whereas an amide, analog of FMDP, exhibits rather poor inactivating ability. This observation suggests that a molecular mode of inactivation may be different for the two groups of selective GlcN-6-P synthase inactivators.

We studied this problem by the molecular modelling calculations. Localisation of intermediate states and products in the reaction pathway was done by the semiempirical PM3 method with option SADDLE [40] To shorten computation time we used the methanethiol as a model of cysteine and methyl-trans-epoxysuccinamide as a model of EADP.

A nucleophilic substitution of -carbon in relation to the amide group by the

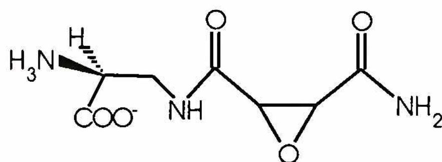
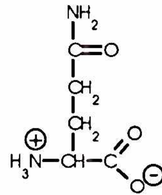


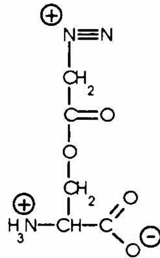
Figure 29. Chemical structure of -amide of N^3-trans-epoxysuccinamoylo-L-2,3-diaminopropanoic acid (EADP) very potent inactivator of GlcN-6-P synthase.

Figure 30. *Chemical structures of intermediate states (in square brackets) and products of a reaction of methanethiole and methyl-trans-epoxysuccinamide [40].*

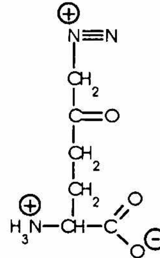


Glutamine

Non-specific inactivators

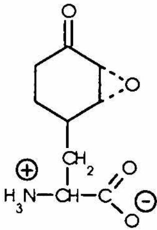


Azaserine

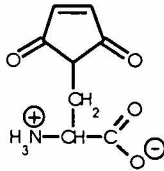


DON

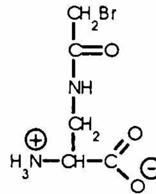
Non-specific inactivators



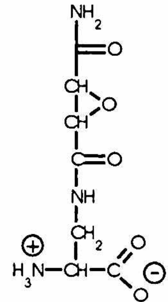
Anticapsin



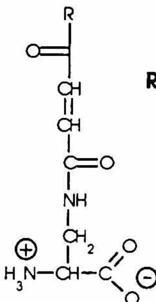
MIDP



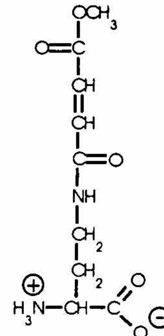
BrAcDap



EADP



R : -NH_2 FCDP
-CH_3 AADP
-OCH_3 FMDP
-OPh FPDP



FMDB

Figure 31. *Chemical structures of glutamine and some known GlcN-6-P synthase inactivators [41].*

sulphur atom is the first step of the reaction. Formation of an intermediate state [IS1] needs relatively high energy and seems to be the factor limiting the whole reaction velocity. An intermediate product IP1 is formed by opening of the epoxide ring. Two different reaction paths are possible due to a particular geometry of the intermediate IP1:

— *path* A with direct proton transfer from the sulphur atom to the epoxide oxygen

— *path* B with proton transfer mediated by the amide group.

When energetic profiles of the two alternative paths are compared, the path A seems to be more probable. However, this path needs significant movement of the cysteine residue in relation to the inactivator. Taking into account that in real situation the cysteine residue is a part of the enzyme, such movement seems to be less probable.

According to the path B, the proton transfer mediated by the amide moiety does not require any conformational changes and seems to be more realistic inside the enzyme active center. In addition, above mentioned structure-activity relationships emphasized a particular role of the unsubstituted ω-amide group. Mono- and dialkyl derivatisation of this group result in a drastic decrease of GlcN-6-P synthase inactivating ability.

4.3. Selective Pharmacophore of Glcn-6-P Synthase Inactivators

There are known a number of natural as well as synthetic inactivators of GlcN-6-P synthase. Chemical structures of some of them are presented in Figure 31. Two of them: the azaserine, and the DON inactivate not only the GlcN-6-P synthase but also some other glutamine dependent aminotransferases. Other inactivators presented in Figure 31. exhibit unexpected selectivity. They inactivate practically only one enzyme: the GlcN-6-P synthase. Recognition of molecular basis of this selectivity should be of significant importance for the design of selective, with low toxicity, antifungal drugs. We tried to elucidate this problem by a molecular modelling.

For this porpoise we used so called the 3D pharmacophore approach. This method assumes that in all compounds, exhibiting affinity to the same target, the same, 3D configuration of some points, critical for this type of activity, exists. A set of distances between these points is called a 3D pharmacophore.

It is known that GlcN-6-P synthase inhibitors or inactivators must have L-α-amino acid system. This factor is indispensable for the binding of the compound to the binding sites in the active center of the enzyme. Thus, we used nitrogen atom of -amino group (point N) and one of the oxygen atoms of carboxyl group (point O) as the two pharmacophore critical points. A carbon atom which is the most probable target for the nucleophilic substitution of the cysteine sulfhydryl group (point C) was chosen as the third critical point (Figure 32). A set of distances between these three points defined a configurational space of the pharmacophore.

The configurational space is divided into hypercubes with a predefined size. Each permissible conformation of the compound studied possesses its representation (hypercube) in the configurational space. The cubes, which represent conformations

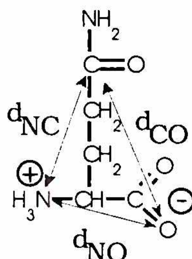


Figure 32. The definition of the pharmacophore for glutamine [41].

permissible for all compounds studied, form the pharmacophore.

Compounds used for the calculation of the pharmacophore were selective inactivators of the GlcN-6-P synthase synthesised in our Laboratory, as well as two typical non-specific inactivators of many cysteine containing enzymes belonging to the amidotransferase class (Figure 31). All these compounds are glutamine analogs acting as active site directed inactivators. Initial 3D structures of these compounds were prepared by means of the molecular mechanics method.

For the first stage of calculations [41] we have chosen the entire set of ten inactivators and glutamine, the natural substrate of the enzyme. The hypercube edge was equal to 0.5 Å. Each of the three pharmacophore distances was constrained to the range of 2-10 Å. As the first structure in the series we used the natural substrate of the enzyme, glutamine, and the initial set of hypercubes was established on the basis of its conformational hyperspace. The whole of glutamine conformational hyperspace was enclosed within 65 hypercubes. As the calculations continued for all remaining molecules this number was consequently decreasing, and on completion of the algorithm, there were only three adjacent hypercubes representing geometries common to each molecule in the series. These hypercubes are presented in red in Figure 33.

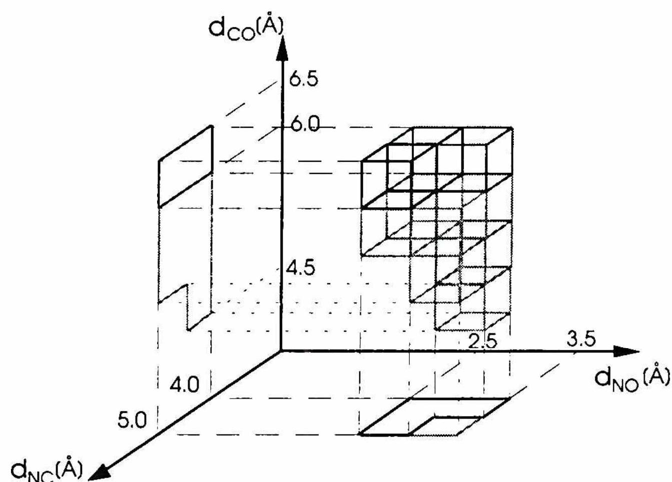


Figure 33. The hypercubes defining pharmacophores of selective inactivators of GlcN-6-P synthase (red) and non-selective inactivators of various thiol enzymes (green) [41].

In order to find out if there is a significant difference between the conformational hyperspaces of specific and non-specific inactivators, we divided the entire set of compounds into two groups and repeated the whole numerical experiment separately for each of them. The first set consisted of glutamine and non-specific inactivators: DON and Azaserine. As the result of calculations for this series a group of ten hypercubes, with a wide range of different distances between pharmacophore, reference points were obtained. These hypercubes are presented in blue in Figure 33.

The calculations for the second group consisted of the glutamine and selective inactivators of GlcN-6-P synthase, resulted in the same three adjacent hypercubes as in the first approach (red in Figure 32).

Two important findings result from a comparison of the two pharmacophores:

— ranges of distances d_{NO} and d_{NC} are the same for the two pharmacophores

— a range of d_{OC} for selective inactivators is a terminal part of the range for non-selective one.

It may suggest that in case of the GlcN-6-P synthase a distance between an orienting and binding centres is particularly large. Glutamine, substrate, as well as non-specific inactivators possess so high conformational lability that might fit to a O-C distance, characteristic for different enzymes. It seems that relatively more rigid molecules of selective inactivators can not adopt conformations in which the O-C distance is lower than 0.6 Å. This situation is schematically depicted in Figure 34.

glutamine and non-specific inactivactors specific inactivactors

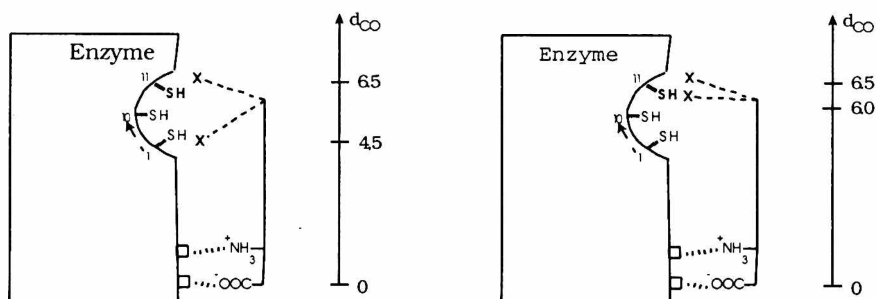


***Figure 34.** The hypercubes defining pharmacophores of selective inactivators of GlcN-6-P synthase (red) and non-selective inactivators of various thiol enzymes (green) [41].*

4.4. FMDP in the Active Center of Glcn-6-P Synthase — Molecular Dynamics Study

Enzyme inactivation is a complex, multi-step process. In case of the GlcN-6-P synthase inactivation by FMDP, before covalent bond formation the inactivator has to be recognised as a glutamine analog, and physicochemicaly bound in a proper

orientation in the active center of the enzyme. The non-covalent binding of the inactivator may be significantly increased by the previous binding of the second enzyme substrate: Fru-6-P. The binding of Fru-6-P induces conformational changes of the enzyme which facilitate binding of glutamine or its analogs [34, 42]. The last step of the inactivation is the formation of a covalent bond between inactivator and the cysteine residue of the enzyme.

Recently, a X-ray structure of glutamine domain of GlcN-6-P synthase from *E. coli* has been presented in Brookhaven Protein Data Bank (entry: 1 GDO). However, it is only a part of the bacterial enzyme. We decided to use this data to analyse the behaviour of selected inactivators in the active center of the synthase.

The pharmacophore of selective inactivator, determined above, is necessary but not sufficient condition for high activity. A serine analog of FMDP: O-FMSer (Figure 35), presents an example of glutamine analog which fits to the pharmacophore, but exhibits no activity against GlcN-6-P synthase. We simulated behaviour of these compounds in the active center of the synthase studied [43].

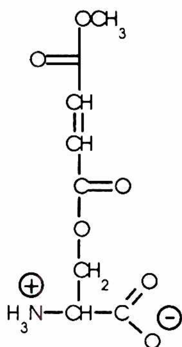


Figure 35. *Chemical structure of O-FMSer, serine analog of FMDP.*

The crystal structure of the glutamine domain of GlcN-6-P synthase, including glutamic acid molecule, were chosen as a starting geometry. For our simulations the molecule of glutamic acid was replaced by FMDP or O-FMSer. In order to take solvent effects into account, a 5 A hydration layer around the peptide molecule was filled with water molecules.

The 200 ps MD simulations of the two systems with subsequent minimisations were carried out to determine: i) the stability of the system, ii) distances between pharmacophore critical points, and iii) interactions between ligands and the protein.

For the two ligands studied, the systems exhibit high stability without any drastic changes of the peptide chain conformation. In addition, no tendency to ligand escape has been observed. The two ligands exhibit similar conformational behaviour during the simulation period. They exist in an extended conformation very similar to the pharmacophore one.

A position of the two ligands inside the active center is determined by a network of hydrogen bonds between their carboxylic and -amino groups, and charged peptide residues: *Arg*-73 and *Asp*-23. The same orienting interactions have

been expected for glutamine: the native substrate of the synthase. However, in addition to these main interactions we found some specific hydrogen bonds patterns which significantly differentiate the two ligands.

In case of FMDP, additional, strong hydrogen bond exist, during almost whole simulation time, between the amide group of *Gly*-99 and the amide hydrogen of FMDP (Figure 36a). It is stressed that this hydrogen bond could not have an equivalent in the case of glutamine. Moreover, relatively weak hydrogen bonds

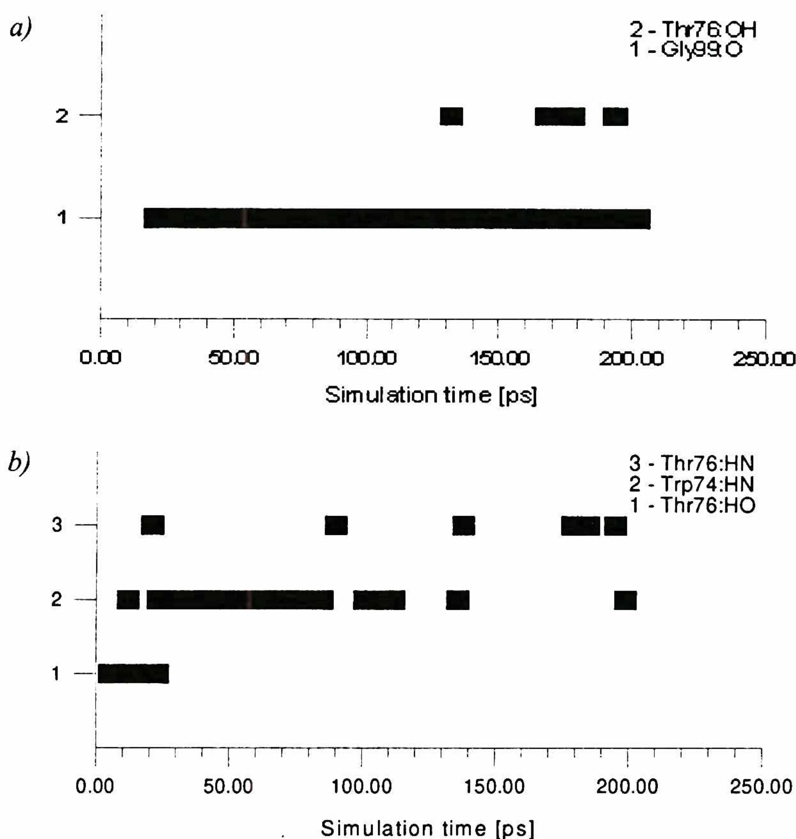


Figure 36. *Histories of hydrogen bonds formed by FMDP during MD simulation [43]: a) by amide nitrogen atom, and b) by amide oxygen atom.*

occur between an oxygen atom of the FMDP amide group and *Trp*-74, as well as *Thr*-76 residues of the peptide chain. All above mentioned amino acid residues are well conserved in the sequence of synthases from different organisms.

Thus, a FMDP molecule is highly stabilised and perfectly oriented inside the active center of GlcN-6-P synthase (Figure 36). It seems that FMDP, and its close analogs, exploit an opportunity created by a particular 3D distribution of polar groups inside the active center of this particular enzyme. This fact may additionally explain their high activity and selectivity. It is important to note that the inactivator

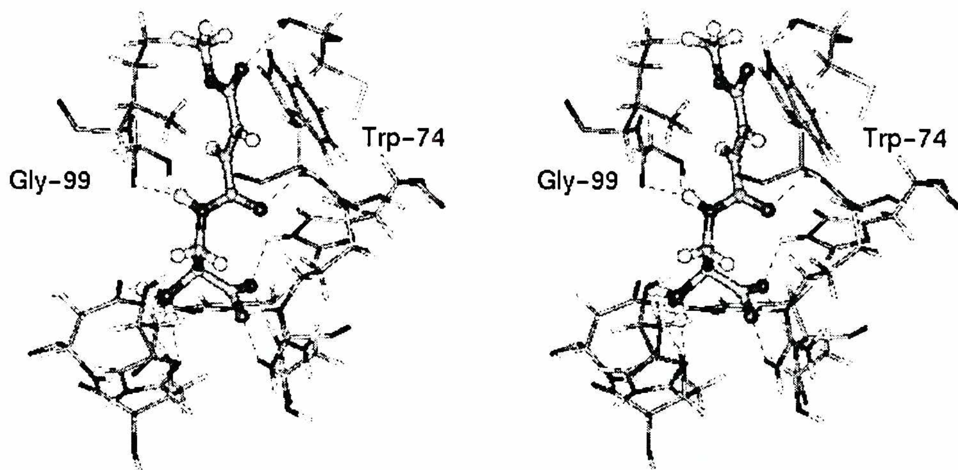


Figure 37. *The hydrogen bond network formed by FMDP with amino acid residues of the GlcN-6-P synthase active center [43].*

exists in the active center in the pharmacophore conformation.

This finding essentially facilitates the rational design of further selective enzyme inactivators based on the calculations prior to the synthesis.

In case of O-FMSer, additional, strong hydrogen bonds have not been found (Figure 38a.). The carbonyl oxygen atom of the ester group forms from time to time hydrogen bond with *Trp*-74, however, it is disrupted by an electrostatic repulsive interaction between the ester oxygen atom of O-FMSer and the amide oxygen atom of *Gly*-99. In our opinion, this observation may at least partially explain the lack of O-FMSer activity. One can find a number of analogous situations in other groups of compounds [44].

In addition, one should have in mind that a starting geometry of the system was made artificially. It means that a substrate recognition step was omitted.

4.5. Conclusions

The main goal of these studies is the design of selective inactivators of the GlcN-6-P synthase. To achieve this goal on rational basis it was indispensable to elucidate the molecular mechanism of the enzyme catalysis. Our earlier enzymological studies allowed us to put forward the preliminary hypothesis. The proposed mechanism involves the nucleophilic attack of a deprotonated thiol group on the carbonyl carbon atom. Our calculations have evidenced, Figure 26., that rather the concerted binding of the sulfhydryl sulphur to the carbonyl carbon, and the sulfhydryl hydrogen to the amide nitrogen, with simultaneous breaking of the S-H bond, occur.

On the basis of the mechanism of the enzymatic reaction it was possible to elucidate the mechanism of the enzyme inactivation by FMDP, EADP, and their derivatives. The quantum chemical calculations revealed that the nucleophilic

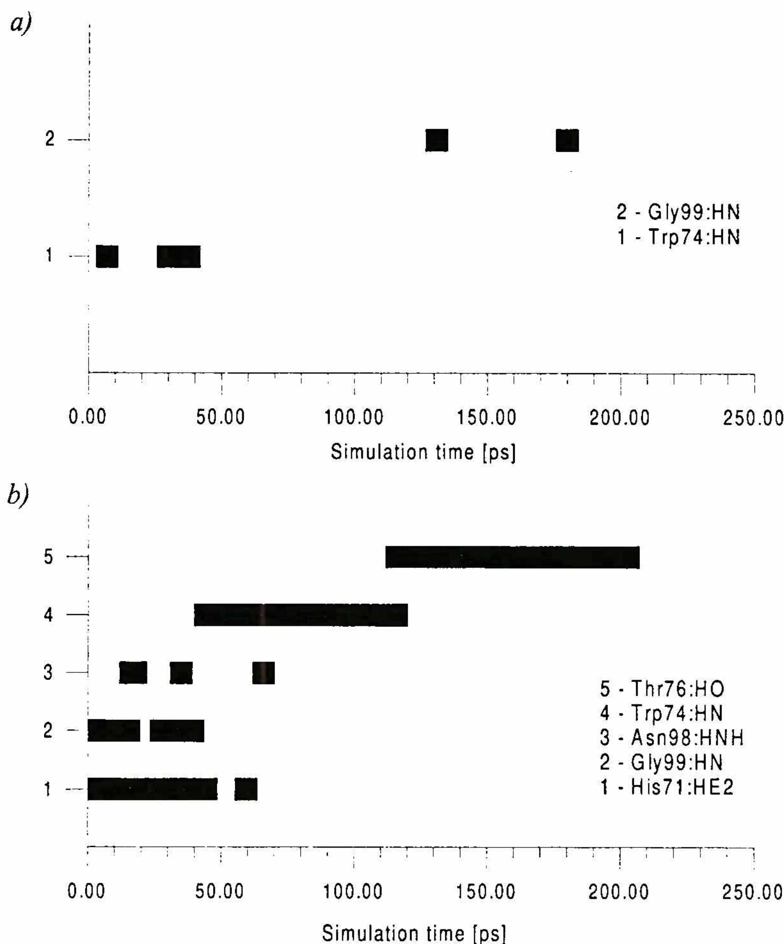


Figure 38. *Histories of hydrogen bonds formed by O-FMSer during MD simulation [43]: a) by ester oxygen atom, and b) by ester carbonyl oxygen atom.*

attack of the enzyme sulfhydryl group is the first step of inactivation common to both type of compounds. The differences in further course of inactivation reactions showed the particular role of an ester and amide moiety in the activity of the FMDP and EADP type compounds, respectively.

The calculation of differences in geometry characteristics of the selective and non-selective amidotransferases inactivators supplied the rational basis for the establishing the structural requirements for the design of selective GlcN-6-P synthase inhibitors. This information could not be obtained by experimental approaches.

The simulations of the inactivators docking to the enzyme active center revealed that for FMDP and its analogs the amide group, formed with β-amino group of diaminopropionic moiety, essentially contributs to the high affinity of the inhibitors to the enzyme. This group participates in the strong and stable hydrogen bond with amide group of the glycine moiety in the enzyme active center

(Figure 36). Replacement of the nitrogen atom by the oxygen (FMDP serine analog), leads to an inactive compound, due to the lack of the mentioned hydrogen bond and the appearance of repulsive electrostatic forces. This important information constitutes a new rationale in the design of GlcN-6-P synthase selective inhibitors.

References

[1] Lown J. W., Chen H. H., Plambeck J. A., *Diminished superoxide anion generation by reduced 5-iminodaunorubucin relative to daunorubicin and the relation to cardiotoxicity of the anthracycline anti-tumor agents*, Biochem.Pharmacol. **28**:2563-2570(1979)

[2] Tempczyk A., Tarasiuk J., Ossowski T., Borowski E., *An alternative concept for the molecular nature of the peroxidating ability of anthracycline anti-tumor antibiotics and anthracenediones*, Anti-Cancer Drug Design. **2**:371-385(1988)

[3] Tarasiuk J., Liwo A., Wojtkowiak S., Dzieduszycka M., Tempczyk A., Garnier-Suillerot A., Martelli S., Borowski E., *Molecular determinants of singlet oxygen binding by anthraquinones in relation to their redox cycling activity*, Anti-Cancer Drug Design. **6**:399-416(1991)

[4] Tarasiuk J., Garnier-Suillerot A., Borowski E., *Lack of competition between cytochrome C and anthraquinone type drugs for the reductive site of NADH dehydrogenase*, Biochem. Pharmacol. **38**:2285-2289(1989)

[5] Tarasiuk J., Garnier-Suillerot A., Stefańska B., Borowski E., *The essential role of anthraquinones as substrates for NADH dehydrogenase in their redox cycling activity*, Anti-Cancer Drug Design. **7**:329-340(1992)

[6] Mazerski J., Martelli S., Borowski E., *Steric hindrance of anti-tumor anthracenediones: molecular dynamics simulation*, Polish J.Chem. **71**:338-345(1997)

[7] Frederick C.A., Williams J. D., Ughetto G., van der Marel G. A., van Boom J. H., Rich A., Wang A. H. J., *Structural comparison of anticancer drug-DNA complexes: adriamycin and daunomycin*, Biochemistry, **29**:2538-2545(1990)

[8] Cirilli M., Bachechi F., Ughetto G., Colonna F. P., Capobianco M. L., *Interactions between morpholinyl anthracyclines and DNA. The crystal structure of a morpholinodoxorubicin bound to d(CGTACG)*, J.Mol.Biol., **230**:878-883(1993)

[9] Mazerski J., Martelli S., Borowski E., *Molecular dynamics simulations of anti-tumor agents Mitoxantrone and Ametantrone intercalated to DNA*, Acta Biochim.Polon. **45**:1-11(1998)

[10] Mechliński W., Schaffner C. P., Ganis P., Avitabile G., *The chemical structure and absolute configuration of AMB*, Tetrahedron Lett. 3873-3876(1970)

[11] DeKruijff B., Demel R. A., *Polyene antibiotic-sterol interactions in membranes of Acheleplasma laidlawii cells and lecithin liposomes. III.Molecular structure of the polyene antibiotic-cholesterol complexes*, Biochim.Biophys.Acta **339**:57-70(1974)

[12] Sowiński P., Pawlak J., Borowski E., Gariboldi P., *1H NMR model studies of AMB: Comparison of X-ray and NMR stereochemical data*, Magn.Reson.Chem. **30**:275-279(1992)

[13] Rinnert H., Maigret B., *Conformational analysis of AMB. I. Isolated molecule*, Biochim.Biophys.Res.Comm. **101**:853-860(1981).

[14] Meddeb S., Bergers J., Caillet J., Langlet J., *Comparative theoretical study of the conformations of AMB methyl ester and AMB polar heads in the presence of water*, Biochim.Biophys.Acta **1112**:266-272(1992).

[15] Bagiński M., Gariboldi P., Bruni P., Borowski E., *Conformational analysis of Amphotericin B*, Biophys.Chem. **65**:91-100(1997)

[16] Bagiński M., Borowski E., *Distribution of electrostatic potential around amphotericin B and its membrane targets*, J.Mol.struct.-Theochem. **389**,139-146(1997)

[17] Mazerski J., Borowski E., *Molecular dynamics of amphotericin B. I. Single molecule*, Biophys.Chem. **54**,49-60(1995)

[18] Bolard J., Legrand P., Heitz F., Cybulska B., *One-side action of AMB on cholesterol-containing membranes is determined by its self-association in the medium*, Biochemistry **30**:5707-5715(1991)

[19] Barwicz J., Christian S., Gruda I., *Effects of the aggregation state of AMB on its toxicity to mice*, Antimicrob.Agents Chemother. **36**:2310-2315(1992)

[20] Mazerski J., Grzybowska J., Borowski E., *Influence of net charge on the aggregation and solubility behaviour of amphotericin B and its derivatives in aqueous media*, Eur.J.Biophys. **18**,159-164(1990)

[21] Ernst C., Grange J., Rinnert H., Dupont G., Lematre J., *Structure of AMB aggregates as revealed by UV and CD spectroscopies*, Biopolymers **20**:1575-1588(1981)

[22] Hemenger R. P., Kaplan T., Gray L. J., *Structure of AMB aggregates based on calculation of optical spectra*, Biopolymers **22**:911-918(1983)

[23] Barwicz J., Gruszecki W. I., Gruda J., *Spontaneous organisation of AMB in aqueous medium*, J.Colloid.Interface Sci. **158**:71-78(1993)

[24] Mazerski J., Borowski E., *Molecular dynamics of amphotericin B. II. Dimer in water*, Biophys.Chem. **57**:99-108(1995)

[25] Bagiński M., Tempczyk A., Borowski E., *Comparative conformational analysis of cholesterol and ergosterol by molecular mechanics*, Eur.Biophys.J. **17**:159-166(1989)

[26] Baran M., Mazerski J., Borowski E., *Comparative study on membrane sterols conformation, Molecular dynamics simulation* - in preparation

[27] Bagiński M., Bruni P., Borowski E., *Comparative analysis of the distribution of the molecular electrostatic potential for cholesterol and ergosterol*, J.Mol.struct.-Theochem. **311**:285-296(1994)

[28] Herve M., Debouzy J.C., Borowski E., Cybulska B., Gary-Bobo C. M., *The role of the carboxyl and amino groups of polyene macrolides in their interactions with sterols and their selective toxicity: a 31P-NMR study*, Biochim.Biophys.Acta **980**:261-272(1989)

[29] Mazerski J., Bolard J., Borowski E., *Effect of the modifications of ionizable groups of amphotericin B on its ability to form complexes with sterols in hydroalcoholic media*, Biochim.Biophys.Acta **1236**:170-176(1995)

[30] Khutorsky V. E., *Structures of amphotericin B-cholesterol complex*, Biochim.Biophys. Acta **1108**:123-127(1992)

[31] Baran M., Mazerski J., Borowski E., *MD simulations of AMB-sterol complex in water*, Abstracts of VIth International Symposium on Molecular Aspects of Chemotherapy, Gdansk, July 1997, p.73

[32] Bagiński M., Haluk R., McCammon J.A., *Molecular properties of Amphotericin B membrane channel: a molecular dynamics simulation*, Molec.Pharmacol. **52**:560-570(1997)

[33] Grzybowska J., Sowiński P., Gumieniak J., Zieniawa T., Borowski E., *N-methyl-N-D-fructopyranosyl amphotericin B methyl ester, new AMB derivative of low toxicity*, J.Antibiot. **50**:709-711(1997)

[34] Chmara H., Andruszkiewicz R., Borowski E., *Inactivation of glucosamine-6-phosphate synthase from Salmonella typhimurium by fumaroyldiaminopropionic acid derivatives, a novel group of glutamine analogs*, Biochim.Biophys.Acta **870**:357-366(1985)

[35] Tempczyk A., Tarnowska M., Liwo A., Borowski E., *A teoretical study of glucosamine synthase*, Part II. Eur.Biophys.J. **21**:137-144(1992)

[36] Tempczyk A., Tarnowska M., Liwo A., Borowski E., *A teoretical study of glucosamine synthase*, Part I. A. Eur.Biophys.J. **17**:201-210(1989)

[37] Chmara H., Smulkowski M., Borowski E., *Growth inhibitory effects of amidotransferase inhibition in Candida albicans by epoxypeptides*, Drug Exptl.Clin.Res. **6**:7-14(1980)

[38] Kucharczyk N., Denisot M. A., LeGoffic F., Badet B., *GlcN-6-P synthase from E.coli: determination of the mechanism of inactivation by FMDP derivatives*, Biochemistry **29**:3668-3672(1990)

[39] Tarnowska M., Ołdziej S., Liwo A., Grzonka Z., Borowski E., *Investigation of the inhibition pathway of glucosamine synthase by FMDP by semiempirical quantum mechanical and molecular mechanics methods*, Eur.Biophys.J. **21**:273-278(1992)

[40] Wojciechowski M., Liwo A., Borowski E., *Theoretical study of the reaction of methyl mercaptate and methyl-trans-epoxysuccinamide as a model of the GlcN-6-P synthase inhibition by EADP*, J.Comp.Aided Molec.Design - in press

[41] Wojciechowski M., Mazerski J., Borowski E., *Constrained search of conformational hyperspace of inactivators of glicosamine-6-phosphate synthase*, J. Enzyme Inhibition **10**:17-26(1995)

[42] Chmara H., Zahner H., *The inactivation of glucosamine-6-phosphate synthase from bacteria by anticapsin, the C-terminal epoxyaminoacid from the antibiotic tetaine*, Biochim.Biophys.Acta **787**:45-52(1984)

[43] Wojciechowski M., *Modelling of GlcN-6-P synthase inhibition by glutamine analogs*, Doctoral Thesis, Technical University of Gdansk, Gdansk 1998

[44] Kubinyi H., *Similarity and dissimilarity: a Medicinal chemist's view*, Perspect.Drug Discovery Design **9/10/11**:225-252 (1998)