Aina Nedal

Post-PKS modifications in the biosynthesis of the antifungal antibiotic nystatin

Doctoral thesis for the degree of philosophiae doctor (PhD)

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Norwegian University of Science and Technology
Faculty of Natural Science and Technology
Department of Biotechnology

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ABSTRACT

The antifungal polyene macrolide nystatin is produced by *Streptomyces noursei* ATCC 11455. The nystatin biosynthesis gene cluster of *Streptomyces noursei* has been cloned and sequenced, and a biosynthesis route has been predicted. In the present work, investigation of genes presumably involved in post-PKS modifications of nystatin is described. The aim of this work was to better understand the nystatin biosynthesis and to further use this information for generation of novel nystatin analogues. Two PKS-modifications of the nystatin molecule were targeted in this study: glycosylation with mycosamine at C-19 and oxidation of the exocyclic methyl group at C-16.

Two genes putatively involved in mycosamine biosynthesis (*NysDIII* and *NysDII*) and one in attachment of mycosamine to the nystatin aglycone (*nysDI*) have been identified in the nystatin gene cluster. Their functions have been suggested, respectively, as a putative mannose dehydratase, aminotransferase and a glycosyltransferase. The deoxysugar mycosamine is proposed to have an important function for the activity of nystatin. To better understand the biosynthesis and importance of mycosamine and to perform modifications of nystatin via this post-PKS modifying step, the mycosamine biosynthesis was studied. The NysDIII protein was overexpressed in *Escherichia coli* and purified, and its *in vitro* mannose 4,6-dehydratase activity was confirmed. To study the function of *nysDII* and *nysDI*, the genes were individually deleted from the *S. noursei* chromosome. Both mutants were shown to produce a mixture of nystatinolide and 10-deoxynystatinolide, albeit at considerably different levels. Complementation experiments unequivocally confirmed the involvement of these two in mycosamine biosynthesis and attachement. Both antifungal and hemolytic activity of the purified nystatinolides were tested, and were found to be strongly reduced compared to nystatin, confirming the importance of the mycosamine moiety for the biological activity of nystatin.

A gene for putative P450 monooxyganse NysN has been identified in the nystatin biosynthesis gene cluster. The function of NysN has been predicted to be oxidation of an exocyclic C16 methyl group on the nystatin molecule in order to afford a C16 carboxyl. The latter group has been implicated in selective toxicity of other polyene macrolides, and thus is considered an important target for manipulation. The *nysN* gene was inactivated in S. *noursei* by both in-frame deletion and site-specific mutagenesis, and the resulting mutants were shown to produce 16-decarboxy-16-methylnystatin, supporting the suggested biological role of NysN as C16 methyl oxidase. The recombinant NysN protein was also expressed in *Escherichia coli,* but its C16-methyl oxidase activity in *vitro* could not be demonstrated. 16-decarboxy-16-methylnystatin was purified from the nysN mutant, and its antifungal activity was identical with nystatin whereby the toxicity was reduced compared to nystatin.

In the work of developing new methods for obtaining nystatin analogues, bioconversion of nystatinolide was performed as a means to modify nystatin aglycone. For this purpose a sub-library of 35 different *Streptomyces* strains isolated from the Trondheims fjord was selected. One strain was shown to be able to add a water molecule (presumed epoxidation) and another strain was able to chlorinate the nystatinolides. An attempt on alternative glycosylation of nystatinolide was performed by using glycosyltransferase hybrids and deoxysugar biosynthesis gene cassettes. However, these experiments did not afford novel nystatin analogues, suggesting strong preference of the NysDI glycosyltyransferase for its natural sugar substrate GDP-mycosamine.

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PAPER II

Nedal, A., Sletta, H., Brautaset, T., Borgos, S.E., Sekurova, O.N., Ellingsen, T.E., Zotchev, S.B. (2007) (Submitted). Analysis of the mycosamine biosynthesis and attachment genes in the nystatin biosynthetic gene cluster of *Streptomyces noursei* ATCC 11455.

PAPER III

Nedal, A., Brautaset, T., Sletta, H., Degnes, K., Ellingsen, T.E., Zotchev, S.B. (2007) (manuscript in preparation). Characterization of the *nysN* gene encoding P450 monooxygenase responsible for post-PKS modification of the macrolactone ring during biosynthesis of the polyene macrolide antibiotic nystatin in *Streptomyces noursei* ATCC 11455.

ABBREVATIONS

ACP	Acyl Carrier Protein
AIDS	Acquired Immune Deficiency Syndrome
AT	Acyltransferase
ATCC	American Tissue and Culture Collection
CYP	Cythocrome P450 monooxygenase
DH	Dehydratase
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ER	Enoyl Reductase
Fdr	Ferrodoxin reductase
Fdx	Ferrodoxin
Gene SOEing	Gene fusion by Splice Overlap Extension
GDP	Guanosine di-phosphate
GT	Glycosyltransferase
GTP	Guanosine tri-phosphate
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromathography
KR	Ketoreductase
KS	Kethosynthase
LC-MS	Liquid Chromatography /Mass Spectrometry
MIC_{50}	Minimal Inhibitor Concentration (50 % inhibition of growth of test organism)
MW	Molecular Weight
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PCR	Polymerase Chain Reaction
PKS	Polyketide Synthase
ppm	Parts per million
RNA	Ribonucleic acid
TE	Thioesterase
TOF	Time-Of-Flight
TTP	Thymidine Tri-Phosphate
UV	Ultra Violet
WT	Wild Type

1. INTRODUCTION

The need for novel, broad-spectrum antifungal agents is increasingly important in today's medical arena due to a significant increase in numbers of immunocompromised patients easily affected by fungal infections. AIDS, poor nutrition, the use of broad-spectrum antibiotics, indwelling surgical devices, and a variety of treatment regimens such as cancer chemotherapy or immunosuppressive therapy prior to transplantation all contribute to the growth of this patient population.

An increase of fungal infections of 400 % over the past two decades is reported, those caused by frequency of *Candida* species becoming the most common (43). The number of antifungal antibiotics currently available is limited, and polyene macrolide antibiotics have so far proved to be the most effective antifungal agents due to their broad spectrum of activity and low frequency of appearance of resistant pathogens. However, polyene macrolides are rather toxic, and it is desirable to develop novel antifungals with improved pharmacological properties.

1.1 HISTORICAL HEADLINES IN THE DISCOVERY OF ANTIBIOTICS

Human infectious diseases were described already in the Old and New Testaments, as the impact of leprosy revealed. It is also known that tuberculosis afflicted Neolithic man 7000 years ago, but early man did not understand even the basic rules needed to contain the diseases. In the VI century an epidemic of plague killed an estimated 100 million people in the Middle East, Europe and Asia. Plague, known as the Black Death, has returned during the fourteenth century, and is estimated to have killed up to half the population of Europe, about 75 million people. Even as late as 300 years ago, when the plague returned from London for the last time in 1665, basic procedures to contain the epidemic were not implemented (3).

As we now enter the third millennium, we are quite privileged having access to an arsenal of developed antibiotics, which can kill or inhibit many of the bacteria that can cause infection (3). But antibiotics have not always been available; the first searches for antibiotics began in the late 1880s, starting with the growing acceptance of the germ theory of disease introduced by Louis Pasteur, a theory which directly linked bacteria and other microbes to a variety of ailments. As a result, scientists began to search for new drugs, the "magic bullets", that would kill these disease-causing microbes without showing toxicity to the patients (160).

1.1.1 Microbial antibiotics to treat diseases

The French biologist Paul Vuillemin was in 1889 the first to use the term *antibiosis* to describe the phenomenon whereby one organism destroys another in order to survive; he also used the term *antibiote* to describe the chemical substance involved. It was Selman Waksman, in 1941, who finally provided the definition of an *antibiotic*, which is still accepted today. This stated that 'an antibiotic is a chemical substance produced by a microorganism that has the capacity to inhibit the growth and even destroy bacteria and other microorganisms' (97).

Antibiotics differ from antiseptics in that they are sufficiently selective to allow their use within the body, rather than just on the surface, the selective toxicity is not absolute but it is quantifiable. Antibiotics that kill the bacteria are called bactericidal, whether they are called bacteriostatic if they merely inhibit replication of the bacteria which remain viable and may start to grow when the concentration of drugs fall (3). One of the first areas of scientific exploration in this field was whether harmless bacteria could treat diseases caused by pathogenic strains of bacteria. In 1877, the great microbiologist, Louis Pasteur, demonstrated that the bacterial disease anthrax, which can cause respiratory failure, could be rendered harmless in animals with the injection of soil bacteria. In 1887, Pasteur and Emmerich demonstrated that bacteria actually could treat the disease and in 1888, E. de

Freudenreich, isolated the first natural antibiotic (pyocyanase) from a bacterium that had antibacterial properties. Unfortunately, pyocyanase proved toxic and unstable, and could not be developed into an effective drug.

In 1909 John Ehrlich discovered the arsenic-containing drug '606' (later called Salvarsan/arsephenamine), the first true successful anti-syphilitic antibiotic. It was to remain the mainstay of treatment until the arrival of another 'wonder drug'; penicillin (Figure 1) discovered by Alexander Fleming in 1928 (97). Penicillin, which was Fleming's second discovery after lysozyme, changed the course of medicine. Penicillin was named after the *Penicillium* fungus that produced it, and Fleming demonstrated that penicillin destroyed a common bacterium, *Staphylococcus aureus*, associated with sometimes deadly skin infections. Penicillin was originally discovered by the French medical student Ernest Duchesne in 1896. Unfortunately, he failed to report a connection between the fungus and a substance that had antibacterial properties, and *Penicillium* was forgotten until Fleming's re-discovery. Though Fleming discovered penicillin, he was not able to purify it in significant quantities. In 1939, H. Florey, E. Chain, and N. Heatley were able to overcome the technical difficulties with purifying penicillin, and demonstrated the efficiency of penicillin in the clinical setting. From the 1940s the penicillin was produced in increased scale, and in 1946 the drug had become widespread for clinical use.

During the period between re-discovery and advancement of penicillin, a few other notable findings in the search for antibiotics have been made. Of greater significance was the work of Lieske, Gratia and Dath in the 1920s, who independently demonstrated that soil microorganisms of the order *Actinomycetales* could, in most instances, produce antibacterial substances. This was demonstrated by inhibition of staphylococcal growth in areas inoculated with a strain of actinomycetes (97). In 1932, Gerhard Domagk turned the attention towards synthetic antibiotics, chemical dyes, for their effects on bacterial infections and found that the dye Prontosil cured diseases caused by the *Streptococcus* bacteria when injected into infected animals. It was later demonstrated that the active

moiety of Prontosil was not the dye part of the molecule, but the sulfonamide group attached to it. Both Prontosil and other sulfonamide derivates proved highly successful, both in efficacy and lack of toxicity. In 1939, the antibiotic gramicidin was isolated from a soil-inhabiting microbe. Gramicidin was the first natural antibiotic extracted from soil bacteria and was able to arrest the growth of *Staphylococcus*, but unfortunately proved highly toxic for systemic use.

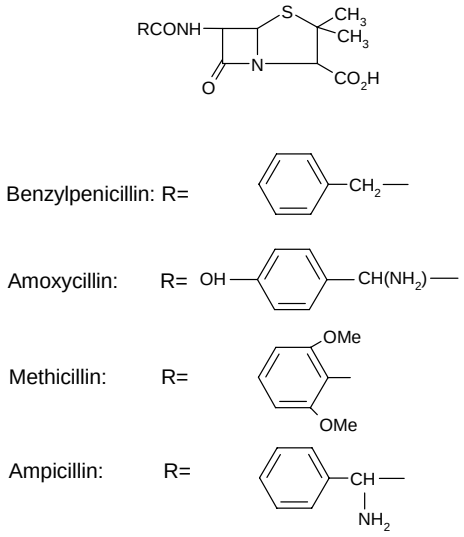


Figure 1: Chemical structures of penicillins.

In 1934, Selman Waksman and his group isolated another antibacterial agent, streptomycin, from the soil bacterium *Streptomyces griseus*. Streptomycin proved effective against several common infections. Most noteworthy, was its ability to kill the bacterium *Mycobacterium tuberculosis*, the microbe causing tuberculosis, which had to that point resisted numerous methods of treatment. Unfortunately, streptomycin had highly toxic side effects and its use induced a fast rate of mutation rendering bacteria resistant to this antibiotic, making it inappropriate for clinical use.

The first antifungal antibiotic, griseofulvin (Figure 2) (25) produced by *Penicillium griseofulvum* and *P. janczewskii Zal* was discovered in 1939. Its structure was elucidated by Grove and McGowan (1947) (57) and Brian *et al*. (1949) (25). It is fungistatic and binds to cell lipids as well as inhibiting nuclear division.

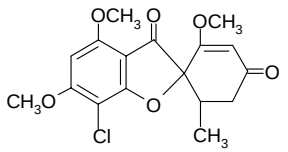


Figure 2: Chemical structure of griseofulvin.

In 1945 Lehman designed the drug Para-amino-salicylic acid to inhibit a specific bacterial target; this drug was active against most virulent strains of tuberculosis. But already in 1951, modifications of thiosemicarbazone derivates led to a more active compound against the tuberculosis bacillus, this compound was named isonicotininic hydrazide or isoniazid (3).

The most productive period of finding and developing antibiotics was between late 1950s and mid 1980s, where the main natural antibiotic groups were discovered by the mid 1950s. In 1961, an important novel antibacterial chemical nucleus was discovered when the nucleus of nalidixic acid (a 4-quinolone) was identified. Many innovative antibiotics have since then been variations of old chemical structures (3). Of New Chemical Entities (NCE's) between 1981 and 2002, 90 antibacterials and 24 antifungal compounds were reported (112), an overview of the distribution between biological, natural and synthetic drugs is given in Table 1.

Table 1: New Chemical Entities of antibacterials and antifungals between 1981 and 2002 (112).

Major Categories or Sources	Antibacterial entities	Antifungal entities
Biological		1
Natural Product	9	
Derived from a natural product and usually a semisynthetic modification	61	2
Totally synthetic drug (random screening/ modification of existing drug)	19	18
Totally synthethic drug / Natural product mimic		3
Made by total synthesis, but the pharmacore is/was from a natural product / Natural product mimic	1	
Total	90	24

1.1.2 Main classes of antibiotics

The main classification of most antibiotics used today is chemical – here some antibacterial and antifungal antibiotics are listed and described (Table 2) (antiviral and antiparasitic antibiotics are not included here due to limitations on the scope of this thesis). After chemical classification antibiotics can be divided in classes depending on their major targets such as cell membranes, cell-wall biosynthesis enzymes and substrates, protein synthesis and nucleic acid replication and repair which act via an anti-metabolite mechanism (3). For antifungal antibiotics the main targets are sterol biosynthesis, nucleic acid and glucan synthesis. The selection of commercial antibacterial antibiotics is still in a far higher number then antifungal antibiotics. There have been less success in developing drugs that act against fungal infections and only limited success has been achieved in discovering antibiotics against viral infections (3). One reason for slow progress in the development of antifungal antibiotics compared to antibacterials is that, like mammalian cells, fungi are eukaryotes. Agents that inhibit protein, RNA or DNA biosynthesis in fungi

will therefore have greater potential for toxicity to the host as well (56). Another reason is that until recently, the incidence of life-threatening fungal infections was perceived as too low to warrant aggressive research by the pharmaceutical companies (55).

1.2 *STREPTOMYCES* BACTERIA AS ANTIBIOTIC PRODUCERS

1.2.1 The genus *Streptomyces*

The genus *Streptomyces* belongs to the order *Actinomycetales* which comprise one of the two major branches of the Gram-positive bacteria. They are characterized as G/C rich organisms often developing mycelia, originally regarded as the hallmark of the actinomycetes. Partial sequence analysis of 16S ribosomal RNA is the most significant taxonomic tool for defining genera and suprageneric groups of actinomycetes (84).

The genus *Streptomyces* can be defined by both genetic and chemotaxonomic characteristics such as 16S ribosomal RNA sequence, cell wall composition, fatty acid and lipid patterns (163, 167). *Streptomyces* are ubiquitous in nature and their growth as vegetative hyphal mass which can differentiate into spores assisting in spread and persistence facilitates their ability to colonize the soil. The spores are a semi-dormant stage in the life cycle that can survive in soil for long periods (49, 101) showing resistance to low nutrient and water availability, whereas the mycelial stage is sensitive to drought (80).

Actinomycetes are producers of two-thirds of the known antibiotics produced by microorganisms, and nearly 80% of those are made by members of the genus *Streptomyces*. Streptomycetes also produce other classes of biologically active secondary metabolites with antitumor, antiparasitic, herbicidal, immunosuppressant activity etc (84). The bacterial antibiotic production is believed to have the function of defending the cells from competitors at the time of development of the aerial mycelium (36).

Table 2: Main antibacterial and antifungal antibiotic classes, examples of drugs, source, antimicrobial use and target (3) [pp57-80, 48, 69].

Antibiotic class	Example drug	Source	Antimicrobial use	Target
Antibacterial antibiotics				
β-lactams	PenicillinG Cephalosporin Amoxicillin	*P. notatum* *A. chrysogenum* Semisynthetic	G+ G+ G+/G-	Cell wall synthesis
β-lactamase inhibitors	Clavulanic acid	*S. clavuligerus*		β-lactamase inhibitor
Glycopeptides	Vancomycin Teicoplanin	*S. orientalis* *A. teichomyceticus*	G+ G+	Cell wall synthesis
Polypeptides	Polymyxin B and E	*B. polymyxa*	G-	Cell wall permeability
Aminoglycosides	Streptomycin	*S. griseus*	G+/G-	Protein Synthesis
Macrolides	Erythromycin Telithromycin	*Sac. erythraea*	G+/G- G+/some G-	Protein synthesis
Streptogramins	Virginiamycin	*S. virginiae* (40)	G+/some G-	Protein synthesis
Lincomycins	Lincomycin Clindamycin	*S. lincolnesis* Semisynthetic	G+	Protein synthesis
Tetracylines	Tetracycline Doxycycline	*S. aureofaciens* Semisynthetic	G+/G-	Protein synthesis
Chloramphenicol	Cloramphenicol	*S. venezuelae*	G+/G-	Protein synthesis
Rifampicin	Rifampicin	*Amycolatopsis mediterranei*	G+/G-	RNA synthesis
Sulphonamides	Sulphanilamide	Synthetic	G+/G-	Folic acid synthesis
Trimethoprim	Trimethoprim	Synthetic	G+/G-	Folic acid synthesis
Quinolones	Nalidixic acid Ciprofloxacin	Synthetic	G+/G-	DNA replication
Metronidazole	Metronidazole	Synthetic	Anaerobic	DNA replication
Antifungal antibiotics (168)				
Polyenes	Nystatin Amphotericin	*S. noursei* (24) *S. nodosus* (30)	*Candidia* *Aspergillus*	Ergosterol, cell membrane permeabilizin
Pyrimidine Analogs	Flucytosine	Synthetic	*Candida* *Cryptococcus*	DNA replication
Azoles	Fluconazole Metronidazole	Synthetic	*Candida* *Cryptococcous*	Ergosterol biosynthesis, DNA replication and transcription
Echinocandins	Caspofungin	Semisynthetic *Glarea lozoyensis*	*Candida* *Aspergillus*	B-(1,3)-glucan biosynthesis

1.2.2 Physiology and regulation of antibiotic production

In *Streptomyces* the antibiotic production is growth phase-dependent. In liquid culture the production starts when the culture enters stationary phase and in agar-grown cultures the production coincides with the onset of morphological differentiation (35). The initiation of antibiotic biosynthesis is determined and influenced by a variety of physiological and environmental factors, such as growth rate, diffusible γ-butyrolactone signaling molecules (72), imbalance in metabolism (70) and various physiological stresses (68, 172). The antibiotic synthesis might also be subject to metabolite repression and/or inhibition by nitrogen sources (generally NH_4^+), phosphate and/or glucose (35, 45, 46).

1.3 BIOSYNTHESIS OF MACROLIDE ANTIBIOTICS IN *STREPTOMYCES*

Secondary metabolites which are not essential for growth tend to be strain specific and have a wide range of chemical structures and biological activities. They are derived by unique biosynthetic pathways from primary intermediates and metabolites, and these pathways are often long and complex; the reactions are catalysed by enzymes with substrate specificities different from those of primary metabolism. The formation of secondary metabolites is directed by organized clusters of 20-30 genes associated with special regulatory mechanisms that seem to control both the timing and the level of gene expression. The control mechanisms are well integrated with the physiology of the producing organisms (158).

Examples of secondary metabolites mainly produced by Gram-positive bacteria of the genus *Streptomyces* are polyketides having applied potential as pharmaceuticals. Examples of such polyketides are erythromycin (antibacterial), nystatin (antifungal), avermectin (antiparasitic), rapamycin (immunosuppressant) and daunorubicin (antitumor). In this thesis the focus will be biosynthesis of antifungal antibiotic nystatin.

1.3.1 Macrolactone ring assembly

The genetics and biochemistry of polyketide biosynthesis in *Streptomyces* bacteria are well characterized (71). Macrolide polyketide compounds are formed in a manner similar to fatty acid biosynthesis via repeated condensations of simple carboxylic acids by modular (type I) polyketide synthases (PKSs). Type I PKS are organized into repeated units (modules) (47) where each of them are responsible for one condensation cycle in the synthesis of a polyketide chain. Manipulations of type I PKS genes have been performed resulting in predictable changes in the chemical structure of macrolides, confirming this mode of operation (82).

Condensation of the next carboxylic acid onto the growing polyketide chain is performed by β-ketoacyl synthase (KS) domain. Modules of PKS type I may also contain domains with β-ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) activities that determine the reduced state of the incorporated extender unit. In each module acyltransferase (AT) and acyl carrier protein (ACP) domains are present responsible for the choice of extender unit and retention of the growing polyketide chain on the PKS, respectively. By completion of the polyketide chain synthesis, it is released from the PKS via the action of a thioesterase (TE). TE is probably also involved in cyclization of the final product.

1.3.2 Post-PKS modifications

Polyketides comprise one of the largest and most diverse groups of secondary metabolites produced by various bacteria, fungi, plants, algae and dinoflagelates etc (127). The polyketides produced by these organisms comprise antibiotics, antifungals, anticancer drugs, immunosuppressants, cholesterol lowering agents, analgesics, antiparasitics, neurotoxins (127) and virulence determinants (133).

The readily synthesized and cyclized polyketide is usually modified via hydroxylation, glycosylation, methylation and/or acylation. These post modifications are believed to be crucial important for the biological activities of macrolides. Tailoring, or post PKS modifications are mostly catalyzed by oxygenases, oxidases, peroxidases, reductases,

dehydrogenases and group transferases. They are crucial for the addition of important functional groups to polyketide skeletons and are crucial to the structural diversity and biological activity of this class of natural products (129).

Oxygenases

Oxygenases catalyse reactions leading to enormous structural diversity of many PKS products, making these enzymes attractive for combinatorial biosynthesis. The most common oxygenase types are cytochrome P450 monooxygenase (CYP450), flavin-dependent mono- and dioxygenase, and anthrone oxygenases, the latter remarkable for their lack of requirement of NADPH/NADH or any other cofactor (129). Most macrolide antibiotic producing bacterias contain one or more soluble cytochrome P450 monooxygenases in the antibiotic gene clusters, where the individual cytochromes exhibit relatively broad substrate specificity (114). A classical reaction for the cytochrome P450 monooxygenases is transfer of a oxygen atom from O_2 to a variety of substrates by use of electrons supplied from NAD(P)H via ferrodoxin (Fdx) and ferredoxin oxidoreductase (Fdr) (114).

Group transferases

The term group transferase refers to enzymes that possess transferase activity introducing novel functional groups and altered profiles on the product relative to the substrate. This enzyme group contains important enzymes such as amino transferases, alkyl (usually methyl) transferases, acyl (usually acetyl) transferases, glycosyltransferases (GTs) and kinases. Methyl- and glycosyltransferases are the most important post PKS modifying enzymes, (129).

Glycosyltransferases perform perhaps the most important biotransformation on Earth, at least in quantitative terms. GTs are responsible for attachment of sugar moieties, often deoxysugars, adding important features and properties to a molecule and they often play an essential role in the biological activity of many natural product-based drugs (52, 85, 92,

104, 153, 170). Some GTs have also shown to have unexpected inherent substrate flexibility either towards their acceptor substrate (usually alcohol or phenols) or (deoxy)sugar donor co-substrates, and sometimes even to both (50, 89, 171, 173).

Methyltransferases use S-adenosylmethionine (SAM) as cofactor and can methylate O, N or C-atoms. O and N-methylations increase the liphophilicity of a molecule and also remove hydrogen-bond donor sites. The methylation reactions can occur either at the polyketide derived aglycone moiety or on sugar residues, either prior to or after the glycosyl transfer (129).

1.3.3 Utilisation of post-PKS modifying enzymes in combinatorial biosynthesis

Combinatorial biosynthesis is defined by C. Khosla as to "involve genetically engineering biosynthetic pathways in such a way that they may be combinatorially reconstructed to produce libraries of novel small molecules that are appropriate for use in screening for new drugs" (83).

Post-PKS tailoring steps are necessary to add functional groups essential for the biological activity and are therefore important in the biosynthesis. Targeted gene inactivation of post-PKS enzymes does not only present a very useful tool for the elucidation of biosynthetic pathways by analysis of accumulated intermediates or shunt products, but is also used in the context of combinatorial biosynthesis to generate new "unnatural" products with altered biological activities.

To further increase the biochemical variability by orders of magnitude, alterations of substrate specificity for enzymes like cytochrome P450 oxidoreductases, methyl transferases and glycosyltransferases as well as the heterologous expression of entire deoxysugar pathways reinforce the chances of finding novel bioactive polyketide compounds with desirable properties.

GTs are and will become more and more important tools for combinatorial biosynthetic approaches (134). The GTs can be used themselves, but glycosidic moieties can be modified by altering the biosynthesis of their deoxysugar moieties as well (52, 79, 86, 92,

104, 153, 170). A different NDP-sugar will be the outcome of such an experiment and the approach is therefore only successful if the natural GTs possesses certain flexibility and can therefore also act on the modified NDP-sugar donor substrates. The modification of deoxysugar biosynthetic pathways, in the context of combinatorial biosynthesis, always goes hand-in-hand with offering foreign NDP-sugar donor substrates to a given GT.

Genetic manipulation of oxygenase genes provides useful information on the functions and characteristics of the encoded enzymes; the ultimate goal being rational design and generation of novel hybrid natural products. The progress toward this goal has been slow, as many experiments have been and still are conducted in a semi-random mix-and-match fashion, especially for post PKS-oxygenases (129). As for GTs, it is also important to identify and characterise additional monooxygenases in order to target the increasing number of novel polyketides obtained by combinatorial techniques (33, 76, 146).

1.4 POLYENE MACROLIDE ANTIBIOTIC GENE CLUSTERS

In recent years the biosynthetic gene clusters for the polyene antibiotics nystatin (Figure 4), pimaricin and amphotericin (Figure 3) have been sequenced (6, 24, 30), and the gene cluster for candicidin (Figure 3) has been cloned and partially sequenced (74).

The PKS proteins of polyene macrolide antibiotics have been analysed and studied in some detail. Nystatin and amphotericin PKS are highly homologous, which is not surprising as the structure of the two polyene macrolides is quite similar. The corresponding PKS proteins show ca 72 % sequence identity. The PKS proteins of pimaricin are organized along the same lines but certain modules are absent according to the contracted size of the macrolactone ring. The candicidin PKS contains 21 extension modules, whereby ten modules have been completely sequences. Candicidin is so far the largest aromatic heptaene studied (32).

The polyene macrolides undergoes relatively few post-PKS modifications. They contain only one deoxysugar (mycosamine), and a methyl branch is converted to a carboxyl group

(116). Whereas amphotericin and nystatin undergoes post-PKS hydroxylation, pimaricin undergoes epoxidation.

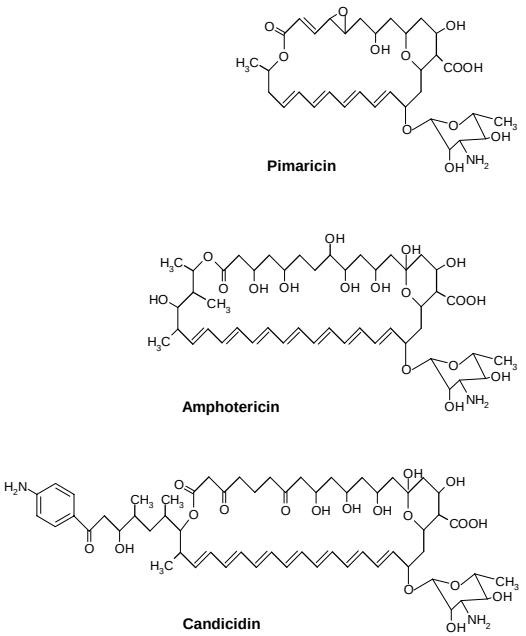


Figure 3: Chemical structures of the polyene macrolides pimaricin, amphotericin and candicidin.

1.4.1 The polyene macrolide antifungal antibiotic nystatin

Nystatin, originally named fungicidin, was the first member of the group of polyene macrolide antifungal antibiotics discovered by Hazen and Brown in 1950 (64). The streptomycete strain identified as a nystatin producer, was characterized in 1953 by Ettlinger (27) as *Streptomyces noursei* named after the farmer (H. Nourse), whose soil contained the initial sample. Nystatin, named after the site of discovery; New York State, was itself isolated, purified and characterized (65). *S. fungicidicus* ATCC 27432 has shown to produce nystatin and *S. albulus* ATCC 12757 have also been reported to produce nystatin (156). However, so far *S. noursei* is the only strain used for commercial production of this antibiotic.

As reviewed by Fjærvik and Zotchev (51), *S. noursei* also produces other biologically active compounds in addition to nystatin, such as antifungal cycloheximidine and the antibacterials phalamycin and nourseothricin. The nystatin complex contains three related polyene macrolides; nystatin A_1 (the major component), A_2 and A_3. Nystatin A_3 contains an additional L-digitoxose moiety attached at C_{35} of the nystatin macrolactone, but the structure of A_2 has so far not been reported (28).

The stereochemical structure of polyene macrolide nystatin (Figure 4) produced by *Streptomyces noursei* ATCC 11455 was completely solved in 1986 (90). It contains a polyketide moiety consisting of a 38-membered macrolactone ring with sets of two and four conjugated double bonds separated by a saturated bond. Nystatin is classified as a tetraene, but because of the two sets of conjugated double bonds it is sometimes referred to as "degenerated heptaene" (39). Nystatin, like other polyene macrolide antifungals, also contains the deoxysugar mycosamine attached to the macrolactone ring at C19 via a β-glycosidic bond and an exocyclic carboxy group at C16. Both appear to be important for biological activity and toxicity (20). The stoichiometric formula of nystatin is $C_{47}H_{75}NO_{17}$, and the corresponding molecular weight (MW) is 926.5 Da.

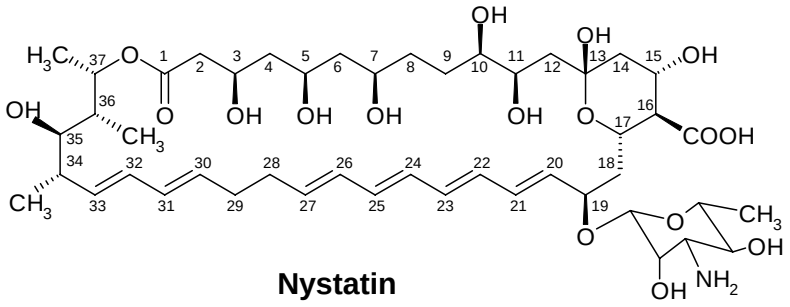


Figure 4: Chemical structure of nystatin.

1.4.2 The nystatin biosynthesis gene cluster

Macrolide antibiotic biosynthesis genes in *Streptomyces* are organized in clusters and cloning and complete DNA sequencing is described for several macrolide antibiotics produced by S*treptomycetes*, including those for avermectin, pikromycin, rapamycin, pimaricin and nystatin (6, 24, 77, 140, 171).

In the 124 kb DNA region of *S. noursei* containing nystatin biosynthesis genes, there were found six genes encoding a modular polyketide synthase (PKS), genes for thioesterase, deoxysugar biosynthesis and attachment, post-PKS modification, transport and regulatory proteins (24). The gene products from the nystatin cluster and putative or experimentally verified functions thereof are listed in Table 3.

PKS modules

Based on amino acid sequence alignments, comparison with known PKS genes and the results of gene disruption experiments, it was suggested that the genes *nysA, nysB, nysC, nysI, nysJ and nysK* encode PKS modules necessary for assembly and cyclization of the nystatin macrolactone ring (Table 3, Figure 5) (22, 24).

Table 3: PKS genes, Post-PKS modifying genes and regulation and transport genes identified in nystatin biosynthetic gene cluster of *S. noursei.*

Gene	Product	Function
PKS genes		
nysA	Type I PKS	Nystatin PKS (loading module)
nysB	Type I PKS	Nystatin PKS (modules 1 and 2)
nysC	Type I PKS	Nystatin PKS (modules 3-8)
nysI	Type I PKS	Nystatin PKS (modules 9-14)
nysJ	Type I PKS	Nystatin PKS (modules 15-17)
nysK	Type I PKS	Nystatin PKS (modules 18 + TE)
nysE	Thioesterase	Release of polyketide chain from PKS
Post-PKS modifying genes		
nysDI	Glycosyltransferase	Attachment of mycosamine to macrolactone
nysDII	Aminotransferase	Mycosamine biosynthesis
nysDIII	GDP-mannose-4,6-dehydratase	Mycosamine biosynthesis
nysF	4′-Phosphopantheteine transferase	Post-translational PKS modification
nysL	P450 monooxygenase	Hydroxylation at C-10
nysM	Ferredoxin	Electron transfer in P450 system
nysN	P450 monooxygenase	Oxidation of methyl group at C-16 of macrolactone
Regulation and transport genes		
nysRI	Transcriptional activator	Positive regulator of nystatin biosynthesis
nysRII	Transcriptional activator	Positive regulator of nystatin biosynthesis
nysRIII	Transcriptional activator	Positive regulator of nystatin biosynthesis
nysRIV	Transcriptional activator	Positive regulator of nystatin biosynthesis
nysG	ABC transporter	Efflux of nystatin
nysH	ABC transporter	Efflux of nystatin

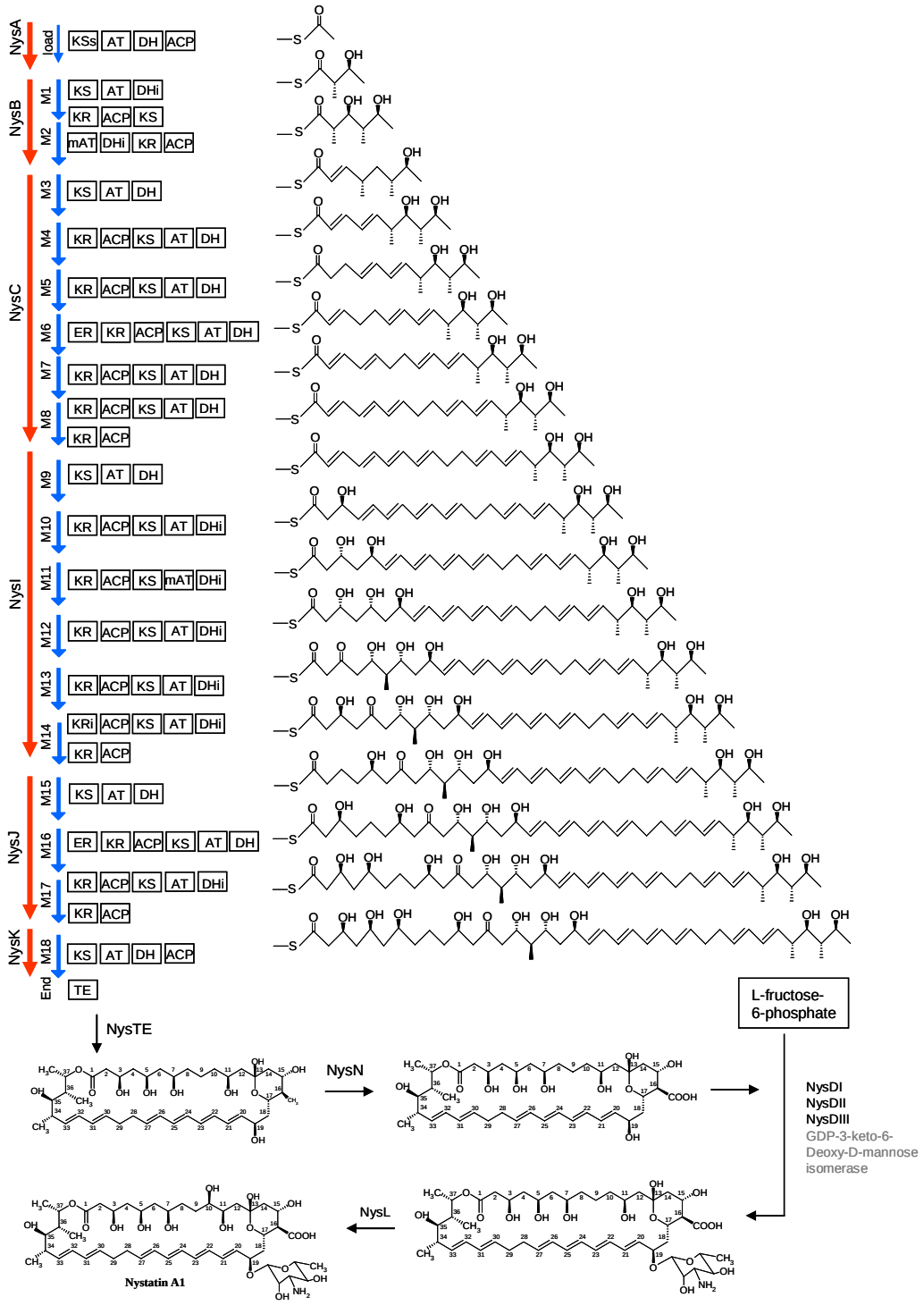

NysA
load
KSs AT DH ACP
NysB
M1
KS AT DHi
KR ACP KS
M2
mAT DHi KR ACP
NysC
M3
KS AT DH
M4
KR ACP KS AT DH
M5
KR ACP KS AT DH
M6
ER KR ACP KS AT DH
M7
KR ACP KS AT DH
M8
KR ACP KS AT DH
KR ACP
NysI
M9
KS AT DH
M10
KR ACP KS AT DHi
M11
KR ACP KS mAT DHi
M12
KR ACP KS AT DHi
M13
KR ACP KS AT DHi
M14
KRi ACP KS AT DHi
KR ACP
NysJ
M15
KS AT DH
M16
ER KR ACP KS AT DH
M17
KR ACP KS AT DHi
KR ACP
NysK
M18
KS AT DH ACP
End
TE
NysTE
NysN
L-fructose-
6-phosphate
NysDI
NysDII
NysDIII
GDP-3-keto-6-
Deoxy-D-mannose
isomerase
NysL
Nystatin A1

Figure 5: Biosynthesis of nystatin macrolactone in *S. noursei* ATCC 11455. Red arrows are PKS proteins, and blue arrows are modules within the PKS module. KS^s is ketosynthase with Cys → Ser active-site substitution; KS is ketosynthase; AT is acetyltransferase; ACP is acyl carrier protein; KR is ketoreductase; DH is dehydratase; ER is enoyl reductase; TE is thioesterase and domains prefixed by ”i” are presumably inactive.

NysA is the protein identified to be the nystatin aglycone biosynthesis initiating PKS module. The protein contains ketosynthase (KS^s), acetyltransferase(AT), dehydratase (DH), and an acyl carrier protein domains. The NysA module has been demonstrated to be absolutely necessary for the initiation of nystatin biosynthesis via construction of an in-frame deletion mutant. The role of NysA KS^s domain remains unclear, but based on the results from site-specific mutagenesis it has been suggested that this domain differs from the KS^Q domains frequently found in the loading modules of PKS type I systems in that the “active site” residue is not significant for its activity (22). It seems as the DH domain has no function in NysA, except probably for maintenance of a protein structural integrity, and being an evolutionary remnant (22). The sequence of AT domain displays a acetate-specific signature (62) containing the conserved Arg117 residue which are typical for such domains with high selectivity for CoA esters of dicarboxylic acids (93). It has been demonstrated that the KS^s domain and AT domain with conserved Arg117 residue are shared by other polyene type I PKSs (7, 30). Based on unclear function of the KS^s domain for the NysA function, it remains unknown whether malonyl-CoA or acetyl-CoA is used as primer for nystatin biosynthesis (22).

While the loading modules for most modular PKS systems are fused to the first extender module, the loading modules of PKSs for polyene macrolides are unique, as they are represented as separate polypetides. This might provide a greater degree of freedom in folding of the polyene PKS loading module and probably affect the choice of the starter unit by these proteins (22).

After loading and chain initiation performed by NysA, elongation is continued by NysB (module 1 and 2) performing the first two elongation steps in nystatin polyketide moiety biosynthesis using propionate extenders. NysC (six modules) is responsible for elongation steps 3-8 incorporating six acetate extenders to the nystatin aglycone. NysI (six modules) performs elongation steps 9-14, where module 11 incorporates a propionyl extender. NysJ (three modules) is performing elongation steps 15-17. NysK (one module) represents the final 18th domain, which contains a TE domain at the carboxyl terminus presumed to be responsible for the release of the mature nystatin polyketide chain from the PKS complex. Most of the nystatin PKS modules contain both KR and DH domains, but many of the latter ones are inactive because of internal deletions or active-site substitutions. This is particularly true for modules involved in the assembly of the polyol region of the nystatin molecule (24). ER domains in module 5 and 15 are responsible for the reduction of a double bond between C28-C29 and C8-C9, respectively. Downstream of NysC there is also found a coding sequence (*nysE*) for a TE, probably encoding a proof-reading enzyme ensuring proper functioning of the PKS complex (24).

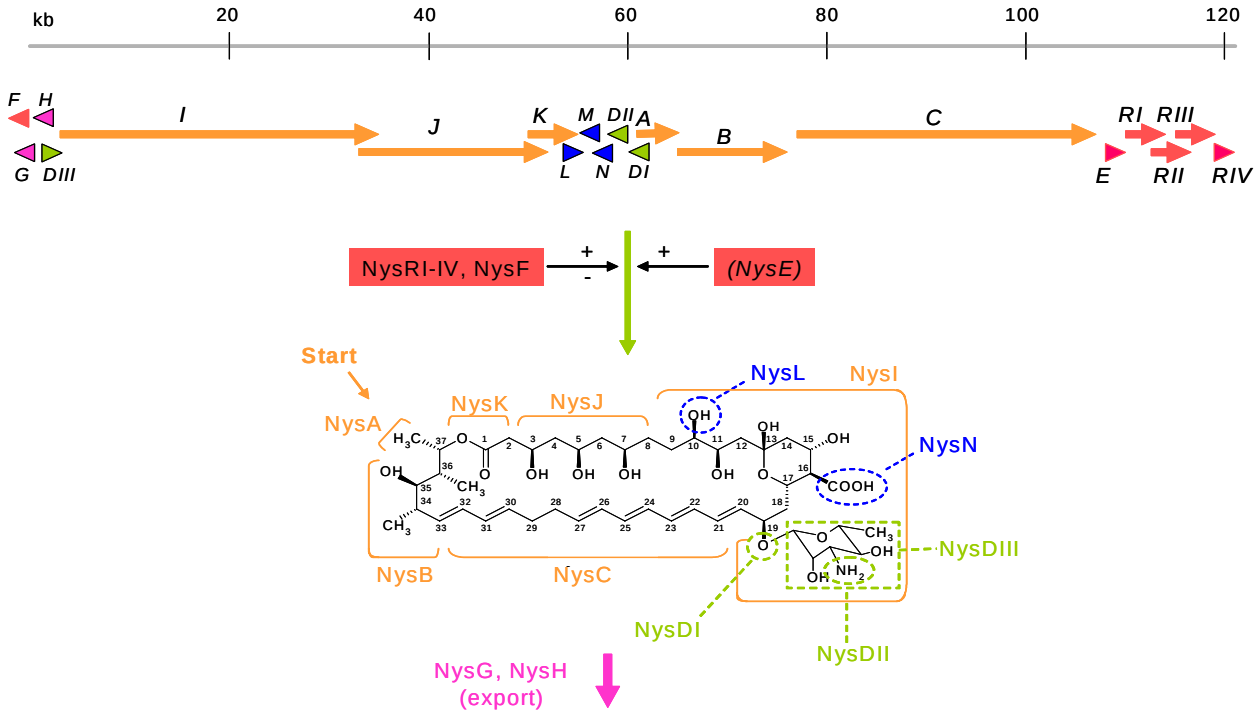


Figure 6: Model describing the organization of nystatin PKS cluster in *S. noursei* ATCC 11455 and the following functions of the genes involved in nystatin biosynthesis. The PKS modules are shown in orange (A, B, C, I, J, K); genes involved in post-PKS functions are in blue (NysN, NysM and NysL); mycosamine biosynthesis (NysDIII and NysDII) and attachment (NysDI) are in green; nystatin export in pink (NysG and NysH); regulation (NysRI-RIV) and editing/maintenance of polyketide synthesis (NysE) in red.

Post-PKS modifications

Based on the structure and organization of the PKS genes in the nystatin biosynthesis gene cluster, it is suggested that at least three post-PKS modifications are required to convert the nystatin macrolactone ring into nystatin. These modifications are represented by formation of a glycosidic bond at C19 between the aglycone and mycosamine, oxidation of a C16 methyl group to a carboxyl and C10 hydroxylation. It is suggested that the oxidation of C16 comes first in a series of post-PKS modifications supported by the fact that nystatin

precursors lacking the carboxyl group at C16 but possessing a C10 hydroxyl and/or a mycosamine moiety has only been identified in trace amounts in *S. noursei* extracts (28).

Two genes found in nystatin biosynthesis gene cluster, *nysL* and *nysN,* encode cytochrome P450 monooxygenases, where NysL is responsible for hydroxylation of C-10 (159) and NysN for oxidation of C-16 (Paper 3). The *nysM* gene apparently encodes a ferredoxin presumably constituting a part of one or both P450 monooxygenase systems serving as an electron donor (114). The three genes putatively responsible for mycosamine biosynthesis and attachment *nysDI, nysDII* and *nysDIII* show homology to other mycosamine biosynthesis genes as for the macrolide antibiotics amphotericin, candicidin and pimaricin produced by *S. nodosus*, *S. griseus* and *S. natalensis* (110). Each of the four polyene macrolide gene clusters were found to contain a gene for GDP-mannose 4,6-dehydratase – *amphDIII*, *canM*, *nysDIII* and *pimJ*, and it has been suggested that mycosamine is synthesized via GDP-D-mannose. GDP-D-mannose is presumably synthesized from fructose 6-phosphate by enzymes involved in primary metabolism as the genes for its biosynthesis have not been found in the polyene gene clusters. A pathway from GDP-D-mannose to GDP-mycosamine would involve the GDP-D-mannose 4,6-dehydratase, a 3,4 isomerization to give GDP-3-keto-6-deoxy-D-mannose, followed by transamination to form GDP-mycosamine. A gene that might encode a GDP-4-keto-6-deoxy-D-mannose 3,4-isomerase has not yet been found in any of the polyene macrolide biosynthesis gene clusters characterized so far. It is therefore presumed that GDP-4-keto-6-deoxy-D-mannose can isomerize to GDP-3-keto-6-deoxy-D-mannose spontaneously, in the absence of a conventional enzyme (5). All four polyene macrolide gene clusters encode aminotransferases (AmphDII, CanA, NysDII, and PimC) with homology to various perosamine synthases that convert GDP-4-keto-6-deoxy-D-mannose to GDP-perosamine (16, 126). For proposed biosynthetic pathway of mycosamine see Figure 7.

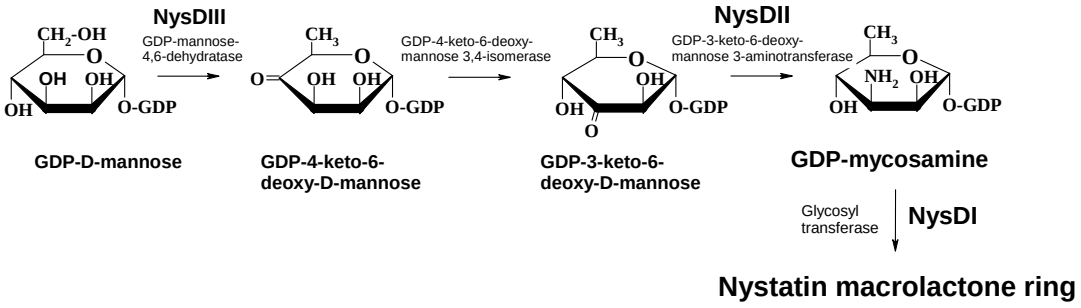


Figure 7: Proposed biosynthetic pathway for mycosamine and attachment to nystatin macrolactone ring.

Regulation of nystatin biosynthesis and its transport

Regulatory genes: In the *S. noursei* nystatin biosynthesis cluster four putative regulatory genes, *nysRI*, *nysRII*, *nysRIII* and *nysRIV,* have been identified. By gene inactivation and complementation experiments Sekurova *et al*. (141) have demonstrated that all four genes were necessary for efficient nystatin biosynthesis. In the control of nystatin production, *nysRI* is more important then *nysRII* and *nysRIII,* as the inactivation of the latter genes has had less effect on antibiotic production then inactivation of *nysRI*. NysRI regulates an endogenous promoter of its own gene, which also seems to be controlling the expression of *nysRII* and *nysRIII.* These three genes seem to be regulating the expression of NysRIV protein, which is suggested to be a transcriptional activator for the nystatin structural genes.

Transporter genes: Two genes, *nysH* and *nysG,* encoding putative ABC-type transporters were found in the nystatin biosynthesis gene cluster of *S. noursei* (24). Individual inactivation of both of these genes performed by Sletta *et al.* (144) has led to reduced level of nystatin production and accumulation of considerable amounts of the nystatin precursor 10-deoxynystatin, lacking a hydroxyl group at C-10. Both nystatin and 10-deoxynystatin were transported out of the cell, indicating an existence of an alternative efflux system. In experiments with ATPase inhibitor (sodium-*o*-vanadate) the production of nystatin and 10-deoxynystatin were affected in different manner suggesting that several transporters for

nystatin-related polyene macrolides exists and that the NysH-NysG efflux system provides conditions favorable for C10-hydroxylation.

1.4.3 Glycosyltransferases; post-PKS modifying enzymes

Glycosyltransferases (GTs) comprise a superfamily of enzymes catalyzing the transfer of monosaccharide moieties to biological substrates such as proteins, lipids and carbohydrates. GTs are present in all organisms studied, from prokaryotes to humans, and are found in every cellular compartment. The carbohydrate components are often vital for survival of the organism as they have specific roles in cell-growth and cell-cell interactions (42), cell adhesion including fertilization (1, 151), modulation of growth factor receptors (150), immune defense (135, 136), inflammation (94), and both viral and parasitic infections (137).

Members of the GT superfamily are named after the sugar moiety that they transfer. The linkage generated between the donor and acceptor is of importance for dividing GTs into subfamilies. Transfer of sugar residue occurs with either retention (by retaining GTs) or the inversion (by inverting GTs) of the configuration at the anomeric C1 atom (Figure 8) (143). Specific enzymes can be classified based on the reaction catalyzed and the substrate specificity, according to the recommendations of the International Union of Biochemistry and Molecular Biology (IUBMB) (17). However, the system does not indicate the intrinsic structural features of the enzymes, or adequately accommodate enzymes which act on several distinct substrates (31).

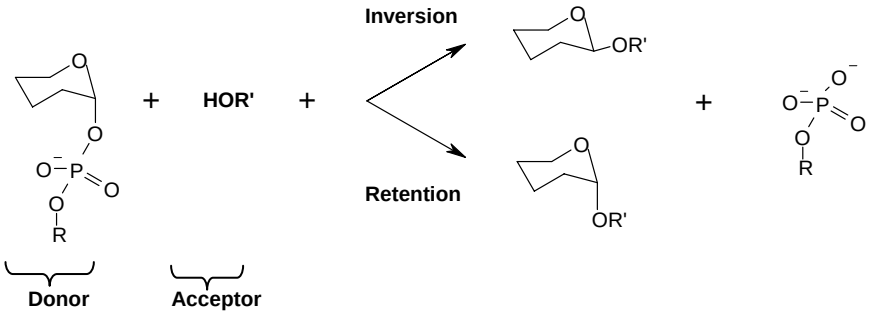


Figure 8: The glycosyl transfer reaction can occur both as inversion or retention of the anomeric configuration of the donor. Glycosyltransferases use sugar donors where the active group typically is a (substituted) phosphate as nucleotide (R = nucleoside monophosphate), nucleoside monophosphate (R = nucleoside) or lipid phosphate (R = lipid). The acceptor, HOR', is varied and R' can both be a sugar, a lipid, a protein, an antibiotic, nucleic acid etc.

The structures of proteins are better conserved than their amino acid sequences (66). Among members in each subfamily there is found sequence homology, but there is no sequence similarity between the subfamilies (123). The structural and functional diversity in combination with distant evolutionary relationships between GTs contributes to the explanation of the low sequence homology between the GTs. On the other hand, the majority of GTs belong to only two structural superfamilies named GT-A and GT-B superfamilies (21, 154), but still more and more enzymes belonging to other families are being discovered.

The structures of the catalytic domains of several GTs have been determined by X-ray crystallography. These studies have revealed a structural basis for ordered binding of the donor and acceptor, and for the proposed catalytic mechanism of the enzymes (13, 111, 124, 125, 154). Even if GTs have significant sequence diversity, some have a great structural similarity (73). They mainly consist of globular proteins with two types of fold termed GT-A and GT-B. Enzymes of GT-A fold comprise two dissimilar domains: the N-

terminal domain which is involved in nucleotide binding and comprise several β-strands that each are flanked by α-helices as in a Rossman-like fold, and the C-terminal domain which binds the acceptor and mainly consists of mixed β-sheets. The GT-B fold enzymes are characterized by two similar Rossman fold subdomains (21), where the N-terminal domain provides the acceptor binding site, and the C-terminal domain provides the donor binding site. The two domains in both GT-A and GT-B fold enzymes are connected by a linker region, and the active site is located between the two domains (123).

Some, but not all, GT-A enzymes require binding of a cofactor (often Mg^{2+} or Mn^{2+}) (123). Those enzymes that need cofactors often contain a three residue side chain DXD (Asp-X-Asp) or an equivalent motif involved in the binding of the metal ion (166). Some GT-B enzymes also need metal ion cofactors, but most do not and thus GT-Bs lack a DXD motif or it is equivalent (123), which further distinguish them from GT-A proteins. Members of the GT-B superfamily have a specific pattern of proline and glycine residues located within the donor-binding domain (73).

The GT-B superfamily includes most of the prokaryote enzymes that glycosylate secondary metabolites to produce biologically active natural products such as erythromycin, daunomycin, vancomycin, novobiocin (165) and nystatin (http://afmb.cnrs-mrs.fr/CAZY/fam/GT1.html). The GT-B superfamily shares highly conserved two-domain architecture, and by comparing structures and sequences of different family members it is possible to identify the regions that determine substrate selectivity. The longest conserved motif is located in the donor binding domain and encodes a folding unit involved in binding the glycosyl donors (73).

NDP-Hexose Formation and GT-Mediated Transfers

Antibiotics are most often glycosylated late in the biosynthetic pathway following PKS assembly of the aglycone, as in erythromycin or daunomycin-producing actinomycetes (53, 147). For GTs the general stoichiometry would be as follows: a TDP-hexose is the donor

substrate undergoing nucleophilic attack by a hydroxyl moiety on the aglycone. The universal biological reagents for hexosyl transfer, the NDP sugars, display the C_1 position of the sugar activated by the nucleoside diphospho moiety. The nucleoside diphospho moiety functions as a low-energy leaving group in GT-catalyzed reactions. Essentially all GT-catalyzed reactions therefore involve capture of the glycosyl substrate at the electrophilic C_1 locus.

TDP-glucose and GDP-D-mannose are common starting points for NDP sugar modification, resulting from TTP and GTP pyrophosphorylase activity on sugar-1-P substrate (78). Most antibiotic biosynthetic gene clusters contain genes encoding enzymes for catalysis of the modification of TDP-D-glucose to afford TDP-deoxy and TDP-aminodeoxy sugars (161).

Two general characteristics of antibiotic GTs are noteworthy. First, the late timing of GT action in the antibiotic biosynthetic processes makes these enzymes amenable for reprogramming and/or combinatorial biosynthetic approaches. The introduction of structural diversity late in the biosynthetic pathway can potentially lead to novel functions in new antibiotics. Second, the vast majority of GTs catalyze activated hexosyl transfer to hydroxyl substituents on co-substrate aglycones; however, amine and carbon nucleophiles exist such as those found in rebeccamycin biosynthesis (139) and in urdamycin biosynthesis.

1.4.4 Cytochrome P450 monooxygenases; post-PKS modifying enzymes

Cytochrome P450s are most often represented by monooxygenases containing protophorphyrin IX as their heme group. They are b-type cytochromes and are named by the Fe^{2+}-carbon monoxide complexes that show an absorption maximum at 450 nm when complexed with carbon monooxide. This characteristic is attributable to an absolutely

conserved cysteine residue which provides the iron-sulphur bond anchoring the heme in the active site (54, 87, 118, 119).

The cytochrome P450s, also named CYPs was first experimentally discovered in 1955 (9, 26). The cytochrome P450s, comprises a whole superfamily of proteins with extremely diverse functions, and is described as "the most versatile biological catalyst known". Although having the same basic structure and chemistry, these enzymes perform an enormous range of oxidation reactions on a wide variety of physiological and non-physiological compounds in organisms as diverse as mammals, archae, bacteria, fungi and plants (98, 109). In most prokaryotes the cytochrome P450s are soluble and confer the ability to catabolize compounds used as a carbon source or to detoxify xenobiotics. Other functions include fatty acid metabolism and biosynthesis of antibiotics (164).

Catalytic mechanism of CYP

Cytochrome P450s catalyze the monooxygenation of a vast array of molecules by transferring one oxygen atom from O_2 into various substrates in the following reaction:

$$R\text{-}H + NAD(P)H + O_2 + H^+ \rightarrow R\text{-}OH + NAD(P)^+ + H_2O$$

Electrons in this reaction are transferred from NAD(P)H to the cytochrome P450 heme group via a redoxin (electron transfer protein) containing either FMN (flavin mononucleotide) or Fe_2S_2 as a cofactor (102).

Classes of cytochrome P450s

Traditionally, cytochrome P450s were grouped in two classes based on the type of redox partners being utilized (how electrons are delivered from NAD(P)H to the active site). Lately, more cytochrome P450s have been discovered and the number of classes have increased (102). More then 500 different cytochrome P450 genes have been cloned up to date. These have been organized into a P450 gene 'superfamily' based on their degree of

relative homology. The superfamily is divided into gene families where the members generally share ≥40 % identity. Members of different families have sequence similarity <40 % (109).

Class I is represented by bacterial and mitochondrial cytochrome P450s, where electrons are shuttled from NADPH to a FAD-containing ferredoxin reductase, which again reduces a Fe_2S_2 redoxin that binds and transfers electrons to the P450 (75). In class II microsomal cytochrome P450s use a single FAD/FMN protein called cytochrome P450 reductase that shuttles electrons from NADPH to the cytochrome P450 heme (157). In the third class, the cytochrome P450s use a fused FMN and Fe_3S_4 electron donor protein as a reductase that is linked to the C-terminal end of the cytochrome P450 (130). The fourth class represents a cytochrome P450 from *Citrobacter braakii* (P450cin) that has recently been found to utilize FAD- and FMN-containing proteins. In contrast to the microsomal cytochrome P450s, the FMN and FAD proteins are separate polypeptides (102). In a fifth class cytochrome P450s that does not require a redox partner for activity are grouped together (102).

Structure

The structures of cytochrome P450 substrate complexes are quite diverse with respect to the substrate structure. The structures have provided important insights into the range of variations required in the active site to achieve substrate selectivity, and there are no structures of cytochrome P450s published where the substrates are very similar (102). According to the variety of reactions catalyzed, the size of their substrate varies. Some cytochrome P450s have open active sites and some have shielded active sites that open only transiently, whereas others bind the substrate only when attached to carrier proteins (122). The cytochrome P450 overall fold and topologies are quit similar (61). The general structure of cytochrome P450s consists of 14 α-helixes named A to L and 5 β-sheets that form a triogonal-prism-shaped molecule with predominately α-helical and β-structured parts (121).

Cytochrome P450s involved in biosynthesis of macrolides

Cytochrome P450 genes are found in a number of macrolide biosynthesis gene clusters. They catalyze the site-specific oxidation of the precursors to many macrolide antibiotics, such as erythromycin (63, 145, 162), tylosin (105), oleandomycin (131), nystatin (24, 159), amphotericin (30), pimaricin (7) candicidin/FR-008 (32, 37). In all reported cases these cytochrome P450s act late in the biosynthesis, after the formation of the macrocycle by polyketide synthase (PKS). Post-PKS modification by cytochrome P450s often constitutes an important contribution to the biological activity of the final molecule (14, 103).

The amphotericin, nystatin and pimaricin gene clusters each contain two cytochrome P450 genes each. *pimG*, *amphN* and *nysN* gene products apparently catalyse oxidation of a methyl group leading to formation of the exocyclic carboxyl that is characteristic for these polyene macrolides (5). Whereas amphotericin and nystatin undergo hydroxylation in the polyol region, pimaricin undergoes an epoxidation. The *ampL* and *nysL* gene products represent hydroxylases acting on the polyol region of their respective polyene macrolactones (103). The cytochrome P450 epoxidase PimD catalyses the C4-C5 epoxidation of 4,5-de-epoxy-pimaricin to pimaricin. PimD, AmphL and NysL belong to the same phylogenetic group responsible for specific oxidations in the polyol region of polyene macrolide precursors (5).

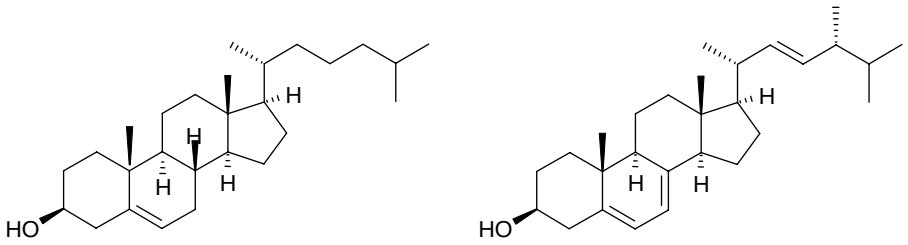
1.5 THE MOLECULAR MECHANISM AND ANTIFUNGAL ACTION OF POLYENE MACROLIDES

1.5.1 Permeabilization of biological membranes

Polyene macrolide antibiotics consist of 20-44 membered lactone rings containing three to eight conjugated double bonds. The conjugated double bonds are crucial for the mode of action of these antibiotics, since they are involved in interaction with fungal membrane sterols (19). The polyene antibiotic molecules form complexes with the membrane sterols

to create hydrophilic channels (44) through which Na^+ and K^+ ions can leak out, eventually leading to fungal cell death (60).

The polyene macrolides have been shown to have a higher binding affinity to ergosterol, which is the main sterol component in the fungal membrane, than to cholesterol present in the mammalian cells (Figure 9). The fact that they bind ergosterol better has been exploited in therapeutic use of polyene macrolides (152). Glycosylated polyenes have a better activity/toxicity ratio compared to non-glycosylated polyene macrolides, probably because non-glycosylated polyenes form larger and more harmful channels in the biological membranes, and induce gross membrane fragmentation. Because of this, only glycosylated polyenes are currently being used in human therapy (19, 106).



Cholesterol **Ergosterol**

Figure 9: Chemical structure of membrane sterols; cholesterol is found in human cell membranes, and ergosterol is found in fungal cell membranes.

Among glycosylated polyenes, the molecular mechanism of action is best studied for amphotericin. Amphotericin associates with sterols to form barrel-stave type pores (11, 100), and it has been found that the barrel consists of eight molecules arranged in a quasi-parallel orientation, with the polyol parts of the molecules pointing inward to constitute the pore lining, and the lipophilic heptaene part directing outward to interact with the membrane sterols (4, 44).

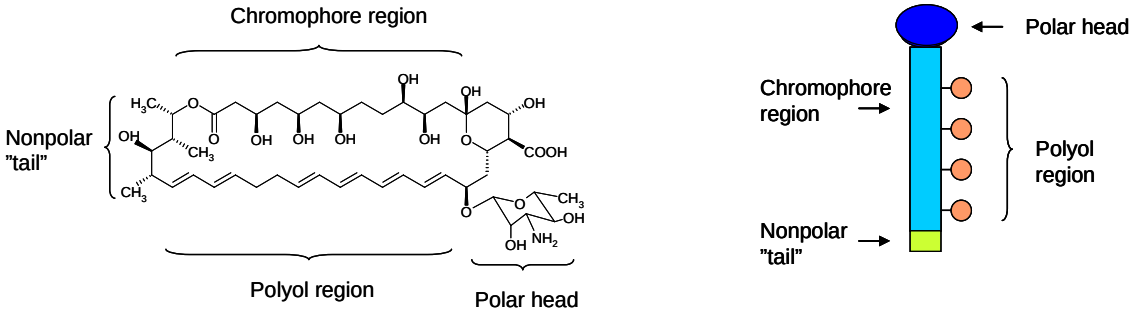


Figure 10: A: Demonstration of polar and nonpolar, chromophore and polyol region of nystatin. B: Schematic presentation of a glycosylated polyene macrolide antibiotic molecule.

Two such channels are thought to align head to head to span the cell membrane, with the mycosamine moieties extending towards the cytoplasmic and extracellular spaces. In these studies it has also been demonstrated that the ionizable carboxyl and amino groups are important for the activity of the molecule. The basis for this was suggested to be interaction, via hydrogen bond formation, between the amino group of mycosamine on one antibiotic molecule and an exocyclic carboxyl on the adjacent molecule (Figure 11) (11).

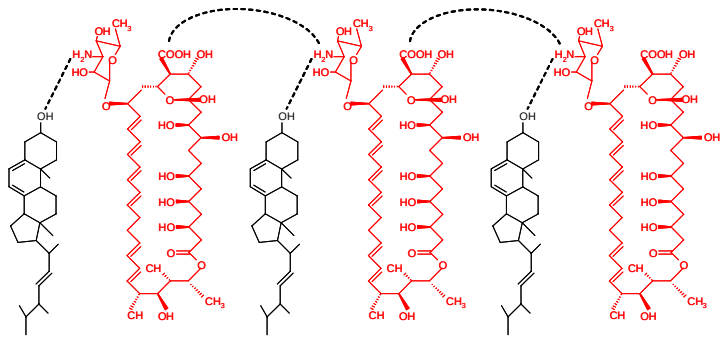


Figure 11: Chemical structures of nystatin (red) and ergosterol (black) with the hydrogen bond formation between the amino group of mycosamine and exocyclic carboxyl group and the ergosterol in channel formation marked with lines.

In a prediction of the mechanism for polyene-membrane complex formation the initial step is supposed to be the contact between the antibiotic aggregates and the outer surface of the membrane where hydrogen bonds between the phosphate groups in the polar heads of the membrane phospholipids and polyene macrolides are formed (107, 169). The next step would be the formation of the polyene-membrane complex and depending on the presence of sterols in the bilayer. It is suggested that the polyol regions of the molecules engaged as dimers form hydrogen bonds with the phosphate groups on the phospholipids, thus triggering the turning of dimmers inside out (107). Whether the antibiotic aggregate penetrate the membrane or not seems to be depending on the membrane sterol content. Thus, the affinity to sterols seems to be mainly responsible for the membrane penetration by the polyene aggregates, which then complex with sterols and arrange themselves into the channels (174).

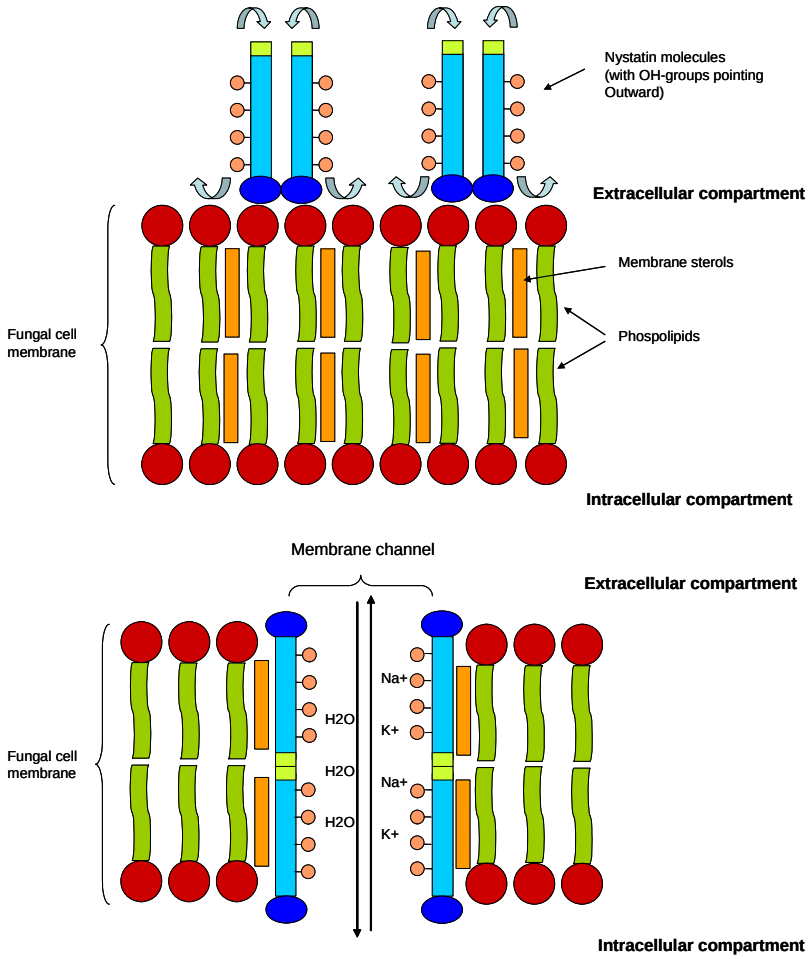


Figure 12: Demonstration of the formation of the membrane channel in the fungal cell membrane by polyene macrolide antibiotics interacting with ergosterol. The channel consists of eight molecules arranged in a quasi-parallel orientation, with the polyhydroxy side pointing inward to constitute the pore lining and the lipophilic heptaene part directing outward to interact with the membrane interior.

1.5.2 Conformation-activity

By comparative analysis it has been demonstrated that the activity of amphotericin is 6 times higher than nystatin against most fungi (8). This observation is in line with the findings showing that the ion conductance of the channel formed by nystatin is approximately 10 times lower compared to that formed by amphotericin (81). As the

chemical structures of these two antibiotics are quite similar, the higher affinity of amphotericin toward ergosterol is often explained by the presence of an additional conjugated double bond, allowing the molecular proximity necessary to maximize the van der Waals interactions between amphotericin and sterols (34, 91).

Previously, it was suggested that the arrangements between the amino-sugar moiety and the macrolide ring are largely dominated by two conformations. These two conformers are described as *open* and *closed* where the positions of the amino-sugar are defined by φ and ψ dihedral angels, where φ is C1´-O-C19-C18 and ψ is C2´-C1-O41-C19. In the o*pen* conform φ is about 150^{o} and ψ is 180^{o}, and *closed* conform φ is about 60^{o} and ψ is 180^{o} (10). In the open conform; the amino and carboxyl groups probably form an intermolecular hydrogen bond with the complementary carboxyl and amino groups of the neighboring amphotericin molecules, thus facilitating amphotericin-amphotericin interactions. The closed conform is probably responsible for the formation of an intramolecular hydrogen bond between the carboxyl and the amino group (100).

It has been demonstrated that while modification of the amino group decreases the antifungal activity, the carboxyl group substitutions had little effect on this property (38). Matsumori *et al*. (100) have shown that the relative position of mycosamine to a macrolide ring moiety have a strong influence on the sterol selectivity in channel activity of amphotericin. This has been demonstrated by using conformation restricted derivates of amphotericin, where the complex shows variable ergosterol selectivity depending on the orientation of the mycosamine. A derivate where the mycosamine is twisted with respect to the macrolide has higher selectivity for ergosterol then a derivate where the mycosamine adopts a parallel orientation with respect to the macrolide ring (100). Rather small conformation alterations of mycosamine can be attributable for the sterol preference of amphotericin (100).

The sterol dependence of membrane permeabilizing activity of amphotericin has so far been explained by different binding affinity between the antibiotic molecule and sterols (67, 91). The affinity is probably a result of two types of interactions; 1) a hydrogen bond between 3*β*-OH of the sterol and the amino-sugar moiety of amphotericin either directly or mediated by water molecules (10, 12, 67, 91); 2) van der Waals interactions between amphotericin heptaene chromophore and hydrophobic steroid rings (34, 91).

1.6 APPLICATIONS OF POLYENE MACROLIDE ANTIBIOTICS IN HUMAN THERAPY

Fungal infections represent a growing problem due to the rapid increase in numbers of immunocompromized patients. Changes in medical technology, diseases or factors as HIV, certain types of anticancer chemotherapy and administration of immunosuppressive drugs after organ transplantation make this group of patients especially prone to life-threatening invasive mycoses.

Fungi constitute a group of about 250,000 species, whereby more than 300 species have been reported to be potentially pathogenic to humans (58). *Aspergillus*, *Candida*, *Cryptococcus*, *Cladosporium*, *Microsporum* and *Trichophyton* spp are causing approximately 90 % of human fungal infections (43, 120). Dasgupta (43) reports a 400 % increase in the incidences of fungal infections over the past two decades.

Antifungal agents now constitute 15-16 % of the total market in the infective medicine area (43); and it is reported that the market for the antifungals will be expanding at a rate of 7,8 % per annum (Business Communications Co, March 2003). Although synthetic drugs contribute to a major proportion of the antifungals used, antifungals based on natural products, such as polyene macrolides, also take a considerable share in the actinomycotic market (41). The need for new, safe and more effective antifungals remains one of the major challenges to the pharmaceutical industry today (59).

Polyene macrolide antibiotics have proved to be very effective antifungal agents due to their broad spectrum of activity and low frequency of appearance of resistant pathogens. Amphotericin is often the treatment of choice of many mycosis cases, but problems with toxicity, resistance and non-availability of an orally absorbable form are important drawbacks. Since the polyene macrolides nystatin and amphotericin were discovered in 1950s (64, 148), more than 200 other polyene macrolides with antifungal activity have been discovered (117). However, only amphotericin, nystatin, candicidin, pimaricin, methyl partricin and trichomycin are currently being used in human therapy. Polyene macrolides are in fact rather toxic, and may cause serious side effects such as renal tubular damage and thromobophletitis, especially upon intravenous administration (96). The toxicity can probably be explained with the affinity of polyene macrolides to the cholesterol in the mammalian cell membrane. New polyene macrolide derivates, formulations and ways of administrations aimed at reducing toxicity are under development. Some polyenes have been shown to inhibit HIV replication, to hamper the development of prion-related diseases, and to be useful in cancer therapy as well (174). The development of novel polyene macrolide analogs and further investigation into their structure-function relationship is necessary in order to reduce the toxicity and improve certain pharmacological properties of these compounds (174).

2. AIMS OF THE STUDY

Considering the fact that systemic fungal infections is an increasing medical problem due to a growing number of immunocompromised patients and a slow pace in development of new antifungal drugs, research aimed at discovering more active and less toxic antifungal antibiotics is necessary. Nystatin is a commercial polyene macrolide antibiotic used to treat superficial fungal infections. Unfortunately, it is too toxic to be used against systemic infections as it can cause renal failure, hypokalemia and thrombophlebitis.

Prior to the present study, a gene cluster encoding the biosynthetic pathway for antifungal macrolide antibiotic nystatin in *Streptomyces noursei* has been cloned and sequenced in our laboratory. One aim with the current study was to deepen our understanding of the nystatin biosynthesis, more specifically, the post-PKS modification steps. Another aim was to afford production of new nystatin analogues through inactivation of the post-PKS modification genes.
As these modifications have been proposed to be important for the activity and toxicity of the polyene macrolide antibiotics, I have in this thesis investigated the post-PKS biosynthesis genes in the nystatin gene cluster presumably involved in mycosamine biosynthesis and oxidation of the exocyclic methyl group.

Through chemical modification and testing of the resulting analogues, the mycosamine moiety of some polyene macrolides has shown to be important for their biological activity. It is presumed that it has an important function in channel formation in the fungal membrane. The aim was to confirm the functions of the three genes predicted to be involved in mycosamine biosynthesis and attachment in the nystatin biosynthesis. In addition, I aimed at confirming the importance of mycosamine for the biological activity and toxicity of nystatin.

A gene for cytochrome P450 monooxygenase NysN identified in the nystatin biosynthetic gene cluster have been predicted to perform oxidation of the C16 exocyclic methyl group on the nystatin molecule. The resulting carboxyl has been implicated earlier in selective toxicity of polyene macrolides. My aim was to confirm the role of NysN and to obtain novel nystatin analogue having C16 methyl group instead of the carboxyl. I was also intended to study the biological activity of this new analogue.

In addition, I aimed at discovering new ways for modifications of the nystatin or its aglycone by means of bioconversion, construction of hybrid glycosyltransferase genes and expression of heterologous deoxysugar biosynthetic genes in *S. noursei*.

All these studies may be crucially important for obtaining novel nystatin analogues with improved pharmacological properties.

3 SUMMARY OF RESULTS AND DISCUSSION

3.1 BIOSYNTHESIS OF DEOXYAMINOSUGARS IN ANTIBIOTIC-PRODUCING BACTERIA (PAPER 1)

Deoxyaminosugars comprise an important class of deoxysugars synthesized by a variety of different microorganisms; they can be structural components of lipopolysaccharides, extracellular polysaccharides, and secondary metabolites such as antibiotics. Genes involved in the biosynthesis of the deoxyaminosugars are often clustered and are located in the vicinity of other genes required for the synthesis of the final compound. Most of the gene clusters for aminosugar biosynthesis have common features, as they contain genes encoding dehydratases, isomerases, aminotransferases, methyltransferases, and glycosyltransferases. In the present mini-review, the proposed biosynthetic pathways for deoxyaminosugar components of both macrolide and non-macrolide antibiotics are highlighted. The possibilities for genetic manipulations of the deoxyaminosugar biosynthetic pathways aimed at production of novel secondary metabolites are discussed.

3.2 MYCOSAMINE BIOSYNTHESIS (PAPER 2)

Two genes putatively involved in mycosamine biosynthesis (*NysDIII* and *NysDII*) and one in attachment of mycosamine to the nystatin aglycone (*nysDI*) have been identified in the nystatin gene cluster of *Streptomyces noursei* ATCC 11455. NysDIII was suggested to be responsible for the conversion of GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose, NysDII was supposed to be an aminotransferase, and NysDI a glycosyltransferase (24). To confirm the functions of *nysDIII* in the mycosamine biosynthesis the protein was expressed heterologously and its function was confirmed in an enzyme assay. The *nysDII* and *nysDI* genes were individually deleted from the chromosome by double homologous

recombination and the *nysDII* and *nysDI* genes further expressed in-trans in the two mutants, respectively.

3.2.1 *nysDI* and *nysDII* in-frame deletion and complementation

The glycosyltransferase NysDI, suggested to be responsible for the attachment of mycosamine to the nystatin aglycone, was inactivated by in-frame deletion through double homologous recombination, yielding the *nysDI* deficient mutant NDD1. NDD1 was producing nystatin aglycone, nystatinolide (MW780) at 28 % (± 2), 10-deoxynystatinolide (MW764) at 63 % (± 4) and nystatin 1.2 % (± 0.2) relative to the nystatin production level in the wild type strain. By complementation of the NDD1 mutant with a single copy of *nysDI* expressed under *ermE**p promoter, the nystatin biosynthesis was restored to 55 % (± 5) relative to nystatin production level in wild type, confirming the suggested function of NysDI as glycosyltransferase, attaching mycosamine to nystatin aglycone. Simultaneously, the production level of nystatinolides which was higher in NDD1 then in wild type was noticeably reduced. A lower production of nystatin by the complemented mutant is probably not surprising, considering that the *nysDI* gene was expressed in trans, and from a heterologous promoter.

The aminotransferase NysDII, presumed to introduce the amino group of mycosamine during the mycosamine biosynthesis, was inactivated by in-frame deletion through double homologous recombination, yielding the *nysDII* deficient mutant NDD2. NDD2 was producing nystatinolide at 0.31 % (± 0.15), 10-deoxynystatinolide at 0.09 % (± 0.15) and nystatin at 0.05 % (± 0.02) relative to the nystatin production in wild type. The production levels were surprisingly low, and by complementation the nystatin production was only partially restored to 0.26 % (± 0.12), whereby the production level of nystatinolide was reduced to 0.03 % (± 0.01). Although the very low production level, the nystatin production is increased by complementation and the nystatinolide production level is reduced confirming the suggested function of NysDII in mycosamine biosynthesis. Based on these results we suggest that the NysDI glycosyltransferase has a strong preference for GDP-

mycosamine as a substrate. It is still not clear why the level of the nystatinolide production is low in the *nysDII* mutant compared to the *nysDI*-deficient strain. One explanation could be that NysDII is a structurally important part of the nystatin biosynthetic complex, and that absence of NysDII prevents its efficient assembly and functioning. However, complementation would have alleviated this problem, which was apparently not the case. One hypothesis may be that intermediate(s), appearing as a result of inactivation of *nysDII* might somehow have a negative effect on nystatin biosynthesis.

3.2.2 Antifungal and hemolytic activity of nystatinolides

In order to confirm the importance of mycosamine for antifungal activity of nystatin, minimal inhibitory concentration (MIC_{50}) values were determined for both nystatinolide and 10-deoxy nystatinolide produced by *S. noursei* NDD1. As test organism, *Candida albicans* ATCC 10231 was used. The MIC_{50} value for nystatinolide was measured to be 60 μg/ml, for 10-deoxynystatinolide 20 μg/ml and for nystatin 1.2 μg/ml. The amino group on mycosamine is presumed to play an important role in interactions between polyene macrolide molecules, which organize themselves into channels spanning the cellular membrane of the target organism (174). Apparently, the antifungal activity of both nystatinolides was significantly reduced compared to nystatin, confirming an importance of mycosamine for the biological activity. Interestingly, the antifungal activity of 10-deoxynystatinolide was higher than that of nystationolide (lower MIC_{50} value). Considering the fact that MIC_{50} values for nystatin and 10-deoxynystatin are identical (159), it is tempting to speculate that the mechanism behind antifungal activity of nystatin aglycones differ from that of nystatin.

In order to test the toxicity of the new derivatives *in vitro,* their hemolytic activity was measured in comparison with that of the nystatin. The results of these experiments showed that both 10-deoxynystatinolide and nystatinolide had more than 90 % reduced hemolytic activity compared to the parental molecule nystatin under the conditions tested (data not

shown, see Materials and methods for details). These results indicate that the mycosamine sugar moiety contributes significantly to the hemolytic activity of nystatin *in vitro*.

The fact that both nystatin aglycones displayed greatly reduced hemolytic activity compared to nystatin suggests that these molecules fail to form stable channels in the eukaryotic cell membranes. The latter is probably due to the lack of efficient interaction between the molecules, which probably is ensured through formation of hydrogen bonds between carboxy and amino groups of the neighboring molecules (11).

3.2.3 Recombinant expression of NysDIII in *E.coli* and verification of GDP-D-mannose 4,6-dehydratase activity *in vitro*

Inactivation of the *nysDIII* homologue *amphDIII*, presumably also encoding a GDP-mannose 4,6-dehydratase, has previously been performed in the amphotericin producer *Streptomyces nodosus*, and the resulting mutant produced amphotericin aglycone (29). For confirmation of the function of *nysDIII*, this gene was instead expressed in *Escherichia coli*, purified and used in enzyme assays to confirm the activity of its product. NysDIII and NysDII were expressed in commercial expression vectors containing both Flag- (Sigma-Aldrich) and His-tag (QIAGEN). NysDIII was successfully expressed with Flag-tag located C-terminally, and with His-tag located N-terminal. The enzymes were purified according to the protocol (Sigma-Aldrich and QIAGEN) and several described methods for enzyme assay were used for proving activity of NysDIII (2, 99). The absorption due to the enolate anion of GDP-4-keto-6-deoxy-D-mannose was measured over time in samples containing purified enzymes. In the assays, purified proteins with both Flag- and His-tag were used. The GDP-mannose 4,6-dehydratase activity could be measured spectroscopic in all assays with all purified proteins. The activity of the His-tag expressed enzymes was measured to be up to 0.18 $\Delta A \cdot min^{-1} \cdot \mu g^{-1}$ protein, for the Flag-tag expressed enzymes up to 0.005 $\Delta A \cdot min^{-1} \cdot \mu g^{-1}$ protein. HPLC analysis was performed whereby the conversion of GDP-D-

mannose to GDP-4-keto-6-deoxy-D-mannose by NysDIII expressed with C-terminal Flag-tag, was confirmed (Figure 1).

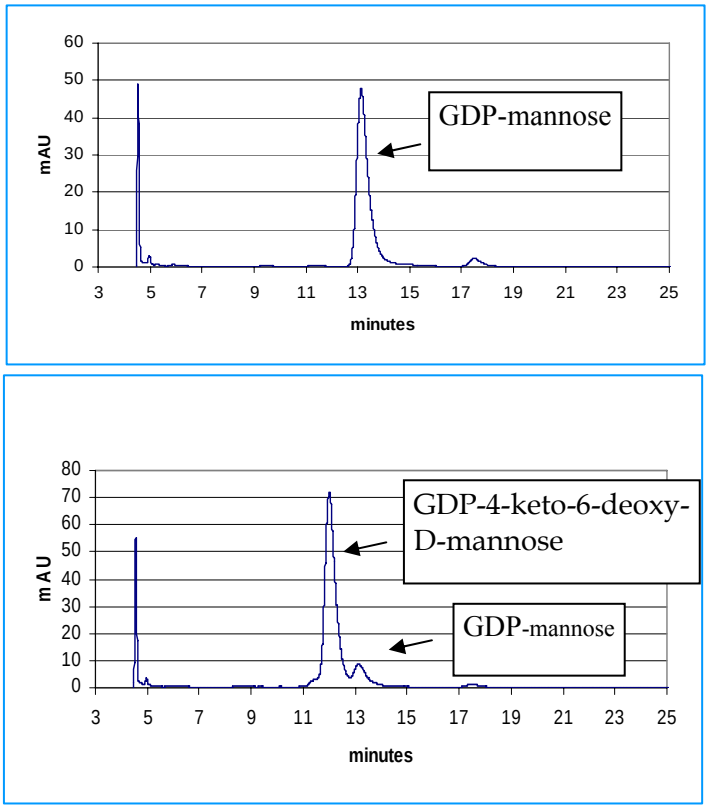


Figure 1: HPLC results confirming the activity of NysDIII converting GDP-mannose to GDP-4-keto-6-deoxy-D-mannose. Top panel shows the substrate GDP-mannose, and the bottom panel show reduction of GDP-mannose which is converted to GDP-4-keto-6-deoxy-D-mannose by addition of NysDIII.

3.2.4 Recombinant NysDII protein

To further confirm the proposed aminotransferase function of NysDII, this protein was also expressed in the same way as NysDIII with both Flag- and His-tag. NysDII was as NysDIII successfully expressed with Flag-tag His-tag located N-terminally. NysDII was used in a

coupled enzyme assay (99) together with NysDIII (as NysDII perform its function after NysDIII). The activity of NysDII in a coupled reaction with NysDIII could not be detected by HPLC analysis of any of the Flag- or His-tag expressed NysDII proteins which may be explained by suboptimal assay conditions, or possibly requirement of an enzyme for isomerization of GDP-4-keto-6-deoxy-D-mannose to GDP-3-keto-6-deoxy-D-mannose. Although it was postulated that isomerization might occur spontaneously (2), experimental verification of this hypothesis with respect to GDP-4-keto-6-deoxy-D-mannose is still lacking.

3.3 MODIFICATION OF THE NYSTATIN MACROLACTONE RING AT C16 (PAPER 3)

Biosynthesis of polyene macrolide antibiotic nystatin by *Streptomyces noursei* involves oxidation of the C16 exocyclic methyl group on the macrolactone ring, leading to formation of a carboxyl group. Previously, it has been suggested that a cytochrome P450 monooxygenase encoded by the *nysN* gene is responsible for this modification (24). However, the experimental evidence of the biological function of *nysN* has so far been lacking. To study the function of *nysN*, this gene was inactivated by deletion and site-directed mutagenesis resulting in production of 16-decarbox-16-methylnystatin, confirming the proposed function of NysN as C16 methyl oxidase. The antifungal activity of 16-decarbox-16-methynystatin was shown to be similar to that of nystatin, while its hemolytic activity was considerably lower.

3.3.1 Inactivation of *nysN*

To study the function of *nysN*, this gene was inactivated by deletion through double homologous recombination in the previously constructed non-nystatin producing strain *S. noursei* NDA59 which can be complemented with *nysA* to restore nystatin production (22) . The resulting deletion mutant, NDDN, was shown to produce 16-decarbox-16-

methylnystatin as expected, but at low levels. 16-decarbox-16-methylnystatin was produced at 1-2 % relative to nystatin production in wild type, whereby the 16-decarbox-16-methylnystatin comprises 80-90 % of the total polyene production and nystatin 10-20 %.

As the production level of 16-decarbox-16-methylnystatin level was low, it was speculated that less severe mutations could contribute to higher yields so that purification of 16-decarbox-16-methylnystatin could be simplified. Two different point mutations were introduced into NysN. Details on the construction of the site-specific mutants can not be revealed due to the process of patenting. Subsequently, the mutations were introduced into *S. noursei* by means of double homologous recombination. Both resulting mutants, named NDDN1 and NDDN2, were shown to produce 16-decarbox-16-methylnystatin, but unfortunately at low levels as well; 1-2 % of nystatin production in wild type, whereby the 16-decarbox-16-methylnystatin comprise 80-90 % of the total polyene production and nystatin 10-20 %. To explain the very low production levels in the *nysN* inactivation mutants, it is suggested that accumulation of decarboxylated polyene macrolides inhibits their biosynthetic pathway.

3.3.2 Recombinant NysN protein

To further confirm the function of *nysN*, the NysN protein was heterologously expressed and purified with His-tag expression system. Despite low level of 16-decarbox-16-methylnystatin production, a few milligrams of this compound were purified and used as a substrate in an enzyme assay as described by Volokhan *et al.* (159). But unfortunately, the biological function of NysN as a C16-methyl oxidase could not be measured in such an assay. This may be explained with suboptimal assay conditions and that 16-decarbox-16-methylnystatin might not be a true substrate for NysN.

3.3.3 Measurement of antifungal and hemolytic activity of nystatinolides

In order to confirm the importance of mycosamine for antifungal activity of nystatin, MIC_{50} values were measured for 16-decarbox-16-methylnystatin produced by *S. noursei* NDDN. As test organism *Candida albicans* ATCC 10231 was used. The MIC_{50} value for 16-decarboxy-16-methylnystatin was measured to be 2.1 (± 0.2) µg/ml / 1.6 (± 0.2) µg/ml, and the MIC_{50} value for nystatin was 1.5 µg/ml in the same experiment. These results demonstrate that 16-decarboxy-16-methylnystatin have a similar antifungal activity as nystatin and suggest that the C-16 carboxyl does not significantly affect this activity.

In order to test the *in vitro* toxicity of 16-decarboxy-16-methylnystatin, we carried out determinations of the hemolytic activity (Materials and Methods) in comparison with nystatin. The results of these experiments showed that 16-decarboxy-16-methylnystatin had significant reduced hemolytic activity compared to the parental molecule nystatin under the conditions tested.

3.4 BIOCONVERSION AS A MEANS TO MODIFY NYSTATIN AGLYCONE (unpublished results)

3.4.1 Bioconversion of nystatinolide with strains from Trondheimsfjord

In the work aimed at generation of novel nystatin analogues, nystatinolide, produced by *S. noursei* NDD1 (Paper 2) can be an excellent target for modification by attaching other sugar molecules by heterologous glycosyltransferases. By feeding of nystatinolide to bacteria strains harboring glycosyltransferases, new compounds might be produced if the glycosyltransferases manage to glycosylate the aglycone with sugars other than mycosamine.

3.4.2 Material and methods

A sub-library of 35 different strains isolated from the Trondheim fjord (TF) sediments was selected for this work. This sub-library contained 29 streptomycetes demonstrating

antibacterial activity (proof of secondary metabolite production) and six strains that have been found to produce unknown polyene compounds, probably displaying antifungal activity. From some of these strains it was possible to amplify parts of genes presumably encoding glycosyltransferases by PCR. Primers were designed by A. Luzhetskyy at Prof. A. Bechtholds lab in Freiburg (95). The primers contain both a consensus and a degenerated region to efficiently detect GT genes involved in the polyketide biosynthesis in different *Actinomycetes* strains. PCR was performed with GC Rich PCR system (Roche) and oligonucleotides delivered by MWG Biotech (Germany). Putative glycosyltransferase gene fragments were successfully amplified from 14 strains in the sub-library.

Conditions selected for bioconversion experiment was based on three methods (18, 113, 142), using two different media; MP5 (25 g/l glycerol, 3 g/l yeast extract, 0.2 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 0.02 g/l $FeSO_4$, deionised water, pH 7.2) (141) and SFM(tg) (20 g/l mannitol, 20 g/l soya flour, 0.20 g/l clerol, 3.40 g/l yeast powder, tap water). As control strains *S. noursei* wild type, the previously constructed non-nystatin producing strain *S. noursei* NDA59 (22) and NDD1 (Paper 2) were used. Pre cultures of the strains were grown in 30 ml MP5 and SFM media in 250 ml baffled flasks, 20-30^{o}C, in Infors shaking incubator at 225 rpm. 50 µl pre cultures were used to inoculate 500 µl medium in deep-well microtiter plates. Plates were incubated at 20-30^{o}C, 800 rpm (and relative humidity at 800 %) in Infors incubator. Nystatinolides were added (300 µg/ml) 24 h after inoculation. The nystatinolide extract used in this experiment was a mix of nystatinolide and 10-deoxy nystatinolide purified from *S. noursei* NDD1 (Paper 2). There were grown two parallels of the strains, one where the strains were added nystatinolide and the other one not (control). There were taken out samples of 100 µl every 24th h after adding nystatinolides, also for the corresponding sample without nystatinolides. Polyenes were extracted by adding 500 µl DMSO to 100 µl sample, shaking for 30 min at RT and centrifuging at 13000 rpm for 5 min. Extracts were then directly analyzed by LC-MS-TOF. UV data was inspected manually to find eventually new tetraene characteristic absorption spectra. MS data were compared for each strain, with and without nystatinolides added.

3.4.3 Results

Significant MS-signals that could be correlated with unique tetraenes was only observed from strain MP21-11 grown in SFM media (Figure 2). Those two tetraenes were found to have m/z at 781.4395 (retention time 12.1) and 797.4307 (retention time 11.3) resulting in the stochiometric formulas $C_{41}H_{66}O_{14}$ and $C_{41}H_{66}O_{15}$. These formulas corresponded to addition of water to 10-deoxy nystatinolide and nystatinolide respectively. Further it was observed that tetraene UV spectra correlating with m/z 781 and 797 have shifted 0.5-1 nm compared to those of the added nystatinolides. Based on these results, a hypothesis would be that a water molecule is added (which means +18 Da as one H atom and one -OH group of each C atom) to one of the double bindings in the diene-region in 10-deoxy nystatinolide and nystatinolide. We can not exclude a possibility that addition of nystatinolides triggered production of new polyenes by the strain MP21-11. Further work will be required in order to analyze these results in detail.

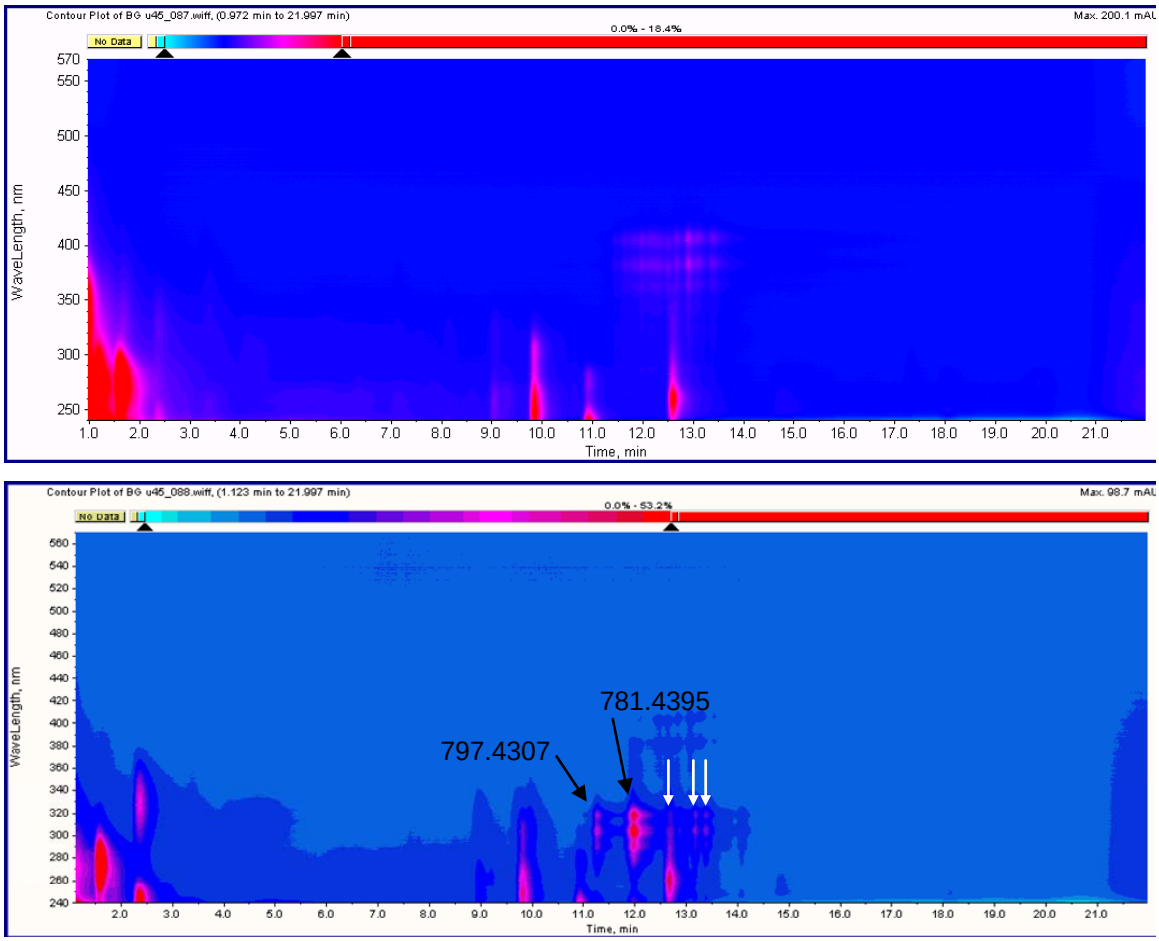


Figure 2: Strain MP21-11 without feeding of nystatinolides (top panel) and with feeding of nystatinolides (bottom panel). Exact mass m/z ($M-H^-$ ion) of putative new nystatin analogues are shown by black arrows; unmodified remaining nystatinolides are shown by white arrows.

For strain EC(S)-29-A grown in MP5 media there were observed traces of a unique tetraene at retention time 11.0 min (Figure 3). The UV-signal for this compound was very weak, and correlates with three observed masses in time; 922.5581 Da, 919.5668 Da and 883.5872 Da where the two latter masses constitutes 90 % of MS-counts. Possible stochiometric formulas for the 919 compound would be estimated to be $C_{47}H_{83}N_2O_{13}Cl$ (0.09 ppm error and good isotope distribution), $C_{50}H_{80}NO_{14}$ (0.7 ppm error and not so good isotope

distribution), $C_{44}H_{85}NO_{16}Cl$ (3.0 ppm error and good isotope distribution) or $C_{47}H_{82}O_{17}$ (3.5 ppm error and not so good isotope distribution). Based on these results it is most reliable that the compound contains a Cl-atom.
Possible stochiometric formulas for the 883 compound were suggested as $C_{44}H_{84}NO_{16}$ (0.6 ppm error and good isotope distribution) or $C_{47}H_{82}N_2O_{13}$ (2.4 ppm error and good isotope distribution). These two formulas correlate well with the loss of H and Cl atoms from the two chlorinated compounds as mentioned above, and it is not unlikely that such loss can be due to ionization.

Both strain MP21-11 and EC(S)-29-A, with and without added tetraene-substrate, were shown to produce heptaene compounds, in trace amounts not sufficient for reliable determination of their molecular masses or purification and testing. Next we tried to use the two identified bioconverting strains for modification of nystatin and amphotericin utilizing same procedures as for nystatinolides. However, modifications similar to those observed for nystatinolide and 10-deoxy nystatinolide could not be identified on these substrates, and other modified derivates were not identified. These results indicate that the strains could not use nystatin and amphotericin as substrates for modifications in the same way as nystatinolide and 10-deoxy nystatinolide.

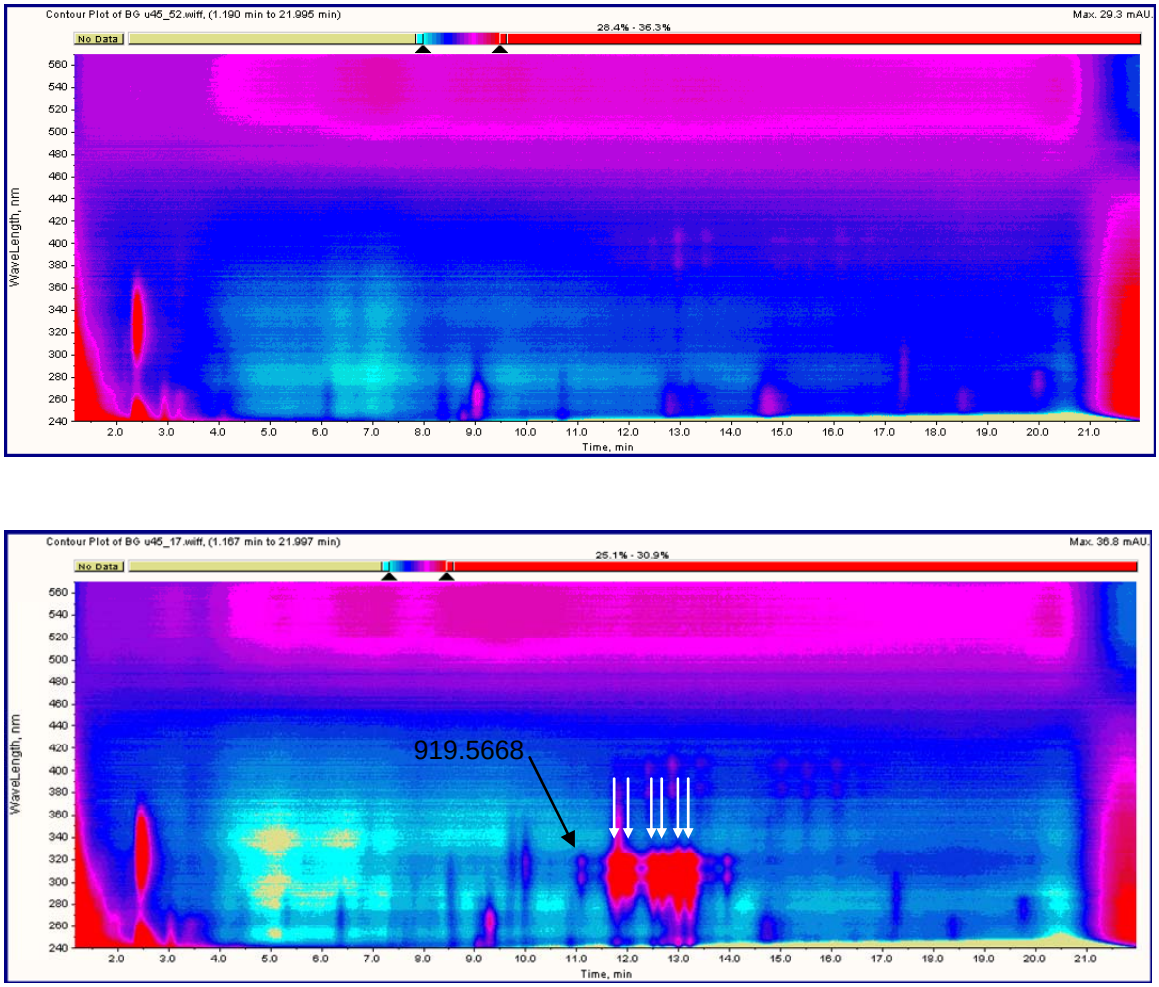


Figure 3: Strain EC(S)-29-A without nystatinolides feeding (top panel), and EC(S)-29-A with feeding of nystatinolides (bottom panel). Exact mass m/z ($M-H^-$ ion) of putative new nystatin analogue is shown by black arrow; unmodified remaining nystatinolides are shown by white arrows.

Characterization of nystatinolide modifying strains by 16s rDNA sequencing

The 16s rDNA from the nystatinolide modifying strains MP21-11 and EC(S)-29-A was amplified by PCR and sequenced. PCR was performed by using Expand High Fidelity PCR System (Roche) (4 min at 94°C followed by 35 cycles of 45 s at 94°C, 20 s at 55°C and 2 min at 66°C followed by a 5 min extension step at 70°C), and primers from MWG Biotech AG (F27: 5'-AGAGTTTGATCMTGGCTCAG-3' and R1492: 5'-

TACGGYTACCTTGTTACGACTT-3'). The amplified fragments were cloned into a pDrive vector (Qiagen PCR Cloning Kit), sequencing was performed by MWG Biotech AG and sequence alignment was performed by BLAST search. Strain MP21-11 showed 99 % sequence identified to *Streptomyces sp*. VTT E-99-1330 (A83) (149) and strain EC(S)-29-A showed 99 % sequence identified to *Streptomyces sp*. VTT E-99-1336 (B329) (149). Both latter streptomycetes have been isolated from water-damaged buildings.

3.4.4 Bioconversion of nystatinolide with *Saccharothrix espanaensis*

Nystatinolide was also fed to a *Saccharothrix espanaensis* that previously has been shown to glycosylate different natural compounds with the deoxysugar rahmnose (Prof A. Bechthold, unpublished data). This work was performed by A. Linnenbrink at Prof A. Bechtholds lab in Freiburg (Germany). But unfortunately no new nystatin/nystatinolide derivates could be observed.

For the main culture a single baffled 300 ml flask containing 100 ml of NL111 medium (20 g of Lab Lemco meat extract, 100 g of malt extract, 10 g of $CaCO_3$ ad 1000 ml tap water, pH 7.2) and 200µl of DMSO with 5 mg of nystatinolide was inoculated with 2 ml of a 24 h preculture in TSB medium (30 g of Bacto™ Tryptic Soy Broth ad 1000 ml tap water, pH 7.2). The main culture was incubated for 8 days on a rotary shaker at 28°C and 180 rpm. For extraction the culture was centrifuged to separate the cell pellet from the supernatant.

LC / MS method:

The analysis was carried out on an Agilent 1100 system (Agilent Technologies, Waldbronn) equipped with a diode array (DAD, G1315B) and a quadrupole mass detector (MSD, G1946D). For nystatinolide electrospray ionization was used.

Column: Zorbax SB-C18 (150 x 4.6 mm; 5 µm), column flow: 0.7 ml/min.

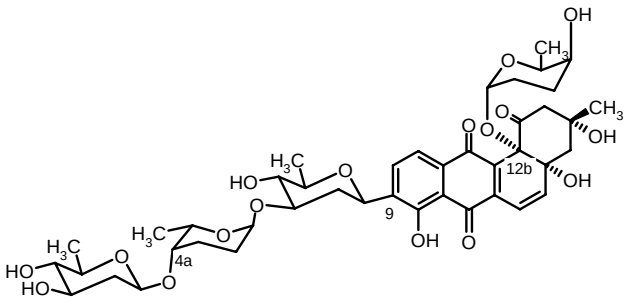
Solvent A: acetonitrile; solvent B: water with 0.5 % acetic acid.

3.5 ATTEMPTS ON ALTERNATIVE GLYCOSYLATION OF NYSTATINOLIDE USING GT HYBRIDS AND DEOXYSUGAR BIOSYNTHESIS GENE CASSETTES (unpublished results)

3.5.1 Construction of hybrids between the glycosyltransferase genes *nysDI, urdGT2* and *lipGTF*

In this work we designed hybrid glycosyltransferases, in the means that these hybrids could glycosylate the nystatinolide. Such hybrids could theoretically attach sugar molecules other than mycosamine, complement the function of *nysDI*, or attach the sugar moiety in another position. For construction of hybrid glycosyltransferases, two heterologous glycosyltransferase genes were used in combination with *nysDI*: *urdGT2* from *Streptomyces fradiae* Tü2717 (69), and *lipGTF* from *Streptomyces aureofaciens* (Tü117) (15). These genes were selected on the basis of chemical structure of the compounds glycosylated by the corresponding GTs, which showed some similarity to the nystatin aglycone. In addition, the UrdGT2 enzyme has previously been shown to have relaxed substrate specificity (69).

Streptomyces fradiae Tü2717 is the producer of urdamycin A (Figure 4), an antibacterial antibiotic and anticancer agent (48). Urdamycin A molecule consists of an angucyclic polyketide core and four deoxyhexose moieties: two D-olivoses (first and last sugar of a C-glycosidically bound trisaccharide chain at the C9 position) and two L-rhodinoses (in the middle of the trisaccharide chain and at the C12b position). Two GTs are involved in the C-glycosyl transfer of D-olivose at C9 (UrdGT2) and in conversion of aquayamycin to urdamycin G (UrdGT1c). It has been demonstrated that these two glycosyltransferases show relaxed substrate specificity for their activated deoxysugar co-substrate and their alcohol substrate, respectively. Both GTs can transfer activated D-rhodinose (instead of D-olivose) to C9, and attach L-rhodinose to the C4a normally occupied by a D-olivose unit (69).



Urdamycin A

Figure 4: Chemical structure of Urdamycin A

Streptomyces aureofaciens Tü117 is the producer of α-lipomycin (Figure 5) (88). α-lipomycin is an acyclic polyene antibiotic where a carbonyl group links a pentaene chain with a derivate of N-methyl-tetramic acid, composing the aglycone. An L-digitoxose moiety is attached to the polyketide part of the aglycone presumably by the glycosyltransferase LipGTF. α-lipomycin is active against Gram-positive bacteria but has no effect on the growth of fungi and gram-negative bacteria (15).

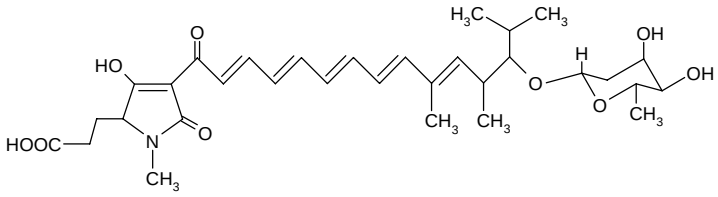


Figure 5: Chemical structure of α-Lipomycin

Since the mycosamine biosynthetic pathway is intact in the *S. noursei* NDD1 mutant; we have decided to introduce our hybrids in this strain. This would allow, besides possible generation of novel glycosylated nystatin analogues, to assess specificity of the glycosyltransferase hybrids. With this in mind, hybrids of the glycosyltransferase *nysDI* and *urdGT2*, and *nysDI* and *lipGTF*, were constructed by PCR Overlap Extension or "Gene SOEing" method. By Gene SOEing two DNA fragments can easily be combined by overlap extension leading to production of chimeric genes. By using pair of primers with

complementary regions, overlapping strands of PCR amplified DNA fragments from various sources can be spliced together by the extension of overlapping strands in a subsequent reaction (155).

As the glycosyltransferases contain two domains connected by a linker region (123), four hybrid combinations were constructed between each of *nysDI/urdGT2* and *nysDI/lipGTF* pairs (Table 1 and 2). As NysDI, UrdGT2 and LipGTF are inverting GT-B fold glycosyltransferases, the N-terminal domain provides the acceptor binding site, and the C-terminal domain provides the donor binding site (123). The linker region in *nysDI*, *urdGT2* and *lipGTF* was predicted based on sequence alignment (Clustal W) in comparison with the glycosyltransferase GtfB, for which a crystal structure is available (108).

Table 1. Overview of combinations for hybrids between NysDI and UrdGT2.

	N-terminal	**Linker region**	**C-terminal**
Hybrid 1: A1+D1	A1*: nysDI*	*nysDI*	D1*: urdGT2*
Hybrid 2: A2+D2	A2*: nysDI*	D2*: urdGT2*	*urdGT2*
Hybrid 3: C1+B1	C1*: urdGT2*	*urdGT2*	B1*: nysDI*
Hybrid 4: C2+B2	C2*: urdGT2*	B2*: nysDI*	*nysDI*

Table 2. Overview of combinations for hybrids between NysDI and LipGTF.

	N-terminal	**Linker region**	**C-terminal**
Hybrid 1: A1+D1	A1*: nysDI*	*nysDI*	D1*: lipGTF*
Hybrid 2: A2+D2	A2*: nysDI*	D2*: lipGTF*	*lipGTF*
Hybrid 3: C1+B1	C1: *lipGTF*	*lipGTF*	B1: *nysDI*
Hybrid 4: C2+B2	C2*: lipGTF*	B2: *nysDI*	*nysDI*

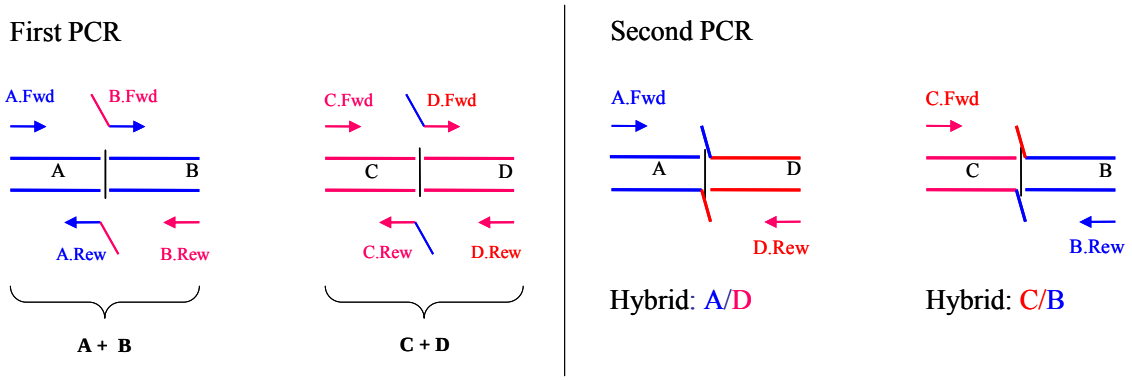


Figure 6: GeneSOEing PCR; A/B represents one gene separated by a linker region, and C/D represents another gene separated with a linker region. A and C represent N-terminal part of the genes, and B and D represent C-terminal parts of the genes. The primers A.Rew, and B.Rew, C.Rew and D.rew are megaprimers (ca 50 nucleotides) containing tails (ca 25 nucleotides) homologous to the gene fragment that the first amplified fragment shall be fused with.

PCR amplification, sequencing and DNA manipulations

Oligonucleotide primers were purchased from MWG Biotech AG. The PCR's were performed with the GC Rich PCR System (Roche Molecular Biochemicals) using the Eppendorf Mastercycler (Eppendorf, Germany), as described previously (23). General DNA manipulations were performed as described previously (138). DNA fragments from agarose gels were purified using Qiaex II kit (Qiagen, Germany). DNA sequencing was performed by MWG Biotech AG.

Construction of hybrid vectors

The ca 1.5 kb PCR amplified hybrids of NysDI/UrdGT2 and NysDI/LipGTF were digested with *Sph*I/*Hind*III or *Not*I/*Hind*III and subcloned via pGEM11Zf + (Promega) before they were joined together with the 0.2 kb *Eco*RI/*Sph*I fragment of pGEM7ZfErm*li (C.R. Hutchinson) into the integrative vector pSOK804 (141).

Analysis

To analyze polyene macrolide production, NDD1 mutant harboring the hybrids (ten parallels of each strain) were incubated for 5 days in a defined production medium, and culture extracts were analyzed for the presence of nystatin-related polyene macrolides using LC-MS-TOF. Neither novel nystatin analogues nor restoration of nystatin biosynthesis were observed in the extracts.

Mutations were identified in some of the hybrids by sequencing but most of the mutations were silent and none of them introduced stop codons. Based on this we still decided to test the hybrids if they had any function or at least any complementing function. This might be the explanation why some hybrids didn't work, even if a mutation does not lead to amino acid change the structure and function might still be altered influencing the activity of the enzyme. Of other reasons why the hybrids didn't work can for instance be that the overall structure of the GT hybrids could have been significantly disturbed, rendering them non-functional. The active site might be interrupted as well, as the linker region was predicted, and experimental data confirming these regions are lacking for the used GTs.

3.5.2 Introduction of L-rhamnose biosynthesis genes into *S. noursei*

Elloramycin (Figure 7) is an anthracycline drug structurally related to tetracenomycin C and containing a methylated L-rhamnose moiety at the C8 (132).

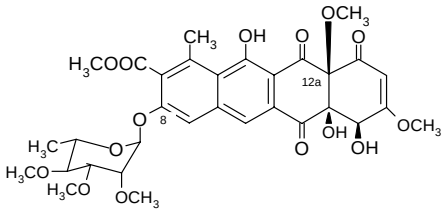


Figure 7: Chemical structure of elloramycin.

L-rhamnose biosynthesis genes from *S. olivaceus* Tü2353 have been cloned and analysed, and successfully used for construction of gene cassettes useful for combinatorial biosynthesis. A construct containing L-rhamnose biosynthesis genes, named pRHAM (132), was received from Prof. J.A Salas group (University of Oviedo, Spain). pRHAM contains four genes encoding the enzymatic functions necessary for the biosynthesis of dTDP-L-rhamnose; *oleL*, *oleS*, *oleE* and *oleU*, in a single transcriptional unit under the control of the *ermE**p promoter from *Saccharopolyspora erythrea* (*ermE*p) (132). All the genes plus the promoter were isolated as a *Hind*III/*Eco*RI fragment and directly cloned into pSOK804 (141). The resulting plasmid was introduced into both *S. noursei nysDI* and *nysDII* deficient mutants (NDD1 and NDD2) (Paper 2).

To analyze polyene macrolide production of NDD2 and NDD1 mutants harboring the L-rhamnose biosynthetic genes, ten parallels of each strain were incubated for 5 days in a defined production medium, and culture extracts were analyzed for the presence of nystatin-related polyene macrolides using LC-MS-TOF. No new nystatin analogues were detected in the extracts, suggesting that NysDI might have a strong preference for GDP-mycosamine as a donor substrate and can not attach L-rhamnose to the nystatin aglycone. The latter notion is supported by the fact that in previous experiments, as for the *nysDII* inactivation mutant (Paper 2), where a deaminated deoxysugar was expected to be attached to the nystatin aglycone, a nystatinolide was produced instead.

3.5.3 Introduction of the N-methyltransferase *OleM1* from *Streptomyces antibioticus*

S. antibioticus is the producer of a 14-unit macrolide oleandomycin (Figure 8). The oleandomycin molecule is composed of a macrolactone ring (oleandolide) with two 6-deoxysugars (L-oleandrose and D-desosamine) attached. The methyltransferase OleM1 is proposed to encode a N-methyltransferase responsible for dimethylation of the amino group at C-3 in D-desosamine (115). In an attempt to afford modification of the mycosamine moiety of nystatin, more specifically, methylation of the amino group, the gene for N-methyltransferase *oleM1* from the *S. antibioticus* was introduced to *S. noursei* wild type.

The *oleM1* was amplified by PCR with oligonucleotides purchased from MWG Biotech AG, encompassing endonuclease sites *Not*I and *Hind*III. As a template, cosAB24 (containing part of the oleandomycin gene cluster; *oleM1* 740 bp, derivate of PKC505 (128)) received from Prof. J.A. Salas group (University of Oviedo, Spain) was used. The PCR product was digested with *Not*I/*Hind*III, and cloned via pGEM11 in *Not*I/*Hind*III sites and further joined together with the 0.2 kb *Eco*RI/*Sph*I fragment of pGEM7ZfErm*li (C.R. Hutchinson), into the integrative vector pSOK804 (141). The resulting construct was introduced into *S. noursei* wild type.

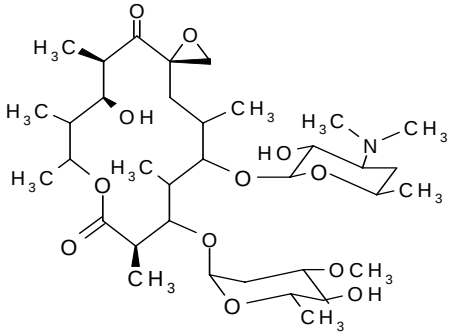


Figure 8: Chemical structure of Oleandomycin

To analyze polyene macrolide production of *S. noursei* harboring the construct with OleM1, ten parallel strains were incubated for 5 days in a defined production medium, and culture extracts were analyzed for the presence of nystatin-related polyene macrolides using LC-MS-TOF. No new nystatin analogues could be detected in the extracts of the recombinant strains. This might be explained by the fact that mycosamine is activated with GDP, while desosamine is activated with TDP. Presumably, OleM1 can not accept GDP-sugar as substrate, or at least not GDP-mycosamine. It might also be that the nystatin glycosyltransferase, *nysDI*, does not accept N-methylated mycosamine as a substrate.

4 CONCLUDING REMARKS AND FURTHER PERSPECTIVES

The work in this thesis has given us a better knowledge of the post PKS modifying genes in the nystatin biosynthesis cluster in *S. noursei*.

The functions of genes involved in mycosamine biosynthesis (*nysDIII* and n*ysDII*) and attachment (*nysDI*) of the antifungal polyene macrolide nystatin produced by *S. noursei* were studied and confirmed. By recombinant expression of NysDIII in *E. coli*, the GDP-D-mannose 4,6-dehydratase activity was demonstrated *in vitro*. NysDII was recombinant expressed as well, but unfortunately the activity could not be demonstrated which can be explained with the absence of an isomerase suggested to be required for isomerisation of GDP-4-keto-6-deoxy-D-mannose to GDP-3-keto-6-deoxy-D-mannose. But importantly, soluble recombinant proteins involved in mycosamine biosynthesis are expressed which can be used in future for *in vitro* synthesis of aminosugars.

Inactivation of *nysDII* and *nysDI* by *in-frame* deletion of the genes individually from the chromosome led to main production of nystatinolide and 10-deoxynystatinolide. Although the production level in ΔNysDII was surprisingly low compared to ΔNysDI, the nystatin biosynthesis was partially restored by introduction of both the *nysDII* and *nysDI* genes in-trans into the respective two mutant strains. A clearly conversion of nystatinolides to nystatin was observed for both mutants confirming the predicted roles of the two genes in mycosamine biosynthesis and attachment. The presumed importance of mycosamine for the biological activity of nystatin was studied as well. Both the antifungal as well as the hemolytic activity of the purified nystatinolide molecules were strongly reduced compared to nystatin confirming the importance of mycosamine for the nystatin activity.

To study and confirm the biological function of the post PKS modifying P450 monooxygenase *nysN* the gene was deleted resulting in production of 16-decarboxy-16-methylnystatin. Unfortunately the production of 16-decarboxy-16-methylnystatin was low

and two less severe mutations in NysN was introduced in an attempt to receive a *nysN* inactivated mutant producing higher levels of 16-decarboxy-16-methylnystatin. Both site specific mutants produced 16-decarboxy-16-methylnystatin, but unfortunately not leading to higher amounts then the deletion mutant. 16-decarboxy-16-methylnystatin was purified from the deletion mutant and its antifungal activity was measured to be the same as for nystatin, whereas the toxicity was measured to be lower.
For amphotericin it is demonstrated a reduction in toxicity by suppression of the charge of the exocyclic group and for rimocidin an amidotransferase activity involved in chemical modifications of the free carboxyl group of two typical polyene macrolide polyenes into an amide group leads to improved pharmaceuticals. It would be very interesting to perform modifications of the C16-carboxy group to reduce the toxicity. And as inactivation mutant of *nysN* producing 16-decarboxy-16-methylnystatin is available, there are many possibilities for further modifications although the challenge will be the low production levels.

Concerning bioconversion as a means to modify nystatin aglycone, we unfortunately could not find a strain that could glycosylate nystatinolide though some harbored GTs. On the other hand two strains successfully modified nystatinolide in an interesting way, though the production levels of those derivates were low and nystatinolide derivates are inactive. In a further perspective, bioconversion and growth conditions could be optimized to increase the levels of modified nystatinolide derivates, purify these compounds and feed them to e.g. the non nystatin producing strain *S. noursei* NDA59 with intact NysDI to eventually glycosylate these compounds.

The work with developing new novel nystatin derivates by use of hybrid GTs, introducing other deoxysugar cassettes, methylation of mycosamine was unfortunately not successful. The nystatin glycosyltransferase NysDI seem to be highly specific in binding and attachment. This is a difficult and challenging project but which can led to very interesting results if it is successful. There are still many possibilities for modifying the nystatin and

nystatinolide derivate, e.g. by trying to attach olivose. The constructed hybrids between NysDI and UrdGT2 can also be tested in *S. fradiae* for modification of urdamycin as the UrdGT2 inactivation mutant is available, and UrdGT2 previously have shown some substrate specificity.

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