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Chemical highlight supporting the Lipid A role in efficient biological adaptation of Gram-negative bacteria face to external stresses

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Abstract

The outer membrane (OM) of Gram-negative bacteria provides an efficient barrier against external noxious compounds such as antimicrobial agents. Associated with drug target modification it contributes to the overall failure of chemotherapy. In the complex OM architecture, Lipid A plays an essential role by anchoring the lipopolysaccharide in the membrane and ensuring the spatial organization between lipids, proteins, and sugars. Currently, the targets of almost all antibiotics are intracellularly located and required for translocation across membranes. We report herein an integrated view of Lipid A synthesis, membrane assembly, a structure comparison at the molecular structure level of numerous Gram-negative bacterial species as well as its recent use as a target for original antibacterial molecules. This review paves the way for a new vision of a key membrane component that acts during bacterial adaptation to environmental stresses and for the development of new weapons against microbial resistance to usual antibiotics.

Introduction

The Gram-negative bacteria envelope comprises an outer membrane (OM) exhibiting an original architecture with an asymmetrical location of lipopolysaccharide (LPS) when usual

phospholipids organize the inner layer, and an inner/cytoplasmic membrane (IM) which contains phospholipids, sensors, transporters, enzymes, and the respiration/energy system (Figure 1A).¹⁻² A documented paper by Simpson & Trent presented the LPS in the OM properties including biological activities during infection, interactions with the immune system, pathogenesis, and OM vesicles, as well as protection against antimicrobial compounds.³

Thus, the structure of the lipid bilayer and protein organization as well as the sophisticated composition of the OM govern the permeability properties of the latter and consequently the susceptibility of the microorganism to antimicrobial agents targeting intracellular processes.⁴ Currently, the increase and spread of antibiotic resistant bacterial strains is a worrying health concern. Importantly, the alteration of envelope permeability is reported in a majority of MDR isolates. Several alterations, termed membrane-associated mechanisms of resistance⁵, exhibit changes in the lipid or protein composition of the OM. This phenomenon requires an analysis of the modification to understand its involvement in the antibiotic susceptibility of a bacterium to a given antibiotic. Considering the literature focused on Lipid A, the aim of this review is to enlighten the role of this important component of OM in bacterial adaptation and physiology. In addition, Lipid A is discussed with regards to the development of molecules that specifically target this key component to generate original antibiotic candidates that are susceptible to combatting multiresistant Gram-negative bacteria.

Thus, this review focuses exclusively on Lipid A in Gram-negative bacteria and its pivotal role in adaptation to environmental changes, and it does not cover the other already well-reviewed role of O-antigen variation in biological activities and the immune response.⁶⁻⁸

I. OM, structure, and components

The marked difference between Gram-negative and Gram-positive bacteria is due to the OM and to the lipopolysaccharide (LPS) located in this outer leaflet whereas the phospholipids

(composed of approximately phosphatidylglycerol 15%, phosphatidylethanolamine 80% and cardiolipin 5%) are confined to the inner leaflet of this OM (Figure 1A).^{4, 9} This membrane must adapt its composition to the variations in the external environment, and the associated functions implicate the presence of complex regulation systems. Consequently, the outer membrane is a lipid bilayer membrane that contains numerous proteins possessing important functions.¹⁰ Among them, lipoproteins provide stability to the membrane by bridging OM to peptidoglycan, and β -barrel proteins serve essential functions in the transport/influx of solutes and receptor-signaling. In addition, membrane biogenesis represents the two main classes of proteins in the OM. Another type of protein possessing structural and enzymatic functions or implicated in plasmid conjugation can also be found (Figure 1B).¹¹⁻¹² The bacteria also possesses elements such as the flagellum, representing a supramolecular motility machine¹³, the pili, which are involved in surface adherence and motility¹⁴ and a large number of protein receptors that are used by phages, bacteriocins, and drugs present in the surrounding medium.¹⁵

Thus, this unique OM controls the exchange process between the bacterial cell and its environment through porins (Omps) possessing specific size-exclusion properties and LPS acting as a selective barrier both of which are involved in the diffusion of nutrients, dissemination of wastes and transport of other molecules such as iron or even more hydrophobic compounds.² Otherwise, OM retains vital components for cell livelihood such as amino acids, sugars, vitamins, and ions, as well as degradative and detoxifying enzymes in the periplasmic space.¹⁶⁻¹⁷ In this context, Gram-negative bacteria use different mechanisms of antibiotic resistance¹⁸⁻²⁰ one of which is based on a decrease in OM permeability which blocks drug penetration and limits its accumulation in association with efflux pumps.^{2, 5, 21}

The antibiotic structure and target modifications modulate the affinity of the drug and the OM barrier with efflux pumps limit the antibiotics internal concentration. Consequently, they contribute together to the overall failure of antibiotic therapy.²²⁻²³ Recently, Vergalli *et al*

showed that the balance between antibiotic influx/efflux is a prominent parameter that controls the translocation across bacterial membrane and governs the antibiotic concentration in the bacterial cell.²⁴⁻²⁵ The OM permeability plays a major role in the susceptibility of Gram-negative bacteria to antibiotics and this behavior could be manipulated by modifications in proteins or the lipid composition of the OM which can significantly lead to resistance or antibiotic susceptibility in several species.^{2, 21}

Figure 1. Schematic illustration of the Gram-negative bacteria envelope

The lipopolysaccharide (LPS) includes a poly- or oligosaccharide responsible for a range of three biologically and chemically distinct domains (Figure 1B, 1C).²⁶⁻²⁷ The structure of LPS consists of an O-antigen chain, a core oligosaccharide, and a lipid component named Lipid A as described below.²⁸

a. O-antigen or O-specific polysaccharide

Complete LPS, which comprises three regions the Lipid A, the core and the O-antigen is called smooth (*S*)-form-LPS while LPS lacking the O-antigen is named rough (*R*)-form LPS or lipooligosaccharide (LOS).^{26, 29} The O-antigen can only be found in smooth-type LPS, where it determines the antigenic properties (Figure 1C) and consists of repeating units of usually not more than five sugar units possessing a hydrophilic character. Present in the LPS structure, the O-antigen leads to a serotype specificity in the bacterial strains that allows it to escape the host defense system, particularly the attack of serum complement.³⁰

b. Oligosaccharide core KDO region

The core region (Figure 1C) represents the link between Lipid A and O-antigen and contains a hydrophobic inner core and a hydrophilic outer core. It consists of 15 sugar residues and confers antigenic properties in the case of a missing O-antigen or “rough” type LPS.³¹ The LPS core starts with at least one residue of 3-deoxy-d-manno-oct-ulosonic acid (Kdo), which is directly linked to one of the sugar moieties of Lipid A (Figure 1B).

c. Lipid A

Lipid A is the proximal portion of LPS and presents a hydrophobic character. It is anchored in the bacterial OM with variable composition of the acyl residues and phosphoryl groups. Lipid A is involved in the endotoxin activities of Gram-negative bacteria and is possibly associated with outer membrane proteins in the recognition binding sites for bacteriocins, and phages, and it also participates in the binding steps on cells.^{26, 32}

Figure 2 summarizes the different biological activities of LPS and Lipid A, which are not always damaging and can sometimes be beneficial for the host.³³

Figure 2. Chemical structure and some biological properties of *E. coli* Lipid A

In addition, Lipid A presents a close contact with some porins leading to a lipid/ protein complex possessing a hexagonal network structure. Lipid A therefore can play a vital role in the maintenance of the integrity of the outer membrane and cell shape.^{32, 34}

II. Structure and biosynthesis of Lipid A

a. Lipid A structure

To elucidate the Lipid A structure, Luderitz and Westphal subjected lipopolysaccharide to an acidic treatment disrupting the Lipid A core covalent link and obtained the first fragment of LPS containing a deletion of the polysaccharide chains of O-antigen or the nucleus. Later, this fragment was called Lipid A and shown to be involved in the endotoxin activity of the bacterium. In 1984, the first synthetic Lipid A molecule was obtained exhibiting similar biological and toxic characteristics to native *E. coli* Lipid A.³⁵⁻³⁷ From a structural perspective, Lipid A represents the anchor moiety of LPS in the OM, and its distinctive phosphoglycolipid structure is highly preserved among Gram-negative bacteria. Basically, the Lipid A structure consists of a 1,4 -bisphosphorylated- β -D-glucosaminyl-(1 \rightarrow 6)-D-glucosamine disaccharide backbone with four to seven fatty acid chains attached to the N-acetylglucosamine sugars.³⁸⁻³⁹ Most Gram-negative bacterial strains present some variations in Lipid A structure corresponding to the aliphatic chains' length and number, nature and

location of acyl groups, types of hexosamines present, degree of phosphorylation as well as presence of substituents, such as pEtN and 4-amino-4-deoxy-1-arabinose (l-Ara4N). Lipid A can also be chemically modified by phosphoethanolamine, aminoarabinose, or glycine residues, which are key for bacterial pathogenesis/virulence.⁴⁰

In *E. coli*, Lipid A is extensively documented and can include more than 10⁶ Lipid A residues and almost 10⁷ glycerophospholipids⁴¹ allowing the determination of the presence of six fatty acids: four hydroxylated fatty acids having a sequence length of 14 carbons and two (*R*)-3-hydroxy fatty acids. The GlcN residues of the decreasing facet (GlcN I) are acylated by no hydroxy fatty acids (12: 0 and 14: 0). It is noteworthy that few molecular species including a further fatty acid connected to the 3-hydroxy acid linked to an amide and a phosphate moiety substituted through ethanolamine phosphate (from GlcN I) were reported.⁴²

In *E. coli* and *Salmonella spp*, the glucosamines are acylated at positions 2, 3, and 3' and phosphorylated at positions 1 and 4'. Thus, the distal glucosamine, usually present two additional secondary acyl chains and the mature Lipid A is mainly hexa-acylated.

The general structure of the core region is constituted by a nonrepeated oligosaccharide which is bonded to a Lipid A glycosamide, made up of 3-deoxy-D-manno-octo-2-ulosonic acid named Kdo residues, or heptoses and hexoses. It is noteworthy that Kdo can be substituted by phosphate or phosphoethanolamine.⁴³

b. Lipid A biosynthesis

The first identification of Lipid A demonstrated that this component could be released from the rest of the LPS molecules by mild-acid hydrolysis conditions. The genetic, molecular and enzymatic study of Lipid A biosynthesis has been extensively performed for *E. coli* and *Salmonella* and is referred to as the Raetz pathway because a large part of the research was reported by Raetz *et al.* (Figure 3).⁴¹

Figure 3. The Raetz pathway of *E. coli* Lipid A biosynthesis

The biosynthesis of *E. coli* Lipid A requires nine enzymes for catalysis (LpxA, LpxC, LpxD, LpxH, LpxB, LpxK, KdtA, LpxL, LpxM) and three protein-bound acyl donor substrates along with UDP-N-acetylglucosamines (UDP-GlcNAc), ATP, and CMP-3-deoxy-D-manno-octulosonic acid (CMP-Kdo). The different enzymes involved in Lipid A biogenesis are either expressed in the cytoplasm or anchored to the IM to achieve the final assembly of LPS in the OM. Conversely, the cytosolic proteins LpxA, LpxC and LpxD catalyze multiple reactions converting UDP-GlcNAc into UDP-2,3-diacyl-glucosamine.^{38, 41, 44-46}

Lipid A synthesis is initiated in the cytoplasm by the acylation of N-acetyl glucosamine linked to a sugar moiety (UDP-GlcNAc) and recruitment of LpxA enzyme to afford UDP-3-O-(acyl)-GlcNAc. This enzyme grafts the acyl chain from *R*- β -hydroxymyristoyl-ACP to the 3-OH group of UDP-GlcNAc through catalysis of a thermodynamically unfavorable reaction.^{34, 41} To drive the reaction forward, LpxC, a Zn²⁺-dependent metalloenzyme, hydrolyzes the 2-acetamido functionality, and thus catalyzes the first committed step. Following de-acetylation, another *R*- β -hydroxymyristoyl moiety is then incorporated by LpxD to generate the 2-amino group, yielding UDP-2,3-diacyl-glucosamine. After the second acylation by LpxD, UDP-2,3-diacylglucosamine is cleaved at its pyrophosphate bond by the specific pyrophosphatase LpxH, which removes the sugar nucleotide carrier from UDP-2,3-diacylglucosamine to generate 2,3-diacylglucosamine-1-phosphate, known as Lipid X.^{38, 41, 44-}

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The resulting product is a tetra-acylated glucosamine disaccharide, which is inserted in the inner leaflet of the IM and is sometimes termed the "Lipid A disaccharide". Following this condensation step, a specific kinase, LpxK, phosphorylates the 4' position of the disaccharide to form the biphosphorylated Lipid IVA which exhibits some of the properties of endotoxins in mouse cells as potent endotoxin-like agonist whereas it is an endotoxin antagonist in human cells. This unusual pharmacology of Lipid IVA is determined by whether the target cells express mouse or human TLR4.

Prior to late-stage acylation, the addition of two 2-keto-3-deoxyoctonate (Kdo) sugar groups of the core oligosaccharide to Lipid IVA sugar is required. This step is mediated by a bifunctional enzyme KdtA that catalyzes two successive glycosyl transfer reactions to form Kdo2-Lipid IVA.^{38, 41, 44-46} Finally, two additional acylations catalyzed by LpxL and LpxM acyltransferases occur and LpxL adds lauroyl and myristoyl secondary acyl chains, respectively, to yield hexa-acylated Lipid A. The final compound is enzymatically flipped and oriented towards the periplasm *via* the addition of O-antigenic polysaccharide, and it is subsequently transported to the OM.^{38, 41, 44-46}

As we previously mentioned, Lipid A is an essential component for Gram-negative bacteria toxicity. The characterizations of its key differences provide a reminder of the fundamental aims for understanding their biological role. Figure 4 summarizes the most common structures of Gram-negative bacterial Lipid A reported in the literature.

Figure 4. Most common structures of Gram-negative bacterial Lipid A

Thus, the chemical structure of Lipid A greatly varies from one Gram-negative bacterial strain to another. However, many similarities can be observed at the backbone level, as well as low structure variability. Generally, the well-conserved structure is formed by a bisphosphorylated $\beta,1\rightarrow6$ -linked disaccharide of D-glucosamine (GlcN and GlcN3N), basically consisting of four residues of *R*-3 hydroxyl groups with, ester and amide functions. This backbone also carries a large panel of fatty acids chain length that depends on the nature of the bacterial species (see supplementary data).

III. LPS and antibiotic resistance

Infectious diseases are a major human health threat: from the discovery of penicillin and other antibiotics, the resistance of Gram-negative bacteria has recently emerged as a serious global health problem (<https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance>). Worrisome bacteria such as *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii* use diverse

mechanisms of resistance, including antibiotic degradation, target modification, permeability modulation and efflux associated or not with the acquisition of genetic mobile elements that increases the resistance intensity. Interestingly, Gram-negative bacteria are resistant to many antibiotics that are effective against Gram-positive organisms due to the intrinsic non-susceptibility associated with the OM permeability barrier and the lack of drug accumulation close to its target.^{5, 18, 24, 47} Numerous works have examined the mechanisms of resistance of Gram-negative bacteria. Nevertheless, the close relationships between the Lipid A structure and antibiotic bacterial resistance remain poorly understood.¹⁸⁻²⁰

Some of antibiotics reach the cytoplasm by permeating the OM such as macrolides (erythromycin), fusidic acid, aminoglycosides (kanamycin gentamycin) and cationic peptides. Other hydrophobic drugs like tetracycline and quinolones use two different pathways for penetration: the lipid- and porin-mediated pathway in the case of small hydrophilic compounds.^{2, 4, 48-51}

Regarding polymyxin that targets OM, some studies have demonstrated that polymyxin-resistant *S. Typhimurium* mutants bind only 25% of the amount of polymyxin bound by susceptible strains and tolerate up to 100 times higher concentrations of polymyxin B. It has been reported that LPSs isolated from mutants also show less binding of polymyxin B, and an analysis of their respective LPSs demonstrated that they contained 4 to 6 times more 4-aminoarabinose and phosphoethanolamine (PEtN). These modifications, due to the esterification of Lipid A phosphates by these moieties, strongly decrease the negative total charge of the LPS molecule (e.g. from 1.5 to minus 1 for L-Ara4N or from minus 1.5 to 0 for PEtN) that reduce the electrostatic repulsion between adjacent LPS molecules.^{4, 52} The resulting more closely packed LPS layer confer a reduced susceptibility of the mutants not only to polymyxin B but also to PMBN, EDTA and other cationic agents by reducing the affinity for these compounds.^{48, 52-55}

In the same context, studies of *K. pneumoniae* polymyxin resistant strains have reported that the phosphate groups of Lipid A contain five times more L-Ara4N than the susceptible strain. This decrease of the negative charge of OM reduces the interaction with polymyxins, consequently leading to increased resistance.⁵⁶ Han *et al* demonstrated for the first time, that polymyxin-resistant strains of *P. aeruginosa* use deacylation and not the phosphate Lipid A modification to confer high-level polymyxin resistance. Lipid A deacylation is performed by removal of the fatty acyl chain at position 3 which induces a decrease in the hydrophobic interactions with polymyxins. This represents a kind of ‘innate immunity’ response to polymyxins by a compensatory mechanism to the amino-arabinose modification for polymyxin-resistant strains.⁵⁷

Moreover, it has been reported that strains containing a full-length LPS have an intrinsic resistance to hydrophobic antibiotic classes such as macrolides and aminoglycosides.^{52, 55, 58-62}

It is also well-known that six fatty acid tails in Lipid A confer a low OM permeability against hydrophobic drugs. About the relationship between permeability and some defective Lipid A resistant and mutant strains isolated from *E. coli*, the Lipid A hexa-acylated type is not essential for the normal function of the OM barrier and that the last two fatty acids transfer to the nascent Lipid A are not a prerequisite step.⁶³

Interestingly, *E. coli msbB* mutant strains with defective penta-acylated Lipid A containing only three fatty acids linked to GlcN' presented the same permeability barrier against both hydrophobic antibiotics and large glycopeptides drugs as the parent-type control strain. The *msbB* penta-acylated form devoid of the myristic acid residue of nonreducing glucosamine can functionally replace the hexa-acylated form and preserve the OM barrier that protect against various hydrophobic antibiotics, e.g. rifampin, fusidic acid, erythromycin, clindamycin and azithromycin.⁶³

As previously discussed, the modification or removal of the phosphate groups on Lipid A is one of the mechanisms of resistance against cationic antimicrobial peptides (CAMPs). Powers

and Trent observed that while LPS or LOS-deficient, *A. baumannii* strains are highly resistant to polymyxin; otherwise, they become more permeable and sensitive to other antibiotic classes that are normally ineffective against Gram-negative bacteria.⁶⁴ *A. baumannii* colistin resistant strains show another kind of modifications. They typically use PEtN phosphorylation exactly at the 4'-phosphate or 1-phosphate group of Lipid A but they can also use Lipid A glycosylation with galactosamine (hexosamine) at the 1-phosphate group. This unique modification is similar to the L-Ara4N modification in colistin (Polymyxin E)-resistant enteric bacteria.⁵⁶

PEtN modification is mediated by EptA enzyme that ensures the transport of PEtN from phosphatidylethanolamine to Lipid A mainly to the 1-phosphate group but also to the 4'-phosphate. ArnT is the enzyme responsible for the branching of L-Ara4N to Lipid A at the phosphate at position 4', as well as the 1-phosphate position.⁴³ In the absence of L LARA4N or under other growth conditions, EptA can also modify the Lipid A at the 4' position with a second phosphoethanolamine moiety.³⁴

Some *Enterobacteriaceae* such as *Salmonella* and *Escherichia* use a similar Lipid A modification to confer resistance, with other species involving various alternatives such as *V. cholerae* that can modify the acyl chains of its Lipid A by adding amino acid moieties, or *Bordetella* isolates that switch the phosphates of their Lipid A with glucosamine. These two variations greatly increase CAMP resistance in these strains compared with their sensitive parental ones.⁴³ Altogether, these data suggest that the presence of 4-amino-4-deoxy-L-arabinose in LPS is the key to resistance against peptides. This is the case for *Burkholderia cenocepacia* displaying an original resistance to antimicrobial peptides and other antibiotics thanks to this component. The defective synthesis of 4-amino-4-deoxy-L-arabinose observed in mutants of *B. cenocepacia* strains highly increases susceptibility to polymyxin and melittin and alters OM permeability. The authors suggested that LPS modification that takes place

after the binding of 4-amino-4-deoxy-L-arabinose molecule to Lipid A determines the LPS transport and its correct assembly into the OM.⁶⁵

In the last decade, studies have shown that the alteration of LPS transport is deleterious for most Gram-negative bacteria.^{20, 66-67} The LPS transport from the *E. coli* inner to the outer membrane is managed by seven essential proteins named Lpt (LptA, B, C, D, E, F and G). Unfortunately, this mechanism of transport remains poorly molecularly defined despite reports that functional Lpt proteins play key roles in OM assembly and bacterial survival.⁶⁸⁻⁷⁰

Divalent cations also play an important role in the branching of Lipid A headgroups, and dynamic simulations and NMR studies have indicated that the tight cross-linking of LPS headgroups promotes a slow diffusion rate of LPS and consequently reduces OM permeability.⁷¹ Additionally, the disruption or damage of the OM structure could be obtained by removing Mg^{2+} , which confers an easy route of entry for larger molecules across the membrane.⁷²

OM is characterized by a strong duality/antagonism between providing nutrient permeation needed by growing cells and maintaining a robust barrier against external aggression.² The OM also constitutes a selective barrier via the presence of pore-forming proteins (Omp channel), since reaching the periplasm and cytoplasmic membrane constitute an important gateway for antibiotic efficiency.^{24, 73} Any decrease in the penetration of these compounds can lead to resistance by altering their intrabacterial concentration.⁵ Several reports have related antibiotic resistance to the loss or functional change of porins in a large number of organisms, such as *Escherichia*, *Enterobacter*, *Klebsiella*, *Neisseria*, *Pseudomonas*.^{21, 48, 74} Studies have demonstrated that loss/reduction of the porin number, exchange of one porin for another and alteration of their function due to specific mutations increase the impermeability and contribute to antibiotic resistance. Interestingly, several strains express a high constitutive or inducible β -lactamase activity that amplifies the resistance profile in those porin minus strains.^{4, 21, 24}

IV. Perspective for combatting multidrug resistant Gram-negative bacteria:

The lipid metabolism inhibitors

Fatty acids are key components of biological membranes and are synthesized/modified through a repetitive cycle of four reactions involving condensation, reduction and dehydration of carbon-carbon bonds.⁷⁵ Significant differences between bacterial and human fatty acid synthesis systems have been reported, including the typical organization and structure of enzymes involved as well as their specific locations and roles played by the fatty acids, making this system an attractive target for antibacterial drug discovery.⁷⁶⁻⁷⁹

In bacteria, during fatty acid biosynthesis, the chain elongation step consists of the condensation of acyl groups derived from acyl-ACP or acyl-CoA with malonyl-ACP involving condensing enzymes (FabH type) (Figure 5A).^{76, 80} Thus, *E. coli* FabH selectively uses acetyl-CoA to initiate the pathway producing straight fatty acids chains. Interestingly, *Bacillus subtilis* contains two FabH isozymes (FabHA and FabHB) that are selective for branched chain acyl-CoAs and produce mainly branched fatty acids chains.^{76, 81-82}

Figure 5. A) Bacterial fatty acid biosynthesis pathway. B) Structure and biological activities of known FabF/B, FabH inhibitors.

Different compounds having inhibitory effects on bacterial syntheses are described as antibiotics, such as cerulenin⁸³, thiolactomycin⁸⁴⁻⁸⁷ platensimycin and platencin (Figure 5B).^{76, 80}

Cerulenin was originally synthesized and isolated from a fungus *Cephalosporium cerulean*.⁸⁸⁻⁸⁹ Studies have demonstrated that this fungal toxin inhibits the eukaryotic and prokaryotic ketoacyl-acyl carrier protein synthases or condensing enzymes⁸⁰ and presents a great selectivity for FabB⁸² in *E. coli* while FabF is less sensitive.^{75, 90-92}

Investigation of the cerulenin mode of action indicated that this antibiotic specifically blocks fatty acid and sterol biosynthesis in cell-free system.⁸⁸ Most fatty acid synthesis inhibitors

recurrently inhibit polyketide synthesis, and cerulenin appears to be a powerful inhibitor of this pathway.⁹³ Thus, cerulenin inhibits fatty acid synthesis by forming a covalent bond with the catalytic cysteine located inside the active site and its tail plugs the long hydrophobic cavity that normally captures the growing acyl chain of the natural substrate.^{76, 82, 91, 94} Alteration of lipid synthesis leads to the disruption of bacterial membrane with harmful consequences.⁹⁵ Cerulenin inhibits FabF/B with an IC₅₀ ranging from 0.1 to 2 µg/mL whereas the IC₅₀ against FabH is 150 µg/mL.⁸²

Several natural β-ketoacyl-ACP synthases (KAS) inhibitors have been described such as thiolactomycin isolated in 1982 from a soil sample and obtained from fermentation broth of *Nocardia* species.^{81, 87, 96-97} This compound presented an original thiolactone structure that was chemically different from the usual antibiotics and was able to inhibit the incorporation of acetate into cellular fatty acids of *E. coli*.⁹⁶ Thus, FabB was the main target of thiolactomycin that alters the activity of all three β-ketoacyl-ACP synthase condensing enzyme systems (I to III) and acetyl coenzyme A (CoA).⁷⁵ Thiolactomycin inhibited KasA and KasB with IC₅₀ values of 20 µM and 90 µM respectively and showed a moderate activity against a broad spectrum of both Gram-positive and negative bacteria, as well as mycobacteria.^{75, 81, 85, 93, 98-99} These preliminary data have stimulated studies to improve the antibacterial activity of this natural product and Merck has reported the isolation from *S. platensis*¹⁰⁰⁻¹⁰² of a metabolite, namely, platensimycin possessing a dual mode of action similar to that of thiolactomycin but presenting 200-fold higher antibacterial activity against *S. aureus* than the latter.⁷⁶ It has been recognized that this molecule exhibits a potent broad-spectrum activity against a wide range of Gram-positive bacteria such as methicillin-resistant *S. aureus* (MRSA) and vancomycin resistant *enterococci* (VRE) (Table S1). Furthermore, *in vivo* efficacy and low cytotoxicity have been reported in mice.^{100, 103-104} Platensimycin is able to inhibit the elongation-condensing enzyme ketoacyl-acyl carrier protein synthase II (FabF) required for bacterial cell membranes building.^{93, 103-105} More precisely, platensimycin has demonstrated a selective

FabF inhibitory activity with IC_{50} value of 0.29 nM, respectively, whereas it presented a weak inhibition of FabH with an IC_{50} value of 247 μ M.^{76, 93}

Unfortunately, very weak antibacterial activity was noticed against Gram-negative pathogens, partially due to the efflux mechanisms as demonstrated by using a *tolC* mutant strain of *E. coli* exhibiting low susceptibility to platensimycin^{101, 105} (Figure 5B). To conclude, platensimycin does not seem to be an attractive drug candidate due to its poor pharmacokinetic properties compromising its further clinical development.¹⁰⁴

Platencin, a derivative of platensimycin antibiotic, was then isolated from a new strain of *S. platensis* MA733 collected from a soil sample from Spain⁷⁶ and reported to have strong antimicrobial activity against sensitive and resistant Gram-positive strains with MIC values ranging from 0.06 to 4 μ g/mL. Conversely, moderate activities against Gram-negative bacteria were observed in association with the efflux pump mechanism.^{76, 101-103} This compound exhibited potent inhibitory activity targeting both FabF and FabH, two essential condensing ketoacyl-[acyl carrier protein (ACP)] synthase II compounds involved in fatty acid synthesis.^{93, 101, 106} The IC_{50} against the FabF and The FabH were between 4.58 μ M and 9.17 μ M respectively.^{76, 101-102} This natural product was evaluated as a strong drug candidate due to its potent *in vivo* efficacy and complete absence of toxic side-effects.⁷⁶

The synthesis pathway of Lipid A offers promising targets for new drugs, as it is involved in the viability/physiology/virulence of Gram-negative bacteria. It has been shown that the loss of Lipid A contributes to bacterial death and to increasing the antibiotic susceptibility of bacteria.¹⁰⁷⁻¹⁰⁸ The inhibition of one of the first six enzymes of Kdo2-Lipid A synthesis has been shown to be lethal for *E. coli*¹⁰⁹⁻¹¹⁰ and/or renders the bacteria susceptible to other antibiotics that normally do not penetrate the outer membrane.^{41, 107, 111-112}

Thus, the strategy of identifying new Lpx inhibitors has a vital role in the development of new antimicrobial agents.¹⁰⁷

➤ LpxA

UDP-N-acetylglucosamine acyltransferase or LpxA is the first enzyme of LPS biosynthesis pathway that catalyzes the reversible transfer of R-3-hydroxy-acyl moiety from its donor acyl carrier protein (ACP) thioester to the UDP-N-acetyl-glucosamine. This enzyme is necessary for bacterial survival and the absence of any homologous protein in humans renders it as a promising target for anti-bacterial drug development.¹¹³⁻¹¹⁶ Thus, LpxA is a well-conserved essential enzyme found in most Gram-negative bacteria such as *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *S. enterica* serovar Typhimurium except for *A. baumannii* which can grow without LPS under laboratory growth conditions.¹¹⁶⁻¹¹⁷

In the last decade, only peptide inhibitors which could represent a potential tool for the development and the screening of compounds binding the LpxA enzyme have been reported.¹¹⁸ However, due to the lack of delivery system, these peptide inhibitors could not be considered as antibacterial agents targeting cytoplasmic LpxA. Thus, no LpxA inhibitor having antibacterial activity has been publicly disclosed¹¹⁹ since unrealistic due to their impossibility to cross bacterial membranes and their low stability towards proteases activity.¹²⁰

The first peptide inhibitor with a high affinity to LpxA, called **peptide 920** (NH₂-SSGWMLDPIAGKWSR-CO₂H) was discovered by Williams *et al.* using a phage display and identified as potential inhibitor of *E. coli* LpxA (*EcLpxA*) with a K_i of 50 nM.¹²¹ Thus, even if no antibiotic activity of **peptide 920** was observed *in vivo* probably due to its inability to cross the membranes¹²² the enzymatic evidence revealed that this compound is a largely competitive inhibitor with respect to the substrate R-3-hydroxymyristate-ACP but not with the substrate UDP-3-O(R-hydroxymyristoyl)-GlcNAc.^{116, 121} It was suggested that **peptide 920** interacts with LpxA in a region that overlaps mainly with the R-3-hydroxymyristoyl-ACP substrate-binding site observed in the crystal structure of the **peptide 920–EcLpxA** complex.^{121, 123} Based on these assumptions, Dangkulwanich *et al.* designed a small library of peptides by varying the number of peptides involved and by using **peptide 920** as a scaffold.

Table 1 summarizes the results encountered with IC_{50} ranging from 50 nM to >100 μ M. Thus, the peptides **CR19-CR22** can be considered as potent hits for the design of LpxA inhibitors and **CR20** ($IC_{50} = 50$ nM) appeared as the most effective inhibitor targeting LpxA mainly in the binding site of acyl-ACP (Table 1). Further experiments revealed that in the co-crystallization complex **CR20-LpxA** the peptide **CR20** is more ordered in the active site of LpxA than **peptide 920** and that its interaction with LpxA is secured by a majority of polar interactions mediated by water molecules.¹²⁴

Table 1: Sequence and biological activity of identified LpxA peptide inhibitors

Peptide	Sequence	IC_{50}
Peptide 920	SSGWMLDPIAGKWSR	60 ± 9 nM
CR19	GWMLDPIAGKWSR	77 ± 3 nM
CR20	WMLDPIAGKWSR	50 ± 6 nM
CR21	SSGWMLDPIAGKWS	12 ± 1.4 μ M
CR22	SSGWMLDPIAGKW	9 ± 2.2 μ M
CR23	GWMLDPIAGKW	10 ± 0.3 μ M
CR24	GWMLDPIA	>100 μ M
CR25	WMLDPI	>100 μ M
CR26	WMLD	>100 μ M

Despite the challenge of the peptide delivery into bacterial cytoplasm, Jenkins and Dotson have identified three peptides called **RJPD31**, **RJPD33**, **RJPD34** with ordered sequence QHFMVPDINDMQ-NH₂, TNLYMLPKWDIP-NH₂, SENNFMLPLLPL-NH₂ respectively¹²⁵ and even though these peptides exert potent antibacterial activity, they show poor bioavailability and susceptibility.¹²⁶ Among them the most interesting was **RJPD33** presenting a dual specific affinity for LpxA and LpxD by competing with mutually exclusive manner with *R*-3-hydroxymyristoyl-ACP substrate and potentially inhibiting the two

acyltransferases of *E. coli*. Thus, this peptide inhibits LpxD ($K_D = 6\mu\text{M}$) and LpxA ($K_D = 20\mu\text{M}$)¹²⁷ but is considered as a weaker inhibitor of EcLpxA compared to **Peptide 920**.¹²² It is noteworthy that to date and despite its relatively weak binding affinity and its lack of antibiotic activity *in vitro*, **RJPD33** remains an attractive lead scaffold for further optimization of peptidomimetics due to its dual specificity against two essential enzymes in the lipid A pathway.¹²²

On the other hand, the crystal structure of EcLpxA in complex with **RJPD33** was determined at 1.9 Å indicating that the peptide binds in a unique modality that mimics (*R*)-hydroxyacyl pantetheine (vitamin B5) binding to LpxA. By superposing the two complexes of EcLpxA-**RJPD33** and EcLpxD-**RJPD33**, it was also noticed the presence of complementary peptide binding pocket within LpxD.^{120, 122, 125}

Otherwise, Kroeck *et al* developed a virtual screening of small zinc molecules ligands against *P. aeruginosa* LpxA and LpxD using a target-structure-based methodology performed by molecular docking surface plasmon resonance (SPR) bioanalysis and high-resolution X-ray crystallography.¹¹⁸ They successfully identified several compounds (**5-9**) able to bind the active site of LpxA in the acyl chain binding pocket such as compound **5** and **6** that are the first examples of small molecules presenting a specific affinity for LpxA and LpxD. Thus, it was demonstrated that the polar functional groups of **5** directly bind with backbone amide groups of LpxA and LpxD whereas the naphthalene moiety remains located in the hydrophobic pocket. Moreover, due to the slightly larger acyl-chain binding pocket in the case of LpxD the aromatic ring of the ligand sinks deeper into the LpxD structure than the LpxA one.¹¹⁸

Nevertheless, the binding affinities of **5** and **6** is low compared to **RJPD33** and **Peptide 920**, with K_D ranging from 2.1 to 36.7 μM (Figure 6) and preliminary studies indicated that no significant inhibition of the *P. aeruginosa* bacterial growth was observed even by using concentrations up to 256 $\mu\text{g/mL}$.¹¹⁸

Figure 6. LpxA and LpxD inhibitors structure **5-15** (ND = K_D not determined)

Recently, Han et al reported novel inhibitors of LpxA (Figure 6) issued from a screening of Novartis compound collection. These small molecules possessed an antibacterial activity against efflux pump-deficient *E. coli* ($\Delta tolC$) with no obvious eukaryotic cell cytotoxicity or hemolysis at 100 μM . Unfortunately, they did not inhibit the growth of *E. coli* ATCC 25922 clinical isolate even at 128 $\mu\text{g/mL}$ presenting an IC_{50} and K_D of 1.4 μM and 0.1 μM for **10** and 4.8 μM and 110 μM for **11**, respectively.¹¹⁹

In vitro assays confirmed that these compounds are LpxA inhibitors with an antibacterial activity mediated by the target inhibition but, intriguingly, by two distinct mechanisms of action. Thus, compound **10** acts by binding the apo form with a competitive inhibition to 3-OH-acyl-ACP and a noncompetitive to UDP-GlcNAc, whereas compound **7** inhibits LpxA only in the presence of the product UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc. The X-ray co-structure of LpxA-**6** demonstrated that the molecule binds to apo LpxA by blocking a large portion of the acyl chain binding pocket. On its side, compound **7** was demonstrated to bind the LpxA-product complex in a product-dependent manner.¹¹⁹

In *P. aeruginosa*, neither compound **10** nor compound **11** was active even at 128 $\mu\text{g/mL}$. This is probably due to active site differences in *P. aeruginosa* LpxA that would impair the inhibitor binding.¹¹⁹

To improve the cellular activity against wild type *E. coli* strains, they designed and optimized several analogues of compound **11** which presented a favorable structure-based property against Gram-negative bacteria with higher solubility and lower logD and molecular weight compared to compound **10**. From the designed compounds, the most potent was the compound **12** (Figure 6) having an amino group on the pyridine ring and a MIC of 16 $\mu\text{g/mL}$ against wild-type strains as well as an IC_{50} of 0.6 μM .¹¹⁹ Taken together, it was demonstrated that the introduction of three different functional groups (chloro, methoxy, and amino groups) resulted in an 8-fold improvement in both biochemical inhibition and antibacterial activity.¹¹⁹

Another virtual pharmacophore-based screening with various drug-likeness filters and molecular docking have allowed to identify 3 LpxA inhibitors **13-15**, efficient against *Moraxella catarrhalis* and that bind near to the catalytic base and make interactions with the key active site residues.¹¹³

Finally, it is crucial to mention that LpxA shares a homotrimer architecture and conserved active site features, especially in the acyl-chain binding pockets that reside in the dimer interface of the β -helix trimer core¹¹⁸ as well as functional similarities with LpxD that could confer dual targeting to inhibitors offering an opportunity of increased potency and reduced likelihood of resistance formation.^{118, 125}

➤ **LpxC inhibitors**

Another attractive target consists of the UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase or LpxC, a constitutively expressed enzyme present in the cytoplasm and exclusively conserved in Gram-negative bacteria. LpxC is a zinc-dependent hydrolase that catalyzes the deacetylation of UDP-3-O-(R-3-hydroxymyristoyl) GlcNAc, the second committed step in Lipid A synthesis (Figure 3).^{41, 128-131} LpxC requires one or more zinc ions to be catalytically active and NMR and X-ray crystallography studies indicate that LpxC has a “ β - α - α - β sandwich” structure and encapsulates the acyl chain of the substrate with a unique hydrophobic passage.^{112, 132} Many potent LpxC inhibitors have been identified, most of which contain a hydroxamate group that targets the catalytic zinc ion.¹³³⁻¹³⁷

In the late 1980s, screening achieved by Merck research laboratories identified the small oxazoline hydroxamic acid compound **16** (Figure 7) which exerts antimicrobial activity against *E. coli* strains with MIC values ranging from 200 to 400 μ g/mL by inhibiting Lipid A synthesis and LPS production.^{132, 138}

Figure 7. Structure and biological activities of LpxC inhibitors **16-21**

Compound **16** was then selected and assayed individually against all nine enzymes involved in Lipid A synthesis. Interestingly, this compound showed a specific inhibition of LpxC activity with an $IC_{50} = 8.5 \mu\text{M}$ for the *E. coli* enzyme and a K_i of $24 \mu\text{M}$.^{107, 137-138}

It has been reported that LpxC inhibitors with effective antimicrobial activity against *Enterobacteriaceae* and *P. aeruginosa* share two structural similarities consisting of the presence of a hydroxamic acid group binding unit and a linear and rigid lipophilic tail interacting with the enzyme hydrophobic tunnel occupied by the fatty acyl chain.^{109, 131, 139-140}

More than 200 analogues of **16** have been synthesized, and the most efficient compound was identified as derivative **17** (Figure 7) which has a inhibition constant up to 50 nM and significantly increases the potency 100-fold due to the attachment of methoxy and electron-donating hydrophobic groups to the phenyl moiety.^{138 141} This compound was identified to be

a potent inhibitor of *E. coli* LpxC with an IC_{50} of 30 nM and MIC values against *E. coli* bacterial strains ranging from 1 to 3 $\mu\text{g/mL}$.^{131, 142} Unfortunately, LpxC inhibition is not a general phenomenon among different species such as *P. aeruginosa* or *Aquifex aeolicus*.¹⁰⁷ It

was shown that the molecule was active against a mutant of *P. aeruginosa* in which the *envA1* gene encoding LpxC was inactivated and replaced by the *E. coli* ortholog. In the same context, the *E. coli* mutant complemented with the *P. aeruginosa lpxC* gene was resistant

towards the compound. To extend the LpxC inhibitor spectrum, Jackman *et al.* designed a series of substrate analogs incorporating a hydroxamic acid functional group.¹⁴³⁻¹⁴⁴ Thus, the most efficient compound labeled **18** (Figure 7) led to moderate inhibition of *E. coli* LpxC,

with a K_i value of 650 nM ¹⁴⁵ (pH 5.5-7.4), when it exhibits an excellent inhibitor activity against *A. aeolicus* enzyme with a K_i of 1 nM and 650 nM at pH 7.4 and 5.5, respectively.^{143,}

¹⁴⁵ Nevertheless, while **18** LpxC inhibitors exhibited almost similar inhibition level among the Gram-negative strains including *P. aeruginosa*, this compound failed to demonstrate any attractive useable activity probably due its long hydrophobic acyl chain which limits its membrane diffusion.^{112, 132, 143}

In 2003, more than 1200 hydroxamate-containing compounds were prepared by Andersen *et al.* and one of them, namely, **19** possessing a threonyl-hydroxamate head group, presented the best inhibition profile against *A. aeolicus* LpxC.^{132, 137, 146} It is also noteworthy that **19** is a powerful antibiotic against a wide range of Gram-negative pathogens such as ciprofloxacin or tobramycin.^{107,146,147-148} This was the first LpxC inhibitor capable of inhibiting the growth of *P. aeruginosa* with an $IC_{50} < 2.1$ nM.¹³⁷ Interestingly, Tan *et al.* evaluated the activity of colistin in combination with **19** against *P. aeruginosa* biofilms and observed synergistic activities at subinhibitory concentrations *in vitro* and *in vivo*¹⁴⁹, using a checkerboard assay and biofilm conditions, synergy (FIC 0.34) was reported not only against colistin-susceptible *P. aeruginosa* biofilms but also resistant strains.¹⁴⁹ To determine the role of efflux in modulating the susceptibility of *P. aeruginosa* to **19**, Caughlan *et al.* compared wildtype PA01 with mutants lacking MexAB-OprM or MexXY-OprM efflux pumps and demonstrated that the deletion of *mexB* contributed to a 4-fold increase in susceptibility, suggesting that compound **19** is a substrate of the MexB pump. Conversely, *mexXY* deletion did not affect the susceptibility, whereas the simultaneous deletion of both genes led to a slightly larger shift (8-fold).¹⁵⁰ Furthermore, the X-ray structure analysis of co-crystals of LpxC and **19** showed the presence of a specific residue of the molecule located at the exit of the hydrophobic passage of LpxC.^{109, 151}

Sulfonamide derivatives of hydroxamic acid have been developed from the screening of a metalloenzyme inhibitor library and identified compound **20** that shows bactericidal properties against *E. coli* with almost 3-log killing in 4 h.^{133, 152} Analyses of *E. coli* mutants showing a decreased susceptibility towards **20** indicated the emergence of resistance mutations in the *fabZ* gene involved in fatty acid assembly and in the *lpxC* gene involved in Lipid A biosynthesis.

Recently, several biopharmaceutical companies such as Achaogen have conducted extensive research projects for the discovery of novel LpxC inhibitors. In this context, compound **21**

(Figure 7) was reported as one of the most promising molecules selected for a phase 1 human clinical trial.¹⁵³⁻¹⁵⁴ It presented significant activity against *E. coli* and *P. aeruginosa* PA01, with MIC values of 0.125-0.06 µg/m.¹³⁷ This compound was potent against 100% of the *P. aeruginosa* tested isolates with MIC values ≤ 2 µg/mL.¹⁵⁵ Nevertheless, even if **21** was active against a large panel of Gram-negative nosocomial bacteria, a failure of efficiency was noted against *A. baumannii*, *B. cepacia* and *S. maltophilia* bacterial strains, limiting its future development as a general antibiotic.^{137, 154-155} The variations in observed susceptibility support the possibility of great enzyme flexibility rather than intrinsic resistance caused by a lack of penetration or efflux in the tested bacteria.¹⁴¹

➤ **LpxD**

As well as the two first enzymes of lipid A biosynthesis, the UDP-3-O-(3-hydroxymyristoyl)glucosamine N-acyltransferase or LpxD is considered as an attractive target for novel antibiotics.¹⁵⁶ This enzyme for which crystal structure was solved for *E. coli* and *P. aeruginosa*, catalyzes the third step of Lipid A biosynthesis e.g. the transfer of 3-hydroxymyristic acid from the *R*-3-hydroxymyristoyl-acyl carrier protein to the 2-OH group of UDP-3-O-(3-Hydroxymyristoyl) glucosamine.¹⁵⁷⁻¹⁵⁸

First, we must cite the already mentioned peptide inhibitors **RJPD31** (QHFMVPDINDMQ-NH₂) and **RJPD33** (TNLYMLPKWDIP-NH₂) (targeting both LpxA and LpxD) as well as **RJPD34** (SENNFMLPLLPL-NH₂) (exclusively binding LpxD) which led Jenkins to identify new small molecules inhibiting the LpxD reaction with an IC₅₀ of 3.2 µM and a MIC value of 3.13 µg/mL against *E. coli* TolC minus strain.^{120, 156, 159-160}

Ma *et al.* reported recently that the aromatization of **22** lead to more stable oxidized compounds **23** demonstrating an improved antimicrobial activity against *E. coli* TolC minus strain with a MIC of 1µg/mL whereas a MIC up to 128 µg/mL was observed against wild type strain (Figure 8). To ensure its effectiveness as a LpxD inhibitors, it was observed that the

overexpression of *lpxD* gene in *E. coli* TolC minus increases the MIC of **23** by 8-fold whereas overexpression of the rest of *lpx* genes did not affect the susceptibility.¹⁶¹ Furthermore, it was determined by using SPR assay that compound **23** directly binds the *E. coli* apo-LpxD.¹⁶¹

Figure 8. Synthetic LpxD inhibitors **22-23** and potent natural LpxD inhibitors **24-32**

Due to the adverse effect of synthetic molecules, the *in silico* study of Rath *et al* suggested that natural products such as ajoene **24**, allicin **25**, cinnamaldehyde **26**, curcumin **27**, neral **28**, gallotannin **29**, isoorientin **30**, isovitexin **31** and vitexin **32** (Figure 8) with both anti-oxidant and antibacterial properties can be attractive hits in the search of LpxD inhibitors against *Bordetella petrii*.¹⁶² They demonstrated that isoorientin **30** was an appropriate candidate to target *B. petrii* LpxD, by contacting a strong interaction with the protein-ligand complex conferring its stability.¹⁶² Moreover, it was shown that this natural product was the lone molecule possessing a perfect balance between a high inhibitory effect and a lowest binding energy and inhibition constant.¹⁶²

➤ **LpxH**

UDP-diacetylglucosamine pyrophosphohydrolase LpxH is the fourth enzyme of the lipid A biosynthesis pathway which catalyzes the formation of 2,3-diacetylglucosamine 1-phosphate (Lipid X) and UMP.¹⁶³⁻¹⁶⁶ In 2015, Nayer *et al.* have successfully synthesized the first LpxH inhibitors as substituted sulfonyl piperazine and pyrazole derivatives such as **33** bearing a N-acetyl indoline group and presenting a weak antimicrobial activity against *E. coli* ATCC 25922 and *P. aeruginosa* PA01 (MICs up to 64 µg/mL) whereas an important inhibitory effect was observed against *E. coli* ATCC 25922 TolC minus strain (0.25µg/mL) (Figure 9).¹⁶⁷ For further understanding dealing with the mechanism of action of **33**, the genome of 15 mutant resistant *E. coli* MG1655 TolC minus strains were sequenced indicating only modifications of four amino acids namely G48, L84, F141 and R149 in the LpxH.¹⁶⁷ The MIC of these mutants increased from 0.25 to >128 µg/mL. To define the potential of LpxH as a

target of interest, Lee *et al.* synthesized series of sulfonyl piperazine LpxH inhibitors analogous to **33** leading to the strongest identified to date LpxH inhibitor **34** able to inhibit 74 % of the LpxH activity at a 1 μ M concentration whereas compound **33** demonstrated only an efficient rate of inhibition of 45% under the same experimental conditions.¹⁶⁸

Figure 9. Synthetic LpxH inhibitors **33-37**.

Recently, a diffusion disk assay suggested that **33** may be an effective inhibitor of *K. pneumoniae* LpxH¹⁶⁹ and Kwak *et al.* report the crystal structure of *K. pneumoniae* LpxH/**33** complex.¹⁷⁰ It was reported that the modification of the phenyl or N-acyl indoline groups of **34** lead to compounds able to inhibit the LpxH of *K. pneumoniae* at low concentration (0.1 μ M). Additionally, Bohl *et al.* found an IC₅₀ of 1.2 \pm 0.2 μ M for **33** against LpxH-mCherry and demonstrated its ability to block the conversion of UDP-DAG to Lipid X.¹⁷¹ Importantly, it was encountered that **33** was unable to inhibit the LpxH of *H. influenzae* and *P.aeruginosa* even at high concentration up to 100 μ M.¹⁶⁹ This may suggest an intrinsic non-susceptibility due to difference in the target site or lack to reach the internal concentration required for action (by permeability barrier or efflux pump).

Recently, among the extended N-acyl chain analogs, the compound **35** lead to the strongest inhibition of LpxH activity with 64% which slightly close to the most active compound with phenyl group such as **36** and **37** leading to an inhibition of 79 and 48%, respectively.¹⁷² Compound **35** appeared as the most efficient with a MIC value against *K. pneumoniae* of 1.6 μ g/mL higher than that encountered for **33** and slightly close to that of **34** ranging from 64 and 2.8 μ g/mL, respectively (Figure 9).^{169, 172}

Even if some promising inhibitors of the Lipid A biosynthesis pathway have been discovered some others Lpx enzymes such as LpxB, M also constitute a promising way for finding novel antibiotics against Gram-negative.

Conclusion

Lipid A is a key membrane component that plays a role during the biogenesis of bacterial OM where it strongly interacts with other membrane partners such as porins, architectural proteins, export systems, etc, in the external lipid bilayer. In this location, it contributes to the stringent permeability properties of outer membrane with respect to many toxic molecules, including antibiotics, defensins, disinfectants, detergents, etc. Due to these multiple involvements in crucial bacterial events, Lipid A has been recently suggested as an original target for the development of new antibacterial agents as outlined in Figure 10.

Figure 10. Potent strategies targeting Lipid A for the development of new antibacterial agents and possible bacterial resistance mechanisms.

One of the first molecule reported acting on lipid A biosynthesis, cerulenin, affects the early steps of its synthesis and although not clinically used some resistance mechanisms have been described anticipating the emergence of bacterial response against a possible therapy. In a different aspect, two macrocyclic peptides thanatin and murepavadin that has a high effect on *Pseudomonas*, target the LPS transport pathway via interaction with Lpt A, LptD, and other partners, involved in the addressing of newly synthesized LPS to OM.¹⁷³ Interestingly, Luther *et al.* have recently generated hybrids using a polymyxin part fused to murepavadin derivatives¹⁷⁴ that are active against Gram-negative bacteria. Sousa *et al.* discussed these important breakthroughs in the view of new molecules able to destabilize the assembly machinery of OM components of Gram-negative bacteria showing resistance to usual antibiotics.¹⁷⁵

Another possible target is the flippase MsbA involved in the IM translocation of nascent LPS chains.¹⁷³ Regarding this point, it must be noted that ATP is the energy driving force of the machinery and possible inhibitors may induce some side effect on eucaryotic transporters and mitochondria physiology. Consequently, the dose-effect relationship must be studied with special attention for this compound group for future clinic development.

Regarding the recent molecules, platensimycin and platencin, their antibacterial activity is impaired by expression of membrane efflux pumps that expel the drug.⁷⁶ Importantly, the producer, *Streptomyces platensis*, exhibits different resistance mechanisms against these molecules and these mechanisms may appear during clinical use requiring specific attention for use.¹⁷⁶ Recent assays using encapsulated-platensimycin suggest that this delivery process strongly increases the antibacterial activity against *S. aureus*.¹⁷⁷ Descriptions of LpxC inhibitor resistant strains have been significantly scarce in terms of antibiotics resistance.^{137,}¹⁷⁸ The development of this kind of specific cargo to ensure the intrabacterial delivery can be an attractive way for this candidate medicament but additional assays are required to define the appropriate capsule and their innocuity.

It is important to mention that these new antimicrobial agents face some efficient resistance mechanisms such as efflux or impermeability, developed by Gram-negative bacteria against clinically used antibiotics, which confer a partial or complete protection. From to this key observation, it must be mentioned that two ways could be envisaged for this new type of antibacterial weapons, - a direct effect on bacterial physiology linked to membrane roles of Lipid A, or - a facilitator effect such a combination with usual antibiotic for restoring its antibacterial effect on resistant bacteria.

Polymyxin molecules and squalamine have been previously used in combination with different antibiotics, for treatment and for identification of resistance mechanisms: the attempt being to increase outer membrane permeability facilitating the penetration and bypassing the membrane associated mechanism of resistance that impaired internal antibiotic concentration.

Considering the large clinical use of bi-therapy based on β -lactams associated with β -lactamase inhibitor to treat β -lactamase producer strains, the association of efflux pump inhibitor to antibiotic to impaired efflux resistance activity proposed during recent years, or the recent murepavadin-polymyxin hydrids.¹⁷⁴ The combination of a drug that target Lipid A with usual antibiotic can be an attractive way to restore antibacterial action in resistant

bacteria. Conjoint synergy between the partners can be also studied for improving antibacterial action. Covalent link or association of the two entities depends on the target location in the bacterial cell, membrane, periplasm, cytoplasm and can be select appropriately. Regarding OM biogenesis, it is also possible to target the bacterial machinery involved in the OM proteins assembly, Bam system, as recently mentioned.¹⁷³ However, similarly to lipid A synthesis/assembly, the bacterial adaptation must be carefully examined and again, the combination could be promising depending on the bacterial species and the antibiotic partner. Importantly, the measure of internal concentration of active molecules is now possible by using appropriate methods (spectrofluorimetry or mass spectrometry).²⁴ This point is strategic for the determination of respective concentration used in combination for anticipating future toxic aspects or selection of resistance mechanisms. In this aspect, various detector-regulators cascades (e.g. TCS, HTH, etc.) are present in bacterial cell envelope and cytoplasm, including sensors, transmitters, regulators.¹⁷⁹ These sophisticated systems can sense the presence of toxic compounds or their deleterious action on bacterial physiology and trigger an adaptative response to protect against the antibacterial activity. Consequently, the dose use is a critical point for a non-selective treatment which can induce the emergence of resistance respectively to the involved pathogens.

The divergence in the antibacterial activity observed between the new compounds described previously also reflects the variation in the structure of the target exhibited in the various bacterial species. Moreover, the conditions that exist in the infectious sites, e.g., pH, osmotic pressure, salt concentration, etc., contribute to the modulation of the biosynthesis cascade and final structure of lipid A. Thus, these key parameters can alter the bacterial susceptibility to compounds and ask for an adapted chemotherapy. Interestingly, this may present an ecological advantage by having a species selectivity the molecule will not target the bacteria belonging to usual microflora and will not affect the natural microbiome. This strongly argues for a personalized antibacterial strategy preserving the balance of human flora.

After several decades of prominent position of membrane proteins for the research and development of antibacterial agents, the lipid A can pave the ways to define appropriate pharmaco-association, or -modulation, of various molecules that can be used for generating “intelligent antibiotic” strategies of the future.

Ancillary Information

Supporting Information

Table S1: MIC values of platensimycin and platencin against Gram-positive and Gram-negative bacteria

Figure S1: Lipid A chemical structure for different Gram-negative bacteria

Figure S2: A) Chemical biphosphate analogue of *E. coli*-type Lipid A B) Effects of structural modifications (OH->H, P->H, O->OH, P->H) of *E. coli* Lipid A on biological activity. C) Effects of acyl modifications of *E. coli* Lipid A on biological activity.

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Jean Michel Brunel was born in Marseille, France. He graduated from the School of Chemistry of Marseille (ESCM) in 1991. He obtained his Ph.D. degree in 1994 from Aix Marseille III University in the field of enantioselective synthesis and organophosphorus chemistry. In 1994, he joined the group of Pr. H. B. Kagan (Université Paris Sud) as a postdoctoral fellow working on the enantioselective catalytic oxidation of sulfides (1994–1996). In 1997, he joined the CNRS as Chargé de Recherche and is now working with the faculty of pharmacy (UMR-MD1, U1261 INSERM). His research program is focused on the development of novel synthetic methods in organic chemistry as well as the synthesis of new classes of antimicrobial agents.

Abbreviations used

OM, Outer Membrane; IM, Inner Membrane; Kdo, 3-Deoxy-d-Manno-Oct-Ulosonic Acid; CAMPS, Cationic Antimicrobial Peptides; KAS, β -KetoAcyl-ACP Synthases.

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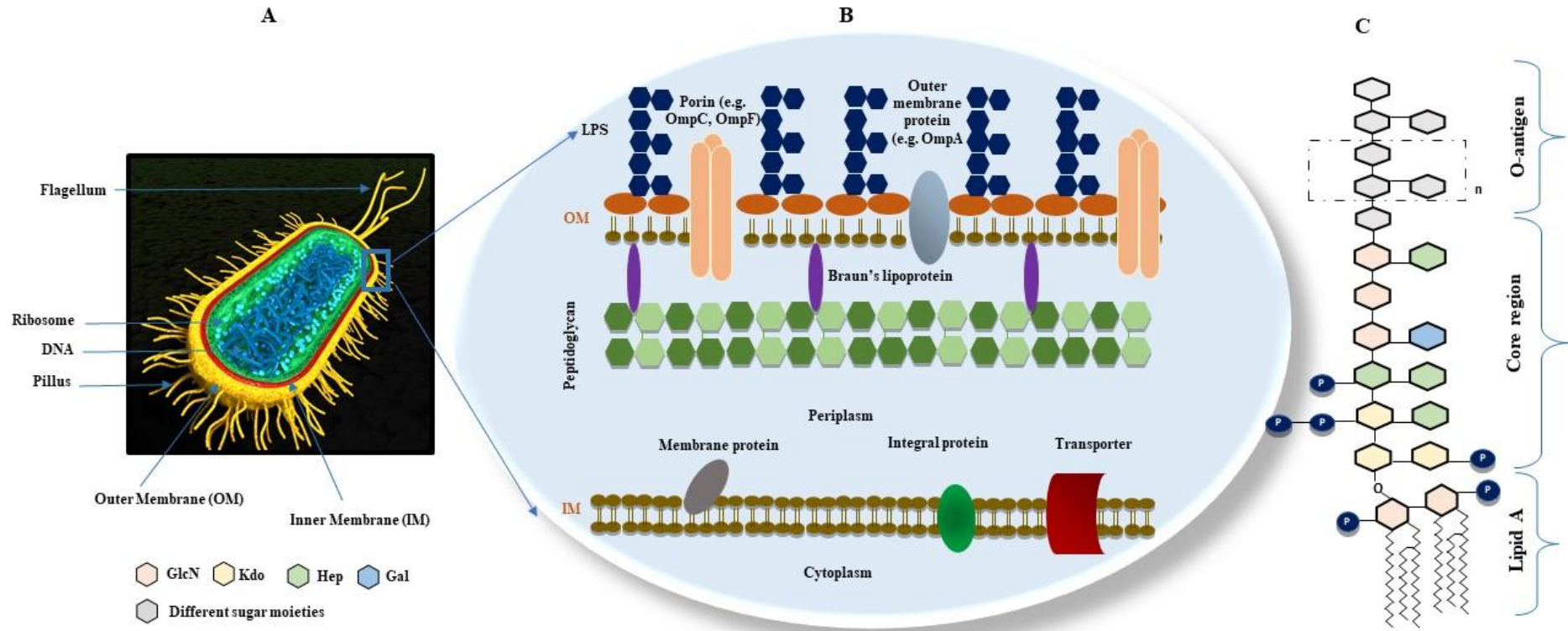


Figure 1

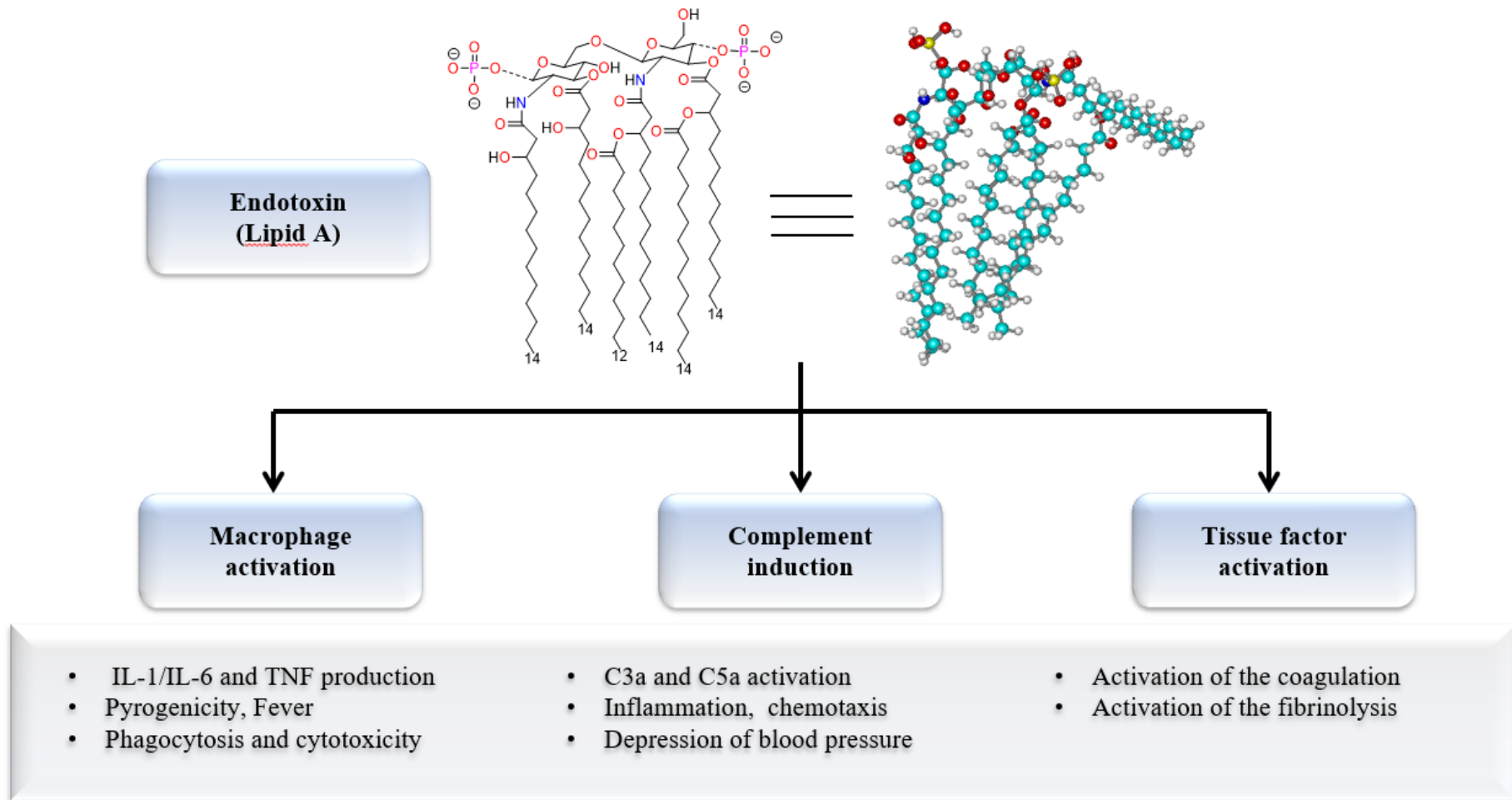


Figure 2

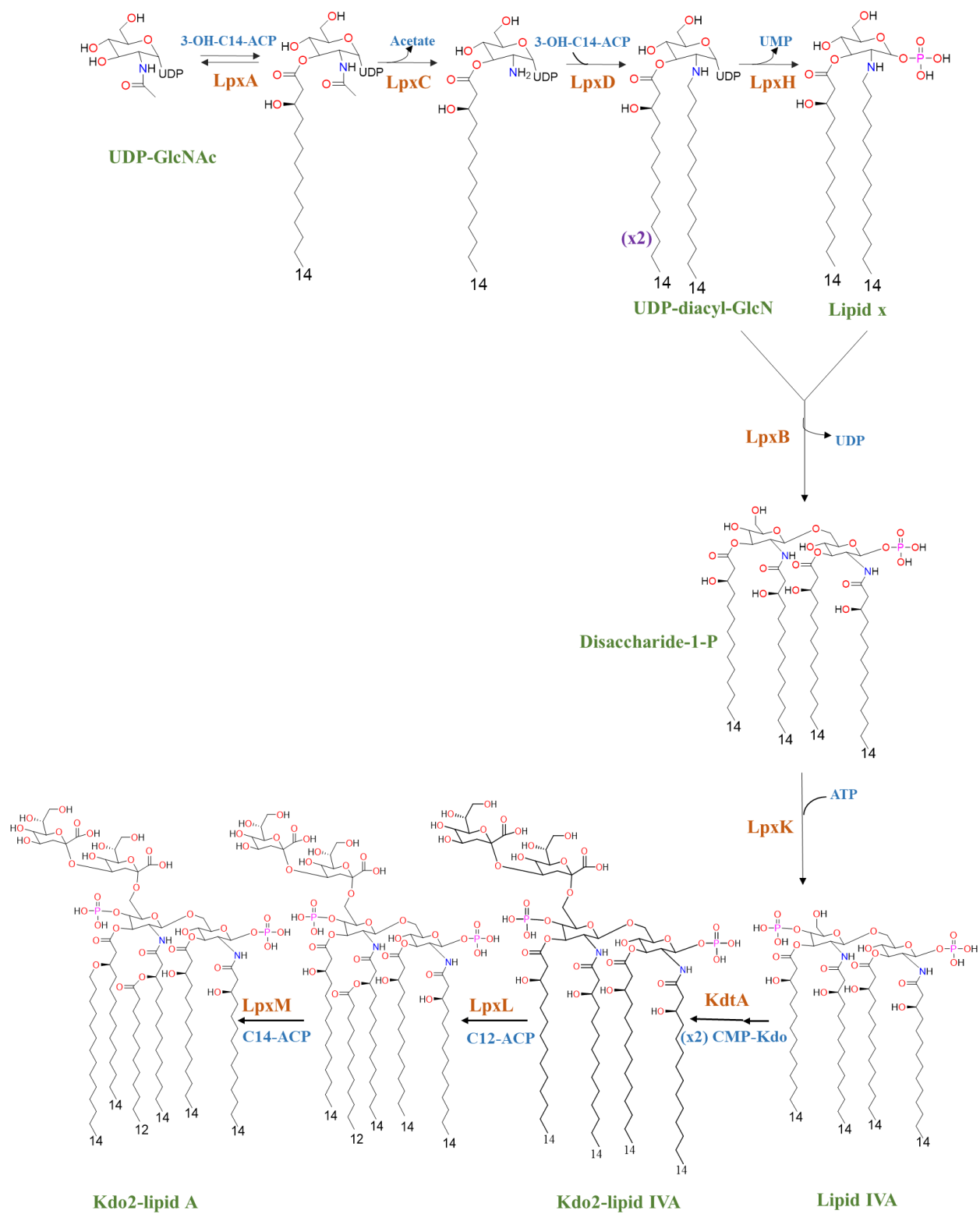


Figure 3

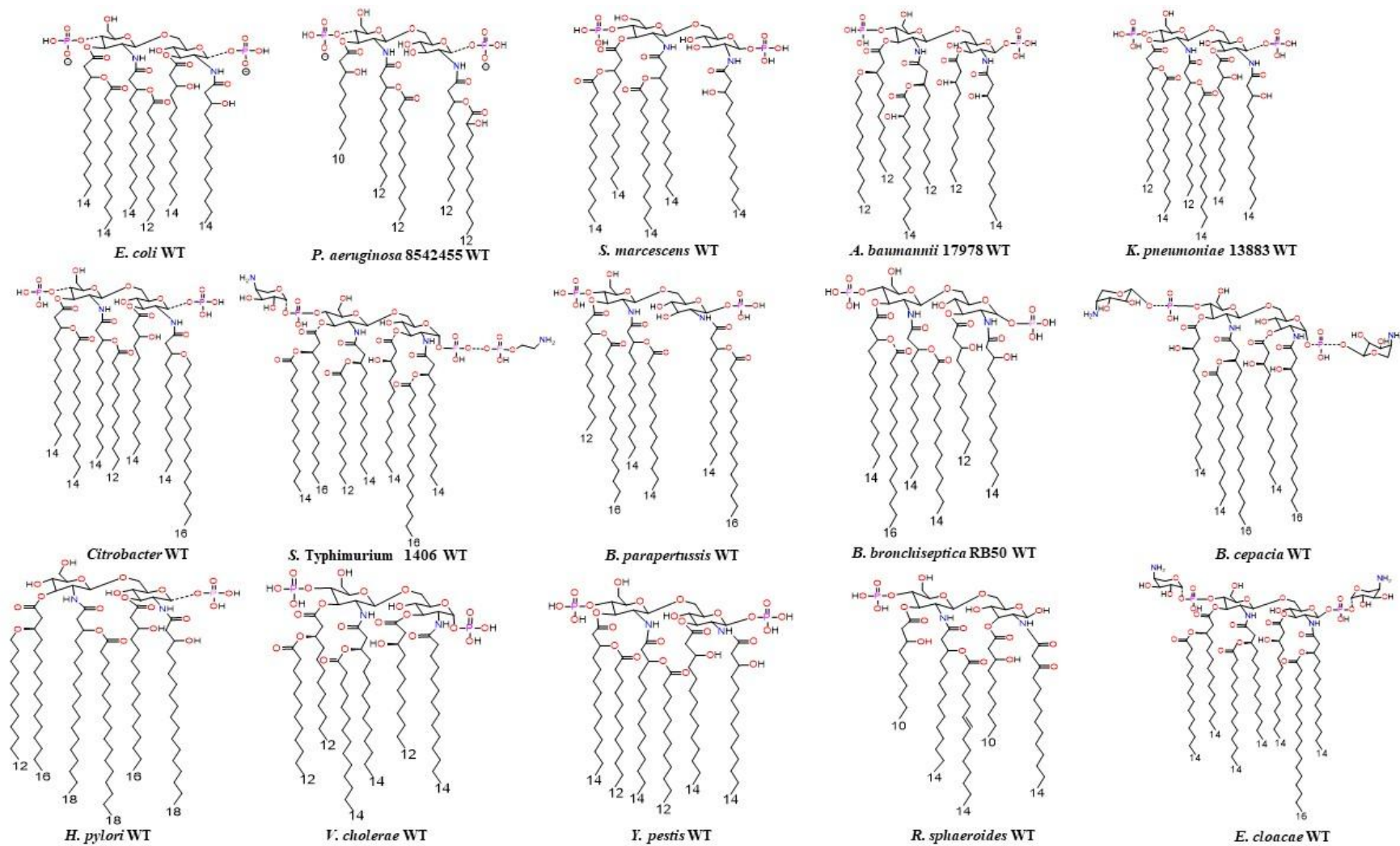


Figure 4

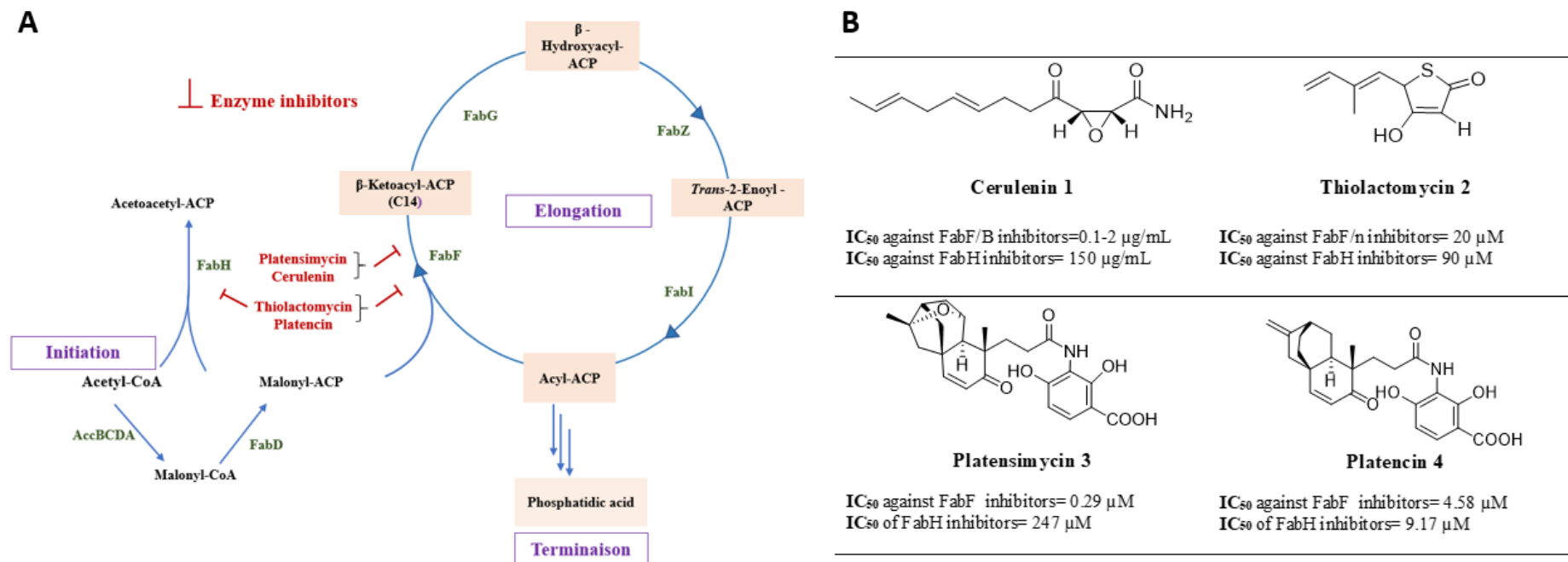
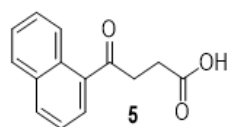
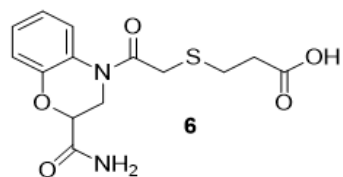
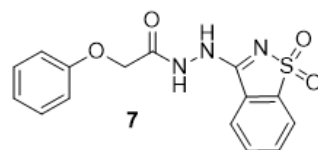
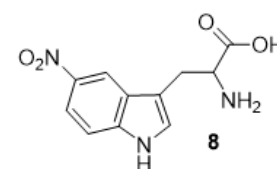
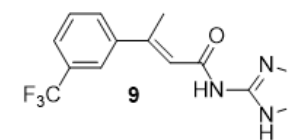
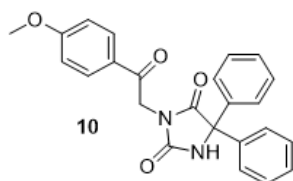
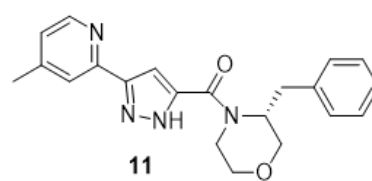
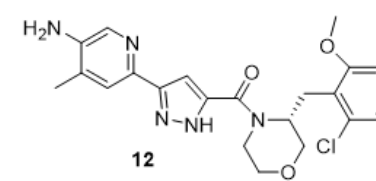
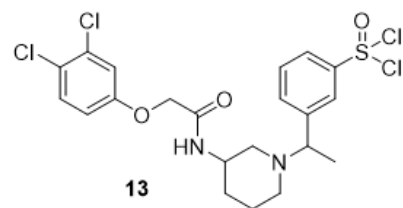
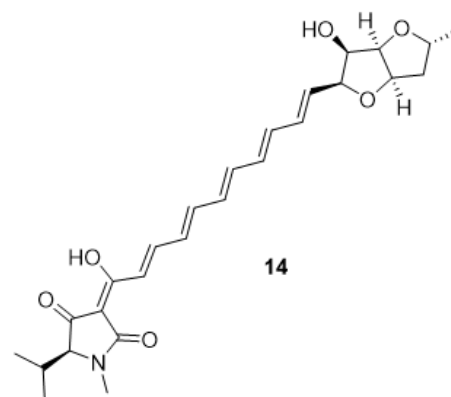


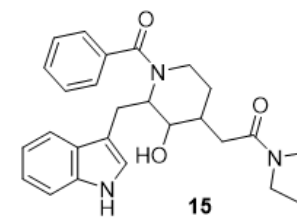
Figure 5

LpxA K_D = NDLpxD K_D = NDLpxA K_D = 19.5 μ MLpxD K_D = 36.7 μ MLpxA K_D = 16.7 μ MLpxD K_D = NDLpxA K_D = 13.6 μ MLpxD K_D = NDLpxA K_D = 2.1 μ MLpxD K_D = ND IC_{50} = 1.4 μ M K_D = 0.1 μ MMIC *E. coli* ATCC 25922 = >16 μ g/mLMIC *E. coli* Δ tolC = 0.5 μ g/mL IC_{50} = 4.8 μ M K_D = 110 μ MMIC *E. coli* ATCC 25922 = >128 μ g/mLMIC *E. coli* Δ tolC = 2 μ g/mL IC_{50} = 0.6 μ M K_D = NDMIC *E. coli* ATCC 25922 = 16 μ g/mLMIC *E. coli* Δ tolC = <0.125 μ g/mL

13



14



15

Figure 6

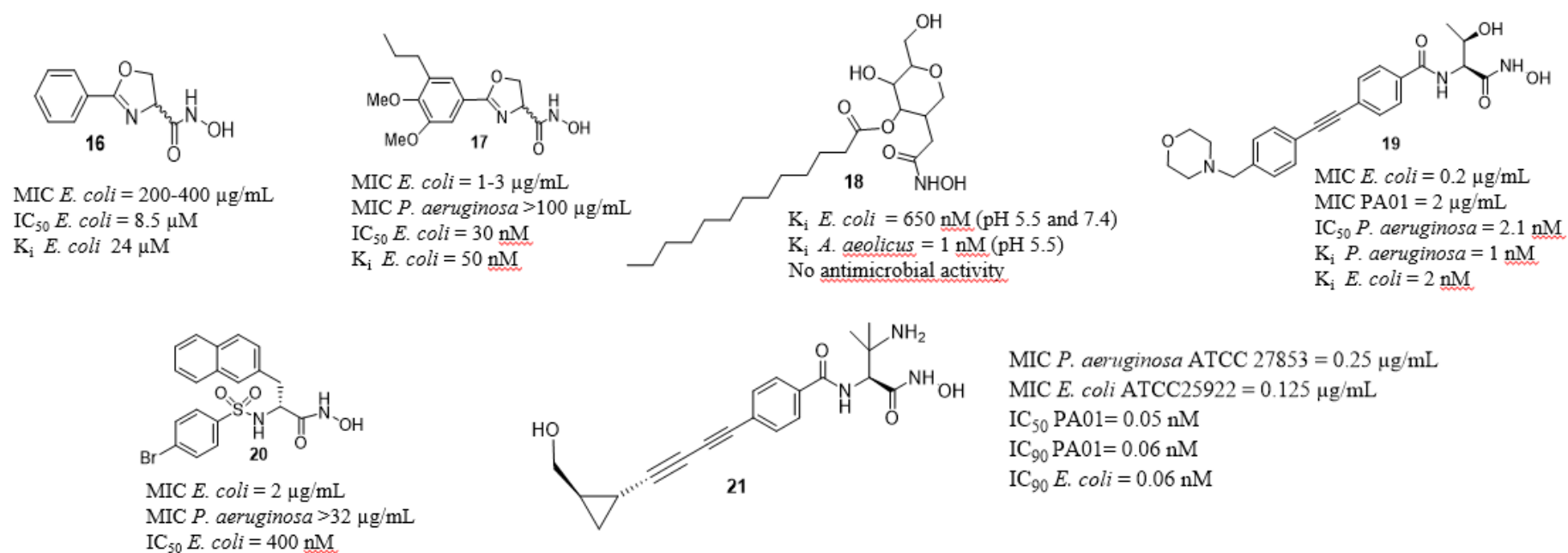
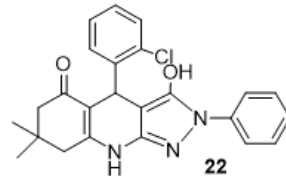
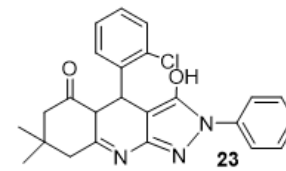


Figure 7



IC₅₀ *E. coli* Δ*tolC* = 3.2 μM
MIC *E. coli* Δ*tolC* = 3.13 μg/mL



MIC *E. coli* = 128 μg/mL
MIC *E. coli* Δ*tolC* = 1 μg/mL
Δ*tolC* Plac::lpxD = 8 μg/mL

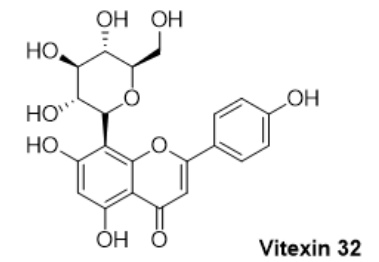
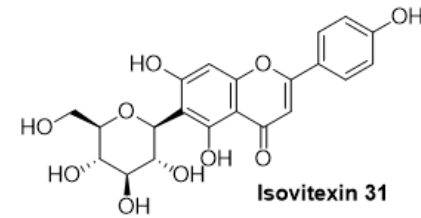
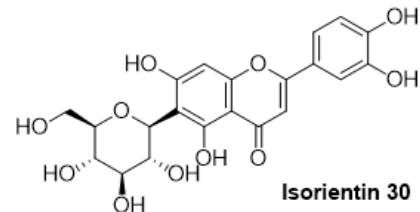
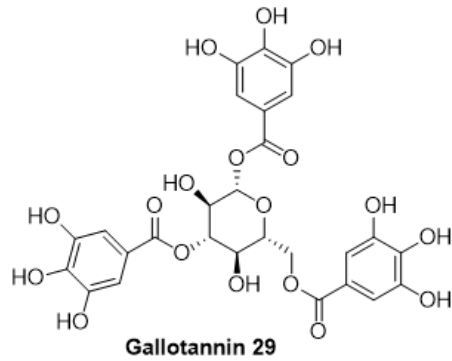
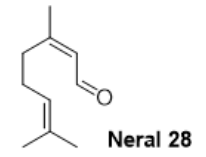
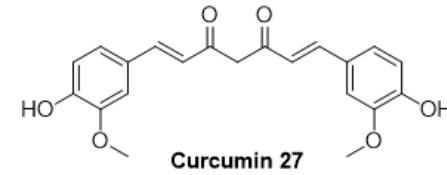
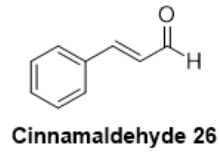
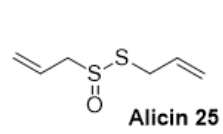
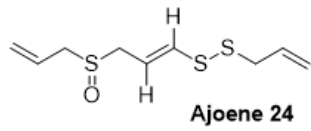
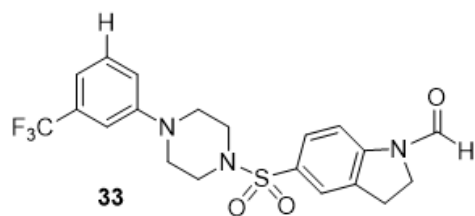


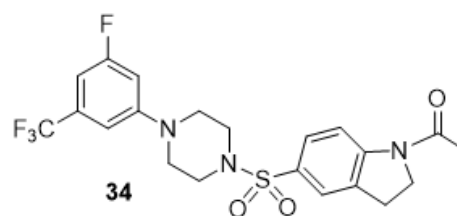
Figure 8



IC_{50} against *K. pneumoniae* = 0.36 μ M

IC_{50} against *E. coli* = 0.14 μ M

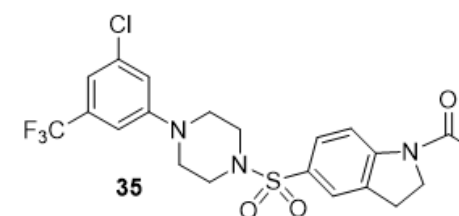
MIC against *K. pneumoniae*, *E. coli* ATCC 25922 and PA01 => 64 μ g/mL



IC_{50} against *K. pneumoniae* = 0.11 μ M

IC_{50} against *E. coli* = 0.083 μ M

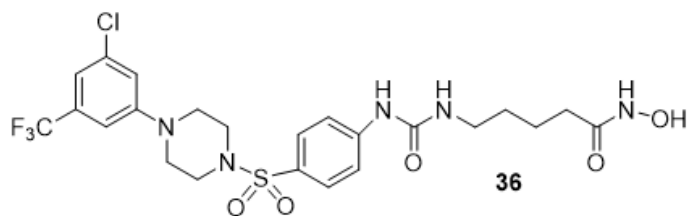
MIC against *K. pneumoniae* = 2.8 μ g/mL



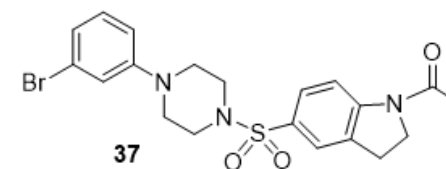
IC_{50} against *K. pneumoniae* = 0.026 μ M

IC_{50} against *E. coli* = 0.046 μ M

MIC against *K. pneumoniae* = 1.6 μ g/mL



MIC against *K. pneumoniae* = 32 μ g/mL



MIC against *K. pneumoniae* = ND

Figure 9

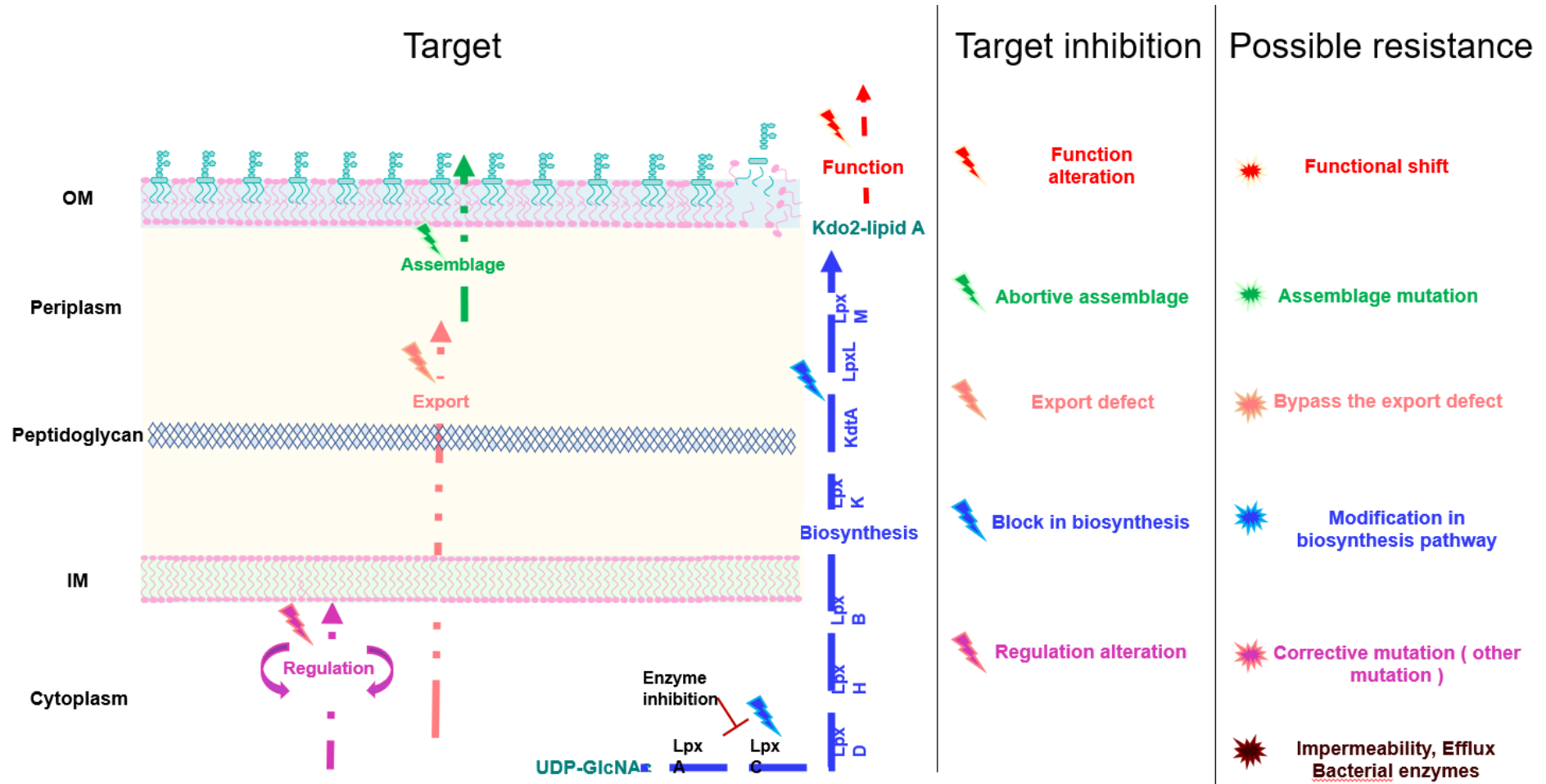


Figure 10

Table of Contents graphic

