UNIVERSITY OF LIMOGES

FACULTY OF PHARMACY

****

YEAR 2012 THESIS N°

Molecular dynamic simulations of the interaction of natural compounds with lipid bilayer membranes

THESIS TO OBTAIN THE STATE DEGREE OF DOCTOR IN PHARMACY

Presentation and public defence

on Wednesday, September 19 2012

by

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born on March 16 1988 in Tulle

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ACKNOWLEDGEMENTS

My first thanks are addressed to Professor Jean-Luc Duroux. It is an honor to have you as the president of my jury. I appreciated your lectures during my studies as much as your personality and human qualities. You have always been very kind with me, welcoming me in your lab and your research team and helping me anytime you had the opportunity.

I would like to express my deep and sincere gratitude to Doctor Patrick Trouillas, my thesis supervisor. More than three years ago, you made me discover molecular modeling and you gave me the scientific curiosity, which only increases over the years. I really appreciate your everyday patience and motivation to teach me your scientific expertise. I am very grateful that you are always supporting me in all aspects of my scientific carrier. Thank you for everything.

Doctor Serge Battu, I am honored that you accepted to be in my jury. Your scientific knowledge and your conception of pharmaceutical studies always bring helpful and interesting discussions. Thank you for having accompanied me all along my studies.

Doctor Claude Calliste, I am grateful that you accepted reviewing my work. I am thankful for all your help, the experimental results presented here, and nice discussions about our scientific projects. I really appreciate your human qualities and the nice environment you bring at the lab.

I would like to thank Doctor Laure Milhet for accepting to be in my jury. For two years you have taught me your profession with patience and pedagogy. I now like working in a pharmacy (which was a challenging task)! Thank you for this essential experience you made me discover. I also thank your parents, Amélie and Bernadette for this.

Next, I thank all my teachers from Faculty of Pharmacy of University of Limoges and Faculty of Physics and Chemistry of Paul Sabatier University in Toulouse. They gave me the passion to study all these sciences and to continue doing it in the future.

Big thanks for my colleagues and friends, with a special one for Doctor Florent Di Meo. You taught me a lot, you gave me your passion for theoretical chemistry and you helped me to gain self-confidence. Thank you for all the nice moments we spent together. I also thank Thomas Desmier, Doctor Pavlína Košinová, Doctor Anouar El Hassane, Manu, Nico, Imene, Assia and all the colleagues from LCSN – EA1069.

I thank Doctor Michal Otyepka, Doctor Karel Berka and the entire lab in Olomouc for your stimulating help and support in Science. My work would not be the same without you.

I would also like to thank all my friends, Bibi, Mary, Tibo, Mathilde, the Nours family, Maxime, those from indus', and all other potards and carabins I enjoy the company, IRL or on the internets.

My next thanks are for all my family that has supported me during all these years and has given me the opportunity to do what I always wanted to do with my life. My thoughts are with those who left too early.

Finally, Gaelle, you have been on my side for five years now and I cannot thank you enough for all the love and support you bring me every day.

CONTENTS

INTRODUCTION ON NATURAL COMPOUNDS: A NEVER-ENDED SOURCE OF INSPIRATION FOR THERAPEUTICS

Natural compounds have been used for centuries for therapeutic purposes, and they became of considerable importance over the last years.[1–3] Over the 1184 new therapeutic molecules approved between 1981 and 2006, only 30% of chemical structures were totally synthetic (not inspired from natural compounds) whereas 46% were directly produced from compounds extracted from Nature; the remaining structures being synthetic but inspired by natural products. Combinatorial chemistry has been used to create new molecular templates for more than 20 years but has only leaded to few novel therapeutic templates. Moreover, even if this technique mainly screens small molecules, 63% of approved small drugs were based on natural compounds.[3]

Natural molecules are mainly discovered in plants, bacteries and fungi, but new sources are being investigated e.g., molluscs, bryozoa, fishes or lichens. They also span a wide range of therapeutic classes. The field of oncology counts several drugs which are either extracted directly from plants or produced by semisynthesis.[5] For instance, the well-known Vinca alkaloids (vinblastine, vincristine) extracted from *Catharanthus roseus* (also known as *Vinca rosea*) are powerful antimicrotubule drugs, as well as taxanes (paclitaxel, docitaxel) which are semisynthetic compounds from *Taxus baccata*. Natural topoisomerase inhibitors are also used against haematological and solid tumors, e.g. the epipodophyllotoxins etoposide and teniposide from *Podophyllum peltatum* or the anthracyclines doxorubicin and epirubicin from *Streptomyces peucetius*. [4],[5] Antimicrobial molecules are mainly issued from microorganisms themselves. This is especially true for antibacterial compounds: for almost every class, the first discovered molecule was extracted from a bacterial or fungal strain. While antiviral drugs are mainly synthetic, some antiparasitic drugs are of natural origin, e.g.

the antimalarian artemisinin from *Artemisia annua* and quinine from *Cinchona* among others.[6] The antimicrobial activity of vegetal molecules was also investigated.[7] Finally, another field in which natural products play an important role is cardiovascular health. Digoxin and its derivatives from *Digitalis lanata* treat atrial fibrillation, while ergotamine derivatives from *Claviceps purpurea* are used against acute migraine attacks.[9] Also, compounds present in food are crucial to prevent cardiovascular diseases, namely fatty acids, omega-3, lycopene, and natural antioxidants such as vitamins and polyphenols, which are detailed later in Chapter 2. [8]

The huge diversity of the natural chemical structures provides an infinite source of inspiration for future drugs. The complexity of the structures is mainly due to the ability of metabolic pathways of plants to synthetize structures containing quaternary carbons and tertiary nitrogens (Figure 1).[10] While this complexity provides novel chemical templates for a broad spectrum of pathologies, it also represents drawbacks because the total synthesis for drug mass production can be both challenging and expensive. To obtain fresh vegetal samples, sample variability, structural determination and extraction procedures constitute a non-exhaustive series of brakes for the discovery of natural products. All these reasons explain why major pharmaceutical companies do not focus on natural products. Molecular modeling has appeared as a promising tool to overcome some of these difficulties. This relatively new science had an increasing potential and now becomes mature enough to rationalize many questions about natural compounds and their biological activities.

Chapter 1 of this manuscript briefly introduces the theoretical concepts of molecular modeling (molecular dynamics and quantum chemistry). The following two chapters detail two separate theoretical studies tackling the mechanisms of biological action for two natural compounds. Namely, chapter 2 shows how molecular modeling allows rationalizing the antioxidant properties of a lipocarbazole derivative. Chapter 3 points out a series of clues

highlighted by the theoretical study to better understand the mode of action of the new antibacterial plantazolicin. Both studies mainly focus on interaction with lipid bilayer membranes.

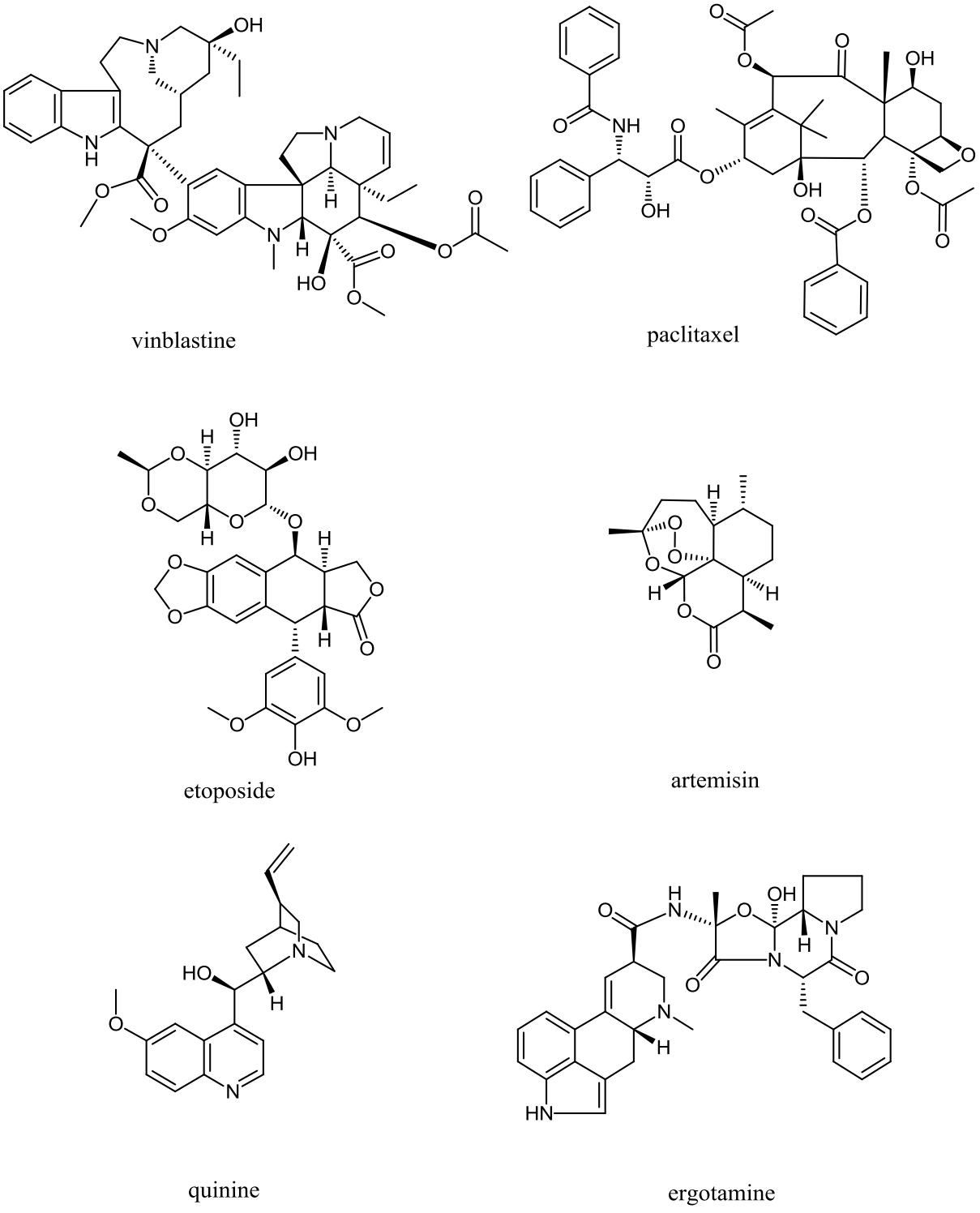


Figure 1: Chemical structures of various natural therapeutic compounds

1 CHAPTER 1: MOLECULAR MODELING, A TOOL TO UNDERSTAND THE MECHANISM OF ACTION OF BIOLOGICAL ACTIVITIES

1.1 Born-Oppenheimer approximation

In the end of the 19th century, Max Planck observed phenomena that were not explained by classical physics. He postulated that the emission of light was not described by a continuum energetic spectrum but as a series of discrete energy quanta. This was further generalized by Einstein, de Broglie and others, light and other particles being considered both as waves and corpuscles. Quantum mechanics was born. In 1926, Erwin Schrödinger formulated an equation to describe the motion of electrons: [11]

$$\boldsymbol{H}\Psi = E\Psi$$

where $\boldsymbol{H}$ is the Hamiltonian operator, E the energy of the system and Ψ the wave function containing all information on the studied particles. This time-independent Schrödinger equation is the basis of most of theoretical chemistry and molecular modeling methods of calculation. It describes any system of particles including molecules made of electrons and nuclei. As the mass and the speed of electrons are very different from those of nuclei, the Born and Oppenheimer approximation[12] propose to decouple and treat separately both motions of electrons and nuclei. This gives rise to two main families of methods in theoretical chemistry. When treating electrons (quantum chemistry, QM), they are considered in a field of fixed nuclei; when treating nuclei (molecular mechanics, MM), they are considered in a field of forces representing all interactions including those of the electrons.

1.2 Notions of quantum chemistry and applications

Treating electrons allows accessing valuable chemical information. An accurate evaluation of ground and transition state energies allows tackling precisely the reactivity. This can drive the choice of catalyzers or activating groups. Light absorption and emission properties can also be calculated in the UV, visible, and IR ranges. It helps for example to rationalize colors of molecules, to discover good candidates as active compounds in sunscreen creams, or to identify products of chemical reactions. Many other properties are accessible including NMR data, polarizability, ionization potential and electron affinity.

Solving exactly the Schrödinger equation is only possible for systems containing one electron; for polyelectronic systems approximations have to be applied and their study has only become possible in the 70's with the developments of computational facilities. Afterwards, according to Moore's law, computer power has doubled every 18 months. This has allowed tackling bigger and bigger molecular systems together with better and better precision. Over the past decades the number of methods of calculations dramatically increased, which are classified as semi-empirical, Hartree-Fock (HF), post-HF and density functional theory (DFT) methods. The accuracy of a quantum chemistry calculation depends on the method used: the less approximation in the method, the more accurate the prediction, but the bigger the computational time. Therefore the challenge of a theoretical chemist is always to choose the best compromise between accuracy and computational time.

Whatever the method of calculations, QM can only treat relatively small molecular systems (up to 100, 1000 and 10000 atoms with post-HF, DFT and semi-empirical methods, respectively). In order to treat bigger systems, MM appears as an adequate alternative.

1.3 Notions of molecular mechanics

1.3.1 Force field

MM treats the motion of particles with classical (Newton) physics instead of quantum physics. Atoms are pictured as balls and bonds as springs. MM is less computational demanding and thus allows treating large molecular systems. Here the interactions are parameterized empirically. All these parameters constitute the so-called force field. In order to obtain accurate predictions, the force field must be carefully built; the parameters are (i) obtained from experiments, (ii) calculated with high-level quantum methods or (iii) fitted to reproduce macroscopic values. The parameters are divided into two main groups:

- Bonded interactions, which include bond stretching, angle bending and dihedral torsions (Figure 2). The first two can be treated as harmonic oscillators defined by two parameters, namely the equilibrium bond distance (or angle) and strength of the spring.
- Non-bonded interactions, which include (i) electrostatic interactions between neighboring atoms for which integer or partial charges are assigned, (ii) van der Waals long range interactions usually described by the classical Lennard-Jones potential and (iii) hydrogen bonding interactions between polar hydrogen atoms and lone pair containing atoms e.g., O and N.

All parameterizations of the force field for a given molecular system are crucial to obtain accurate results. Therefore, for each new study, a dedicated methodology must be carefully established.

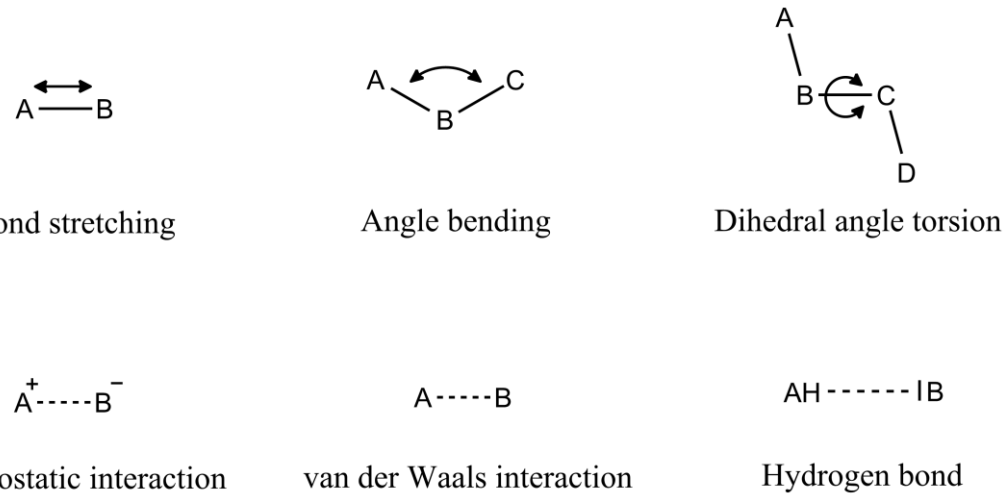


Figure 2: interactions described in force fields.

1.3.2 Molecular dynamics

A MM calculation allows finding the nearest energy minimum and corresponding geometry from a given 3D structure, according to optimization algorithms. The movement of molecules vs. time (trajectories) can also be obtained by solving Newton's second law of motion ($F = ma$), which can be expressed in its differential form as:

$$-\frac{dV}{d\boldsymbol{x_i}} = m_i \frac{d^2 x_i}{dt^2}$$

where V represents the potential described by the force field and $-\frac{dV}{d\boldsymbol{x_i}}$ the force acting on an atom of mass m_i along a coordinate x_i. From an original structure, it is possible to calculate the potential acting on each atom and to deduce their speed, thus their positions after a given time step. This loop is repeated from the new positions. In order to accurately describe molecular motion, a time step smaller than the fastest movement must be used. Since the fastest vibration of a hydrogen atom is approximately 13 fs (1 femtosecond = 10^{-15} seconds), a 2 fs time step is generally used. Additionaly, since biological relevant processes (e.g. protein rearrangement, DNA folding or permeation of molecules through membranes) occur at least

in the range of hundreds of nanoseconds, hundreds of millions of steps are required. This only became possible in the last few years thanks to the new developments of computer hardware, algorithms and software.[13]

Introducing time provides access not only to valuable time-dependent properties such as diffusion or viscosity, but also to a better sampling of the properties evaluated e.g., mainly conformational search of complex structures like peptides, or proteins. For these systems, an extensive sampling is required to explore the complex potential energy surface and to find the relevant conformation(s).

1.4 MD applications for biological macromolecular systems

1.4.1.1 Protein folding and function

Protein structures are generally obtained from experimental X-ray crystallography; however this technique requires proteins in a crystal solid phase. This raises two main concerns: (i) the 3D-conformations in the crystallized protein differ significantly from those in aqueous phase and (ii) only a static structure is obtained, thus dynamical properties are not easily accessible. MD simulations may solve these problems. Starting from X-ray structures, MD simulations allow relaxing the structures in order to provide more realistic conformations. Concerning the latter problems, MD simulations provide atomistic insights of dynamical processes. Binding sites can be accurately predicted or confirmed, mechanisms of action of membrane transporters can be rationalized and their permeability calculated, and conformational changes like those induced by kinases can be accurately described.[13]

1.4.2 DNA

DNA is a flexible polymer that can adopt a wide range of conformations. While this flexibility is crucial, it is only hardly available experimentally and MD has proven to be the

most valuable tool to describe structural changes in DNA. However, the complexity of this problem requires an extreme precision in the force field parameterization. Due to accumulation of errors, accuracy is especially important when multi-microsecond simulations are performed. Although force fields describing proteins and membranes are nowadays able to reproduce accurately experimental results, this is not systematically the case with DNA, for which the force fields are currently under heavy development. There are however very promising studies e.g., predicting thermodynamics and kinetics of drug-DNA binding and presence of high affinity interaction sites. [14]

1.4.3 Membranes

Lipid membranes are an exciting topic since the interaction of numerous compounds with biological membranes is crucial, being involved at different stages. First, all molecules in the digestive tract have to cross the intestinal membranes in order to reach blood and target organs. Membrane crossing depends on many parameters including (i) size, (ii) charge and (iii) lipophilicity of the molecule. Even if membrane crossing can be evaluated by global parameters such as logP, an atomistic description is required to fully deal with the mechanisms of action. Second, some compounds may interact with key proteins embedded in membranes (e.g., cytochromes[15], [16] or G-protein-coupled receptors) and trigger important mechanisms in cells. Third, natural molecules may influence chemical reactions taking place in membranes such as lipid peroxidation reaction (LPO).[17]

The field of MD simulations of membranes deserves a constantly growing interest. Most of these simulations involve membrane proteins. They represent 50% of the drug targets and are interesting subjects for all-atomistic simulations, as other proteins.[18] However, several limitations exist: (i) high-resolution structures are required to perform accurate simulations

but only a few hundreds are known and (ii) long simulation times (i.e. µs) are required to sample their dynamics.

Another interesting topic in membrane simulations is the interaction of small compounds with lipid bilayers. Most of them involve the insertion of antimicrobial peptides into lipid bilayer membranes.[19–24] Small organic compounds are less extensively studied despite the fact that they represent the largest part of therapeutic molecules, therefore being of considerable interest. A few drugs with various activities were studied: the anesthetic articaine[25], β-blockers, steroid hormones[26], ibuprofene[16], coumarin[27] or the antioxidant quercetin and its metabolites[28].

1.5 Conclusion

The huge diversity and complexity of natural products requires long and challenging research protocols in order to access their therapeutic potential and their valuable outcomes. Once identified, molecular modeling and in particular MD simulations can in principle provide the atomistic description of most biological molecules, thus allowing a better understanding of their biological activities and *in fine* the prediction of these activities. This work describes lipid bilayer membrane interactions of two natural molecules, namely the antioxidant lipocarbazole and the antibacterial plantazolicin.

2 CHAPTER 2: THE ROLE OF THE ANTIOXIDANT LIPOCARBAZOLE IN LIPID PEROXIDATION INHIBITION

2.1 Antioxidants

2.1.1 Oxidative stress

2.1.1.1 Free radicals

Free radicals are atoms or molecules with one unpaired electron providing an open shell electronic configuration. Such species are usually created by homolytic bond cleavage. The enthalpy required for symmetrical bond breaking is relatively high, of the order of tens of kcal.mol^{-1}. Thus the formation of free radicals requires high-energy sources including UV light, high temperature or aerobic metabolism. Free radicals are constantly produced by the human metabolism. They can be originated exogenously (from cigarette smoke or intoxications) or endogenously. Free radicals are formed by respiration, where molecular oxygen O_2 is reduced in water, and can escape the respiratory chain. Inflammatory reactions are also a powerful source of free radicals, which are used as weapons against the infecting organism and as signal to attract other immunity cells. Due to their unpaired electron, free radicals are highly unstable and reactive; they are capable to induce chain reactions accompanied by many deleterious effects. To limit these effects, various endogenous and exogenous antioxidant systems regulate the constant free radical production: enzymes (e.g., superoxide dismutase, glutathione peroxidase, and catalase) and small compounds (e.g., vitamins C and E, glutathione, polyphenols). In the case of imbalance of this regulation/production equilibrium, the free radical concentration increases in tissues, originating oxidative stress. The high reactivity of these species is responsible for severe damages in tissues.

2.1.1.2 Reactive oxygen species (ROS)

Most of free radicals produced by the metabolism contain one or more oxygen atom(s) and are called reactive oxygen species (ROS). When the source is endogenous, ROS are formed along the mitochondrial respiratory chain from molecular oxygen. During this process, four electrons and four protons are added to O_2 to form two H_2O molecules.

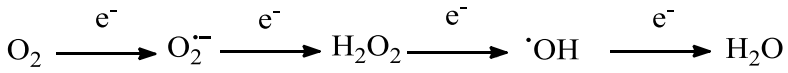
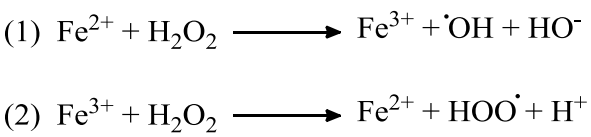


Figure 3: oxygen species produced during respiration

Even though the oxygen molecules are bound to enzymes during the whole process, ROS can escape. The $^{\bullet}OH$ radical is the most toxic; it is so reactive that it can attack all solute and propagate from water molecule to water molecule. Moreover when attacking lipid chains it can produce lipoperoxyl free radicals $ROO^{\bullet}$. The superoxide anion $O_2^{\bullet-}$, rarely react by itself due to its very short lifetime but may produce free radicals. It is estimated that every cell of the human body is exposed to 10^{10} molecules of $O_2^{\bullet-}$ every day.[29] The less unstable ROS is H_2O_2, but when H_2O_2 encounters ferrous iron Fe(II), it catalyzes the production of $^{\bullet}OH$ and $HOO^{\bullet}$. These reactions are called (1) Fenton and (2) Haber-Weiss reactions, which are also possible with copper:



Molecular oxygen exists in two states. The most stable and common is the triplet oxygen $^3O_2$, having two unpaired electrons of same spin on its valence layer (Figure 4a). The less stable that can be created under specific conditions is the singlet oxygen $^1O_2$ (Figure 4b). The difference in stabilizing energy between both states is 22.5 $kcal.mol^{-1}$, so the interchange

requires high-energetic processes. This molecule is also very reactive and is considered as a member of the ROS family.

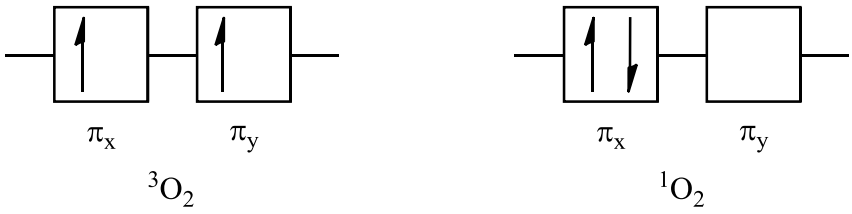


Figure 4: Electronic configuration of the valence layer of triplet and singlet dioxygen.

Although H_2O_2 and $^1O_2$ are not sheer free radicals they can generate other ROS.

2.1.1.3 ROS in inflammation

As mentioned earlier, ROS are produced massively during inflammatory reactions, causing oxygen consumption. This phenomenon is called respiratory burst. Several chain reactions then occur, first involving NADPH oxidase, a membrane enzyme present in phagocytes, which catalyzes the production of $O_2^{\bullet -}$.

$$O_2 + e^- \xrightarrow{\text{NADPH oxidase}} O_2^{\bullet -}$$

$$2\ O_2^{\bullet -} + 2\ H^+ \longrightarrow H_2O_2 + O_2$$

$$O_2^{\bullet -} + H_2O_2 + H^+ \longrightarrow {}^{\bullet}OH + O_2 + H_2O$$

Polymorphonuclear neutrophils are capable to synthetize hypochlorite from H_2O_2 and chloride ions, which can lead to $^1O_2$ production:[30]

$$H_2O_2 + Cl^- \xrightarrow{\text{Myeloperoxidase}} ClO^-$$

$$H_2O_2 + ClO^- \longrightarrow {}^1O_2 + H_2O + Cl^-$$

Phagocytes, neutrophils and eosinophils use ROS to destroy foreign organisms (bacteria, viruses, parasites and cancer cells). The first two types of immune cells are able to perform

phagocytosis. In this process, ROS are released into the phagosome to kill and digest bacteria and viruses. Eosinophils release ROS into the extracellular fluid to act on cancer cells or parasites.

The ROS that are released are toxic for the tissues but they participate in another process: the gradient of ROS in the extracellular fluid is sensed by transmembrane receptors of other immune cells, which guide their movement to the inflammation site. This phenomenon is called chemotaxis.

2.1.1.4 Reactive nitrogen species (RNS)

Radical nitrogen species (RNS) are also formed in the human organism: nitric oxide $NO^{\bullet}$ is relatively non-reactive and non-toxic and it plays an important role in vasodilatation. However, it can react with superoxide anion to form the peroxinitrite anion $ONOO^-$, which is toxic by binding to various biomolecules:

$$O_2^{\bullet -} + NO^{\bullet} \longrightarrow ONOO^-$$

2.1.1.5 Lipid peroxidation

ROS are known to intensively target DNA, but also proteins and membranes. Membranes are viscous fluids that delimitate cells and keep their integrity (i.e. volume, shape, osmolarity) and ensure exchanges with outer cell media mainly via membrane proteins.

Mammalian membranes consist of lipids (phospholipids, cholesterol and glycolipids), proteins, glycoproteins and other organic compounds. Membrane composition strongly depends on species, cells and layers (internal and external layer have different functions). Various factors influence membrane properties including temperature, length, number of unsaturations of lipid chains and cholesterol concentration (i.e. increasing cholesterol concentration increases membrane viscosity). [31], [32]

The main types of phospholipids (PLs) present in mammalian membranes are phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines and sphingomyelin. PLs are amphipathic molecules which consist of one hydrophilic polar head group and two lipophilic hydrocarbon tails (Figure 5). This structure allow PLs to organize in bilayers, where polar head groups face the outer polar aqueous environment while lipid chains are confined into the inner part (Figure 5b).

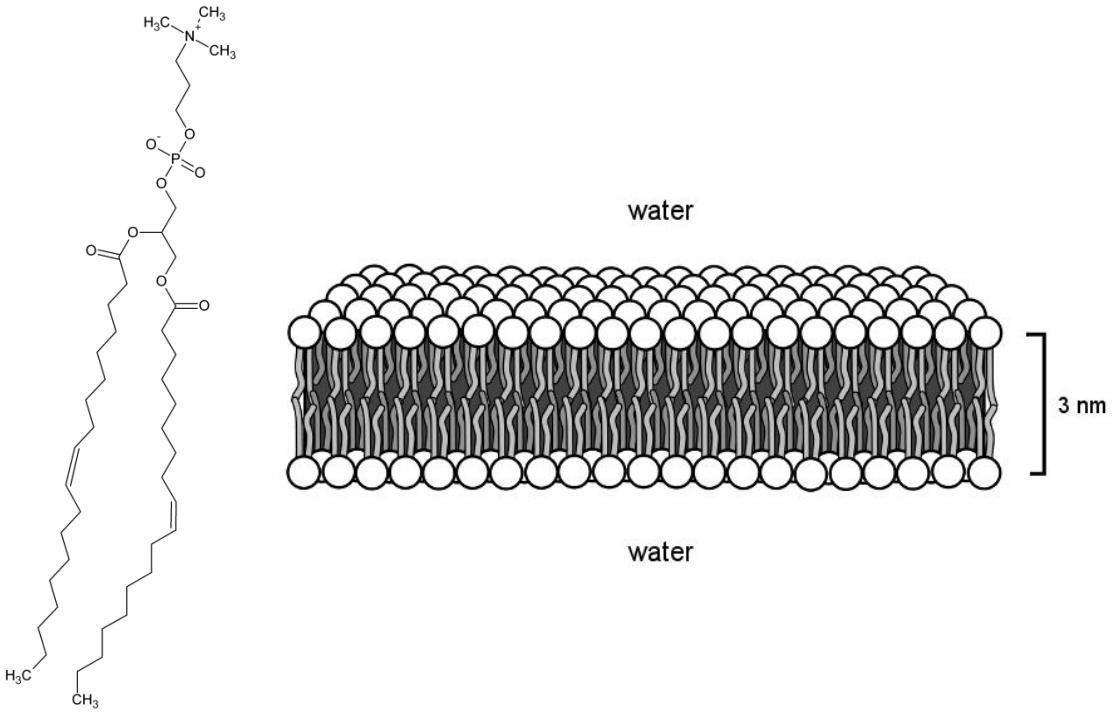


Figure 5: Phospholipid (e.g., dioleoylphosphatidylcholine) and organization of phospholipids in bilayers

Lipid peroxidation is a radical chain reaction happening in three steps: initiation, propagation, termination. [17] The hydroxyl radical $^{\bullet}OH$ is the most common initiator of this reaction. It attacks preferentially C-H groups located close to double bonds of phospholipids, by hydrogen atom transfer (HAT) from the lipid to the free radical. This process generates a carbon-centered radical on which molecular oxygen can be added to form a peroxyl radical.

This radical propagate from lipid chain to lipid chain by HAT processes. During this propagation stage, many other cross-reactions can happen between radical lipid chains and their stable neighbor. The most common cycle is detailed in Figure 6. Other products can form especially malondialdehyde and small dienes, which are both toxic and useful to follow the reaction.

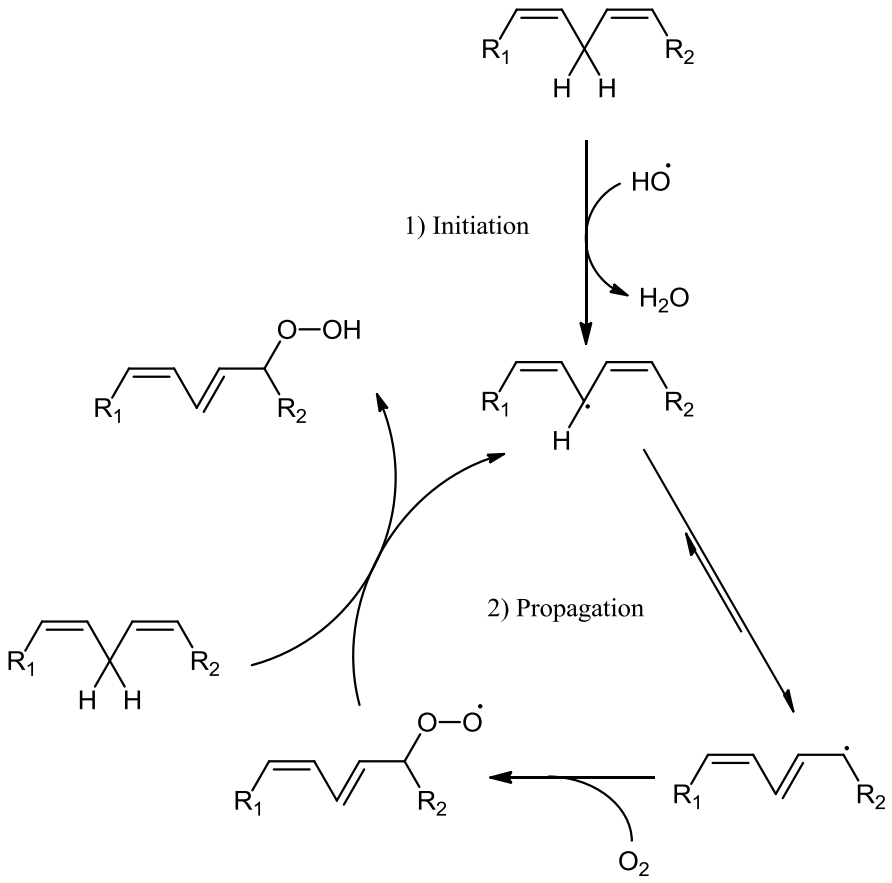


Figure 6: Initiation and propagation steps of a typical lipid peroxidation reaction involving polyunsaturated fatty acids.

The termination stage occurs when the reactive species react together to form stable products. However, these non-reactive molecules differ from the original phospholipids as they possess much more oxidized functions and/or shorter chains. The bilayer structure is disorganized, usually less fluid and loses its crucial ability to protect the cell. It was also shown that termination products induce apoptosis.[33]

LPO can also occur in the absence of oxidative stress with low ROS concentration via the action of enzymes (lipoxygenases, LOXs or cycloxygenases, COXs) to synthetize leukotrienes and prostaglandins.

2.1.1.6 Pathological effects

Even though ROS production is sometimes beneficial for the organism (especially in inflammation) it always causes damages to tissues. As described before the effects on membrane are important, but proteins and nucleic acids (DNA, RNA) are also deteriorated. These damages are key points in cardiovascular diseases, cancer and aging. [30]

LPO not only affects cell membranes but also low density lipoproteins (LDL). Native LDL transport fatty acids and cholesterol to peripheral organs where they are recognized by transporters and removed from blood circulation. In case of oxidation, bonding to the receptors becomes impossible and LDL are eliminated by macrophages, which become spumous cells. These cells aggregate in arteries to form atheroma.

DNA in each human cell is attacked by ROS 10^4 times per day, leading to an average of 20 injuries (single and double strand cleavage). Most of the time, these defects are repaired by endonucleases, exonucleases and glycosylases. However, repairs are not always successful and mutations may appear. Depending on the cell type, there exists a mutation threshold beyond which cancer processes are initiated. In addition, ROS can accelerate the development of existing tumors.[34]

The two main theories formulated to explain aging are programmed genetics aging and free radical aging. Both related effects might occur in synergy. The latter theory rationalizes aging as an accumulation of deteriorations of proteins (enzymes essentially) and DNA caused by ROS.[35]

The oxidative stress clearly appears responsible for many pathological effects, but the human metabolism possesses a series of inhibiting machineries, most of them involving antioxidants.

2.1.2 Natural antioxidants

An antioxidant is a compound or a mixture of compounds capable to delay or inhibit the oxidation of biological molecules. Since the oxidation process is multifactorial, antioxidants have several targets. They can (i) inhibit the formation of ROS, (ii) metabolize ROS so that they become harmless molecules or (iii) scavenge free radicals.

Antioxidants can be divided into two groups: endogenous and exogenous. Endogenous antioxidants are mainly proteins (enzymes). Exogenous antioxidants are relatively small organic molecules, most of them scavenging free radicals. They are usually obtained from food but a few antioxidant drugs also exist.

2.1.2.1 Proteins preventing the formation of ROS

As mentioned above, the reduced forms of iron and copper (Fe(II) and Cu(I)) are able to reduce H_2O_2 into $^{\bullet}OH$. The subsequent Fe(III) and Cu(II) are able to oxidize H_2O_2 into $HOO^{\bullet}$ through the Haber-Weiss cycle. They are then regenerated into Fe(II) and Cu(I). The reduced forms are much more reactive than the oxidized forms. Therefore, enzymes able to oxidize iron and copper or to complex them are antioxidants. Lactoferrin complexes two Fe atoms, hemopexin binds to free heme which contains Fe(II), albumin complexes Fe(II), Cu(I) and HClO, and ceruloplasmin complexes eight copper atoms but also oxidizes Fe(II) to Fe(III).

2.1.2.2 Enzymes metabolizing ROS

Three enzymes are in charge of transforming ROS into harmless species. The first one is superoxide dismutase (SOD). Dismutation is a chemical reaction where the reactant is both

oxidized and reduced. SOD efficiently catalyzes the dismutation of $O_2^{\bullet -}$, 10^4 to 10^5 times faster than spontaneous dismutation:

$$2\ O_2^{\bullet -} + 2\ H^+ \xrightarrow{\text{SOD}} H_2O_2 + {}^3O_2$$

As a result of this reaction H_2O_2 is produced, showing the importance of SOD to act in synergy with other antioxidant systems.

Catalase is one of those partners, being able to dismute H_2O_2 into water and dioxygen:

$$2\ H_2O_2 \xrightarrow{\text{catalase}} {}^3O_2 + 2\ H_2O$$

Its antioxidant activity is relatively weak because it is only present in red blood cells and peroxisomes, not in extracellular fluids.

Glutathione peroxidase (Se-GPx), a selenoprotein, is another of those SOD partners. It is expressed in nearly all mammalian tissues which reduces H_2O_2 as well as hydroperoxides (ROOH) issued for instance from LPO. Alcohols formed (ROH) are much more stable than ROOH and cannot propagate LPO. The Se-GPx cofactor, glutathione (GSH), is a tripeptide containing one cysteine residue. Two GSH molecules can be oxidized into a dimer linked with a disulfur bond (GSSG). Glutathione is then regenerated by glutathione reductase, which uses $NADPH,H^+$ as cofactor (Figure 7).

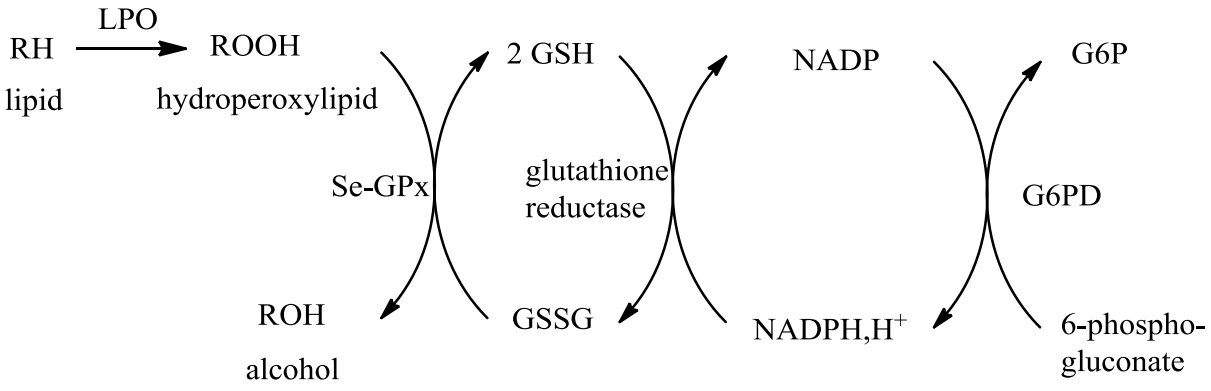


Figure 7: Antioxidant mechanism of glutathione peroxidase.

These three enzymes are efficient protectors against daily free radical attacks. However, in the case of oxidative stress, they can be saturated. Direct free radical scavenging by small organic molecules like vitamins then becomes crucial.

2.1.2.3 Vitamins

The mechanism of free radical scavenging by antioxidant vitamins mainly consists in HAT from the antioxidant (Antiox-H) to the free radical ($R^{\bullet}$):

$$\text{Antiox-H} + R^{\bullet} \longrightarrow \text{Antiox}^{\bullet} + RH$$

During this process, the antioxidant becomes a radical itself. So to be active the antioxidant must be able to efficiently delocalize the unpaired electron (mainly by π-conjugation) to stabilize this radical and to make it less reactive than $R^{\bullet}$.

Vitamins are organic molecules required by the metabolism in small concentrations. They cannot be synthetized by the organism and are thus exogenous. Three of them are dedicated to scavenge free radicals: α-tocopherol (vitamin E), ascorbic acid (vitamin C) and β-carotene (part of vitamin A) (Figure 8).

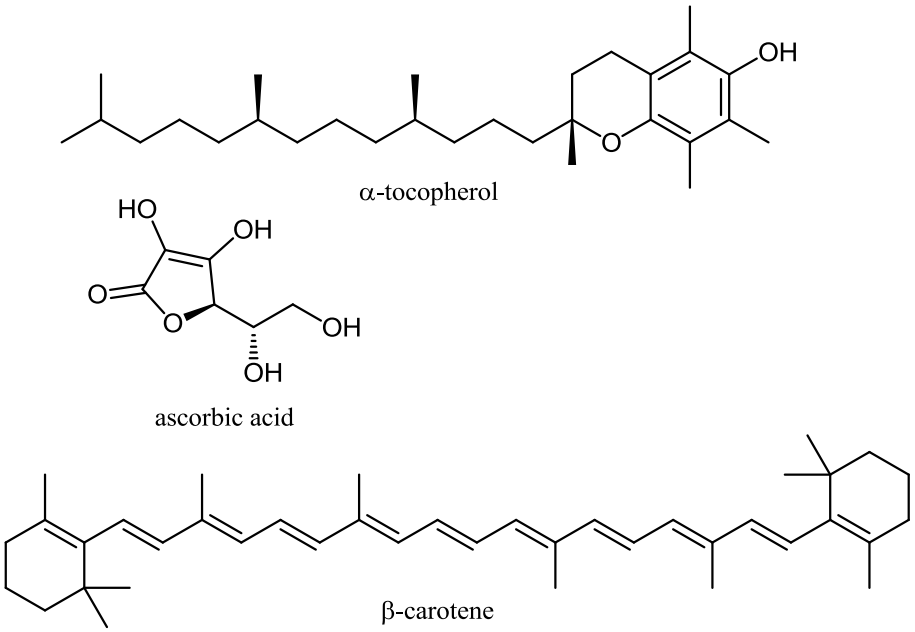


Figure 8: Chemical structures of antioxidant vitamins.

α-tocopherol (vitamin E) is an hydrophobic molecule with a long saturated carbon chain and a phenolic head group. It is thus embedded in lipid bilayers, where it is able to efficiently scavenge lipid peroxyl radicals ($ROO^{\bullet}$) produced during LPO. It is one of the best defense systems of the metabolism against LPO (i) blocking the formation of lipid degradation products such as malondialdehyde, (ii) preventing the oxidation of essential polyunsaturated fatty acids (e.g., linoleic acid), (iii) reducing dramatically the concentration in exhaled air of *n*-pentane, a lipid degradation product due to cigarette smoking,[36] (iv) protecting LDL against oxidation *in vivo*[37] and (v) significantly reducing the occurrence of cancers in animals[30].

Ascorbic acid (vitamin C) is a polar hydrophilic molecule present in extra- and intracellular fluids. It is the first barrier against free radicals in aqueous media. It can also act below the surface of lipid bilayers as an efficient vitamin E regenerator. It efficiently acts against oxidation of cholesterol in LDL[38] and cancer occurrence in animals[30]. In addition, it is able to prevent the formation of nitrosamines, powerful oncogenes formed in the stomach from amine functions of proteins and nitrates or nitrites present in food (Figure 9).

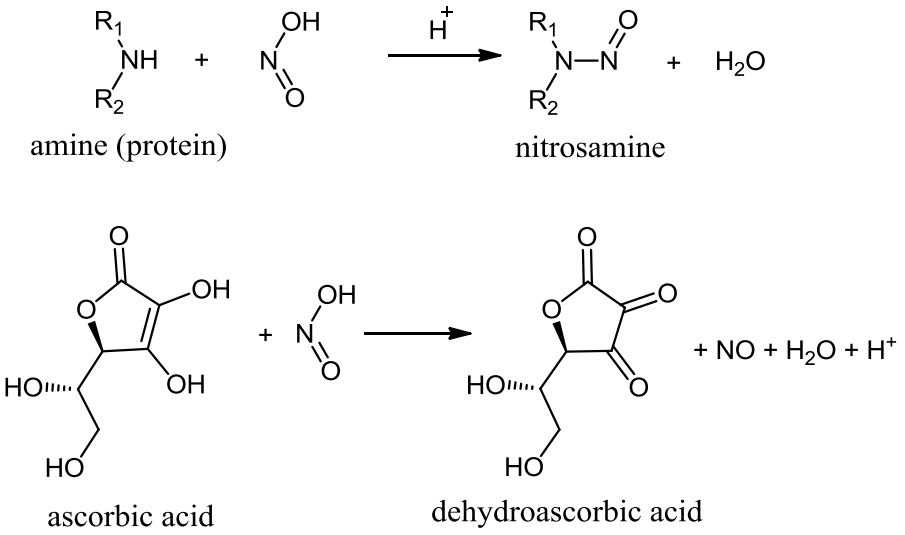


Figure 9: Protective effect of ascorbic acid against the formation of nitrosamines.

β-carotene belongs to the group of carotenoids (vitamin A). It exhibits antioxidant activity thanks to two different mechanisms. First, this hydrophobic molecule is present in membranes and is able to scavenge lipid peroxyl radicals ($ROO^{\bullet}$). The subsequent radical formed from β-carotene is stabilized by the conjugated double bonds. Second, it can very efficiently quench $^1O_2$:

$$\beta\text{-carotene} + {}^1O_2 \longrightarrow \beta\text{-carotene}^* + {}^3O_2$$

where β-carotene* is an excited form of β-carotene, stabilized by the π-conjugation, thus being less reactive than $^1O_2$. β-carotene is only present in very low concentrations in LDL and its antioxidant activity against cardiovascular diseases has not been proven. It exhibited anticarcinogenic effects in many studies on *in vivo* animal models. It was active on (i) various modes of cancer induction, (ii) various organs, (iii) at least one of the three stages of cancer development (i.e., initiation, promotion and progression) and (iv) at doses ranging from 22 to 70 ppm.

2.1.2.4 Polyphenols

At the beginning of their discovery, polyphenols were called “vitamin P”. It is now known that the lack of polyphenol does not induce any short-term disease and they are not anymore classified as vitamins. They constitute a large group of antioxidant molecules present in food (fruit, vegetables, wine, spices, oils, tea…). The polyphenol family regroups hundreds of compounds with different chemical structures, sizes and solubility. As a consequence they exhibit a wide range of biological activities. Their phenol groups confer them a strong capacity to scavenge free radicals. Some polyphenols also exhibit the special ability to chelate metals or to inhibit oxidant enzymes (xanthine oxidase, NADPH oxidase, myeloperoxidase, COXs, LOXs).

Polyphenols exhibit a strong antioxidant activity *in vitro*, either in the water phase[39], in lipid bilayer membranes[28], [40] and LDL[39], [41]. Quercetin (Figure 10), a powerful polyphenolic antioxidant, was shown to localize at the interface between water molecules and the lipid bilayer, thus being (i) a protector against LPO initiators and (ii) a potential vitamin E regenerator.[28] The correlation between *in vitro* and *in vivo* is currently under debate. It appears that the bioavailability of polyphenols could be very low, thus their effects on the whole organism are uncertain. This has been verified for quercetin, but it appears than anthocyanins from berries and catechin from tea are more bioavailable.[42] The non-absorbed fraction of polyphenols exhibits protective effects against cancers of the digestive tract.[43] Another mechanism of action is the metabolism of this fraction by the colonic microbiota into more bioavailable and antioxidant metabolites.[42]

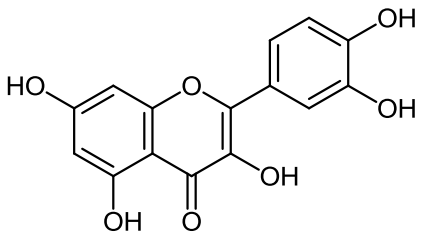


Figure 10: Chemical structure of quercetin, a model antioxidant polyphenol

Renaud and de Lorgeril showed that the mortality caused by cardiovascular diseases was much lower in southern Europe (especially in south-west of France) than in northern countries as UK even if saturated fat consumption was normalized. The only two foodstuffs that correlated with these differences were saturated fat and wine; saturated fat increased mortality while wine decreased it. This was called the "French paradox".[44] Even if the French paradox is controversial, many studies were then performed on this topic, and polyphenols contained in high concentration in red wine seem to participate in these protective effect.[30]

2.1.2.5 Natural antioxidants in drugs

The main source of natural antioxidants is plant food including fruit, vegetables, beverages and spices. Most of the most powerful antioxidants are also available in medicines as supplementation. In France, vitamin E is present as α-tocopherol acetate in capsules (TOCO 500®) or in scar cream (CICATRYL®). Vitamin C is available alone (VITAMINE C UPSA®, LAROSCORBINE®) or in association with other compounds indicated in asthenia (BEROCCA®, SARGENOR®, GURONSAN®), drugs inducing drowsiness (CODOTUSSYL MAUX DE GORGE®) and drugs indicated in fever (EFFERALGAN VITAMINE C®, FERVEX®). β-Carotene is indicated in functional asthenia in association with vitamins C and E (BETASELEN®) or in vascular protection in association with anthocyanosides (DIFRAREL 100®). Many antioxidant cocktails also include metal ions required by antioxidant enzymes such as selenium for Se-GPx.

Polyphenols are mainly available as plant extracts for vascular protection. Examples of medicines or supplements include extracts of grape seeds (ENDOTELON®), *Ginkgo biloba* (TANAKAN®), red grape leaves, silymarin from *Silybum marianum* (LEGALON®) and diosmine (DIOVENOR®).

2.1.3 Clinical studies

Several epidemiological studies have evaluated the role of antioxidants in cardiovascular diseases or cancer. MONICA is a large prospective and transcultural study conducted by the World Health organization (WHO), which evaluated the risk factors of cardiovascular diseases.[45] It showed that vitamin E blood concentration was strongly inversely correlated with the mortality by coronary pathologies. This result was then confirmed by several other studies.[30]

The most recent reference study concerning the beneficial effects vitamin supplementation is SU.VI.MAX.[46] This randomized, double-blind, placebo-controlled trial included 13,017 French adults. During 7.5 years on average, people involved in the study took a daily dose of 120 mg of ascorbic acid, 30 mg of vitamin E, 6 mg of β-carotene, 100 μg of selenium, and 20 mg of zinc, or a placebo. The results were far from expectations: there was no significant impact on total cancer incidence, ischemic cardiovascular disease incidence or all-cause mortality. However, when the results were separated according to sex, cancer incidence and all-cause mortality was significantly lower in men supplemented with the antioxidant cocktail. This result clearly showed that antioxidants play a beneficial role for health. The no-effect results obtained for women seem to be explained by a diet already rich in antioxidants, thus the supplementation was inefficient. Vitamin C and β-carotene also seem to exhibit protective effects although they were weaker antioxidants than vitamin E.

The European Prospective Investigation into Cancer and Nutrition (EPIC) study enrolled more than half a million people all across Europe. It shows that the intake of fruit and vegetables associated with physical activity reduces all-cause of early death including cancers.[47], [48] This study led to the public recommendations of eating five fruit and vegetables per day. The effects of antioxidants were not actually measured, however as fruit and vegetables are a major source of antioxidants, this study is another clue of their protective role in cancer.

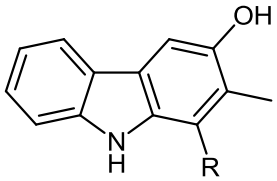
A meta-analysis showed that on 130 studies, 120 showed an inverse correlation between antioxidant intakes and several types of cancer.[49] However, many studies showed no effect of antioxidant vitamins on cancer progression. Therefore it is very likely that a diet rich in fruit and vegetables inhibit early stages of cancer occurrence caused by ROS. Due to the high number of parameters, establishing accurate relationships between heath care and antioxidants is a complex problem that is still under intense debates. However, it clearly appears that a diet rich in fruit and vegetables is healthy and that the presence of antioxidants participates in the beneficial actions.

Natural antioxidants are of prime importance to inhibit the deleterious effects of oxidative stress. The lack of antioxidants leads to health problems as cardiovascular diseases and cancer. There exist a few medicines to supplement the organism in antioxidants but most of them contain polyphenols, for which bioavailability is questionable. Therefore, the research for new natural antioxidants would allow extending the spectrum of drugs indicated in (i) diets low in fruit and vegetables or (ii) inhibition of deleterious effects of acute oxidative stress (anaphylactic shock, intoxication).

2.2 Lipocarbazole, a new natural antioxidant

2.2.1 Introduction

As, mentioned above, LPO is one of the most important sources of free radicals and is implicated in various diseases.[17], [50] Molecules able to inhibit this process are therefore of interest. Recently, a series of novel compounds was synthetized[51] and isolated from *Tsukamurella pseudospumae* Acta 1857, bacteria collected at Stoke Bardolph Water Reclamation Works near Nottingham, UK. These compounds were so-called lipocarbazoles.[52] Due to their structural analogy with carazostatin[53], an effective *in vivo* antioxidant, their biological properties were investigated including antioxidant capacities. In solution, lipocarbazole A3 and A4 (Figure 11, compounds **3** and **4**) are good free radical scavengers, better than vitamin C (see 2.2.3.1 on page 38).



1: R = $(CH_2)_7CH=CHCH_2CH=CHCH_2CH=CHCH_2CH_3$

2: R = $(CH_2)_7CH=CHCH_2CH=CH(CH_2)_4CH_3$

3: R = $(CH_2)_7CH=CH(CH_2)_7CH_3$

4: R = $(CH_2)_{16}CH_3$

5: Carazostatin: R = *n*-C_7H_{15}

6: “Carbazole”: R = H

Figure 11: Chemical structures of carbazole derivatives

An effective LPO inhibitor must i) scavenge efficiently free radicals from both thermodynamic and kinetic points-of-view and ii) incorporate lipid bilayer membranes. Both capacities allow the compound to inhibit the LPO chain reaction *in situ*. Pr. Süssmuth et al. suggested that lipocarbazoles may efficiently act as free radical scavengers in biological membranes. In the present study the capacities for free radical scavenging and for lipid bilayer

penetration were investigated for lipocarbazole A3 (**3**). Thermodynamics of free radical scavenging reactions were obtained using QM calculations, whereas MD simulations described incorporation, position and orientation of the molecule in lipid bilayers.

In order to evaluate the role of the lipid side chain of **3**, MD simulations (5µs total) were performed for this compound and its lipid-side-chain-less counterpart derivative (**6**).

2.2.2 Methodology

2.2.2.1 Force field and membrane model

All molecular dynamic simulations were carried out using the GROMACS package, version 4.5.4[54], [55]. Two compatible united-atom force fields were used: GROMOS 53a6[56] and Berger's[57] for water and heteromolecules, and for phospholipids, respectively. The membrane model bilayer consisted of one hundred and twenty eight 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine,[1] (DOPC, Figure 5A)[59] surrounded by approximately five thousand four hundred water molecules (SPCE model) (Figure 12). Na^+ and Cl^- ions were added to the system by replacing water molecules using the Genion program, at a physiological concentration *C* (0.9% = 0.154 mol.L^{-1}). Since Genion calculates the number of ions to be added with respect to the volume of the box and since membrane is empty of water molecules, the corrected concentration C_{corr} was calculated as:

$$\boldsymbol{C_{corr} = C.\frac{z_{box} - z_{membrane}}{z_{box}}}$$

[1] Phosphatidylcholines represent the main type of phospholipids in human membranes[58]

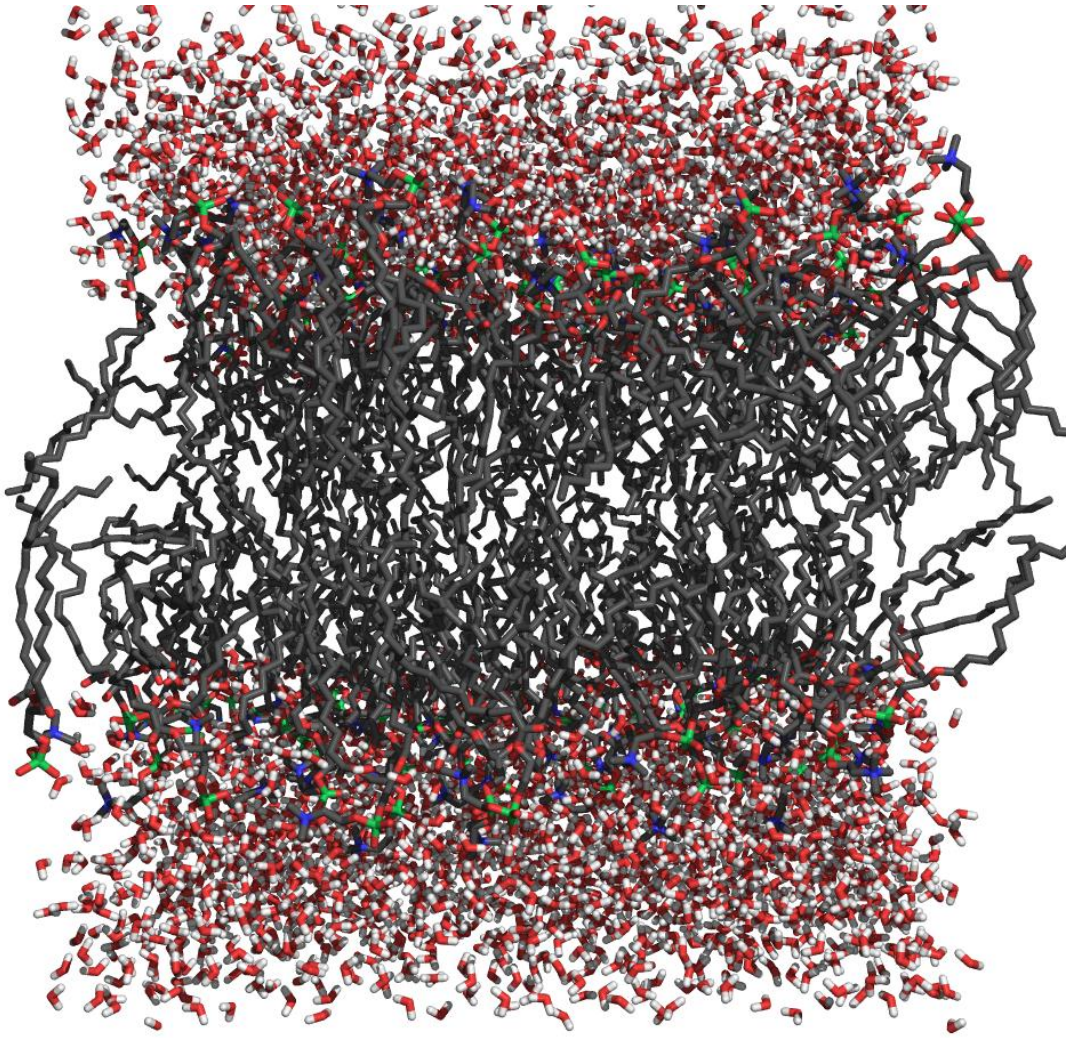


Figure 12: Lipid bilayer model used in simulations.

2.2.2.2 New molecule parameters

There is no standard way to derive force field parameters for heteromolecules. These parameters must be consistent with the force field used for the rest of the molecular system. Here, bonded and van der Waals (vdW) parameters were obtained from the PRODRG webserver[60], except for constants of torsion angles between conjugated systems[2], these values were consistent with the Gromos 53a6 force field.

The set of parameters for electrostatic interactions (partial charges) must be carefully chosen. The charge definition appeared to strongly affect the results concerning the position

[2] We are currently studying the methodological development of such parameters.

of a given molecule in the lipid bilayer. The partial charges defined empirically by PRODRG were significantly lower than those issued from the GROMOS force field and were shown to describe poorly the partitioning between aqueous and cyclohexane phases.[61] The partial charges obtained from the automated force field topology builder (ATB) webserver[62] were close to those from PRODRG. The restrained fit of electrostatic potential (RESP[63]) partial charges were successfully used by AMBER force fields. The ESP charges were obtained from B3LYP/aug-cc-pVTZ[64] calculations obtained on geometries optimized at the same level, with Gaussian 09 software[65]. RESP fit was carried out with the Antechamber package of AMBER 11[66]. The RESP partial charges were tested on a few aminoacids and were in good agreement with the force field.

2.2.2.3 Free simulations

In free simulations, the molecule of interest is placed at different starting points around or inside the lipid bilayer. During the simulation, it is allowed to move freely. After a time sufficiently long, it is possible to observe molecule motions and the location tendency. Depending on the molecule and interaction with membranes, the average location zone can be more or less extended.

Several free simulations were carried out for every studied molecule, with different starting points (far from, close to and inside the lipid bilayer membrane). Energy minimization using steepest-descent algorithm was performed before production simulations. Then, hundreds ns long MD simulations were performed. The Leap-frog Verlet integrator was used with a 2 fs time step. The cut-off for electrostatic and vdW interactions was set to 14 Å. Long range electrostatic interactions were calculated with the particle mesh Ewald (PME) algorithm[67]. Temperature and pressure were set to be as close as possible from the physiological conditions, the temperature being maintained at 310 K by velocity rescaling[68] every 0.1 ps and the pressure being regulated anisotropically (each axis independently) at 1

atm by Parrinello-Rahman barostat[69] with a time constant of 5 ps and a 4.5×10^{-5} bar^{-1} compressibility. All bonds were constrained by LINCS algorithm[70]. Periodic boundary conditions were used along the three axes.

2.2.2.4 Free energy profiles

While free simulations provide an insight on the position and orientation of molecules, free energy profiles allow accessing valuable information including (i) the global energy minimum along z-coordinate (i.e, the vertical axis through the membrane), (ii) free energy barriers for membrane incorporation and crossing, and (iii) free energy differences between inside and outside the lipid bilayer and thus partitioning between lipid and water phases. The z-constrained method[26], [71–74] was used to compute free energy profiles, using the same parameters than for free simulations. In this method, several simulations (windows) were conducted; in each window a part of the molecule of interest was constrained at one specific distance on the vertical z-axis, between the center of the membrane and the center of the water phase (40 Å). The constrained position difference between adjacent windows was set to 0.1 ± 0.02 nm. Equilibrated starting structures were taken from 20 ns long simulations, and were then computed for 40 ns each. The forces acting on constrained molecule at a given z' depth were then averaged over time and integrated along the bilayer orthogonal axis z to build free energy profiles:

$$\Delta G(z) = -\int_{outside}^{z} \langle \vec{F}(z') \rangle_t dz'$$

2.2.3 Experimental antioxidant activity

2.2.3.1 Free radical scavenging

The free radical scavenging activity of lipocarbazole A3 and A4 (Figure 11, compounds **3** and **4**) in solution was evaluated by the DPPH assay. DPPH (1,1-diphenyl-2-picrylhydrazyl,

Figure 13) is a relatively stable and soluble free radical which loses its color when reduced by antioxidants. In this assay, different concentrations of antioxidants are placed in a methanol solution with a given DPPH concentration. The concentration of antioxidant required to scavenge 50% of DPPH radicals (IC_{50}) is then calculated; the lower the IC_{50}, the higher the antioxidant activity.

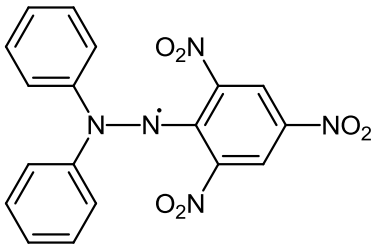


Figure 13: Chemical structure of DPPH

Here, compounds **3** and **4** are more active than ascorbic acid as DPPH free radical scavenger (Table 1).

Table 1: Experimental DPPH scavenging inhibition concentrations [49]

	IC_{50} (µmol.L^{-1})
lipocarbazole A3 (3)	8.73
lipocarbazole A4 (4)	10.49
ascorbic acid	4.40
propyl gallate	13.36

The DPPH assay allows evaluating free radical scavenging activities in relatively polar solutions. It usually partially correlates with lipid peroxidation inhibition in biological membrane but cannot provide a direct evaluation of this activity.

2.2.3.2 Lipid peroxidation inhibition

Lipid peroxidation inhibition was evaluated experimentally on small unilamellar DOPC vesicles, measuring UV absorption of lipid degradation products (namely the dienes). The concentrations required to inhibit 50% of LPO (IC_{50}) are reported in Table 2. Compound **3**

was very active, even more active than reference antioxidants such as α-tocopherol (vitamin E) and quercetin (Figure 10). It was 230 times more active than its lipidless counterpart (**6**).

Table 2: Experimental lipid peroxidation inhibition concentrations

	IC_{50} (mol.L^{-1})
lipocarbazole A3 (3)	$1.6 \pm 0.3 \times 10^{-7}$
carbazole moiety (6)	$3.7 \pm 0.7 \times 10^{-5}$
α-tocopherol	8×10^{-7}
quercetin	2×10^{-7}

2.2.4 Bond dissociation enthalpies

Over the past few years, free radical scavenging by polyphenols (ArOH) has been extensively investigated using quantum chemistry calculations.[75],[76] O-H bond dissociation enthalpy (BDE) was shown to be the major descriptor to rationalize free radical scavenging; the lower the BDE, the higher the capacity of H-atom transfer and the higher the antioxidant-activity of the corresponding OH group. The BDE is a thermodynamic intrinsic parameter calculated as the following difference in enthalpy:

$$BDE(ArO\text{-}\mathbf{H}) = H^{298K}(ArO^{\bullet}+H^{\bullet}) - H^{298K}(ArOH)$$

The O-H BDEs but also those of the NH groups were calculated for **3** and **6** using density functional theory (DFT) with the B3P86 functional and 6-31+G(d,p) basis set (Table 3). The calculations were performed both in the gas phase and in solvent, using PCM. We clearly confirmed that the lowest BDE is obtained for the OH groups, making the N-H bond cleavage unlikely to scavenge free radicals. The O-H BDEs of both compounds are exactly the same, highlighting that both compounds exhibit the same sheer free radical scavenging capacity.

*Table 3: Bond dissociation enthalpies of **3** and **6** in gas and water*

BDE	**Lipocarbazole A3**		**Carbazole moeity**	
(kcal.mol^{-1})	R-OH	R-NH	R-OH	R-NH
Gas	79.5	92.5	80.0	88.3
Water	77.0	95.0	77.0	87.1

2.2.5 Position and orientation in lipid bilayer

In order to investigate the position and orientation of both **3** and **6**, unbiased free simulations were carried out. For each compound, three simulations of 300 ns were performed, either starting in the water phase or in the middle of the lipid bilayer. In all cases, both compounds incorporated the lipid bilayer after a few nanoseconds. After 40 ns, all simulations were equilibrated and simulations of each compound converged to the same location.

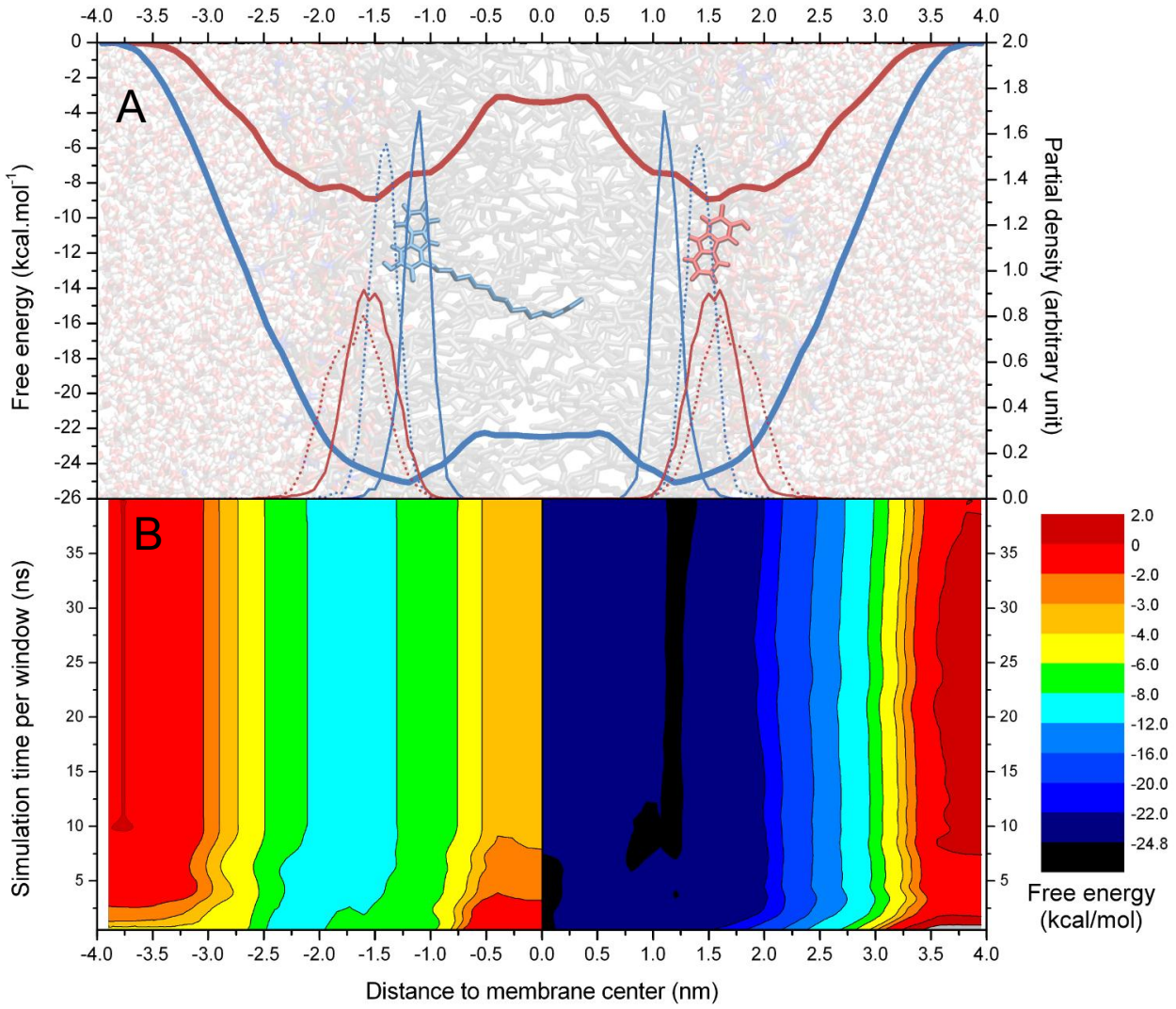


Figure 14: A) partial densities (thin lines) and free energy profiles (bold lines) of carbazole (red) and lipocarbazole (blue). Densities of carbazole moieties are drawn in solid lines and those of active OH group in dotted lines. B) Free energy profiles convergence as a function of window time length. Bottom left: carbazole; bottom right: lipocarbazole.

The equilibrium distances of center of mass (COM) of the carbazole moiety with respect to the center-of-membrane were 1.10 ± 0.05 nm and 1.53 ± 0.13 nm for 3 and 6, respectively (Figure 14A). The OH groups were mainly orientated towards the surface of the membrane for both compounds. However, they were located at 1.41 ± 0.07 nm and 1.66 ± 0.17 nm with respect to the center of membrane, respectively. This means that the active moiety of **3** is

inserted deeper inside the membrane compared to that of **6**. The OH group of **6** (the lipidless compound) is clearly embedded in the region of ester groups of DOPC, in contact with water molecules and with a limited contact with lipid chains. The inhibition of the propagation step of LPO is thus very unlikely. The only way to inhibit LPO for this compound is to scavenge free radical initiators coming from the outer part. The location of **3** allows better probability to act also in the propagation step.

2.2.6 Free energy profiles

In order to calculate the free energy profiles of compounds **3** and **6**, the carbazole moieties were constrained. A similar profile shape was observed for both compounds (Figure 14A), allowing to conclude that both molecules are more stabilized inside the membrane rather than in the water phase. Moreover no energetic barrier was observed for membrane incorporation, suggesting an easy penetration. Three main differences appeared between both compounds. First, the free energy profile confirmed that **3** is inserted deeper inside the bilayer, confirming free simulations. Second, the difference in free energy between the optimized location and the water phase was dramatically lower for **3** (-25.0 kcal.mol^{-1}) compared to **6** (-8.93 kcal.mol^{-1}). Here we can definitely conclude that compound **3** is more likely inserted in membrane with respect to **6**, indicating that the concentration of the former compound in the membrane is much higher than that of the latter. Third, a barrier to cross the center-of-membrane was observed, which is lower for **3** (2.82 kcal.mol^{-1}) than for **6** (5.84 kcal.mol^{-1}). Consequently, **3** has also a capacity of flip-flop from one side of the bilayer to the other. As LPO occurs everywhere between lipid chains, **3** can inhibit the propagation step more efficiently while being present in both sides.[3] The convergence of free energy profiles was assessed (Figure 14B). As for other small molecules like coumarin[27], 10 ns long

[3] This flip-flop was not observed during free simulations because it would require MD simulations at much longer time scales.

windows were sufficient for convergence. However, for bigger molecules longer windows are required.[28] The final profile of **3** was almost reached after 12 ns, but slowly converged until 40 ns.

2.2.7 Conclusion

In this study, the capacity of lipocarbazole to inhibit LPO and the role of its lipid chain in this process is fully rationalized. Even if the lipid side chain does not improve the thermodynamic ability to scavenge free radicals, it plays a key role in positioning lipocarbazole in membrane: i) it pulls the active OH group deeper, increasing the contact with lipid chains affected by LPO, ii) it enhances the free energy difference between polar and apolar phases, thus increasing lipocarbazole concentration inside the membrane, and iii) it lowers the central free energy barrier in membrane, allowing a better contact between lipid chains of the membrane and the active OH group. These results are in perfect agreement with experimental lipid peroxidation inhibition.

This study also opens many perspectives for future works. Compounds **3** and **6** can serve as a reference to understand the role of lipid side chains in compounds incorporating membranes. As we have shown, lipocarbazoles are more active than α-tocopherol as LPO inhibitors and are probably bioavailable, as carazostatin. One can imagine that they could be used as natural food preservatives or as supplementation in antioxidants.

3 CHAPTER 3: INTERACTION WITH MEMBRANE OF PLANTAZOLICIN, A NOVEL ANTIBACTERIAL COMPOUND

3.1 Mechanisms of action of antibacterials

3.1.1 Definition

An antibacterial is a molecule able to cause the death or to slow the growth of bacteria. In the former case the drug is called bactericidal, in the latter it is a bacteriostatic. The term "antibiotic" is also used as a synonym of antibacterial in the common language, but it actually refers to compounds that are active on other types of living organisms such as parasites, viruses or fungi.

3.1.2 Bacterial diseases

Bacteria are present in extremely high concentrations in every environment on earth; for instance, each human being carries ten times more bacteria on his skin and in his intestines than he has constitutive cells. This profusion of organisms is naturally correlated with diversity: a huge number of different species exists but only a very small part of these species are known[77].

Most of the studied bacteria are harmless; some are even beneficial. Many species of the gut flora live in symbiosis with humans, synthetizing vitamins, metabolizing carbohydrates, protecting intestinal barrier and enhancing immune response[78]. However, some species may parasite their host therefore becoming pathogens. When being in contact with the human body, certain bacteria cause diseases, including *Rickettsia*, *Chlamydia* or *Mycobacterium*. Some species are harmlessly present on the skin or in cavities and require a wound or an

immunodeficiency to become pathogen (e.g., *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli*).

Some of the bacterial-caused diseases are infectious diseases causing large world health concerns. In 2010, (i) 5.75 million cases of tuberculosis (*Mycobacterium tuberculosis*), (ii) 317 thousand cases of cholera (*Vibrio cholera*), (iii) 228 thousand cases of leprosy (*Mycobacterium leprae* and *Mycobacterium lepromatosis*), (iv) 129 thousand cases of pertussis (*Bordetella pertussis*) and (v) 11 thousand cases of tetanus (*Clostridium tetani*) were reported [79].

Bacteria are also responsible for numerous diseases related to hospitalization (95% of the cases), these diseases being named nosocomial infections. These infections are attributed to bad hygiene at hospital. In 2006 in France, three main types of nosocomial infections were identified, representing 60% of the cases, namely infections of the urinary tract (30.3%), the lower respiratory tract (14.7%) and surgical sites (14.2%).[80] The three main bacteria species involved in nosocomial infections are *Escherichia coli* (25%), *Staphylococcus aureus* (19%) and *Pseudomonas aeruginosa* (10%).[80] There are two major new concerns with these infections. First, more and more strains develop resistances to antibacterial drugs. Second these strains have spread all over the world over the past decades. Around 52% of *S. aureus*-related cases were resistant to meticillin, an antibacterial used to measure the sensitivity of *S. aureus* to other beta-lactamase-resistant penicillins. In the same way, 25% of *Pseudomonas* was ceftazidime-resistant and 15% of enteric bacteria were cefotaxime- or ceftriaxone-resistant.[80] These data clearly illustrate the constant fighting between deleterious bacteria and humans, and the need to search for new antibacterial molecules active on resistant strains. In this perspective, the bacterial physiology and the mechanism of action of antibacterial compounds must be fully rationalized at the molecular level.

3.1.3 Anatomy of the bacterial cell wall

A key element to tackle the mechanism of action of antibacterial compounds is bacteria's constitution and machinery. In particular, it is of crucial importance to perfectly know the differences that exist between bacterial (prokaryotic) and human (eukaryotic) cells, in order for antibacterials not to target the patient's organs. The nucleus is absent in the former type and the genetic material is constituted of a folded circular DNA named the nucleoid (Figure 15). Despite this major difference, the cell machinery for DNA replication and protein production exhibits similarities in both cell types. However, the lack of organelles and the structural differences between eukaryotic and prokaryotic macromolecules (proteins, DNA, RNA) can be exploited for antibacterial compound targeting.

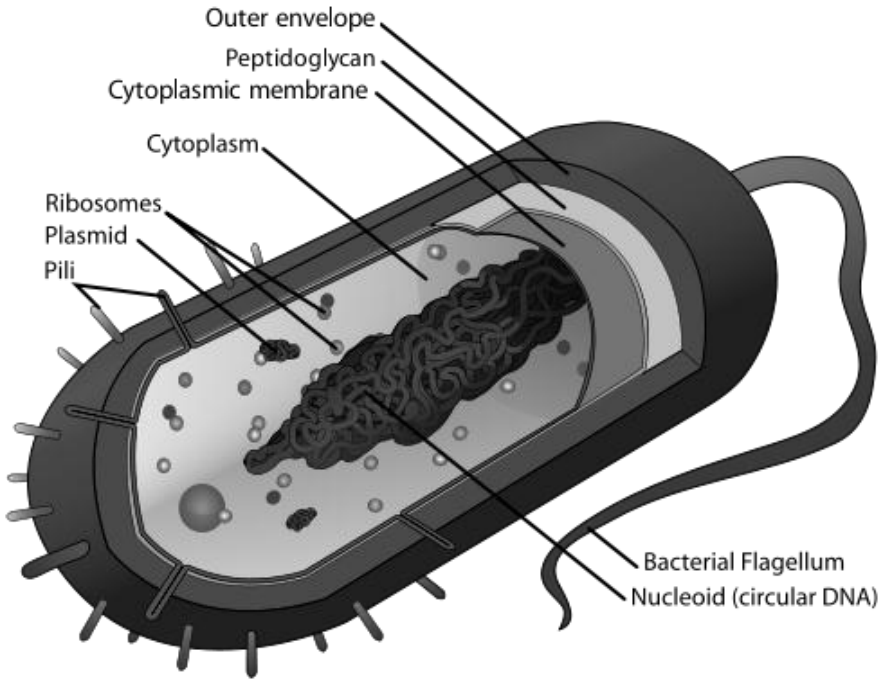


Figure 15: General scheme of bacteria.

Another major specificity of bacterial cells consists in their cell wall. This is a crucial constitutive descriptor of bacteria's survival. Therefore, the cell wall is a target of choice for antibacterial molecules.

3.1.3.1 Gram staining

In 1884, Hans Gram has developed the staining technique allowing to distinguish between two main types of bacteria, namely Gram-positive and Gram-negative. This highlights the difference in the cell wall structure.[81] Gram-positive species exhibit a single phospholipid (PL) bilayer (the cell membrane), generally surrounded by a thick layer of peptidoglycan. Gram-negative bacteria possess two PL bilayers, embedding a thin layer of peptidoglycan.

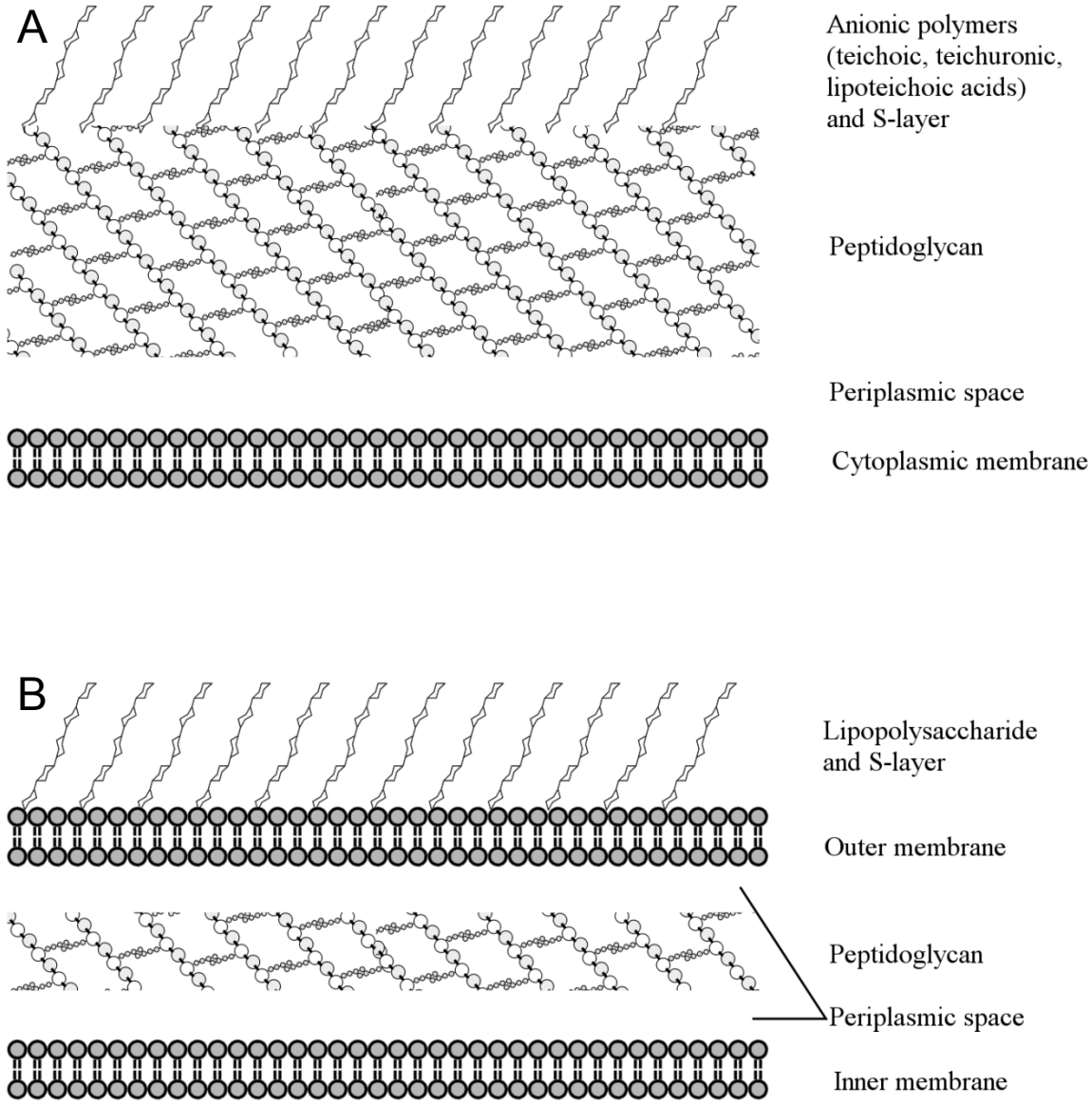


Figure 16: Schemes of cell walls of A) Gram positive and B) Gram negative bacteria.

3.1.3.2 Lipid bilayer(s)

The constitution of bacteria lipid bilayers exhibits a huge diversity, depending on (i) Gram staining, (ii) position of the bilayer (inner or outer) in case of Gram-negative species, (iii) leaflet of the bilayer, (iv) species and strains and (v) growth conditions.

The averaged membrane composition of Gram-positive bacteria is 60% of proteins, 20% of RNA and 20% of lipids. Around 75% of the lipids are PLs, having shorter lipid chains (15 to 17 carbon atoms) compared to eukaryotic membranes (18 to 20 carbon atoms in linear chains). The proportion of polar head groups varies a lot regarding species, strains and growth conditions. For common species of the gender *Bacillus*, the main type of head groups are negatively charged (phosphatidylglycerol or diphosphatidylglycerol), but neutral PLs as phosphatidylethanolamine are also present[82]. In *S. aureus*, positively charged PLs (lysylphosphatidylglycerol) also compose the bilayer[83]. It is important to note that these compositions contrast with eukaryotic bilayers containing mainly zwitterionic (globally neutral) PLs.

Membranes of Gram-negative bacteria also exhibit specificities. The outer membrane consists of proteins, phospholipids and lipopolysaccharides (LPS, see 3.1.3.4), while the inner membrane only consists of proteins and phospholipids. The main type of phospholipids is phosphatidylethanolamine (neutral). Phosphatidylglycerol and diphosphatidylglycerol (negatively charged) are also always present.[84], [85]

3.1.3.3 Peptidoglycan

Peptidoglycan, also known as murein, is a substance composing bacteria cell wall. In all species, it consists of a polymer of sugar and short peptide moieties. The sugar chains (glycan strands) consist of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) linked by β1⟶4 bonds. They are cross-linked by peptide chains constituted of three to five aminoacids

(Figure 17). The number of aminoacids and their chemical structures depend on species. Some are different from the 20 L-aminoacids known for eukaryotes (e.g., D-isoglutamine, D-alanine, D-glutamic acid).

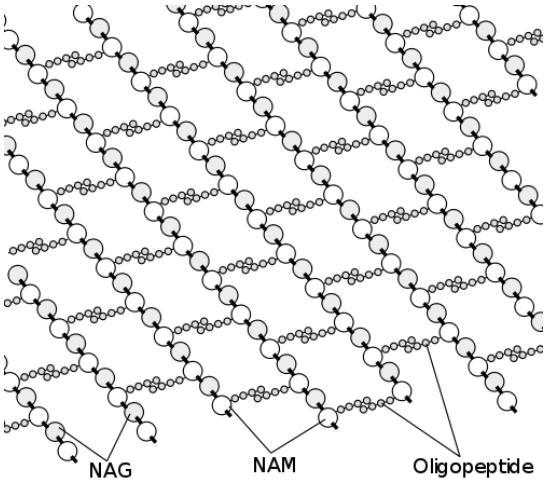


Figure 17: Scheme of peptidoglycan polymer

The biosynthesis of peptidoglycan is a complex process involving numerous steps. The first stage occurs in the cytoplasm and is decomposed into (i) synthesis of the precursors UDP-NAM and UDP-NAG, (ii) stepwise addition of aminoacids to UDP-NAM to form UDP-NAM-peptide, (iii) linkage of the lipid transporter (undecaprenyl phosphate) to UDP-NAM-peptide and (iv) addition of NAG from UDP-NAG to undecaprenol-diphosphate-NAM-peptide to form the complete monomer. In the second stage, the disaccharide-peptide monomer is transported through the lipid bilayer; although the mechanism of this transportation is not fully understood yet. The lipid transporter is then removed, and the polymerization of peptidoglycan starts with the linkage of disaccharide units together via the action of glycosyl transferases to form the glycan strands. In the same time, oligopeptide units are cross-linked between glycan strands by transpeptidases and carboxypeptidases.[86], [87] The enzymes involved in this polymerization process are part of the family of penicillin-binding proteins (PBPs), which are the targets of β-lactamine antibacterial drugs.

The thickness of the peptidoglycan layer is very different between Gram-positive and negative bacteria. In Gram-positive, it is 15 to 60 nm thick and represent 90% of the dry weight of the cell, while it is only 2 to 7 nm thick in Gram-negative cells with a dry weight of 10%.[88] This explains the difference of fixation of Gram's stain. The main function of peptidoglycan is to keep intact the shape and integrity of cells by opposing a force to the turgor (i.e., the internal osmotic pressure). Peptidoglycan is constantly remodeled and repaired, in order to protect itself from degradation or to adapt the shape of the cell in case of mitosis or sporulation.

Even if the chemical structure of peptidoglycan has perfectly been elucidated, its macromolecular conformation is controversial. Several hypotheses have been proposed for the macromolecular architecture, but for each of these schemes even the orientation of the glycan strands remains uncertain.[88] It is known that pores exist in this structure, and their diameters were measured experimentally with an average radius of 2.1 nm regardless the species.[89] Small molecules and proteins of relatively low dimensions are expected to diffuse through the peptidoglycan layer by these canals. This is actually the case of most antibacterial compounds and peptides.

3.1.3.4 Outer envelopes

Bacteria outer envelopes serve as a supplementary protection of the cell. Their composition differs significantly between Gram-positive and negative species. In Gram-positive, anionic polymers are combined within the peptidoglycan layer. They consist of teichoic, teichuronic and lipoteichoic acids. These molecules contain phosphate groups responsible for the linkage to peptidoglycan and have strong negative charges (important for porosity). [86]

In Gram-negative bacteria, the outer membrane contains lipopolysaccharides (LPS), macromolecules constituted of several lipid chain anchors and a long sugar polymer. It protects cells against various chemical stresses and is recognized by B cells, inducing a strong immune response.[90]

In all species, an additional layer is present on the surface: the S-layer. It consists of identical proteins or glycoproteins, although the proteins may differ between species. It is 5 to 25 nm thick layer, which is organized in a quasi-crystalline phase providing special abilities to protect the cell against chemical attacks, to filter molecules through 2 nm pores or to trap ions.[91], [92] It is either linked to peptidoglycan and anionic polymers in Gram-positive bacteria or to lipopolysaccharides in Gram-negative bacteria.

3.1.4 Antibacterial mechanism: a classification by target

In order to be active against bacteria, a compound must fulfill several criteria, namely being capable (i) not to be inactivated, (ii) to approach and penetrate the bacteria, (iii) to reach the target, and (iv) to act on this target. In addition, to be a good candidate for a drug, the molecule must (i) reach the targeted organ, (ii) be non-toxic at therapeutic doses and (iii) exhibit a short excretion half-life (i.e. in the range of a few hours). Discovering molecules having bactericidal or bacteriostatic activity is not an easy task, and finding a compound that is also a good candidate for a future medicine is even harder. The best source of inspiration remains Nature: for billions of years micro-organisms have been creating new antibacterials to fight each other. Studying mechanisms of action of known antibacterial molecules (used as drugs or not) allows to guess the activity of new compounds like plantazolicin. When mechanisms of action are fully rationalized at the molecular level, modifications to the chemical structure can be proposed to enhance their activity or/and lower their toxicity.

Approximately 10,000 antibacterial compounds are known, but only 150 are used in human or animal medicine, which can be sorted in four categories.[93]

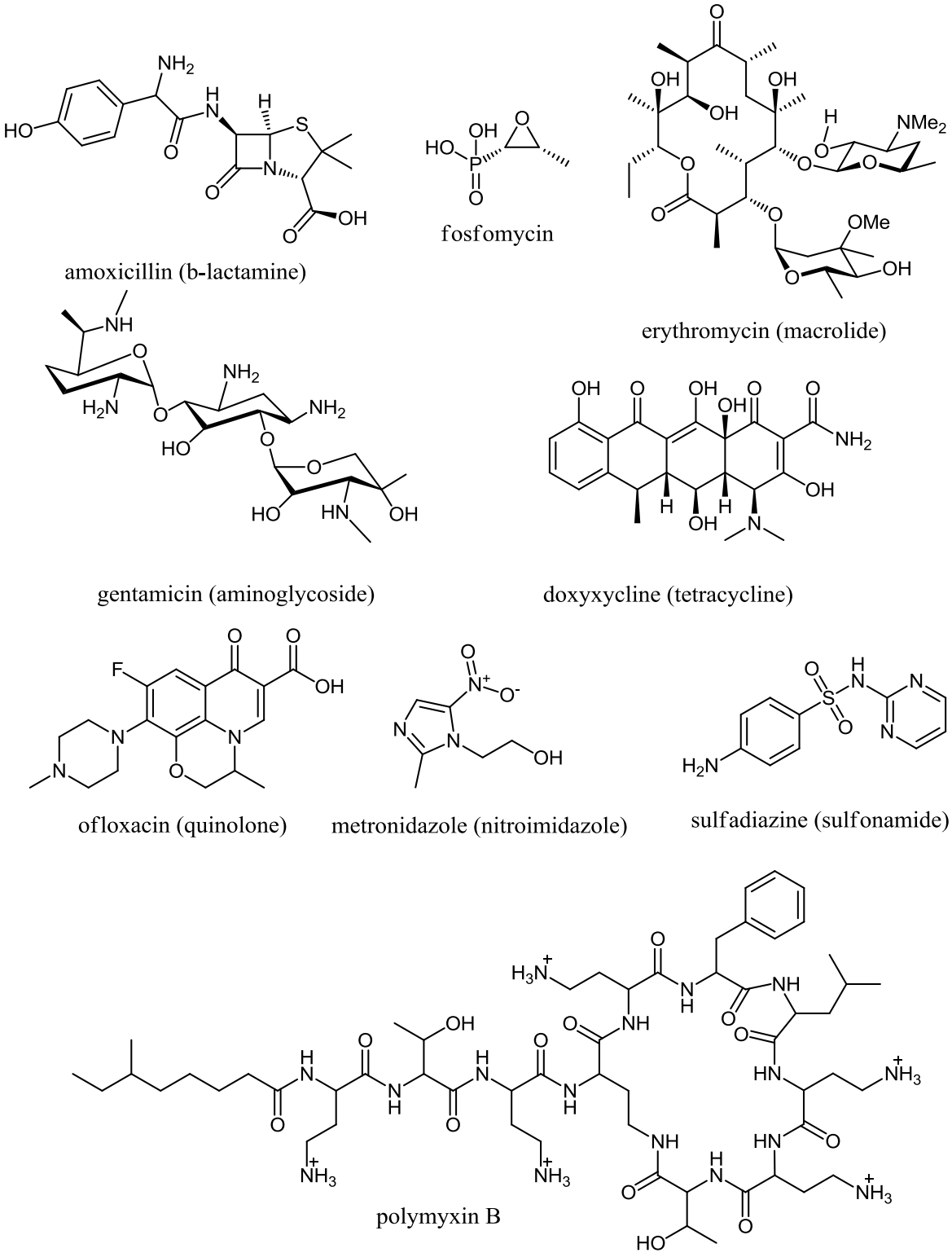


Figure 18: Chemical structures of antibacterial drugs and their families.

3.1.4.1 Antibacterials inhibiting the cell wall synthesis

The main family of this class is β-lactamines. In 1928, Alexander Fleming discovered the first antibacterial: penicillin. By luck, this molecule was non-toxic and very effective against almost all bacteria. This revolutionized healthcare and almost doubled life time expectancy. This family was then expanded with numerous semi-synthetic compounds and still represents a large part of antibacterials used nowadays. β-lactamines exhibit structural analogies with the dipeptide D-ala-D-ala that is present in peptidoglycan monomers, and act as a suicidal compound to block the enzymes responsible for peptidoglycan polymerization (i.e. penicillin-binding proteins transpeptidases and transglycosylases). As a direct consequence, peptidoglycan is not regenerated and this leads to the death of the cell.

Glycopeptides constitute another family; they bind to the dipeptide D-ala-D-ala, thus blocking the action of transpeptidases and transglycosylases. Their important size (approximately 1500 $g.mol^{-1}$) prevents them from crossing the outer membrane of Gram-negative species.

Fosfomycin is a very small molecule (138 $g.mol^{-1}$) which enters the cytoplasm via active transportation and then inactivates one enzyme (UDP-N-acetylglucosamine-3-enolpyruvyltransferase) implicated in the early stages of peptidoglycan monomer synthesis.

3.1.4.2 Antibacterials inhibiting protein synthesis

Macrolides, aminoglycosides, and tetracyclines belong to this class. They bind to the ribosome sub-units RNA, inducing a wrong protein synthesis or stopping it, which leads to the death of the bacterium. Rifampicin acts at early stages of protein synthesis by inhibiting the transcription of DNA to RNA, by RNA polymerase. The difference of spectrum (i.e. the range of species on which the molecule is active) of these compounds is related to their ability to cross the bacterial membranes.

Macrolides are big hydrophilic molecules, thus they are unable to cross the outer membrane of Gram-negative bacteria. This explains why this family of compounds is only active on Gram-positive bacteria.

The mechanism of action of aminoglycosides involves the cytoplasmic membrane in a two-step process. These small compounds are able to reach the cytoplasmic membrane by diffusing through outer membranes and peptidoglycan. The first step named EDP I (energy-dependent phase I) is slow and requires the membrane potential to be sufficient for a few molecules to cross the membrane. This potential is correlated with a concentration threshold of aminoglycosides at the outer surface of the membrane.[94] The mechanism of the second step (EDP II) is not fully elucidated, but it involves binding of few antibacterial molecules to ribosomes and synthesis of misfolded proteins. These proteins are then responsible for an unbalanced production of reactive oxygen species, inducing an oxidative stress on the cytoplasmic membrane. Defects in the lipid bilayer allow the penetration of a much higher concentration of aminoglycosides in the cytoplasm, increasing the bactericidal activity.[95]

3.1.4.3 Antibacterials acting on DNA

This class regroups three families of antibacterials with very different mechanisms of action:

- Quinolones inhibit topoisomerase II, an enzyme that unfolds, cuts and sticks back DNA strands during replication and transcription.
- Nitroimidazoles in which the nitro group is reduced in anaerobic bacteria, produce derivatives able to oxidize the DNA.
- Sulfonamides and trimethoprim inhibit the synthesis of folates, precursors of purine bases of DNA.

All these compounds are relatively small and amphipathic, and thus are able to diffuse passively through the bacterial cell wall.[96]

3.1.4.4 Antibacterials inducing membrane destruction

Only one family of this class of compounds is available as medicines, namely polymyxins, which are active on Gram-negative bacteria. These compounds consist of a linear tripeptide bound in one side to a heptapeptide ring and in the other side to a lipid side chain. The peptide moiety contains numerous non-standard and positively charged aminoacids, mainly (*S*)-2,4-diaminobutanoic acid. The positive charges drive the approach through the negatively charged LPS. Once in contact with the cytoplasmic membrane, some PLs are selectively pulled away from the bilayer, leading to a membrane disorganization, an osmotic imbalance and the cell death.[97]

Polymyxins are only used as last resort treatment against multi-resistant Gram-negative bacteria because they are relatively nephro- and neurotoxic[98] and only bioavailable after intravenous injections. Moreover their mechanism of action is physical in this way that it is based on intermolecular interactions. This powerful property means that the apparition of resistances would imply a deep change in the PLs present in the cytoplasmic membrane or a complete reorganization of the cell wall. The apparition of resistances usually requires a few random DNA mutations. They modify protein chemical structure so that they become insensitive to antibacterial drugs. In the case of membrane reorganization, a huge number of mutations is required in order to modify the numerous proteins involved in PL or cell wall syntheses. The higher the number of mutations, the smaller the probability of resistance. Therefore looking for new peptide-like antibacterials active on membranes is an area of research that has gained a growing interest over the past years: nowadays, more than 1,600 antibacterial peptides have been reported.[99]

The majority of antibacterial peptides act on membranes by permeabilization. To do so, the compound requires i) a 12 to 50 aminoacid length, ii) a strong positive charge ranging from +2 to +9, and iii) amphipathic character i.e., hydrophobic aminoacids on one side (typically more than half of the total aminoacids) and polar and charged aminoacids on the other side. The electrostatic interactions between the positively charged aminoacids of these peptides and the negatively charged LPS or PLs drive these compounds peptides close to the membrane. Afterwards, the peptides may adopt particular conformations, mainly α-helices.

At a threshold concentration membrane perturbation is initiated, following different possible mechanisms. First, stable pores can be formed either by the central canal of single α-helix or by multiple molecules together (carpet-like, toroidal or barrel-stave models). This leads to the loss of membrane potential and the leakage of the cytoplasmic content.

Second, pores can appear when an electric field is created by the charge density of polycationic peptides. At least 0.2 V is required to trigger the formation of 2-4 nm wide pores. This mechanism is called the electroporation model (Figure 19A).

Third, the sinking-raft model (Figure 19B) proposes that antibacterials may aggregate like a raft floating at the surface of the membrane. This induces curvature modifications, inducing pressure gradient changes. To re-equilibrate this superficial pressure, transient pores are formed, so that antibacterials sink i.e., reach the cytoplasm.

Forth, peptides may induce lipid segregation. As mentioned earlier, bacterial membranes consist of a mixture of negatively-charged and neutral (zwitterionic) PLs. Cationic peptides are able to interact preferentially with anionic PL, somehow sorting them, creating weak points in the membrane structure and thus inhibiting important biological processes taking place in membranes.

Fifth, some amphipathic peptides may act as detergents, removing micelles from the bilayer. [83]

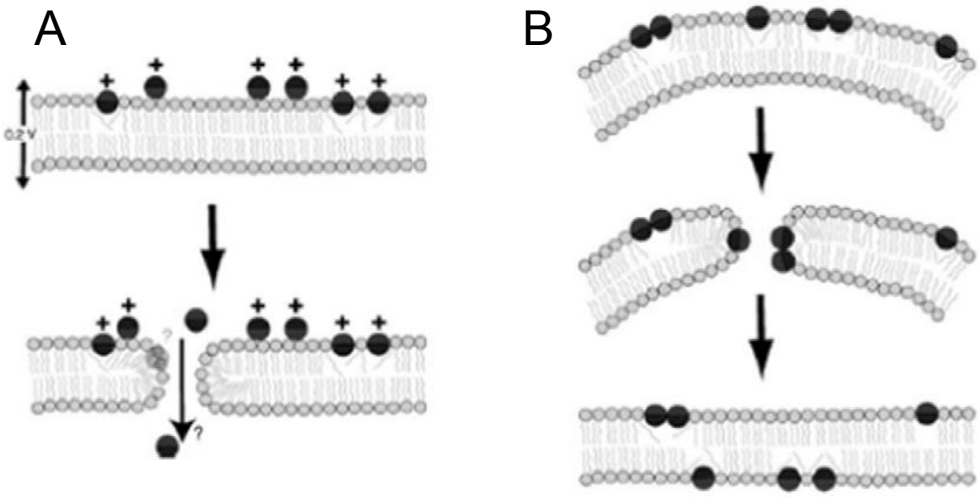


Figure 19: Schemes of A) the electroporation model and B) the sinking-raft model. Adapted from [83].

Among the known antibacterial peptides, several exhibited very promising potential as wide-spectrum compounds in primary tests. They especially bring a new hope against multi-resistant strains. But currently, except polymyxins none was FDA-approved. However, a few peptides already reached the phase III clinical trials e.g., Omiganan that was shown to reduce catheter infections by 40% [100], $rBPI_{21}$ is used against meningococcaemia and septic shock. DPK-060 also exhibited a broad spectrum activity in treatment for atopic dermatitis and eczema during phase I/IIa.

3.2 Plantazolicin, a new natural antibacterial

3.2.1 Introduction

Antibacterial drugs are nowadays broadly used, but often misused either against viral infections including colds or the flu, or because of a bad compliance. This misuse is responsible for the natural selection, in the Darwin's sense, of antibacterial-resistant

strains.[101] The dissemination of multiple resistant strains is now a world-wide health problem, because only a few or no efficient antibacterial treatments exist against them.[102] Three main ways are employed to overcome this problem: (i) to limit apparition of resistances by a reduced usage of antibacterial drugs, (ii) to limit dissemination with a better infection control and (iii) to search for new antimicrobial agents. The latter is crucial to always have one step in advance in the war against resistances.

Drug design offers two choices: either improve an existing compound or search for a new class of derivatives. Novel families of antibacterial are of main interest, because the probability of existence of resistances is lower for new families of compounds than for old and broadly used antibacterials. The simplest way to discover new families of active compounds is to be inspired by Nature. *Bacillus amyloliquefaciens* FZB42 is a bacteria producing a large variety of antibacterial secondary metabolites: polyketides (bacillaene, difficidin, and macrolactin), lipopeptides (surfactin, fengycin, and bacillomycin D), and siderophores (bacillibactin).[103] It was recently found that a FZB42 mutant (RS6), which was unable to produce the compounds mentioned above, was still producing an antibacterial molecule active on closely related gram-positive bacteria.[104] This compound was named plantazolicin.

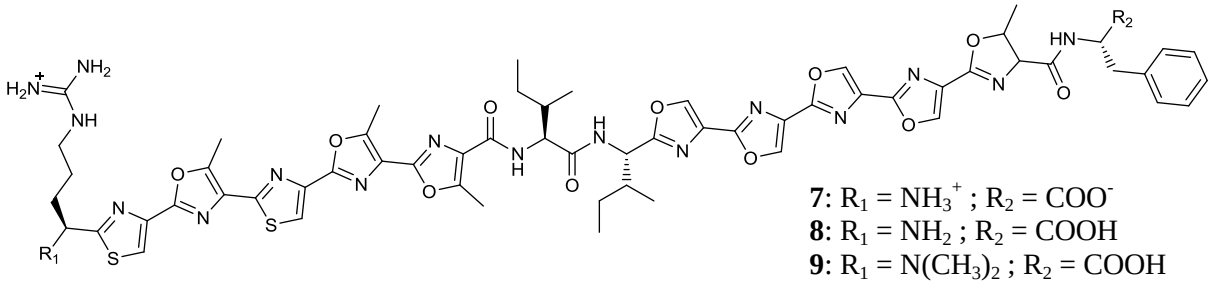


Figure 20: Different chemical structures of plantazolicin used in simulations.

The chemical structure of plantazolicin was recently fully elucidated[105] (Figure 20). It belongs to the thiazole/oxazole-modified microcin (TOMM) family, which includes numerous

compounds with various activities[106] e.g., thiostrepton (50S ribosome inhibitor), trunkamide (anti-cancer compound), microcin B17 (DNA gyrase inhibitor), goadsporin (secondary metabolism inducer), yersiniabactin (siderophore) or Ritonavir (HIV-1 protease inhibitor). TOMM-type compounds are synthesized from peptides with high concentration in cysteine, serine and threonine, which undergo posttranslational cyclodehydration and dehydrogenation to form oxazole and thiazole rings. These modifications confer these compounds (i) a rigid structure, (ii) pharmacophore properties and (iii) a resistance to proteases.

The mechanism of action of plantazolicin as active on closely-related gram positive bacteria has not been elucidated yet. The main part of this peptide is hydrophobic, thus it has been hypothesized that its activity can be somehow related to interactions with membrane. The mechanisms of action of antibacterial peptides may involve membrane in various ways. QM calculations and MD simulations can provide an atomistic description of both (i) the 3D structural feature of the molecule and (ii) its capacity to interact with lipid bilayer membranes.

3.2.2 Methodology

The methodology used in this study was very similar to the methodology used in the study of lipocarbazole. The differences and complementary information are mentioned here.

To investigate the conformation of plantazolicin, MD simulations and QM energy minimizations were performed in aqueous media. MD simulations were carried out in a truncated octahedronal box full of water molecules. The sodium and chloride ions were added at physiological concentration (0.154 $mol.L^{-1}$) taking the C_{corr} correction into account. The edges of the box were 7 Å from the solute. Temperature and pressure were kept constant at 310 K and 1 atm, respectively. In order to investigate more extensively the complex potential energy hypersurface, a simulated annealing procedure was used. In this case, temperature

regulation consists in successive artificial heating/cooling loops, a loop being in this case i) 100 ps heating from 310 K to 400 K, ii) 800 ps slow cooling from 400 K to 310 K and iii) 100 ns equilibration at 310K. Such a 1 ns cycle was repeated hundred times.

QM calculations were performed at the density functional theory (DFT) level. In order to properly take dispersion into account the ωB97XD[107] functional was used with the 6-31+G(d,p) basis set. Solvent effects were taken into account with an implicit method, namely polarizable continuum model (PCM) in which the molecule is embedded in a shape adapted cavity surrounded by a continuum dielectric characterized by its dielectric constant (ε = 78.35 for water).

3.2.3 Conformational analysis: folding in water

Although the chemical structure of plantazolicin is well-elucidated[105], its 3D-conformation is more difficult to guess (or to obtain) while it is crucial in order to tackle its interaction with membranes. There are numerous degrees of freedom in plantazolicin, which does not allow a systematic analysis of the potential energy hypersurface at the QM level. In this case, MD simulations of plantazolicin appeared adequate to obtain the 3D conformations. Starting from a linear conformation, MD simulations (100 ns) provided many different possible conformations. However the structure always converged to a folded conformer in which the charged C-terminal and N-terminal moieties interact, and π-conjugated rings of both parts of the structure face together (Figure 21a). In order to confirm the stability of this conformation, QM calculations were performed using a DFT functional that includes dispersive effects (ωB97XD).[4] The folded conformation (Figure 21b) appeared 41.5 kcal.mol^{-1} more stable than the linear conformation, electrostatic and π-stacking interactions being the driving force explaining this difference.

[4] This kind of methods of calculations (i.e., including dispersive contributions) is mandatory to properly describe non-bonding interaction such as π-stacking interactions.

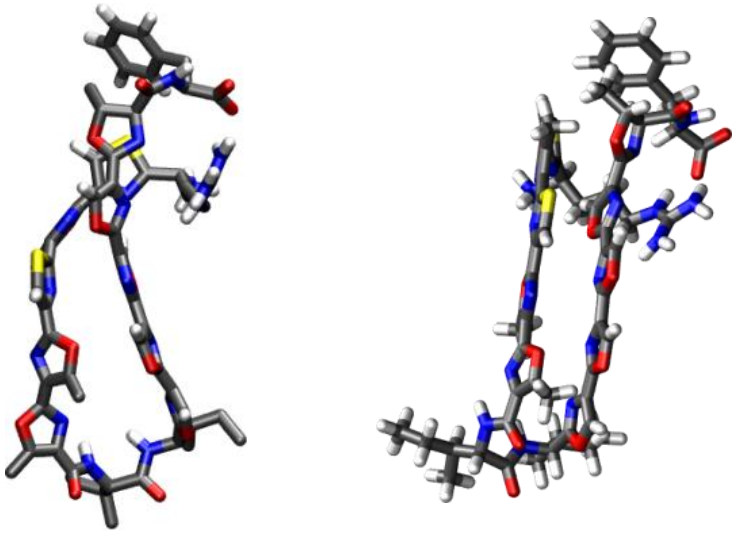


Figure 21: Plantazolicin conformations as obtained from A) MD and B) QM calculations.

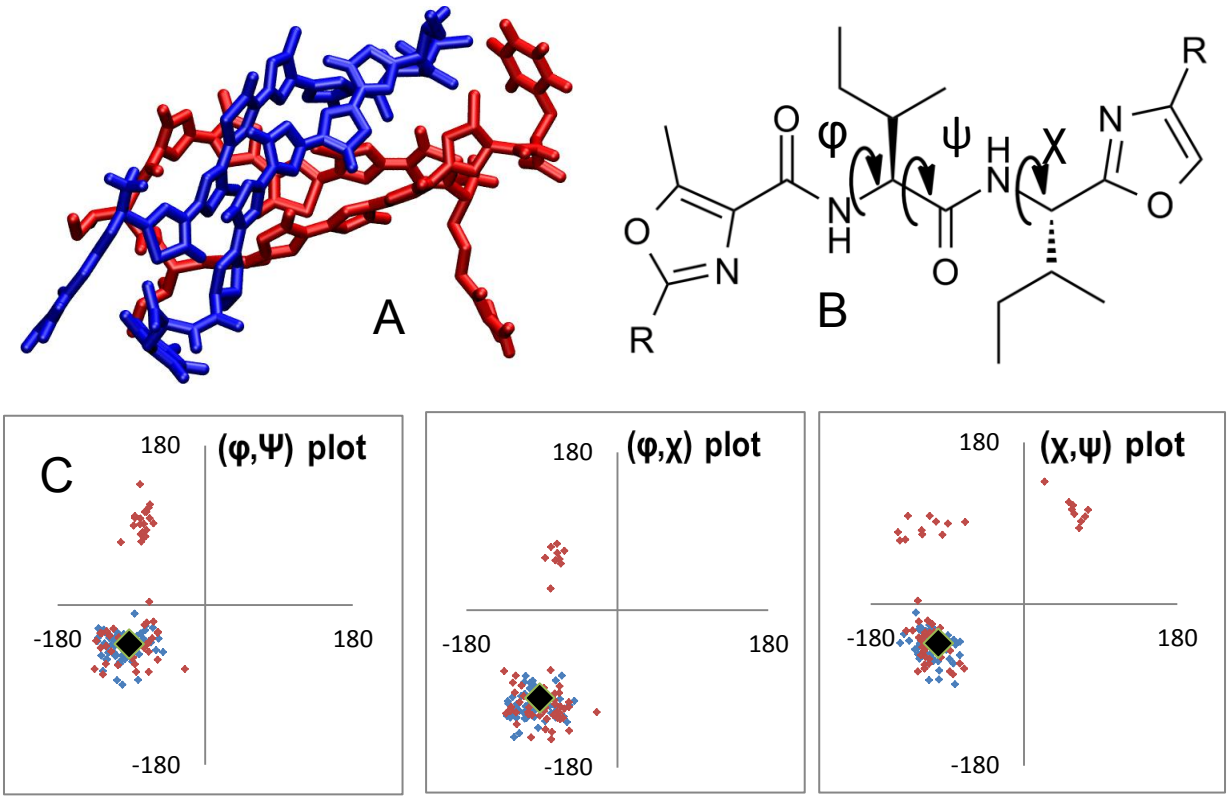


Figure 22: A) Conformation of dimer as obtained by a 100 ns MD simulation. B) Definition of the three dihedral angles analyzed, being responsible for molecule folding. C) Ramachandran plots of these three dihedral angles after each simulated annealing cycle. Black dots correspond to the dihedral values as obtained from the optimized DFT calculation (Figure 21B).

Regarding the results obtained for monomers, the formation of dimers was envisaged. Starting from a head-to-tail linear dimer arrangement, free MD simulation was achieved in water. Due to the high number of degrees of freedom (inducing many possible conformations), simulated annealing was used to explore fully the potential energy hypersurface. Both molecules folded very fast in a similar way compared to monomer simulations. Both molecules stayed close to each other but the final conformation did not appear much interlinked (Figure 22A). In the dimer, both partners adopted a conformation close to that of monomer. To confirm that the potential energy hypersurface was completely explored three dihedral angles responsible for plantazolicin folding were analyzed over the entire simulated annealing simulation (Figure 22B & C). The obtained optimum values (φ = -101° ± 21°, ψ = -47° ± 16°, χ = -109° ± 16°) were in perfect agreement with the QM optimization.

3.2.4 Interaction with membrane

In order to evaluate the behavior of plantazolicin around membrane, several simulations with different starting points were carried out. When plantazolicin was placed in the water phase, in its linear conformation and parallel to the lipid bilayer surface (Figure 23B), it interacted very quickly with the polar phase of the lipid bilayer (phosphates and choline groups) lying on the surface during 100 ns. Interestingly, it did not fold as in water and stayed mainly linear during the time scale studied here. In the same way, when the initial conformation was folded instead of linear (Figure 23C), plantazolicin also interacted very quickly with the polar head groups of the lipid bilayer and lied in this position during 175 ns.

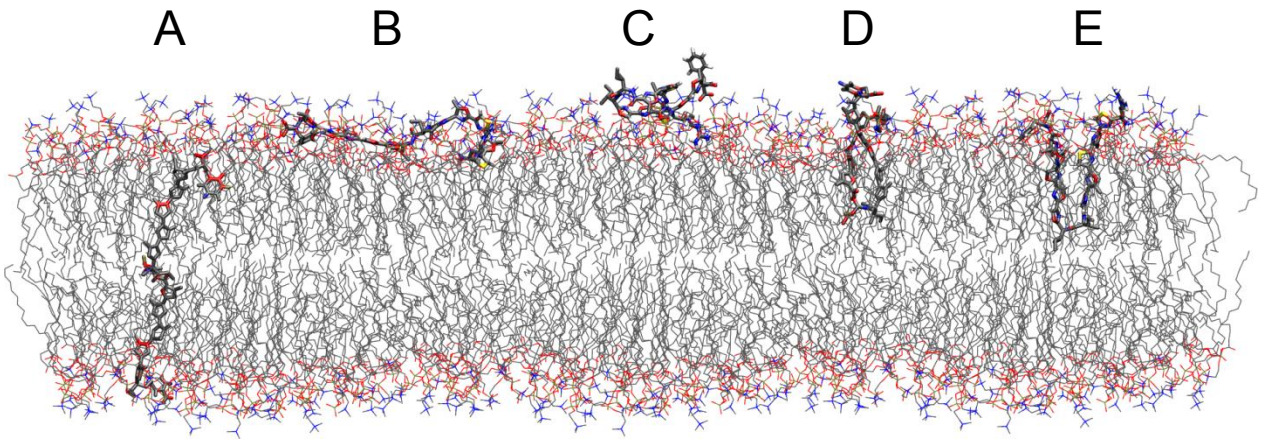


Figure 23: Conformations of free simulations of plantazolicin

Starting positions inside the lipid bilayer were also investigated. In the case of a linear transmembrane conformation (Figure 23A), due to the formal charges at each edge of plantazolicin and because the length of the molecule equals the thickness of the lipid bilayer, it did not fold and stayed transmembrane, even after a 100 ns MD simulation.

Another simulation was carried out in which a folded plantazolicin was fully inserted into the lipid bilayer, terminal charged groups close to phosphates and cholines (Figure 23E). After 200 ns, the molecule moved laterally but stayed at around the same depth in the folded conformation. When the folded plantazolicin was placed only half inserted into the lipid bilayer (Figure 23D), the molecule incorporated fully into the bilayer membrane to reach a similar location as the previous starting point (Figure 23E).

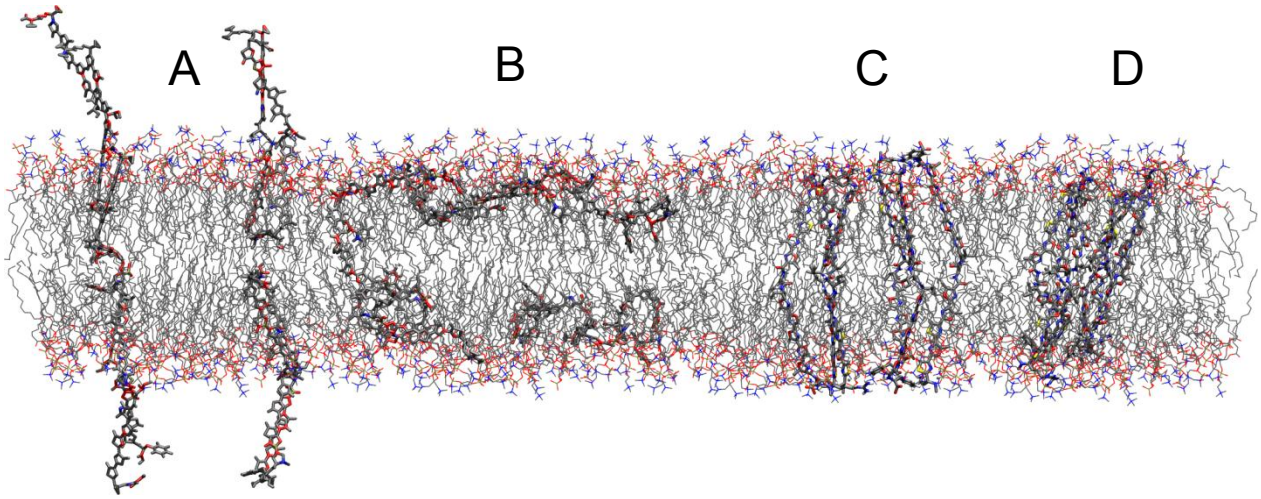


Figure 24: Starting and ending conformations for plantazolicin pore formation. Water is omitted for clarity. A and B: 2-member pore hypothesis. C and D: 6-member pore hypothesis.

3.2.5 Pore formation (prospective study)

To rationalize the antibacterial activity of plantazolicin and by analogy with gramicidin A[108–110], pore formation was suggested. MD simulation may partially confirm or infirm this hypothesis at the molecular level. As other polypeptides, two plantazolicin were suggested to adapt together to form a pore according to Figure 24A. A 100 ns simulated annealing procedure was conducted starting with different orientations of two plantazolicin partners. No dimer or α-helix conformations were observed, probably due to the relative rigidity of plantazolicin (Figure 24B).

It is known that pores can be constituted of several molecules. A MD simulation was performed with six plantazolicin molecules (compound **9**) placed head-to-tail in the membrane, forming a 16 x 31 Å wide pore (Figure 24C). A 100 ns simulated annealing simulation was performed, but after only 13 ns, the pore completely collapsed because plantazolicin molecules packed together (Figure 24D). This result indicates that a pore constituted by several molecules is unsuitable. Due to the capacity of plantazolicin molecules to stack together by dispersive interactions, the formation of a stable pore is highly unlikely. This result was recently confirmed experimentally, showing that no electrical conduction was observed through a lipid bilayer in presence of plantazolicin.

3.2.6 Perspectives

These new highlights on the 3D structure of plantazolicin (namely the folding capacity) now require a thorough analysis of NMR data, including those from NOESY (nuclear Overhauser effect spectroscopy).

The lipid bilayer membrane model used here (DOPC) is an appropriate model for eukaryotic cell membranes. As mentioned previously bacterial membranes differ from eukaryotic cells and also differ between species, strains, growth conditions… The charge

difference seems to play a key role in the interaction between antibacterial peptide and bacterial membranes[83]. This could explain the difference observed in the activity of plantazolicin on different Gram+ species[104]. Some bacteria such as *Staphylococcus aureus* acquire resistances to antibacterial peptide by increasing the concentration of positively charged PLs. As a future improvement, theoretical models of charged membranes are under building, which would allow rationalizing these charge effects.

Several mechanisms of action have been suggested for antibacterial peptides, they are described in detail by Teixera *et al.*[83]. Most of them are complex, requiring relatively long time scales i.e., far beyond the 100 ns time scale investigated with MD simulations. To investigate the ms time scale, less-detailed (non-atomistic) membrane simulations (so-called coarse-grained) could also be used in principle, in order to support experimental data.

3.2.7 Conclusion

Although plantazolicin is "only" active on a few species, as other antibacterial peptides, it has an exciting potential. We showed that molecular modeling is a powerful tool allowing to explore the problem at the atomic scale. We have just opened the way to unravel the mechanism of action of this molecule, towards the full understanding of all remaining questions, e.g. why is it active only on closely-related species? How does *B. amyloliquefaciens* protects itself from it? Can we improve its activity by changing its chemical structure? May an association with another drug increase its activity?

EPILOGUE

Molecular modeling is a rather new science for the pharmaceutical and biological domains, which now becomes mature enough to rationalize the most intimate details of therapeutic actions. It allows (i) to understand the mechanisms of action of bigger and bigger

systems at the atomic level, (ii) to rationalize the reactivity of molecules and propose efficient synthesis pathways and (iii) to help identifying new compounds by predicting spectroscopic data (IR, UV/Visible and NMR). It is also relatively inexpensive, safe and green (toxic or hazardous materials are not used). Thanks to these new possibilities and advantages, molecular modeling has the potential to become a very useful scientific tool in the pharmaceutical domain in the next few years.

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SUMMARY

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

BDE	=	Bond dissociation enthalpy
COM	=	Center of mass
COX	=	Cycloxygenase

DFT	=	Density functional theory
DOPC	=	Dioleoylphosphatidylcholine
DPPH	=	1,1-diphenyl-2-picrylhydrazyl
EDP	=	Energy-dependent phase
EPIC	=	European prospective investigation into cancer and nutrition
ESP	=	Electrostatic potential
FDA	=	Food and drug administration
HAT	=	Hydrogen atom transfer
HF	=	Hartree-Fock
IR	=	Infrared
LDL	=	Low-density lipoproteins
LOX	=	Lipoxygenase
LPO	=	Lipoperoxidation
LPS	=	Lipopolysaccharide
MD	=	Molecular dynamics
MM	=	Molecular mechanics
MONICA	=	Multinational MONItoring of trends and determinants in CArdiovascular disease
NAG	=	N-acetylglucosamine
NAM	=	N-acetylmuramic acid
NMR	=	Nuclear magnetic resonance
NOESY	=	Nuclear Overhauser effect spectroscopy
PBP	=	Penicillin binding protein
PCM	=	Polarizable continuum model
PL	=	Phospholipid
PME	=	Patricle mesh Ewald
QM	=	Quantum mechanics
RESP	=	Restrained fit of electrostatic potential
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
SU.VI.MAX	=	Supplémentation en vitamines et minéraux anti-oxydants
TOMM	=	Thiazole oxazole modified microcin
UDP	=	Uridine diphosphate
UV	=	Ultraviolet
WHO	=	World health organization

SERMENT DE GALIEN

Je jure en présence de mes Maîtres de la Faculté et de mes condisciples :

- d'honorer ceux qui m'ont instruit dans les préceptes de mon art et de leur témoigner ma reconnaissance en restant fidèle à leur enseignement ;

- d'exercer, dans l'intérêt de la santé publique, ma profession avec conscience et de respecter non seulement la législation en vigueur, mais aussi les règles de l'honneur, de la probité et du désintéressement ;

- de ne jamais oublier ma responsabilité, mes devoirs envers le malade et sa dignité humaine, de respecter le secret professionnel.

En aucun cas, je ne consentirai à utiliser mes connaissances et mon état pour corrompre les moeurs et favoriser les actes criminels.

Que les hommes m'accordent leur estime si je suis fidèle à mes promesses.

Que je sois couvert d'opprobre et méprisé de mes confrères, si j'y manque.

ABSTRACT

Based on the structures of natural molecules, many new derivatives are continuously synthetized to enhance therapeutic. To understand the mechanism of action of these compounds, one of the key steps is the capacity to incorporate/cross lipid bilayer membranes. Molecular dynamics (MD) has recently appeared as a powerful tool to rationalize these interactions at the molecular level. Here we report on two different types of derivatives. The first class concerns antioxidants. One of the most important oxidative processes in the organism is lipid peroxidation in cell membrane. To efficiently stop lipid peroxidation, potential antioxidants have to (i) scavenge free radicals and (ii) incorporate inside the membrane. Lipocarbazole exhibits an efficient antioxidant activity against lipid peroxidation in vitro, which can be correlated to its actual position and orientation inside the membrane. The second class concerns antibacterials. Plantazolicin A exhibits antibacterial activity against Gram+ strains. In order to understand this activity (i) the 3D re-arrangement and (ii) its interaction with bacterial membrane must be fully understood. Using a model of lipid bilayer membrane, the capacity of this compound to form pores is evaluated here.

Keywords: membrane, natural compounds, antioxidant, antibacterial, molecular dynamics, mechanism of action.

Simulations de dynamique moléculaire de l'interaction de composés naturels avec les membranes lipidiques.

RÉSUMÉ

Les molécules naturelles, et encore plus particulièrement les composés modifiés par hémisynthèse, présentent un potentiel thérapeutique inépuisable. Un des points clés de la compréhension de leur mécanisme d'action biologique est leur capacité à incorporer/traverser les bicouches lipidiques. La dynamique moléculaire (MD) apparaît comme un outil puissant permettant de décrire les interactions entre un soluté et une bicouche lipidique à l'échelle atomique. Dans ce travail nous décrivons ces interactions pour deux composés naturels. Le premier composé naturel (lipocarbazole) est un antioxydant. Un des processus oxydatifs les plus importants dans l'organisme est la réaction de peroxydation lipidique dans les membranes cellulaires. Pour inhiber efficacement cette réaction, les polyphénols doivent (i) piéger les radicaux libres et (ii) s'incorporer au sein de la membrane. Le lipocarbazole étudié ici a démontré une forte capacité à inhiber la peroxydation lipidique par rapport au carbazole correspondant, mais également par rapport à la vitamine E. Les simulations de dynamique moléculaire permettent de rationaliser le rôle de la chaîne lipidique dans l'interaction avec la bicouche lipidique de ce nouveau composé. La seconde molécule est la plantazolicin A. Elle a montré une activité antibactérienne sur des souches Gram+. Pour comprendre cette activité, (i) sa conformation tridimensionnelle et (ii) son interaction avec la membrane bactérienne doivent être parfaitement comprises. En utilisant un modèle de bicouche lipidique, cette étude théorique apporte des éclaircissements concernant la possible existence d'un pore transmembranaire.

Mots-clés : membrane, molécules naturelles, antioxydant, antibiotique, dynamique moléculaire, mécanisme d'action.