

# STRUCTURAL ENZYMOLOGY OF THE BIOSYNTHESIS OF POLYKETIDE ANTIBIOTICS

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Cover: Doxorubicin, the most common anthracycline used in chemotherapy today.

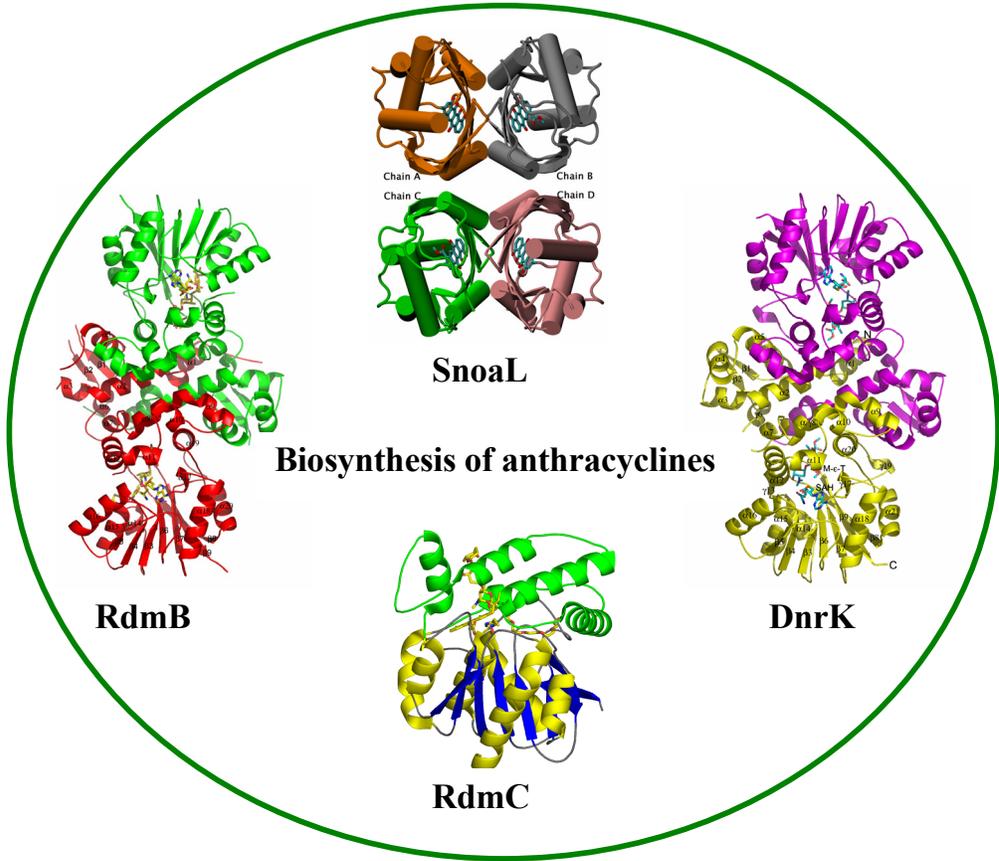
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# ABSTRACT

Anthracyclines are an important group of aromatic antibiotics that exhibit antitumour activity, which makes them useful in treatment of various cancers. They are synthesised in the polyketide biosynthetic pathway as secondary metabolites by different *Streptomyces* species. An increasing number of anthracyclines have however been shown to exhibit cardiotoxic side-effects. The genetics and enzymology of this pathway has recently attracted considerable interest, not at least with the possible prospect for the production of novel antibiotics.

In this thesis some of the enzymes involved in biosynthesis of anthracyclines have been studied by protein crystallography and biochemical methods. The structure of SnoaL, a stereospecific cyclase was determined to a resolution of 1.35 Å as a complex with a product analogue. SnoaL belongs to a hitherto uncharacterised family of enzymes with  $\alpha+\beta$  barrel like fold and catalyses a novel form of intramolecular aldol-condensation. The structure of the methylesterase RdmC in complex with product analogue shows the common  $\alpha/\beta$  hydrolase fold and contains a catalytic Ser-His-Asp triad. RdmB is a hydroxylase built up by a Rossmann-like fold common to methyltransferases. The enzyme utilizes the SAM moiety in a novel way as a cofactor in the hydroxylation reaction. DnrK is a methyltransferase with a structure very similar to that of RdmB.

RdmB and DnrK are thus two enzymes sharing the same fold but catalysing different reactions. They are illustrative examples of two enzymes evolved through divergent evolution. A common feature to all the enzymes studied in the thesis is that they bind their anthracycline substrates mainly through hydrophobic interactions with the involvement of only a few hydrogen bonds. Many of the enzymes have a very broad substrate specificity which might be due to these features.



# LIST OF PUBLICATIONS

The thesis is based on the following papers and manuscripts, referred to by their roman numerals I - VI.

- I**            **Jansson A**, Niemi J, Mäntsälä P, Schneider G. (2003) Crystal Structure of Aclacinomycin Methyltransferase with Bound Product Analogues: Implications for Anthracycline Recognition and Mechanism. *J Biol Chem.* Oct 3; 278(40):39006-13.
- II**            **Jansson A**, Niemi J, Mäntsälä P, Schneider G. (2003) Crystallization and preliminary X-ray diffraction studies of aclacinomycin-10-methyltransferase and aclacinomycin-10-hydroxylase from *Streptomyces purpurascens*. *Acta Crystallogr D Biol Crystallogr.* Sep; 59(Pt 9):1637-9.
- III**           **Jansson A**, Niemi J, Lindqvist Y, Mäntsälä P, Schneider G. (2003) Crystal structure of aclacinomycin-10-hydroxylase, a *S*-adenosyl-*L*-methionine-dependent methyltransferase homologue involved in anthracycline biosynthesis in *Streptomyces purpurascens*. *J Mol Biol.* Nov 21; 334(2):269-80.
- IV**            Sultana A, Kallio P, **Jansson A**, Niemi J, Mäntsälä P, Schneider G. Crystallisation and preliminary crystallographic data of SnoaL, a polyketide cyclase in nogalamycin biosynthesis. Accepted for publication in *Acta Crystallogr D Biol Crystallogr.*
- V**            Sultana A, Kallio P\*, **Jansson A\***, Wang J, Niemi J, Mäntsälä P, Schneider G. (2004) Crystal structure of the polyketide cyclase SnoaL suggests novel mechanism for enzymatic aldol condensation. *EMBO J.* Apr 8 (published on-line ahead of print).
- VI**            **Jansson A\***, Koskiniemi H\*, Mäntsälä P, Niemi J, Schneider G. Crystal structure of a ternary complex of DnrK, a methyltransferase in daunorubicin biosynthesis, with bound products. *In manuscript.*

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# LIST OF ABBREVIATIONS

|                   |   |
|-------------------|---|
| SAM               | <i>S</i> -adenosyl- <i>L</i> -methionine  |
| SAH               | <i>S</i> -adenosyl- <i>L</i> -homocysteine  |
| AknT/A            | Aclacinomycin T/A   |
| DcmaT/A           | 10-decarbomethylaclacinomycin T/A   |
| DmaT/A            | 15-demethoxyaclacinomycin T/A   |
| DbrT/A            | 11-deoxy- $\beta$ -rhodomycin T/A   |
| $\epsilon$ -T     | $\epsilon$ -rhodomycin T  |
| M- $\epsilon$ -T  | 4-methoxy- $\epsilon$ -rhodomycin T   |
| NAME              | Nogalonic acid methylester  |
| AAME              | Aklanonic acid methylester  |
| SnoaL             | Nogalonic acid methylester cyclase  |
| RdmE              | Aklavinone-11-hydroxylase   |
| RdmC              | Aclacinomycin methylesterase  |
| RdmB              | Aclacinomycin-10-hydroxylase  |
| DnrK              | 4-O-methyltransferase   |
| PEG               | Polyethylene glycol   |
| Å                 | Ångström ( $10^{-10}$ m)  |
| CoA               | Coenzyme A  |
| FDA               | Food and drug administration  |
| Bp                | Base pair   |
| RNA               | Ribonucleic acid  |
| DNA               | Deoxyribonucleic acid   |
| GSH               | Reduced glutathione   |
| GSSG              | Oxidized glutathione  |
| MAD               | Multi-wavelength anomalous diffraction  |
| SIRAS             | Single isomorphous replacement with anomalous scattering  |
| $R_{\text{sym}}$  | $\frac{\sum_{\text{hkl}} \sum_i  I_i - \langle I \rangle }{\sum_{\text{hkl}} \sum_i \langle I \rangle}$ , where $I_i$ is the intensity measurements for a reflection and $\langle I \rangle$ is the mean value for this reflection. |
| $R_{\text{work}}$ | $\frac{\sum   F_{\text{obs}}  -  F_{\text{calc}}  }{\sum  F_{\text{obs}} }$ ; $ F_{\text{obs}} $ is the observed and $ F_{\text{calc}} $ is the calculated structure factor amplitudes.   |
| $R_{\text{free}}$ | The R-factor as above but calculated for a subset of reflections that is not used in the refinement   |

# 1 INTRODUCTION

Together with the emergence of new infectious diseases, an increase of bacterial strains resistant to existing antibiotics has been observed. There is therefore an enormous challenge to pharmaceutical companies, researchers, and governments to develop new methods for treating both existing and new infectious diseases.

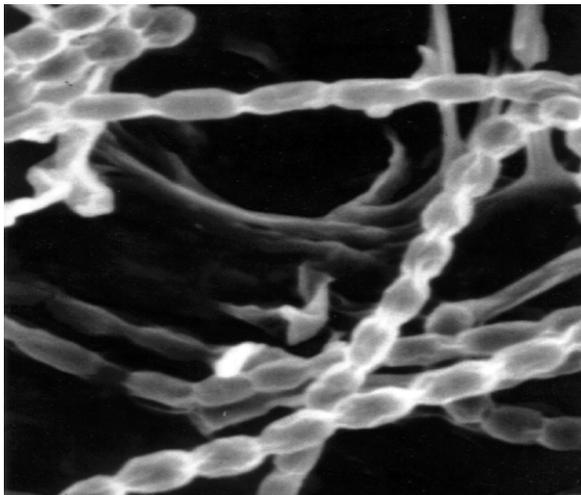
Antibiotics are naturally occurring or synthetic chemical substances that exhibit bacteriostatic or bactericidal effects; that is, they inhibit or kill bacteria. The word antibiotic is derived from Greek, and means "against life" and the concept of antibiotics was first introduced in 1889 by Paul Vuillemin (1, 2). The definition of antibiotics was later refined by Selman A. Waksman in 1949 as "products of the metabolisms of microorganisms, with molecular mass < 2000 Dalton, which in small quantities inhibit growth of other microorganisms" (3). This narrow definition later had to be revised since antibiotics were introduced in for example cancer chemotherapy. Although Alexander Fleming was credited with the discovery of antibiotics, they were not isolated and synthesised for clinical applications until the 1940s, the first example being penicillin for the treatment of septicemia. The mass production of antibiotics began during the World War II with streptomycin and penicillin, saving the lives of tens of thousands of allied soldiers.

Natural products antibiotics belong to a group of compounds called secondary metabolites, generally characterised by being produced at low specific growth rates, and by the fact that they are not essential for the growth of the producing organisms in pure culture. Antibiotics are, however, critical to the organisms in their natural environment, as they are needed both for survival and for competitive advantage (4). Generally these microbial compounds are too complex for total synthesis to be a viable way of obtaining large quantities for commercial exploitation. Fermentation of bacterial or fungal strains followed by extraction and purification are instead preferred by pharmaceutical companies (5). Many antibiotics are also obtained by chemical modification of natural substances; often such derivatives are more effective against infecting organisms or are better absorbed by the body.

## 1.1 POLYKETIDE ANTIBIOTICS

Polyketides are important natural products exhibiting antibacterial (rifamycin), antifungal (erythromycin), antitumor (doxorubicin), immunosuppressant (FK506) and cholesterol-lowering activities (lovastatin) (6). They are produced mainly by *Streptomyces* species which belong to the large group of mycelially growing,

filamentous bacteria from soil known as actinomycetes (figure 1.1). These gram-positive, fungi-like bacteria are one of the best known producers of secondary metabolites used as naturally occurring antibiotics.



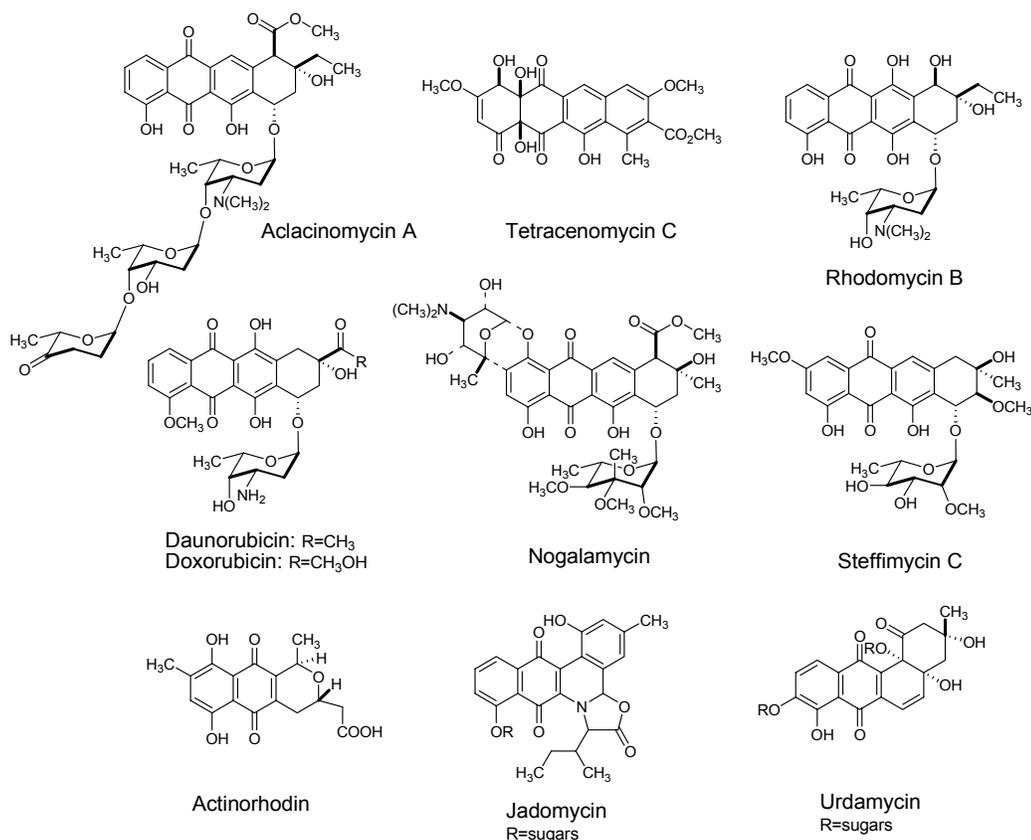
**Figure 1.1:** *Streptomyces* mycelia.

Each core of the polyketide is synthesised biologically under the control of an exceptionally large, multifunctional enzyme called polyketide synthase (PKS), in a manner similar to that of fatty acid synthesis (7), where the carbon backbones of the molecules are assembled by the successive condensation of small acyl units. There are presently three types of polyketides:

Type I modular polyketides are built up by a PKS consisting of large multifunctional proteins with a different active site for each enzyme-catalysed step in polyketide carbon chain assembly (8). The type I polyketides, such as for example erythromycin, are often structurally very intricate due to the vast number of combinations in building blocks and modifications performed by the PKS.

Bacterial type III iterative polyketides were characterised not long ago and the type III PKS is a member of the chalcone synthase (CHS) and stilbene synthase (STS) superfamily of PKS previously only found in plants (9).

Type II iterative polyketides also known as aromatic polyketide, are normally synthesised by a single PKS built up by discrete polypeptides which carry active sites that are used more than once in the biosynthetic pathway (6). Some examples of aromatic polyketides are shown in figure 1.2. The class of aromatic polyketides called the anthracyclines are the subject of discussion in this thesis and are the focus of the following chapters.



**Figure 1.2:** Structures of some aromatic polyketides: aclacinomycin A from *S. galilaeus*, tetracenomycin C from *S. glauscens*, rhodomycin B from *S. purpurascens*, daunorubicin from *S. peucetius* and *S. sp. C5*, doxorubicin from *S. peucetius*, nogalamycin from *S. nogolater*, steffimycin C from *S. steffisburgensis*, actinorhodin from *S. coelicolor*, jadomycin from *S. venezuelae* and urdamycin from *S. fradiae*.

## 1.2 ANTHRACYCLINES

1939 Hans Brockmann and Klaus Bauer isolated the first anthracyclines from the rhodomycin producing strain *S. purpurascens* found in the soil in a forest outside of Göttingen (10). Although the antibacterial properties of the organisms that produce anthracyclines were discovered already at that time, the chemistry of the active metabolites was not investigated until the 1960s. Farmitalia research laboratories (now Pfizer) in Milano, Italy then began screening isolates from soil samples for anticancer compounds in the mid 1950s (11) and the following years were denoted as “the golden age” of antibiotics discovery (12, 13). The anthracyclines are among the most intensely studied natural products over the past quarter century and of all antibiotic substances known today 2/3 (about 5000) come from the *Streptomyces* genus. Many of these antibiotics exhibit high cytotoxicity (14) and have been employed as cytostatics in cancer therapy. Doxorubicin (adriamycin or 14-hydroxydaunomycin), isolated from mutant strain, *S. peucetius* subsp. *caesius* in 1967 by Farmitalia (13) was approved by FDA in 1974 for commercial use as an anticancer agent. In contrast to most antitumour

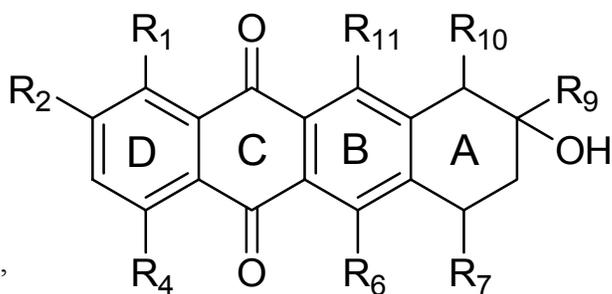
drugs such as daunorubicin (11) used against acute leukaemia, doxorubicin display remarkable activity against a broad range of tumours and is also less toxic (15, 16). To date, doxorubicin has been the most successful and useful anticancer agent developed, being the second most used cancer therapeutic agent, widespread in clinical use. Seven anthracyclines have come into worldwide clinical use, namely: daunorubicin (daunomycin, rubidomycin), doxorubicin (adriamycin), idarubicin, epirubicin, zorubicin and aclacinomycin A (aclarubicin) the last one mainly used in Japan and Asian countries. Many anthracyclines give rise to serious side effects such as cardiotoxicity, and a great challenge for the future is the development of more active variants without displaying these side-effects.

### 1.2.1 The carbon skeleton or aglycone core of anthracyclines

The anthracyclines were found to look like “yellow-red optical active dyes” and because of their resemblance to anthraquinones they obtained the name anthracylinones (17). Since then, “anthracycline” has been the name of the microbial product that contains an anthracylinone moiety, typically as a glycoside. The intense colour of these aromatic compounds ranges from yellow (aklavinone derived) and red ( $\epsilon$ -rhodomycinone derived) to purple and blue.

**Table 1.1**

| Position        | Substituent  |
|-----------------|--|
| R <sub>1</sub>  | H, OH, glycoside   |
| R <sub>2</sub>  | H, OH, glycoside   |
| R <sub>4</sub>  | OH, OCH <sub>3</sub>   |
| R <sub>6</sub>  | H, OH  |
| R <sub>7</sub>  | H, OH, glycoside   |
| R <sub>9</sub>  | CH <sub>3</sub> , CH <sub>2</sub> CH <sub>3</sub> , COCH <sub>3</sub> ,<br>COCH <sub>2</sub> OH, CHOCH <sub>3</sub> ,<br>CHOHCH <sub>2</sub> OH, CH <sub>2</sub> COCH <sub>3</sub> |
| R <sub>10</sub> | H, OH, COOCH <sub>3</sub> , glycoside  |
| R <sub>11</sub> | H, OH  |



**Figure 1.3:** The general structure of the aglycone moiety of aklavinone- or nogalomycinone-type anthracyclines. **Table 1.1** shows the most common substituents in these aglycones

The main structural characteristics of the anthracyclines were determined in 1964 by detailed studies of daunorubicin (18). Anthracyclines belong to the group of aromatic polyketides where the basic structure is a cyclic polyketide backbone that shares the 7,8,9,10-tetrahydrotetracene-5,12-quinone structure. The diversity of these secondary

metabolites lies in the variations in the modifications of the aglycone moiety and in the composition of the attached carbohydrate (figure 1.3 and table 1.1) An interesting feature is that the hydroxyl group at position C11 seems to have an important role, since all anthracyclines with this modification are more toxic for *Streptomyces* itself and cytotoxic for animals than their C11-deoxy analogues (18, 19).

### **1.2.2 The three-dimensional structure of anthracyclines**

The structure of aklavinone (19) shows that the rings B, C and D are planar, and that ring A has a half-chair conformation. The O9 on ring A is axial, while the C13 is equatorial which is similar to the conformation of daunorubicin and doxorubicin. Nogalamycin (20) has the O9 equatorial and the C13 axial, giving the configuration of ring A as 7S, 9S, 10R. The structure of daunorubicin was the first anthracycline determined by X-ray crystallography (21) and the glycosylated structure revealed the conformation of the attached sugar moiety. Many anthracycline structures have also later been determined in complex with DNA both by NMR and X-ray crystallography.

## **1.3 BIOLOGICAL ACTION OF ANTHRACYCLINES**

The antineoplastic activity of the anthracycline drugs has been mainly attributed to their inhibition of DNA biosynthesis in the target cells (14, 22-24) but the cytostatic mechanism has not been completely resolved. However, the primary mode of action of most anthracyclines is believed to be their strong, but non-covalent intercalation with DNA (25, 26). This causes the inactivation of topoisomerase II, presumably by stabilising the normally reversible topoisomerase II-DNA complex and thereby inhibiting DNA religation after the double-strand break introduced by topoisomerase II. Partially unwinding and deformation of the double-stranded helix in the cells of tumour tissue (27, 28), because of the formation of a drug-DNA-enzyme ternary complex, prevents the replication and translation processes (29, 30), eventually leading to apoptotic cell death. DNA binding is necessary but not sufficient for drug activity and it is at present not really known how this relates to cytotoxicity. Other molecular interactions might play a role as well, such as interactions with helicases, topoisomerase I and other DNA interaction enzymes (31, 32). The topoisomerase II inhibition might however be a primary triggering event for a signalling pathway leading to apoptosis at least in leukemia cells and tumours. Toxicity might additionally be associated to altered cell-permeability in the membranes due to reaction with phospholipids as well as damage to essential cellular machinery by anthracycline-generated oxygen radicals (33). The potential involvement of free radical formation in the cytotoxicity of anthracyclines, both in terms of antitumour effect and cardiotoxicity, is complex.

Because the biological activities of these drugs are probably closely related to their DNA binding affinity and sequence specificity, knowledge of their interaction with the target DNA would help to better understand the structure-function relationship and improve the design of novel agents.

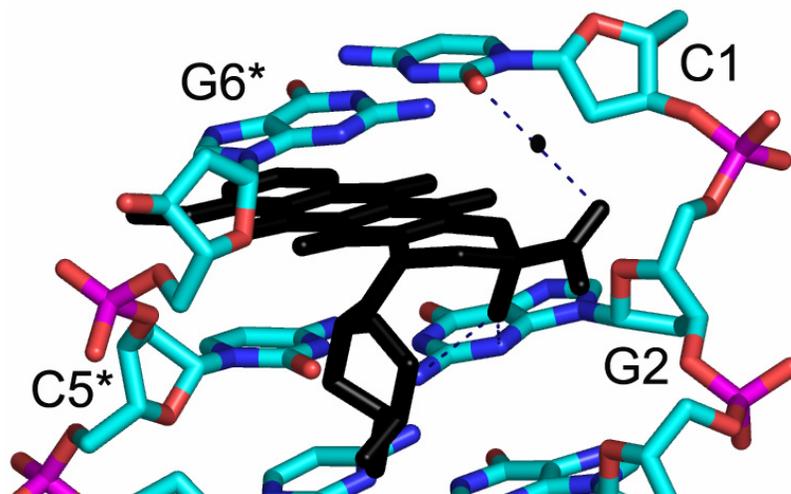
### 1.3.1 Structures of drug-DNA complexes

Numerous biochemical studies including evidence from NMR spectroscopy and X-ray crystallography of the binding of anthracyclines to a short nucleotide sequence have provided a detailed picture of the nature of the drug-DNA complex (34-41). The complex of d(CGATCG) together with daunorubicin was the first anthracycline-DNA complex solved by several groups about twenty years ago (35, 36, 42). The first structure, determined 1980 by X-ray crystallography, consists of DNA and daunorubicin in a 2:1 ratio (42). Ring D is protruding into the major groove and ring A, together with the sugar moiety, is binding to the minor groove (figure 1.4). The DNA shows a B-type conformation and daunorubicin is positioned between the d(CpG) base-pairs, intercalating with the aromatic rings B-D. The carbonyl and hydroxyl groups at position C9 form hydrogen bonds to atoms in the minor groove, the hydroxyl on O9 to N2 and N3 of a guanine base (G2) and O13 via a water molecule to O2 of a cytosine base (C1) in the DNA (figure 1.4). There are also additional van der Waals interaction between anthracycline and DNA.

There are no crystal structures of trisaccharide containing anthracyclines, but two solution structures (NMR) of the trisaccharides aclacinomycin A and B (37). Only a few crystal structures of semisynthetic anthracyclines containing disaccharides are known (43, 44). MEN 10755 is a semisynthetic disaccharide analogue of doxorubicin with a broader spectrum of antitumor activity. In the crystal structure of this analogue in complex with hexameric DNA (44), the aglycone binds in the very same mode as previously seen but the sugar moieties show two different binding conformations. Either they fit into the minor groove or they protrude into the solvent. Their flexibility suggests that they do not seem to be required for DNA binding. However, in the solvent the sugar could interact with other cellular targets such as topoisomerase II and might be important for the tertiary topoisomerase II-DNA-drug complex. Alternatively, it could block the site for particular replication enzymes such as topoisomerase I more efficiently (45).

The NMR structures of aclacinomycin A and B in complex with a sequence of DNA (37) exhibit a different binding mode from that seen in daunorubicin in two respects. Firstly, the more extensive trisaccharide in aclacinomycin is positioned further into the solvent region. Secondly, there is a kink introduced in the DNA complexed with

aclacinomycin which is not seen in the daunorubicin complex. This suggests that aclacinomycin might have a different biological mode of action than daunorubicin in



**Figure 1.4:** The first structure solved of a DNA-anthracycline complex. Daunomycin is intercalated into the d/CpGpTpApCpG), showing the intermolecular interactions. The hydrogen bonds are marked with broken lines and the water molecule as a sphere.

cells and it has indeed been seen that aclacinomycin A has an antagonistic effect on DNA cleavage by topoisomerase II stimulated by daunorubicin, thereby placing doxorubicin (and daunorubicin) and aclacinomycin into two different groups of inhibitors (30, 46). Doxorubicin thus belongs to the group of topoisomerase II poisons that stabilises the non-covalent DNA topoisomerase II complex while aclacinomycin is a member of the group of catalytic topoisomerase II inhibitors that prevent binding of topoisomerase II to the DNA (47).

In conclusion, in the anthracycline-DNA complex structures solved so far, the aglycone intercalates with the DNA in a similar manner, through van der Waals interactions and direct and solvent mediated hydrogen bonds, and in some cases also via monovalent cations. The major differences observed between the DNA-anthracycline complexes are the conformations of the sugar moieties that will vary the binding to the DNA and/or to enzymes in the replication process.

### 1.3.2 Side-effects

Despite the usefulness of anthracyclines as chemotherapeutic agents, there have been major problems associated with undesirable side effects and multi drug resistance (MDR) caused by high dosage (48) which has rendered prolonged treatment ineffective. The drugs are concentrated in leukocytes, which lead to undesired location in for example kidney, liver, spleen, heart and bone marrow resulting in the respective organ-toxic side effects, the most serious of them being cumulative cardiotoxicity (15,

49, 50). The mechanism of anthracycline-induced cardiotoxicity has been studied extensively (51) and although the exact mechanism is not yet clear, most of the toxic side-effects are thought to be caused by anthracycline-generated oxygen radicals, which will lead to altered cell permeability and damage of the cellular machinery. The heart is particularly susceptible to free radical injury, because it contains less detoxifying enzymes such as catalase and superoxide dismutase (52).

There are two major pathways by which anthracyclines could cause free radical formation, generation of reactive oxygen species (ROS) (52-55). First, there is a range of flavin-dependent, NAD(P)H-dependent reductases, cellular P450 proteins, capable of producing one-electron reduction of anthracyclines to semiquinone free radicals. These can readily donate the extra electron to molecular oxygen under aerobic conditions, generating superoxide anion radicals ( $O_2^*$ ). Secondly, anthracycline free radicals may arise via a non-enzymatic mechanism involving reactions with iron. Fe(III) readily interacts with anthracycline in a redox-reaction where the iron atom accepts an electron, generating an iron(II)-anthracycline free radical complex, which can easily reduce oxygen leading to the generation of oxygen free radicals and also the more reactive OH-radicals ( $OH^*$ ). The ease of production and stability of radicals is associated with the number of hydroxyl groups in the anthracycline molecule. The iron chelator dexrazone has been shown to reduce the formation of anthracycline-iron complexes, thus reducing cardiotoxicity (56). Another way of trying to reduce side-effects is to look more into how anthracyclines are transported into the target cells. Liposomal incorporation represents the leading method to passively target anthracyclines to tumours and has given promising results.

Major strategies to improve pharmacokinetics thus lie in targeting the drugs more specifically to the tumour site, both to prevent or minimise damage to other tissue and to limit the dosage to prevent MDR induction. Previous structure-activity studies had shown that minor modifications of the anthracycline structure can result not only in active agents, but, more importantly, analogues with reduced cardiotoxicity and activity on multi drug resistance (57).

## **1.4 BIOSYNTHESIS OF ANTHRACYCLINES**

### **1.4.1 Biosynthetic gene clusters from *Streptomyces***

About two years ago the entire genome of *S. coelicolor* A3(2) was sequenced by Prof. David Hopwood and colleagues at the John Innes Center (58). The genome is very large by bacterial standards, over 8.6 Mbp. There are about 8000 protein coding sequences, about 3500 more than for *E.coli*, and over 20 of the gene clusters (4.5 % of

the whole genome) are predicted to encode biosynthetic enzymes involved in the production of secondary metabolites. An even larger number of secondary metabolic gene clusters was found in the recently sequenced *S. avermitilis* genome (30 clusters covering 6 % of the genome) (59). These two genetic studies have also shown that the genome has a high G+C content and that the genes for the biosynthetic enzymes usually seem to be clustered in *Streptomyces* species. Some of most common types of anthracyclines that have been isolated during the last 35 years come from five different strains which are all shown in table 1.2 and these are also the strains further discussed in the thesis.

Daunorubicin and doxorubicin are the group of anthracyclines that have been shown to be the best candidates as chemotherapeutic antitumor agents and the *Streptomyces* strains producing these compounds are the most intensively studied. Most of the *dnr* biosynthetic genes from *S. peucetius*, the producer of daunorubicin, have been sequenced and characterised in the laboratory of C. Richard Hutchinson (60, 61). Arcamone *et al.* isolated a mutant of *S. peucetius*; *S. peucetius* subsp. *caesius* ATCC 27952, that produced doxorubicin (13) and some genes from this strain have been characterised (61).

William Strohl and his collaborators have worked intensively on the *dau* genes from *S. sp* C5, a daunorubicin producer and cloned, sequenced and characterised most of these genes (6, 62, 63). The primary anthracycline products in most daunomycin-producing strains are in fact baumycins, higher glycoside derivatives of daunorubicin. (6) (figure 1.8). The organisation of the *dau* and *dnr* genes involved in the biosynthesis of these anthracyclines is identical in the two different strains and the overall sequence identity of the genes in the two strains is about 93%.

Aclacinomycins are produced by various strains of *S. galilaeus* and they have shown potent antileukemia activity and low cardiotoxicity, especially aclacinomycin A which was isolated in 1975 (64). The genes from *S. galilaeus* use the prefix *akn* (65) and plenty of them have been cloned and characterised (66).

Rhodomyces, the first anthracyclines discovered (10) are produced in *S. purpurascens* (67). The *rdm* genes have remarkable similarities to their counterparts in the *dau/dnr* cluster and have been extensively used in combinatorial biosynthesis (68, 69).

Nogalamycin produced by *S. nogalater* was too toxic for use in the clinic but the genes producing this substrate, the *nog* genes, have been sequenced, characterised (70) and used in combinatorial biosynthesis (71). Nogalamycin is in many ways unusual compared to other anthracyclines: The amino sugar is attached to C1 and C2 instead of

**Table 1.2: Corresponding genes and their functions from the *dau/dnr*, *akn*, *sno* and *rdm* biosynthetic clusters**

Data for this table is mainly derived from a review by Jarmo Niemi (72).

The gene products of the underlined genes are studied in this thesis.

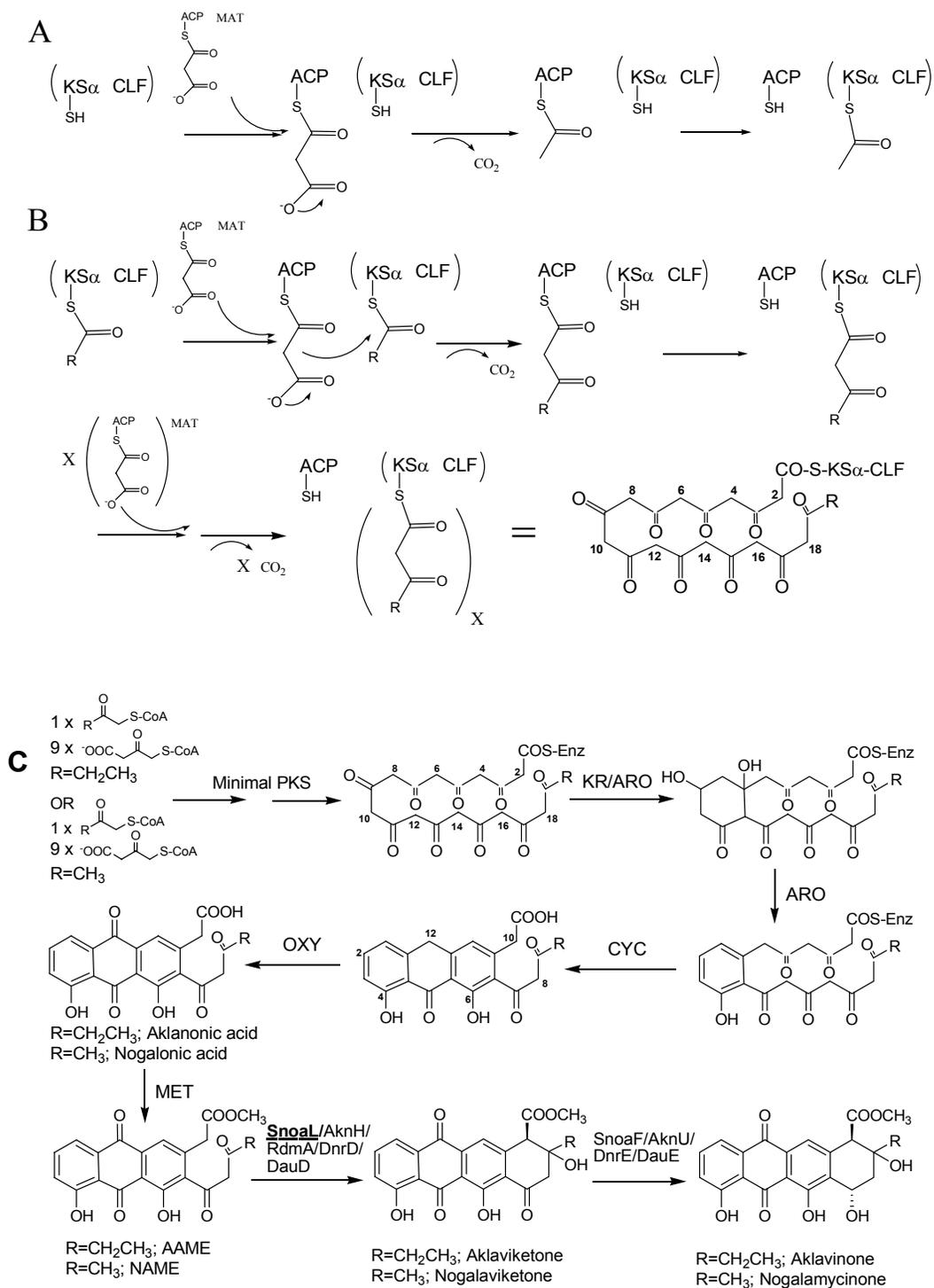
| <b>Biosynthetic function</b>        | <i>rdm</i><br><i>S. purpurascens</i> | <i>akn</i><br><i>S. galilaeus</i> | <i>sno</i><br><i>S. nogalater</i> | <i>dnr</i><br><i>S. peucetius</i><br><i>dau</i><br><i>S. sp C5</i> | <b>Enzymatic function</b> |
|-------------------------------------|--------------------------------------|-----------------------------------|-----------------------------------|--|---------------------------|
| <b>Polyketide biosynthesis</b>      |                                      | <i>aknB</i>                       | <i>snoa1</i>                      | <i>dpsA</i>  | minPKS K5a                |
|                                     |                                      | <i>aknC</i>                       | <i>snoa2</i>                      | <i>dpsB</i>  | minPKS K5β - CLF          |
|                                     |                                      | <i>aknD</i>                       | <i>snoa3</i>                      | <i>dpsG</i>  | ACP for minPKS            |
|                                     |                                      | <i>aknE2</i>                      |                                   | <i>dpsC</i>  | propionate starter unit   |
|                                     |                                      | <i>aknF</i>                       |                                   | <i>dpsD</i>  | ACP acyltransferase       |
|                                     | <i>rdmJ</i>                          | <i>aknA</i>                       | <i>snoaD</i>                      | <i>dspE</i>  | ketoreductase             |
|                                     | <i>rdmK</i>                          | <i>aknE1</i>                      | <i>snoaE</i>                      | <i>dpsF</i>  | aromatase                 |
|                                     |                                      | <i>aknW</i>                       | <i>snoaM</i>                      | <i>dpsY</i>  | cyclase                   |
|                                     |                                      | <i>aknX</i>                       | <i>snoaB</i>                      | <i>dnrG/dauG</i>   | oxygenase                 |
| <b>Post-polyketide biosynthesis</b> |                                      | <i>aknG</i>                       | <i>snoaC</i>                      | <i>dnrC/dauC</i>   | methyltransferase         |
|                                     | <i>rdmA</i>                          | <i>aknH</i>                       | <u><i>snoaL</i></u>               | <i>dnrD/dauD</i>   | cyclase                   |
|                                     | <i>rdmL</i>                          | <i>aknU</i>                       | <i>snoaF</i>                      | <i>dnrE/dauE</i>   | ketoreductase             |
| <b>Aglycone Modification</b>        | <u><i>rdmE</i></u>                   |                                   |                                   | <i>dnrF/dauF</i>   | C11-hydroxylase           |
|                                     | <u><i>rdmC</i></u>                   |                                   |                                   | <i>dnrP/dauP</i>   | C16-methylesterase        |
|                                     | <u><i>rdmB</i></u>                   |                                   |                                   | <u><i>dnrK/dauK</i></u>  | 4-O-methyltransferase     |
|                                     |                                      |                                   |                                   |  | C10-hydroxylase           |
|                                     |                                      |                                   |                                   | <i>doxA</i>  | C13-hydroxylase           |
|                                     |                                      |                                   |                                   |  | C14-hydroxylase           |
|                                     |                                      | <i>aclR</i>                       | <i>snoaL2</i>                     |  | C1-hydroxylase            |
| <b>TDP-deoxysugar biosynthesis</b>  |                                      | <i>aknY</i>                       | <i>snogJ</i>                      | <i>dnmL</i>  | TDP-glucosyltransferase   |
|                                     |                                      | <i>aknR</i>                       | <i>snogK</i>                      | <i>dnmM</i>  | 4,6-dehydratase           |
|                                     |                                      | <i>aclN</i>                       | <i>snogH</i>                      | <i>dnmT</i>  | 2,3-dehydratase           |
|                                     |                                      | <i>aknZ</i>                       | <i>snogI</i>                      | <i>dnmJ</i>  | transaminase              |
|                                     |                                      | <i>aknL</i>                       | <i>snogF</i>                      | <i>dnmU</i>  | 3,5-epimerase             |
|                                     |                                      | <i>aknM/aclM</i>                  | <i>snogG/snogC</i>                | <i>dnmV</i>  | 4-ketoreductase           |
|                                     | <i>rdmD</i>                          | <i>aknX2/aclP</i>                 | <i>snogA/snogX</i>                |  | aminomethylase            |
|                                     | <i>rdmF</i>                          | <i>aknQ</i>                       |                                   |  | 3-ketoreductase           |
|                                     | <i>rdmI</i>                          | <i>aknP</i>                       |                                   |  | 3-dehydratase             |
|                                     |                                      |                                   | <i>snogG2</i>                     |  | C-methyltransferase       |
|                                     |                                      |                                   | <i>snogY/snogL</i>                |  | O-methyltransferase       |
| <b>Glycosyl transfer</b>            | <i>rdmH</i>                          | <i>aknS</i>                       | <i>snogE/snogZ</i>                | <i>dnmS</i>  | glycosyltransferase       |
|                                     |                                      | <i>aknK</i>                       | <i>snogD</i>                      | <i>dnrH/dauH</i>   | glycosyltransferase       |

C7 and there is a unique bond C-C bond to C2. A methylated neutral sugar, nogalose, is attached to C7 and the stereochemistry at C9 is opposite to that observed in most anthracyclines (73). Additionally, there is a methyl group at the C9 position instead of an ethyl group.

#### 1.4.2 Biosynthesis of the aglycone moiety

Each aromatic, iterative class II PKS contains a set of four essential subunits, ketosynthase (KS), chain elongation factor (CLF), acyl carrier protein (ACP) and malonylCoA:ACP transacylase (MAT) (74) which are together referred to as the minimal PKS (minPKS) (6, 75-78). The minPKS enzyme complex catalyses repeated Claisen condensations between acyl thioesters to build up a carbon chain. The KS and the CLF forms a heterodimer ( $KS_{\alpha}$ -CLF, also termed  $KS_{\alpha}KS_{\beta}$ ) that catalyses condensation reactions between successive malonyl units (75). The mechanism of action of the CLF subunit of the  $KS_{\alpha}$ -CLF heterodimer is unknown, although it is thought to play an important role in chain length control (75). ACP firstly becomes malonylated by MAT. ACP then shuffles the malonyl units, attached via a flexible phosphopantetheinyl arm, to the active site of the  $KS_{\alpha}$ -CLF in the form of malonyl-ACP. Decarboxylation of malonyl-ACP is followed by transfer of the acetyl group to the active site of  $KS_{\alpha}$  and  $KS_{\alpha}$ -CLF is thereby primed by an acetate unit (figure 1.6A). This is followed by dissociation of ACP and association of a second equivalent of malonyl-ACP. The chain is extended by a certain numbers of acetyl units until a full-length poly- $\beta$ -ketoacyl chain is synthesised (figure 1.6B). The polyketide precursor for both aklavinone and nogalamycin is built up by malonate extender units. They however differ in the use of starter unit, propionate (aklavinone) or acetate (nogalamycine) (79). The aglycone moieties of *dau/dnr*, *acm* and *rdm* are composed of 21 carbon atoms (80) as opposed to the carbon skeleton of *nog* that comprises 20 carbon atoms (73).

Additional PKS subunits including ketoreductase, cyclase and aromatase are responsible for the further processing of the nascent chain to form specific polyaromatic compounds. A ketoreductase (KR) reduces the C9 keto group, the future C2, to a hydroxyl moiety, after which a bifunctional aromatase (dehydratase/cyclase) (ARO) catalyses the closure of the first ring (81) and another cyclase (CYC) (82) closes the following two rings (figure 1.6C). An oxygenase (OXY) oxidates at the C12 position, thereby producing aklanonic acid (or nogalonic acid) which is the end product of the PKS pathway and the first isolatable intermediate (6, 83, 84).

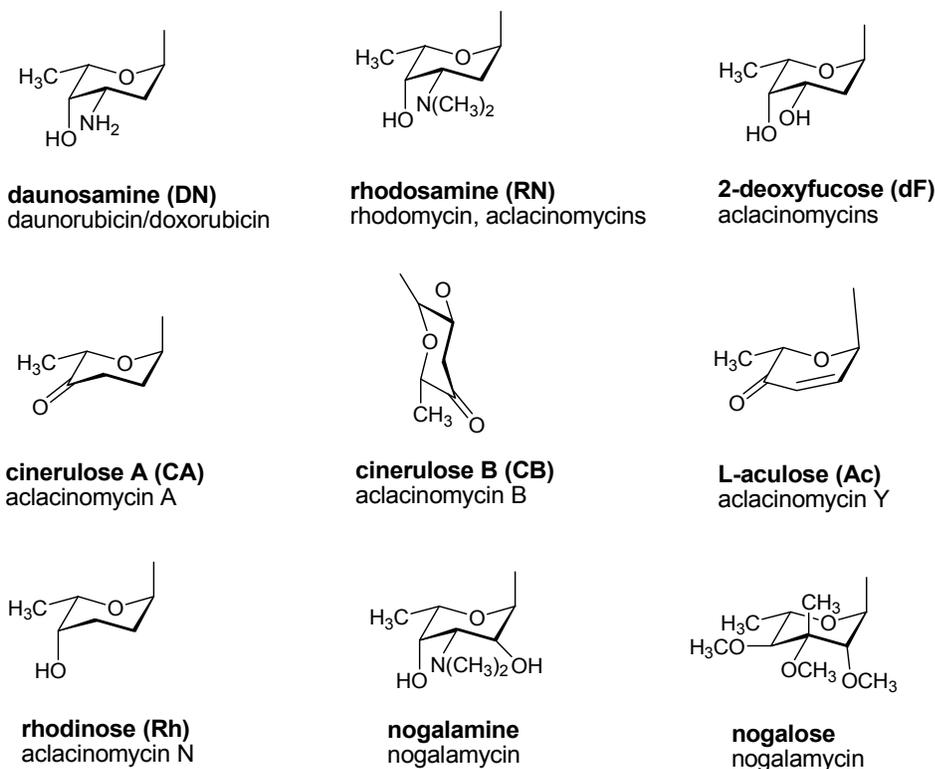


**Figure 1.6:** Biosynthesis of the polyketide aglycone in the PKS (A-B) and post-PKS (C) pathways. The enzymes are named according to the abbreviations in the text. **A** and **B**. The minPKS (CP, KS<sub>α</sub>, CLF) catalysing the priming (**A**) and elongation (**B**) of the polyketide carbon chain. **C**. The enzymes and substrates involved in the further minPKS and the post-PKS pathway.

The so-called post-PKS reactions start with a methyltransferase (MET) converting the tricyclic acid to the corresponding ester, which is a prerequisite for the closure of the fourth ring (6). The first asymmetric centres present in the anthracyclinone are produced in this reaction where SnoaL generates the (9S, 10R)-configuration (85) and AknH, DnrD and DauD the (9R, 10R)-configuration (86) (figure 1.6C). The anthracyclinone biosynthesis is completed with the action of another ketoreductase (SnoaF, AknU, DauE or DnrE) which will lead to products with 7S configurations, aklavinone or nogalamycinone.

### 1.4.3 Biosynthesis of deoxysugar moieties and glycosyl transfer

Anthracyclines themselves are biologically inactive and O-glycosylation with one to five rigid, hydrophobic sugar units at the C10 or C7 position (also C1 and C2 in nogalamycin) is necessary for their antimicrobial and antitumor activity (25). The presence of an amino group in the aglycone or an amino sugar is another prerequisite of biological activity (65). The biosynthetic pathway leading to production of the deoxysugars is poorly understood, although many genes involved have been cloned and



**Figure 1.7:** The possible deoxysugars that are attached to the anthracyclines discussed in this thesis.

characterised (87). The initial building block in the sugars is glucose as a glucose derivative, D-glucose-1-phosphate, which after several yet not very well-characterised enzymatic steps is attached to the aglycone moiety by glycosyltransferases (70, 88, 89).

In *S. peuceitius*, this glucose derivative is converted, with the involvement of six gene products named *dnm* to TDP-daunosamine, the sugar derivative used for glycosylation. The biosynthetic clusters also contain genes for putative enzymes to produce the observed glycosylations, however the actual sequence of action is in many cases still unclear. Two putative glycosyltransferases are present in the *dau/dnr* and *akn* clusters (88-90) and three in the *snoal* cluster (70, 73) see table 1.2.

Deoxysugars are divided into amino- (primary or secondary amine) and neutral sugars (no aminogroup) (figure 1.7). Aclacinomycin A carries a rhodosamine (RN), deoxyfucose (dF) and cinerulose A (CA) at the C7 position and differs in the third sugar residue from other forms of aclacinomycin as AknB and AknY (91, 92) (figure 1.7). Because of its triglycosylated moiety with different sugar residues attached, aclacinomycin is an ideal target for studies on sugar biosynthesis (92). Daunorubicin and doxorubicin both contain one daunosamine (DN) at the same position. Baumycins with more sugars attached have also been observed in *S. peuceitius* and *S. sp* C5 (6). Rhodomycins have a rhodosamine (RN) sugar attached to the aglycone (67). Nogalamycin differs in its glycosylation profile as well as in the aglycone moiety in having a nogalose sugar connected to the oxygen atoms at C1 and C2 of the aglycone by an unusual carbon-carbon bond (73) (figure 1.7).

#### 1.4.4 Tailoring enzymes

##### 1.4.4.1 Unglycosylated substrate

Aklavinone is the key intermediate in the formation of anthracycline aglycones such as rhodomycine, aclacinomycin and daunomycine. After its synthesis, the biosynthetic pathway for the various anthracyclines separate (figure 1.8). Most of the reactions performed by the tailoring enzymes require the substrate to be glycosylated (65). One of the exceptions is the 1-hydroxylation of nogalamycinone performed by *SnoaL2* which is the only modification of the anthracyclinone in nogalamycin biosynthesis (73)

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**Next page - Figure 1.8.** The biosynthetic pathway for the tailoring enzymes. The enzymes which have been studied in this thesis are in red italic in the original strains and blue underlined if applied on other substrates. The final products in the pathways are shown in light green. The different pathways shown in colored squares are from the top: Red - aclacinomycin T/A production in *S. galilaeus*, blue - nogalamycin production in *S. nogalater.*, yellow - rhodomycin B production in *S. purpurascens* and green - doxorubicin and daunorubicin production in *S. peuceitius* and daunorubicin production in *S. sp* C5.



(figure 1.8). The others are the aklavinone-11-hydroxylases RdmE, DauF, and DnrF adding a hydroxyl group to the C11 position of aklavinone (93-95). In the *akn* cluster in *S. purpurascens* only three glycosylation reactions are needed to complete the synthesis of one of the end products, aclacinomycin A (90). Hydroxylation at the C1 position in *S. purpurascens* by the *aclR* gene has however been observed (6, 64).

#### 1.4.4.2 Glycosylated substrate

RdmC, DauP and DnrP are methylsterases (63, 68, 96, 97) involved in the removal of the carboxymethyl side chain at the C10 position of the aglycone (figure 1.8). The free carboxylic acid is relatively unstable and decarboxylation occurs spontaneously in aqueous solution (98). It has so far not been established if these enzymes are involved in this decarboxylation reaction as well but it has been suggested that DnrK could enhance the decarboxylation reaction by influencing the ability of DauP to carry out the reaction (99). DnrK and DauK act as 4-O-methyltransferases (63, 97). Remarkably, a homologous protein present in *S. purpurascens*, RdmB acts as hydroxylase instead, and is responsible for the hydroxyl group added at the C10 position of 15-demethoxyaclacinomycin T and A (DmaT/A) and most likely 15-demethoxy- $\epsilon$ -rhodomycin (figure 1.8) (68, 72, 96). The two enzymes show 55 % sequence identity to each other but no 4-O-methylated products have been found in either *S. purpurascens* or in heterologous anthracycline producers, in which RdmB has been expressed (96), indicating that RdmB is incapable of acting as a methyltransferase. The three last reactions in doxorubicin biosynthesis are catalyzed by DoxA, a cytochrome P450 like monooxygenase. It oxidises the C13 position first to a hydroxyl moiety, then to a keto group and finally it oxidises the C14 position to a hydroxyl group (99, 100) (figure 1.8). This enzyme is essential in both daunorubicin and doxorubicin strains, and a puzzling problem is the fact that although both the strains contain the DoxA enzyme the *S. sp* C5 strain ends with daunorubicin while *S. peucetius* has both doxorubicin and daunorubicin as final product. It is postulated that the production of baumycins competes with the C14 hydroxylation in daunorubicin producing strains and the doxorubicin producing strains could be deficient in baumycin biosynthesis (6, 99). Neither DoxA, DnrK nor DauK are very substrate specific and two routes have been suggested for their reactions (figure 1.8). However, experimental data has suggested that the flux through the daunomycin (*i.e.* 4-methoxy) pathway is preferred to flux over the carminomycin (*i.e.* 4-hydroxy) pathway (100); however both routes are shown in figure 1.8

## 1.5 PRODUCTION OF NEW AROMATIC POLYKETIDES

Anthracyclines have an enormous therapeutic and commercial significance. Consequently, researchers have used both biological and chemical approaches to find new anthracyclines with a higher efficacy to toxicity index, or possessing a wider range of antineoplastic activities than those of either doxorubicin or daunorubicin, the currently most used anthracycline (101). Intense efforts have been made to improve the pharmacological properties of anthracycline compounds by modifying either the aglycone or the amino sugar. This approach has resulted in the preparation of literally hundreds of synthetic or semi-synthetic compounds and some of them seem to have improved anticancer activities (102).

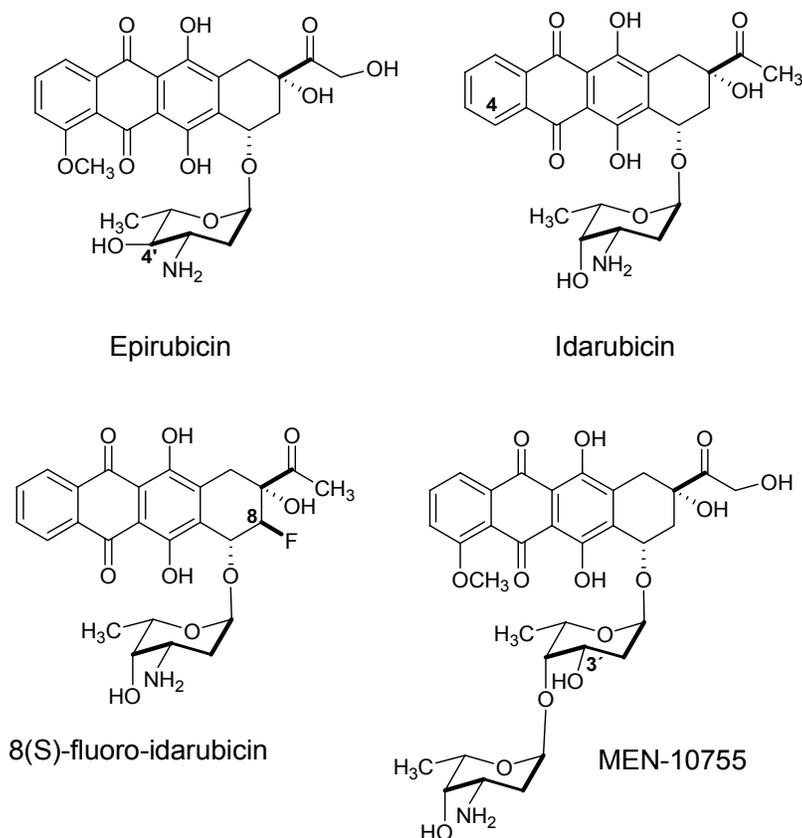
A lot of effort has been put into trying to find new approaches and strategies for the biosynthesis of novel natural products antibiotic and pharmaceutically active biomolecules. Traditionally, the approach has been to screen bacteria isolated from soil and to try to find new sources for anthracycline production (6) which has resulted in the clinically used daunorubicin (11) and aclacinomycin A (64). Another way is to find new use for already existing compounds. Because of the complexity of many naturally occurring antibiotics combinatorial chemistry, *i.e.* synthesis of novel anthracyclines *de novo*, has shown to be very difficult and not competitive to use in large scale (6, 25). Semisynthetic methods however, have lead to production of antibiotics with synthetical modifications (103) examples being menogaril (104) derived from nogalamycin and idarubicin (105) from daunorubicin (figure 1.9). Mutated anthracycline strains have also resulted in novel compounds, some in clinical use as for example doxorubicin, obtained from a mutant of daunorubicin producing *S. peucetius* (13). Recent advances in genetic manipulation of antibiotic biosynthesis in *Streptomyces* have made it possible to generate new antibiotic structures using combinatorial biosynthesis and the hybrid antibiotic approach (106). Hybrid antibiotics can be obtained by cloning heterologous antibiotic biosynthetic genes from one strain into another strain producing a similar compound. Structure based mutagenesis in a random or selective fashion is worth putting more attention into in the future era of proteomics when many enzyme structures are being solved (107).

### 1.5.1 Semisynthetic derivatives

Biosynthetic studies have led to more than 300 new compounds whereas more than 2000 analogues were derived from structural modifications of natural compounds or from total synthesis (103, 108).

Firstly, modifications in ring D and in the sugar moiety were taken in consideration in order to avoid too large changes in the general architecture of the molecule (109). Two

compounds have emerged from this work as clinically useful agents: idarubicin (4-demethoxy-daunorubicin) from daunomycin (33, 105, 110, 111) and epirubicin (4'-epidoxorubicin) from doxorubicin (112-114) (figure 1.9). Some changes in the approach were made after structural knowledge had been obtained for drug-DNA intercalation. The hydroxyl group at C9 was shown to be essential for bioactivity due to the direct interaction with DNA (115). This indicated the importance for ring A as a “scaffold” for the orientation of the substituents at C7 and C9 that are important in binding to DNA.



**Figure 1.9:** Some of the most important semisynthetic anthracycline produced.

It was thought that the introduction of the strong electron withdrawing and poor sterically demanding fluorine atom close to the C9 position might enhance the binding of the drug to the receptor site. The group of Menarini therefore started to synthesise 8- and 10-fluoro derivatives from doxorubicin (116). The 8(S)-fluoro-idarubicin was the most efficient so far tested (figure 1.9), being almost as efficient as doxorubicin in the inhibition of ovarian carcinoma. The same group also reported the synthesis of new derivatives of anthracycline having disaccharides at the C7 position. In these compounds, the amino group of daunorubicin was moved to the second sugar moiety

and the first sugar contained a hydroxyl group at that position, in the hope to gain better DNA binding properties (108) (figure 1.9). Among these analogues, the 2-deoxyfucosyl-daunosaminy-glycoside of 4-demethoxy-daunorubicin, MEN 10755, showed high cytotoxic activity on L1210 leukemia cells as well as considerably reduced cardiotoxicity and is presently entering phase 2 clinical trials (117).

### **1.5.2 Combinatorial biochemistry: hybrid antibiotics**

The concept of hybrid compounds (“hybrid antibiotics”) was introduced by David Hopwood in the mid-1980s (118, 119). Hybrid antibiotics are molecules which combine structural features of two or more antibiotics that are not normally produced by the same organism and are often produced by transferring genes from one antibiotic producer to another strain that produces a structurally related antibiotic. Accumulating information of the biosynthetic genes suggests the possibility of more precise structural control of novel anthracycline production in the near future (81). The genes required for the biosynthesis of antibiotics are close together on the chromosome, building up clusters which has made the cloning quite feasible and facilitated this emerging technology for novel anthracycline production (58).

This approach has mainly been focused on the enzymes involved in building up the carbon chain in the aglycone skeleton, the PKS, by adding, deleting or exchanging domains or entire modules (figure 1.6). Bartel and Strohl *et al.* were among the first trying this approach by subcloning components of the actinorhodin type II PKS from *S. coelicolor* into the anthracycline producer *S. galilaeus* (120). Later Hopwood and colleagues developed an expression system where the PKS type II genes could be mixed and matched to form new combinations (75, 121, 122). At about the same time Katz *et al.* as well as others generated the first genetically engineered modifications of the erythromycin PKS type I, which even today is the most extensively studied (123, 124).

This has left the identity and function of the enzymes that perform the later steps in these pathways relatively unclear. Late-acting, ‘tailoring’ enzymes catalyse a wide range of modifications to polyketide structure, such as hydroxylations, methylations or glycosylations (figure 1.8). It has been shown using combinatorial biochemistry that these enzymes can accept substrates structurally related but different to their natural substrates. The first demonstration of this technology applied to anthracyclines was done in the lab of Pekka Mänstälä. The transfer of genes from *S. purpurascens* into *S. galilaeus*, resulted in aclacinomycin-type anthracyclines possessing a C10 and C11 modified rhodomycin-type aglycone and a glycosylation profile determined by the host *S. galilaeus* (69) (figure 1.8). *S. galilaeus* is well suited for this purpose as the natural aglycone moiety of aclacinomycin is not modified, but aclacinomycin has been shown

to be an acceptable substrate for modifying enzymes from the other anthracycline gene clusters. Several of these novel compounds possessed cytotoxic activity against L1210 mouse leukemia cells. A different approach by the same group was the introduction of *S. nogalater* genes into *S. lividans* TK24 resulting in hybrid products with structural features that could be associated with either parent strain (71). Another straightforward example, by a Korean group, is from *S. peucetius*. The *dnrF* gene coding for an aklavinone-11-hydroxylase (figure 1.8) was cloned into an aclacinomycin host that started to produce hybrid 11-hydroxyl derivatives of aclacinomycin A (125, 126).

Alternative ways to find novel anthracyclines could be based on structural characterisation of the enzymes involved in the biosynthesis of anthracyclines. Knowledge about mechanism and structure could guide the redesign of the enzymes to obtain antibiotics with improved cytotoxic properties. Directed evolution could be utilized by using randomised mutagenesis coupled with screening for the desired function or product (127). Structure-assisted molecular evolution means to mimic the natural evolution process, but also to limit the sequence space to be explored by feeding in structural information in the library design (107). In contrast to this approach, rational design could be applied, where site-directed point mutagenesis, coupled to 3D structure information is used to engineer the enzymes towards desired novel activities.

## 2 THE AIM OF THE THESIS

During the last two decades, a large number of genes in antibiotic producing *Streptomyces* species have been extensively studied. Their functions have in many cases been elucidated and the future now lies in a more careful investigation of the reaction mechanisms of the enzymes of antibiotic biosynthesis. In order to resolve the structure/function relationship a comprehensive knowledge of the three-dimensional structure is needed. This can be used for generating new tools for future combinatorial biosynthesis, where information about catalytic properties and substrate specificity could lead to production of new potential antibiotics.

We have therefore set out to use crystallography as a tool to determine the structures of several enzymes involved in the biosynthesis of anthracyclines. The studies have focused on (a) the cyclases; a highly interesting class of enzymes, of unknown structure and mechanism, and (b) the tailoring enzymes; so far not well studied structurally, but of interest due to their capability of modifying the aglycone moiety.

## 3 RESULTS

### 3.1 SNOAL (PAPERS IV AND V)

SnoaL, nogalonic acid methylester cyclase, is involved in the last cyclisation step in the post-polyketide pathway where closure of ring A leads to formation of the tetracyclic ring system in *S. nogalater* (85) (figure 1.6 and 1.8). The substrate for SnoaL, NAME (nogalonic acid methylester), is the second metabolite in the nogalamycin pathway that can be isolated (84). The final product, nogalamycin contains two sugar residues: a neutral sugar, nogalose, and a dimethyl amino sugar, nogalamine. Homologous genes coding for cyclases in *Streptomyces* are DnrD (*S. peucetius*) (97), DauD (*S. sp C5*) (63), RdmA (*S. purpurascens*) (68), and AknH (*S. galilaeus*) (66) all of them showing more than 60 % amino acid sequence identity to SnoaL. The products of the cyclisation reactions do however differ in stereochemistry at the C9 atom for these enzymes, having 9R, 10R instead of 9S, 10R as for SnoaL (86). In addition to the nogalamycin group, steffimycins are the only anthracyclines known to have the 9S configuration.

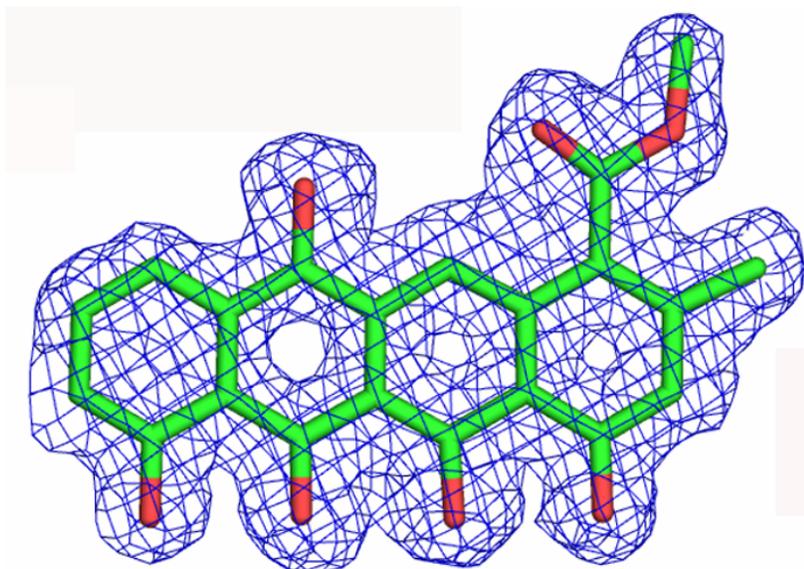
Little is known about the polyketide cyclases and elucidation of the cyclase mechanism is challenging due to the novelty of this class of enzymes involved in stereo- and regiospecific cyclisation and aromatisation.

#### 3.1.1 Single isomorphous replacement

SnoaL was co-crystallised in complex with the substrate NAME and the structure was solved by the SIRAS method (single isomorphous replacement with anomalous scattering) using one uranium derivative. Isomorphous replacement methods utilize the differences in X-ray scattering by one or more heavy atoms incorporated into the protein to obtain phase information. These differences are shown by changes in intensity of some of the reflections. By inspection of the native and difference Patterson maps, information about the position of the heavy atom sites could be obtained. In the case of SnoaL two uranium ions were found, bound to Glu6 and Asp30 (figure 2, paper IV). The structure of SnoaL in complex with the product analogue nogalaviketone was solved to a resolution of 1.35 Å (figure 3.1) and this superb atomic resolution allowed the use of automatic tracing of the whole chain (128).

#### 3.1.2 SnoaL is a member of a $\alpha+\beta$ family

The small 144 amino acid polyketide cyclase is built up by a single domain forming a  $\alpha+\beta$  barrel-like structure with a curved beta-sheet (figure 3.2). This fold has been



**Figure 3.1:** The  $2F_o-F_c$  electron density map for SnoaL to 1.35 Å resolution contoured at  $1.0\sigma$ , covering the dehydrated product analogue.

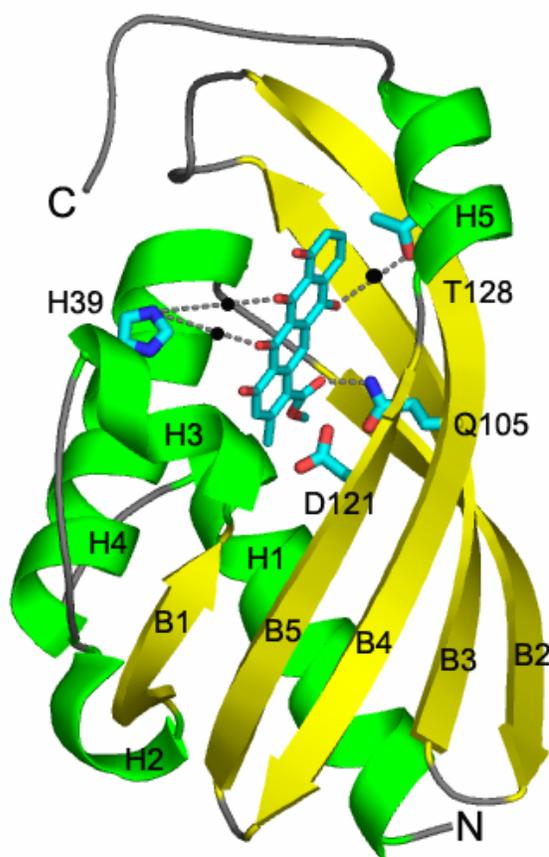
observed in other enzymes, which do however differ from SnoaL in sequence, function and oligomeric state and/or interface formation (129-131). SnoaL thus belongs to the superfamily of the  $\alpha+\beta$  barrel fold and is the first cyclase showing this fold. The quaternary structure of SnoaL comprises a rather weak interacting tetramer built up by a dimer of dimers (figure 5, paper V). In the structure the active site is almost completely covered by one helix and two loops of the monomer and in addition by residues from the other subunit indicating that product binding and/or release is most likely coupled to conformational changes in the enzyme (paper V).

### 3.1.3 Substrate binding pocket

The red-pink color of the crystals suggested that the dehydrated aromatic product analogue is bound in the SnoaL complex (figure 3, paper IV). The analogue is bound mainly by hydrophobic stacking or van der Waals interactions. In the binding pocket there is only one direct hydrogen bond formed between product analogue and the enzyme, Gln105 to the carbonyl oxygen of C14. Apart from this, a few additional hydrogen bonds are formed via water molecules to His39 and Thr128 (figure 3.2 and figure 7, paper V) and Trp122 and Arg120 from the other subunit. Most of the few hydrophilic residues found in the binding pocket are positioned around ring A of the substrate where carbon-carbon bond formation is taking place. A number of these residues are conserved in the sequences of the other *Streptomyces* cyclases, RdmA, DauD, DnrD and AknH, and could thus be important for catalysis.

### 3.1.4 Novel mechanism for aldol condensation

The condensation of two carbonyl compounds in aldol reactions offers an effective method for stereo-specific carbon-carbon bond formation. The key step in an enzymatic aldol addition is the abstraction of a C-H proton, *i.e.* the generation of an enol(ate)/carbanion. Aldolases have been divided into two classes depending on the way of stabilizing this intermediate (132). Class I aldolases contain an essential lysine residue that forms a protonated Schiff-base with the substrate carbonyl carbon to stabilize the enol(ate) intermediate (figure 3.3A). In class II aldolases the active center contains a metal ion, usually zinc that functions as a Lewis acid, *i.e.* electron sink and polarises the carbonyl bond (figure 3.3B). SnoaL catalyses an intramolecular aldol condensation without use of Schiff base formation or metal ion cofactors, and thus represents a novel family of aldolases.

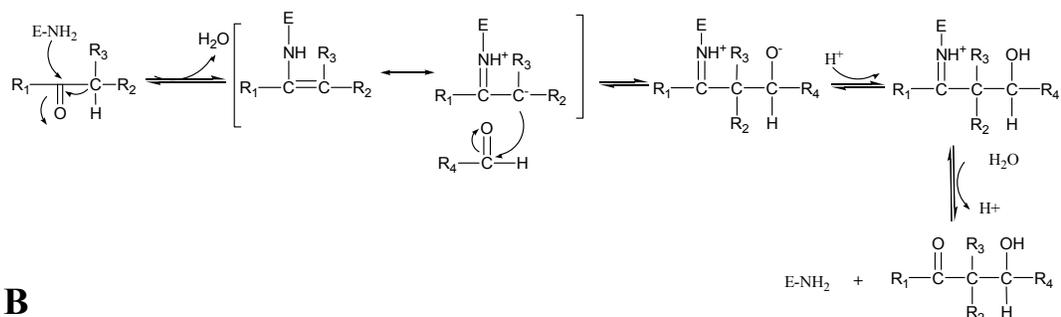


**Figure 3.2:** The SnoaL monomer with bound product analogue. The catalytic residues as well as residues involved in direct or indirect hydrogen bonding to the ligand are shown in the picture. Hydrogen bonds are shown as dotted lines and water molecules are represented as black spheres.

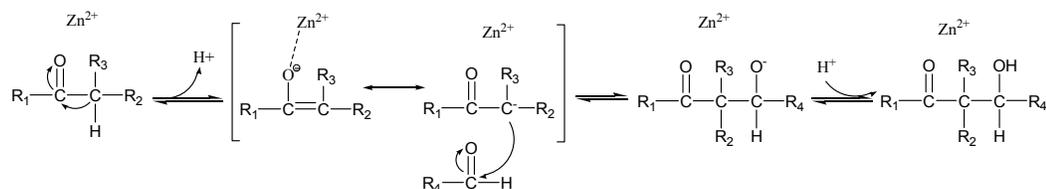
The 3D structure and mutational studies lead to a mechanistic proposal that is based on general acid/base chemistry (figure 3.3C). Firstly, Asp121 abstracts a proton from the C10 position. The negative charge formed on the enolate intermediate is stabilized by

Gln105 and by resonance stabilization via the aromatic system of the substrate. The delocalization of the electrons over the  $\pi$  system in the resulting enol(ate) also makes the abstracted proton more acidic *i.e.* facilitates deprotonation. In the next step, the intermediate performs a nucleophilic attack onto C9 and Asp121 transfers a proton to the carbonyl oxygen of C9. The dehydrated product analogue observed in the structure is formed because a dehydration reaction proceeds presumably non-enzymatically by elimination of water and formation of an additional aromatic ring (figure 3C).

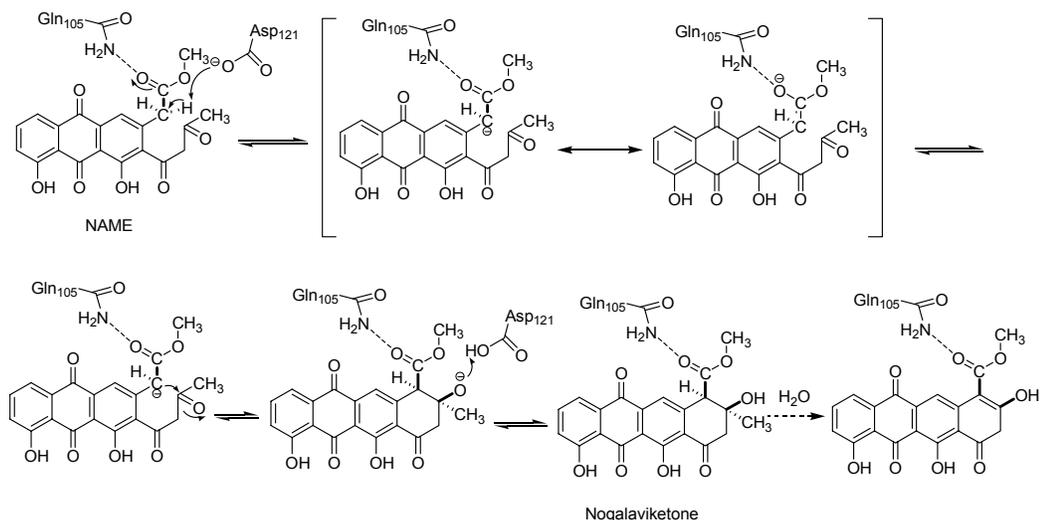
**A**



**B**



**C**

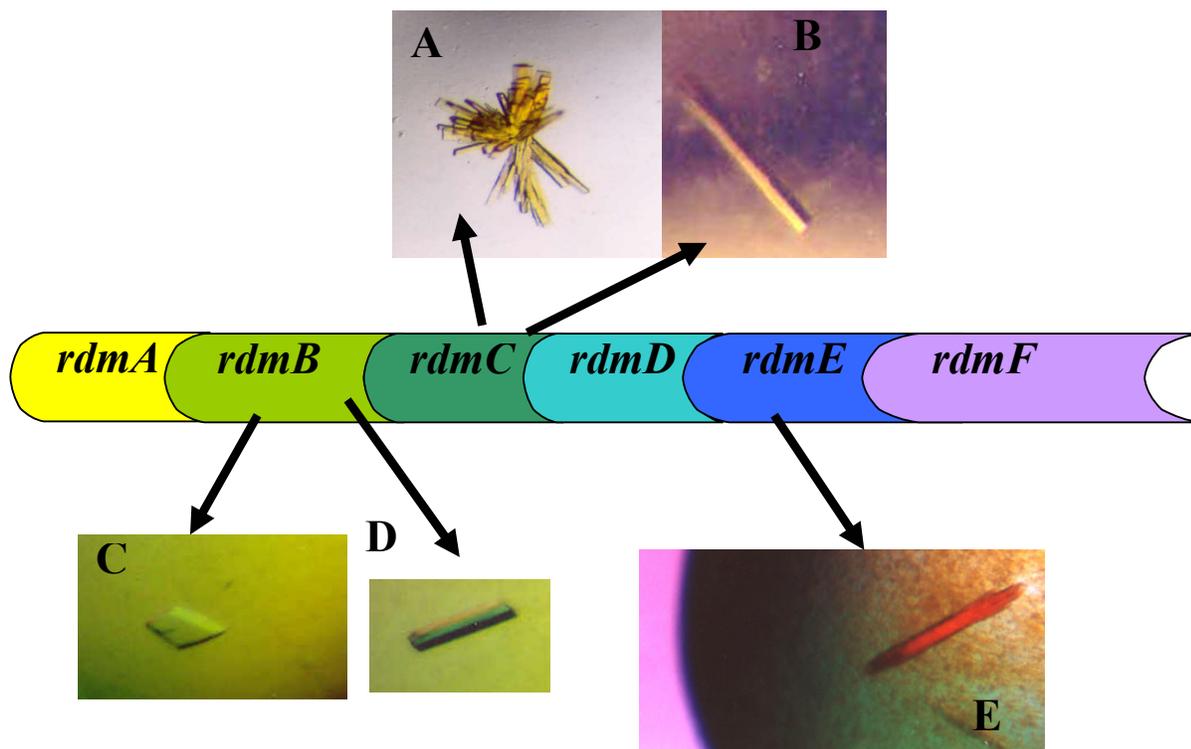


**Figure 3.3:** Aldol condensation reactions. **A.** class I aldolases. **B.** class II aldolases.

**C.** The intramolecular aldol condensation reaction mechanism for SnaaL.

### 3.2 THE *RDM* GENES

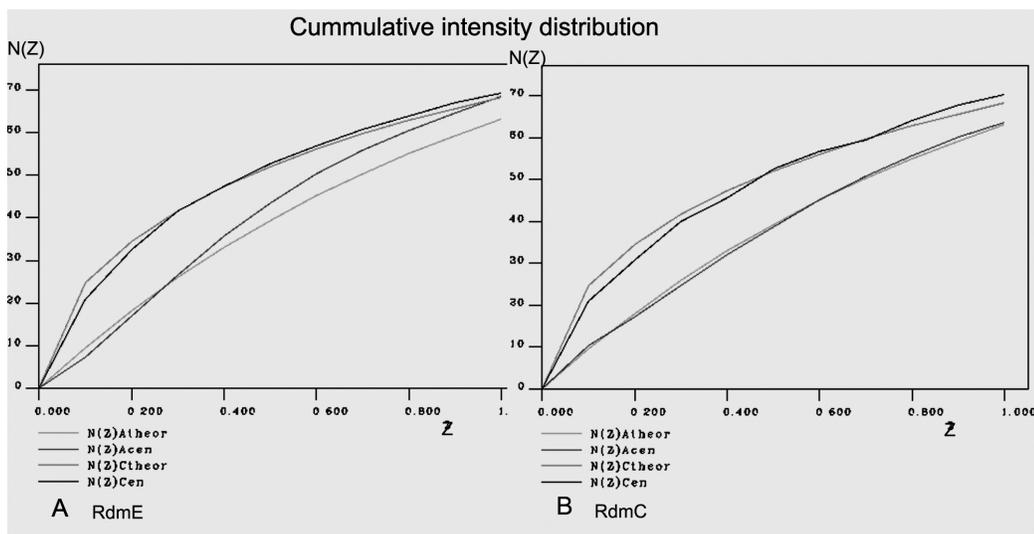
Six genes from *S. purpurascens* belonging to the rhodomycin biosynthetic cluster, *rdmABCDEF*, were cloned and sequenced (68) (figure 3.4). Separate expression of these genes as well as purification and characterization of their corresponding enzymes is essential to gain insight into their roles. Very recently also another 7250 bp gene region, *rdmGHIJKL*, was sequenced and contains five complete genes (Jarmo Niemi, personal communication) (table 1.2) The genes *rdmE*, *rdmC* and *rdmB* encode aklavinone-11-hydroxylase (RdmE), aklacinomycin methyl esterase (RdmC) and aklacinomycin-10-hydroxylase (RdmB), respectively. RdmE and RdmC were successfully expressed in *S. lividans* while RdmB could be expressed in *E. coli*. The functions of other genes of this cluster were deduced by sequence analysis. *rdmA* is a homologue to *dauD/aknH* coding for aklanonic acid methyl ester cyclase, which is catalysing the closure of the fourth ring in the aglycone (63). *rdmD* probably codes for a methyltransferase catalysing N-methylation of the sugar moiety and *rdmF* shows homology to enzymes involved in reduction of the C3 keto group in the in rhodosamine biosynthesis (table 1.2).



**Figure 3.4:** The *rdmA-F* genes surrounded by pictures of crystals obtained. **A and B:** RdmC/AknT **C.** RdmB/SAM **D:** RdmB/SAM/DbrA **E:** RdmE/FAD/aklavinone.

### 3.3 RDME

Aklavinone-11-hydroxylase is a FAD dependent monooxygenase that hydroxylates aklavinone to give  $\epsilon$ -rhodomycinone the precursor to the products daunorubicin, doxorubicin and rhodomycins, utilizing NADPH as a coenzyme (93) (figure 1.8). RdmE from *S. purpurascens* has a size of 60 kDa and consists of 535 amino acids. A homologous enzyme is DnrF (95) (94) from *S. peucetius* (figure 1.8). Phenol hydroxylase, a member of the *p*-hydroxybenzoate hydroxylase family that catalyzes the hydroxylation of phenol with the use of NAD(P)H and FAD (133), shows 23 % sequence identity to RdmE.



**Figure 3.5:** The cumulative intensity distribution plot.  $N(Z)$  is the cumulative distribution function, where  $Z$  represents the intensity relative to the mean intensity (*i.e.*  $Z=I/\langle I \rangle$ , where  $I$  is the intensity). The lower curves represent the centric reflections and the upper curves the acentric reflections, with the grey curves showing the theoretical data for an untwinned crystal and the black curves showing the experimental data. **A.** RdmE crystals: The centric reflections give a curve with the typical sigmoidal shape which is an indication of twinning, **B.** RdmC crystals: The centric reflections give a curve that looks like the theoretical, not twinned data.

RdmE was cloned, expressed in *S. lividans* TK24 and purified (93) and conditions for crystallisation of the ternary complex of the enzyme with FAD and the substrate aklavinone were established. The yellow-orange crystals appeared to belong to space group  $P6_322$  with cell dimensions 183,5 Å, 183,5 Å, 99,8 Å,  $\gamma = 120^\circ$  (figure 3.4). During the screening for heavy metal ion derivatives it was realized that the crystals were merohedrally twinned.

In the case of RdmE, the crystals exhibited a twin fraction of about 0,45 to 0,49; *i.e.* almost perfect twins. This could be suspected by inspecting some of the statistics that

are available to predict twinning, for example the cumulative intensity distribution plot generated from TRUNCATE (134) which in the case of twinning gives rise to a sigmoid shaped curve for the centric reflections (figure 3.5) (135). Twinning is not possible in space groups  $P6_x22$  but a perfect twin for the Laue groups  $P3_x21$ ,  $P3_x12$  and  $P6_x$  might appear to have this higher symmetry. It has been shown that structure determination from such twinned crystals by MIR/MAD is possible (136, 137) provided heavy metal derivatives can be identified. Alternatively, new, untwinned crystal forms might be a more straightforward route towards structure determination.

### 3.4 RDMC (PAPER I AND II)

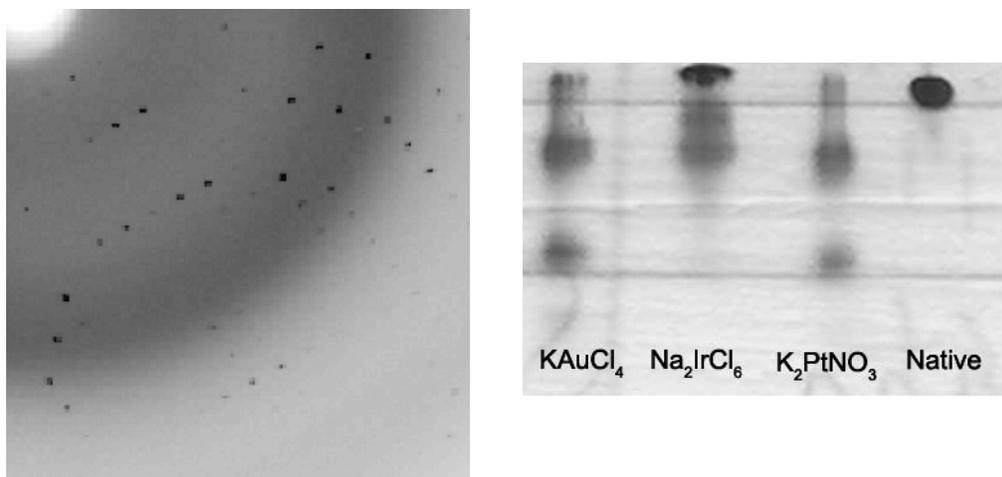
*RdmC* codes for an aclacinomycin methylesterase, RdmC, that is responsible for the removal of the methoxy group at the C15 position of  $\epsilon$ -rhodomycin T ( $\epsilon$ -T) in the natural host *S. purpurascens* (72) (figure 1.8). RdmC has also been shown to hydrolyse aclacinomycin T and A (AknT/A) *in vitro* and *in vivo* in *S. galilaeus*, resulting in the hybrid antibiotics 15-demethoxyaclacinomycin T and A (DmaT/A) and 10-decarboxymethylaclacinomycin T and A (DcmaT/A) (96) (figure 1.8 and figure 1, paper I). Aclacinomycin methylesterase shows 56% sequence identity with 10-carbomethoxy-13-deoxycarminomycin esterases from *S. peucetius* and *S. sp C5.*, DnrP and DauP, respectively (figure 1.8) (63, 97).

#### 3.4.1 Structure determination

RdmC was crystallised in complex with the substrates AknT and AknA, respectively. The crystals appeared overnight as bundles of needles with a bright yellow color indicating substrate binding (figure 3.4). Attempts were made to slow down the crystallisation process by using lower temperature, reduced protein and precipitant concentrations, the sitting drop method or oil to cover the well-solution. Eventually the method of streak seeding (138) was used which in the case of RdmC led to the formation of separated, very thin crystals (figure 3.4).

For flash-freezing of the crystals in liquid  $N_2$  at 100K, 20 % PEG400 was used as a cryo protectant (antifreezer). The RdmC crystals appeared to have rather high mosaicity and crystal annealing (139, 140) was used to improve the crystal diffraction. Practically, this was done by transferring the crystals from the cryo stream back to the cryo solution for a short time and then placing them again into the nitrogen stream. This procedure indeed reduced the mosaicity, giving well resolved spots. It also increased the resolution significantly, from 2 Å to 1.45 Å (figure 3.6).

Purified RdmC was expressed in *S. lividans* TK24, a strain commonly used for overproduction of anthracyclines since it does not produce any itself (96). Since production of selenomethionine substituted protein in *S. lividans* was unsuccessful, the isomorphous replacement method had to be used.



**Figure 3.6:** **A.** The diffraction pattern after annealing is showing well shaped and well-resolved diffraction spots. **B.** Detection of heavy atom derivatives on a native gel. The first three lanes are different heavy atoms ions mixed with RdmC and the fourth lane is the native RdmC protein. All the heavy atoms seem to bind to the protein and thereby give retardation in the mobility of the complex.

One way to readily find potential candidates for heavy atom derivatives is band shift analysis using native gel electrophoresis. In this method, the protein solution is mixed with different amounts of heavy atom salts and after various times of soaking, run on a native gel (141). A shift indicating that the enzyme mixed with heavy atom runs differently compared to the native protein means that the heavy metal most likely has bound. For RdmC, several heavy atoms induced band shifts, for example 5 mM K<sub>2</sub>PtNO<sub>3</sub> soaked for 45 minutes with the enzyme (figure 3.6). This was the single heavy atom compound used for solving the structure by SIRAS (table II, paper I). Five platinum sites were found, all of them attached to imidazol sidechains of histidine residues.

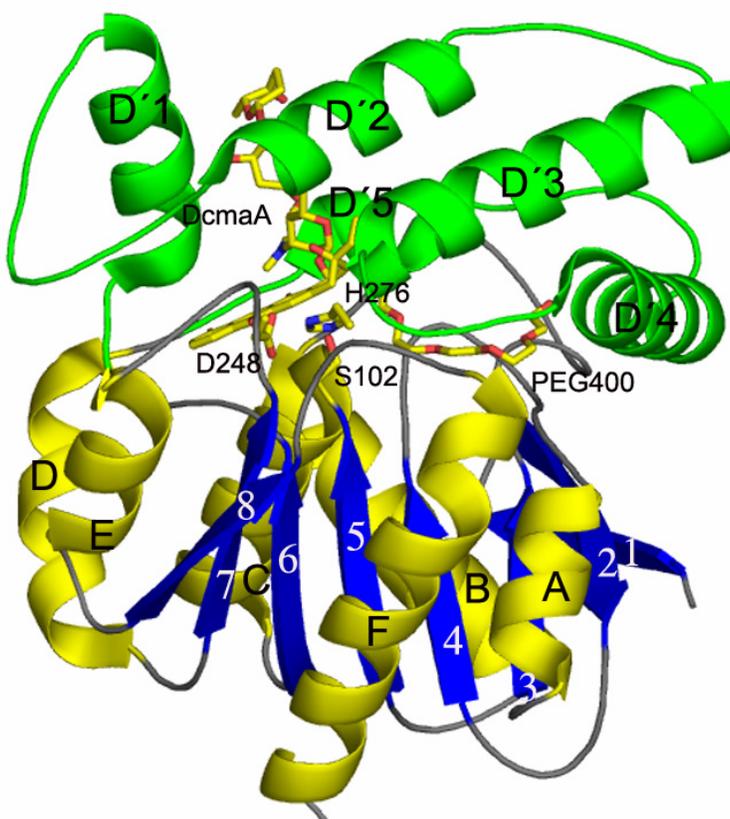
### 3.4.2 $\alpha/\beta$ fold

RdmC consists of 298 amino acids and is a monomer consistent with gel filtration analysis (96). It is built up by two domains; one larger domain comprising a canonical fold with an eight stranded  $\beta$ -sheet flanked by six helices and an all  $\alpha$ -helical (D1-D5) domain forming a lid over the active site which is located in between the domains (figure 3.7 and figure 3A and B, paper I). The large domain shows the general topology of the  $\alpha/\beta$  hydrolase fold, with the sequence motif G-X-S-X-G, typical for the serine

hydrolases (63). This domain also contains the catalytic triad (Ser-His-Asp) and exhibits a twisted form of a half-barrel where strand one and eight are perpendicular to each other (figure 3.7). The  $\alpha/\beta$  hydrolase super family contains a large number of enzymes such as lipases, esterases, hydrolases and proteases, with low or no overall sequence similarity to each other (142). The structures most similar to RdmC are that of the haloperoxidases (143, 144). The major similarity lies in the  $\alpha/\beta$  domain whereas the lid domain differs highly between the  $\alpha/\beta$  hydrolases.

### 3.4.3 Active site and hydrolase reaction

In the final refined structures of RdmC/AknT and RdmC/AknA, both solved at pH 7.5, it is the demethylated and decarboxylated products DcmaT and DcmaA that are



**Figure 3.7:** The RdmC monomer with the product analogue bound. The catalytic triad (Ser-His-Asp) and a molecule of PEG400 are shown in the picture.

observed (figure 1.8 and figure 1, paper I). It is unclear if the decarboxylation happens spontaneously as has been observed in solution (98) or if any enzyme is involved. The electron densities for the product analogues are well defined except for the last sugar of DcmaA which is probably flexible since it is sticking out from the surface of the enzyme (figure 3.7). The binding of DcmaT and DcmaA to RdmC occurs mainly via

hydrophobic amino acids. The only hydrogen bonds formed in DcmaT are between the O4 of the aglycone via water to the main chain of Ile132 and atom N3\* of the sugar to Asp135 in the enzyme (figure 4B, paper I). In DcmaA there is an additional bond formed between O12 of the second sugar and Tyr22. RdmC is more efficient on the monoglycoside AknT, with about 10-30 % activity on the triglycosides (145).

Modelling of the substrates AknT and AknA into the binding pocket provides a structural basis for a proposed reaction mechanism (figure 5, paper I). The nucleophile, Ser102, is activated by its partners of the catalytic triad, His276 and Asp248. The oxygen atom of the side chain of serine attacks the carbonyl carbon and the negative charge of the carbonyl oxygen developing in the transition state and formed in the intermediate is stabilised by the oxyanion hole consisting of the backbone residues of Gly32 and Met103. After protonation of the oxygen atom of the methoxy group and bond cleavage, the acyl-enzyme intermediate is formed. In the final step, a water molecule attacks the acyl-enzyme giving the final carboxylic acid product.

The complexes were crystallised using PEG400 as a precipitant and a PEG molecule was found in the active site cavity. It interacts mainly via hydrophobic interaction with the enzyme, but a small fraction of the molecule is covalently linked to the nucleophile, Ser102 (figure 2 and 4A, paper I). The large cavity adjacent to the substrate is also present in the structure solved from crystals obtained without PEG400. So far, no function for this pocket has been found, although it could partly serve to harbour reaction products (143, 144).

### **3.5 RDMB (PAPER II, III, UNPUBLISHED)**

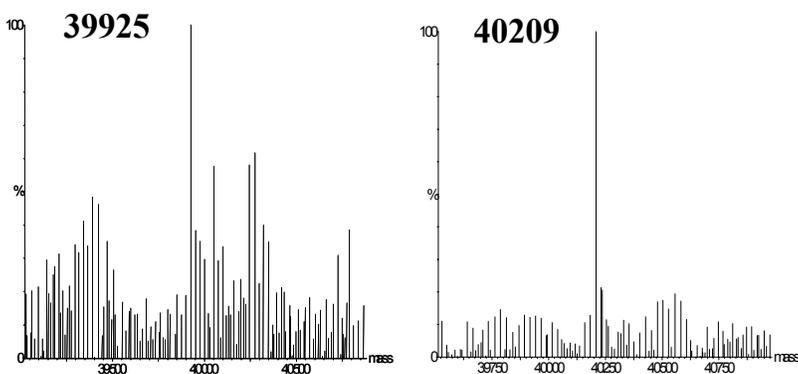
RdmB, aclacinomycin-10-hydroxylase, from *S. purpurascens*, hydroxylates the 10-position of the substrates DmaT and DmaA in *S. peucetius* to give the products DbrT and DbrA, respectively (96) (figure 1.8). The enzyme has been expressed and purified and has been shown to catalyse the same reaction *in vitro* (96). It has therefore been suggested that RdmB converts 15-demethoxy- $\epsilon$ -rhodomycin to  $\beta$ -rhodomycin in its natural host *S. purpurascens* (72) (figure 1.8). RdmB shows high sequence identity with the class of small molecule O-methyltransferases and contains the conserved binding domain for the *S*-adenosyl-*L*-methionine (SAM) cofactor (96). The amino acid sequence is 55% identical to that of the carminomycin 4-O-methyltransferases DnrK from *S. peucetius* (146) and DauK from *S. sp C5* (63) which both methylate the C4 hydroxyl group of the aglycone skeleton (figure 1.8). However, no 4-O-methylated compounds have been found either in *S. galilaeus* or in *S. purpurascens* (96).

### 3.5.1 Binary and ternary complexes

RdmB was crystallised as binary complexes with the cofactors SAM (*S*-adenosyl-*L*-methionine), SAH (*S*-adenosyl-*L*-homocysteine) (paper II, III), the cofactor analogue sinefungin and also as a ternary complex with SAM and the product DbrA (unpublished data) (figure 3.4). Crystals of the ternary complex could not be obtained using the same crystallisation conditions as for the binary complexes indicating that already at that stage some conformational change had probably occurred upon substrate binding.

### 3.5.2 Structure determination

The RdmB/SAM complex was solved using MAD (multiple wavelength anomalous dispersion). RdmB was expressed in an auxotroph *E.coli* strain, a host appropriate for production of selenium-substituted protein (paper III). Incorporation of six selenium atoms could be confirmed by mass spectroscopy analysis (figure 3.8). RdmB/SAM MAD data was collected at three wavelengths to a resolution of 2.8 Å to obtain phase information and a native data set was used to extend the phases to 2.1 Å. Due to non-isomorphism, evident from the differences in cell dimensions of more than 1 %, between all different RdmB crystals, molecular replacement had to be employed to solve the structures of the other binary and ternary complexes.



**Figure 3.8:** Mass spectrum confirming incorporation of selenium atoms into the protein. To the left, the native protein and to the right, the selenomethionine labeled protein.

Diffraction data to 2.3 Å resolution was collected of the ternary complex at 100 K using 25% glycerol to flash-freeze the crystals (table 3.1). The crystals belong to space group  $P3_221$  and contained two subunits per asymmetric unit, giving a solvent content of 57%. The diffraction pattern from crystals of the tertiary complex exhibited anisotropy *i.e.* high thermal motion or partial disorder along the *l*-axis, as indicated both by the ellipsoid shape of the diffraction data as well as by the intensity fall-off-analysis generated by SCALA (134). Numerous attempts to solve the structure of the

RdmB/SAM/DbrA complex by molecular replacement using various programs and the coordinates of the binary complex of RdmB/SAM (figure 3, paper III) as a search model failed. The reason for this might partly be due to the severe anisotropy the data exhibited. Finally, the program PHASER, by Randy Reed (147), which is based on the maximum-likelihood function and utilises anisotropic correction, was used successfully. First, a search model consisting of the N- terminal and middle domains of RdmB gave a solution for this part of one subunit in the asymmetric unit. This solution was fixed and a rotation and translation search was carried out to find the position of the C-terminal domain. Molrep (148) was then used to find the second subunit in the asymmetric unit. Details of data statistics and refinement are found in table 3.1. The model obtained showed, as expected, distinct and large domain movements compared to the structure of the RdmB/SAM complex.

**Table 3.1: Statistics of data collection and structure refinement:**

|   | <b>RdmB<br/>+SAM+DbrA</b> | <b>RdmB<br/>+Sinefungin</b> |
|---|---------------------------|-----------------------------|
| <b><u>Data collection</u></b>               |                           |                             |
| Space group                                 | P3 <sub>2</sub> 21        | C222 <sub>1</sub>           |
| Mol/asu                                     | 2                         | 1                           |
| Cellaxis a                                  | 79.6                      | 62.7                        |
| Cellaxis b                                  | 79.6                      | 87.7                        |
| Cellaxis c                                  | 232.3                     | 118.6                       |
| Resolution (Å)                              | 2.30                      | 2.70                        |
| Wavelength (Å)                              | 0.934                     | 0.980                       |
| No. of observations                         | 436563                    | 89667                       |
| No. of unique reflections                   | 38132                     | 9123                        |
| R <sub>sym</sub>                            | 6.0 (18.7)                | 10.4 (25.2)                 |
| Completeness                                | 99.4 (73.7)               | 98.6 (99.5)                 |
| I/σ   | 14.4 (5.3)                | 12.2 (4.6)                  |
| <b><u>Refinement</u></b>                    |                           |                             |
| R <sub>work</sub> (%)                       | 25.1                      | 22.7                        |
| R <sub>free</sub> (%)                       | 29.6                      | 29.5                        |
| Number of amino acids: chA/chB              | 336/329                   | 340                         |
| Number of atom                              |                           |                             |
| Protein: chA/chB                            | 2607/2569                 | 2554                        |
| Ligands: cofactor/substrate/ion             | 27/55/0                   | 27/0/4                      |
| Waters                                      | 131                       | 26                          |
| B-factor from Wilson plot (Å <sup>2</sup> ) | 45                        | 40                          |
| B-factor (Å <sup>2</sup> )                  |                           |                             |
| ChA. Protein                                | 38.2                      | 38.7                        |
| ChB. Protein                                | 43.1                      |                             |
| ChA. Ligands: cofactor/substrate            | 31.9/57.5                 | 28.5                        |
| ChB. Ligands: cofactor/substrate            | 38.0/59.9                 |                             |
| Waters                                      | 41                        | 45.3                        |
| r.m.s.d from ideal geometry                 |                           |                             |
| Bond length (Å)                             | 0.013                     | 0.007                       |
| Bond angles (deg.)                          | 1.496                     | 1.201                       |
| f.o.m                                       | 0.778                     | 0.776                       |
| Ramachandran plot (%)                       |                           |                             |
| Residues in most favored regions            | 91.9                      | 92.7                        |
| Residues in additional allowed              | 8.1                       | 6.6                         |
| Residues in generously allowed              | 0.0                       | 0.7                         |

### 3.5.3 Conformational changes

According to gel filtration analysis, RdmB is a tetramer (96). However, in the crystal only weak interactions are observed on the tetramerisation interface, indicating that this oligomerisation state might not be very stable in solution. Instead, the stable oligomer is the dimer that is formed by the two NCS related monomers (RdmB/SAM figure 4, paper III and RdmB/SAM/DbrA figure 3.11). The C-terminal domain of RdmB shows a typical  $\alpha/\beta$  Rossman-like fold (149) that binds the SAM molecule with a motif conserved among O-methyltransferases (150). The mainly  $\alpha$ -helical N-terminal domain is involved in the dimerisation interface and the middle domain is small and  $\alpha$ -helical. The extensive dimerisation surface, making up 3000 Å<sup>2</sup> of the total monomer surface, might have evolved via domain swapping involving the first 27 amino acids of helix  $\alpha$ 1 (figure 4, paper III). The structures of the binary complexes of RdmB with SAH or sinefungin are very similar to that of the RdmB/SAM complex.

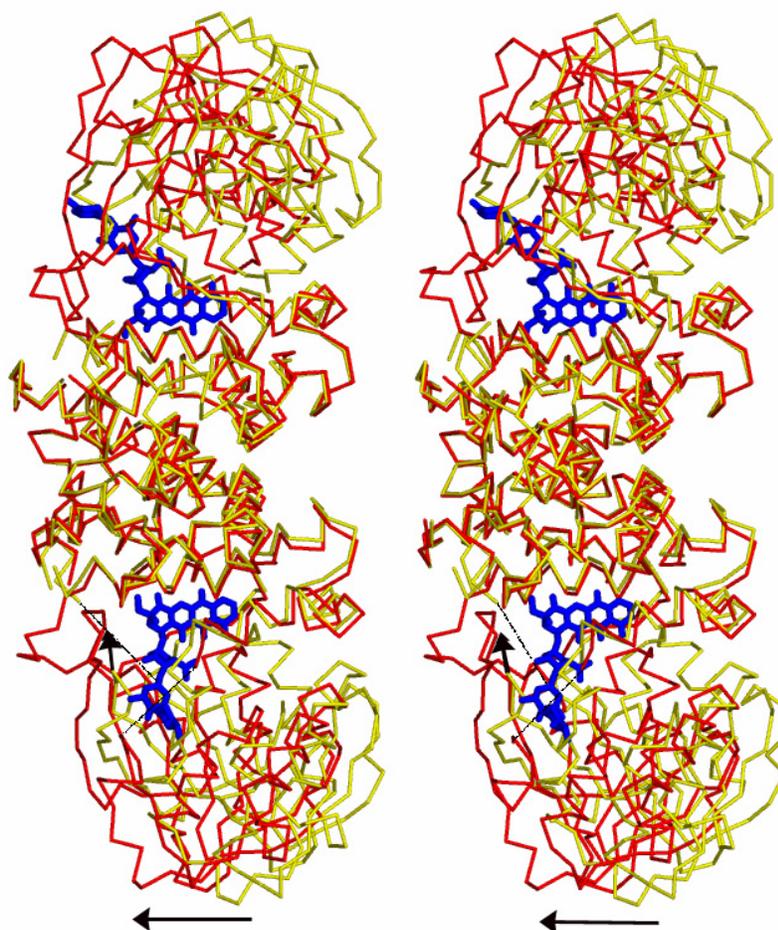
RdmB undergoes rather extensive domain movements when binding both SAM and the product DbrA and they can be described as a rotation of the C-terminal domains towards the N-terminal and the middle domains (figure 3.9). This movement causes a shift in orientation after helix  $\alpha$ 12, giving a tilt of the whole Rossman-like fold. The conformational change also leads to a substantial movement of the loop 339-345 between  $\beta$ 7 and  $\beta$ 8 to better cover the substrate binding site. In addition, the loop containing amino acids 288 to 296 that is not visible in the electron density map of the binary complex (figure 3, paper III) is well ordered in the ternary complex and folds over the substrate binding site. Also residues 165 to 172 that are present in the binary complex are too flexible and thereby not seen in the electron density of the ternary complex.

### 3.5.4 Cofactor and substrate

In the binary complexes the SAM, SAH or sinefungin cofactors and cofactor analogues bind in the same way through a number of hydrogen bonds to the C-terminal end of the  $\beta$ -strands in the Rossman-like fold (figure 5, paper III). There is only one small difference observed in cofactor binding in the binary and tertiary complexes; in RdmB/SAM the carboxyl group of SAM is hydrogen bound to Tyr171, a residue that is located on helix  $\alpha$ 14, which is not visible in the RdmB/SAM/DbrA structure (figure 3.11 and figure 5, paper III).

The substrate binding site in RdmB is located between the C-terminal and middle domains and residues from these domains are involved in binding the product (figure 3.14A). The SAM moiety is positioned underneath the O4 atom of the aglycone and the carbohydrates are protruding in the opposite direction, directly into the solvent. The

binding pocket is built up by mainly hydrophobic residues that interact with the ligand by van der Waals interactions or stack against the aromatic aglycone. The residues involved in the hydrophobic interactions are W109, M308, F159, M163, F311, F145, M312, F346, L344 and L304 around the aglycone and F348, F256 and S341 around the glycosides (figure 3.14A). DbrA is bound to the enzyme by only one hydrogen bond which is formed between O4 of the aglycone and Asn260. The last sugar moiety is flexible and therefore not visible in the electron density maps.



**Figure 3.9:** The RdmB/SAM/DbrA dimer superimposed on the RdmB/SAM dimer by aligning the N-terminal and middle domains. The RdmB/SAM/DbrA dimer is shown in black and the RdmB/SAM dimer is shown in grey. DbrA is shown in black sticks and the domain movement causing a partial closure of the substrate binding site (loop 339-345) is indicated by arrows. The missing residues 288-296 and 165-172 are shown with dotted lines in subunit A.

RdmB has been shown to accept both mono- (15-demethoxyaclacinomycin T) and triglycosides (15-demethoxyaclacinomycin A) as substrates. The structure analysis shows a local unfolding of helix  $\gamma$ 13 and partly  $\alpha$ 14 (residues 165-172) in the ternary

complex which might be due to the fact that the last of the sugars on the substrate is sticking out into the solution and thereby disrupts these secondary structure elements (figure 3.11). This rearrangement might be necessary for the substrate to be able to enter the cavity and/or to provide sufficient space for more than one sugar to fit into the cavity.

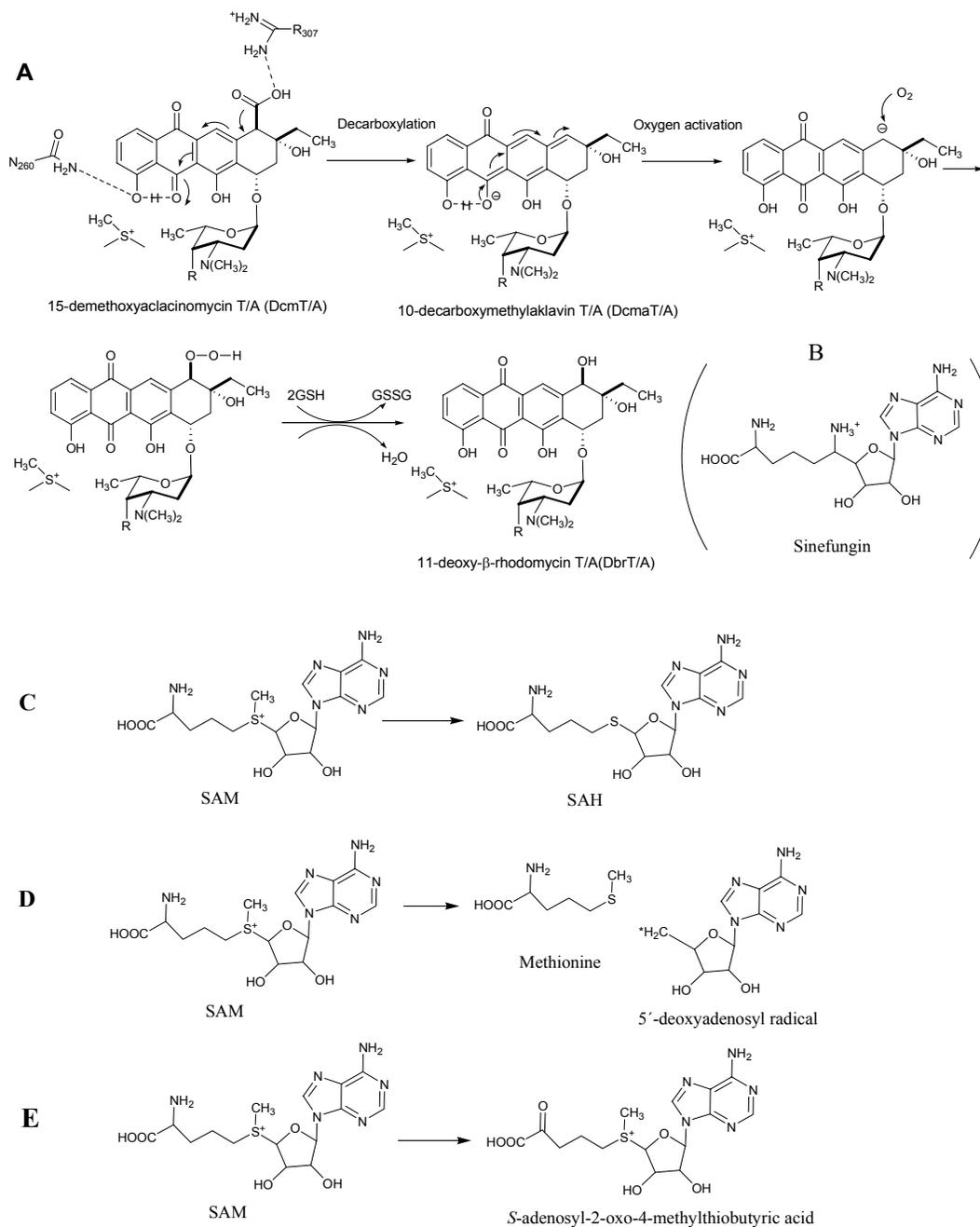
### 3.5.5 Mechanistic implications

RdmB requires oxygen, SAM, which is not consumed, and a reductant (e.g. GSH or DTT) for its hydroxylase activity. There is a ratio of 1:1 of anthracycline hydroxylated to oxidized glutathione produced (Jarmo Niemi, personal communication). The structure of the complex suggests a few key residues required for the reaction, for instance Arg307, which is a possible candidate in interacting with the carboxyl group at the C10 position of the substrate and possibly later with a reaction intermediate (figure 3.10A). Another important residue is Asn260 which is positioned close to SAM and binds to the O4 hydroxyl group. A carboxylated substrate, here DcmA, is needed for RdmB to perform the hydroxylation. The decarboxylation step has to occur on the enzyme active site because RdmB does not accept 10-decarboxy compounds as substrates. After the decarboxylation reaction, the carbanion formed has to be stabilised in some manner. The positive charge of the SAM cofactor most likely helps to distribute the negative charge that is formed into the aromatic ringsystem. This notion is supported by the finding that sinefungin (figure 3.10B), a SAM analogue containing a positive charge, also sustains catalysis, whereas the non-charged SAM analogue SAH does not (Jarmo Niemi, personal communication). The structure of RdmB in complex with sinefungin showed that sinefungin is positioned in the same manner as SAM.

The delocalisation of electrons onto the aromatic substrate can be used for the activation of oxygen (figure 3.10A). There are several hydrophobic residues lining a pocket above the C10 position of the product that could harbor the hydrophobic oxygen molecule (figure 3.14A). Data from mass spectrometry are consistent with the formation of a peroxy intermediate after reaction of the substrate with molecular oxygen (Jarmo Niemi, personal communication). This intermediate is then subsequently reduced to a hydroxyl moiety with the involvement of two GSH molecules that are oxidized to GSSG and production of water. An adjacent cysteine residue, Cys165, appears not to be involved in this step, because a cysteine to serine mutant retains catalytic activity.

The triplet state corresponds to the most energetically stable configuration of dioxygen. In the "triplet state", the two unpaired electrons have the same spin and the molecule is paramagnetic. In the ground-state organic substrates are generally in the singlets state and the insertion of oxygen would therefore be a spin-forbidden process (151).

Oxygenases and hydroxylases usually employ a metal ion (Cu or Fe) or an organic molecule *e.g.* flavin, to activate oxygen. The flavin cofactor FAD will, in the reduced form, as a carbanion, transfer an electron from the singlet flavin to the triplet oxygen to yield a semiquinone-superoxid caged radical pair. This radical pair will, after spin conversion, become a hydroperoxide intermediate on FAD and in the final step a



**Figure 3.10:** **A.** The hydroxylation reaction catalysed by RdmB. For simplicity Arg307 and Asn260 are only drawn once. **B.** The structure of the SAM analogue, sinefungin. **C.** SAM as a methyl donor. **D.** SAM as a radical. **E.** SAM as an amino donor.

hydroxyl will be added to a potential substrate giving an oxidized FAD. This process resembles the reaction between the aromatic substrate and RdmB and the activation of oxygen in RdmB could thus be explained in a similar way as by FAD. Two families of hydroxylases/oxygenases without requirement for cofactors or metal ions have recently been described; quinone-forming monooxygenases and dioxygenases (152). One such example is ActVA-orf6, a “tailoring enzyme” in the biosynthesis of actinorhodins (153). In ActVA-orf6 a set of intramolecular hydrogen bonds between keto groups and hydroxyl groups on the same side of the molecule to the enzyme together with the existing aromatic system of the substrate stabilises the carbanion. The activation of oxygen performed by RdmB could however not be placed within this family of enzymes since the presence of the SAM cofactor is essential for RdmB to perform the hydroxylation reaction.

### 3.6 DNRK (PAPER VI)

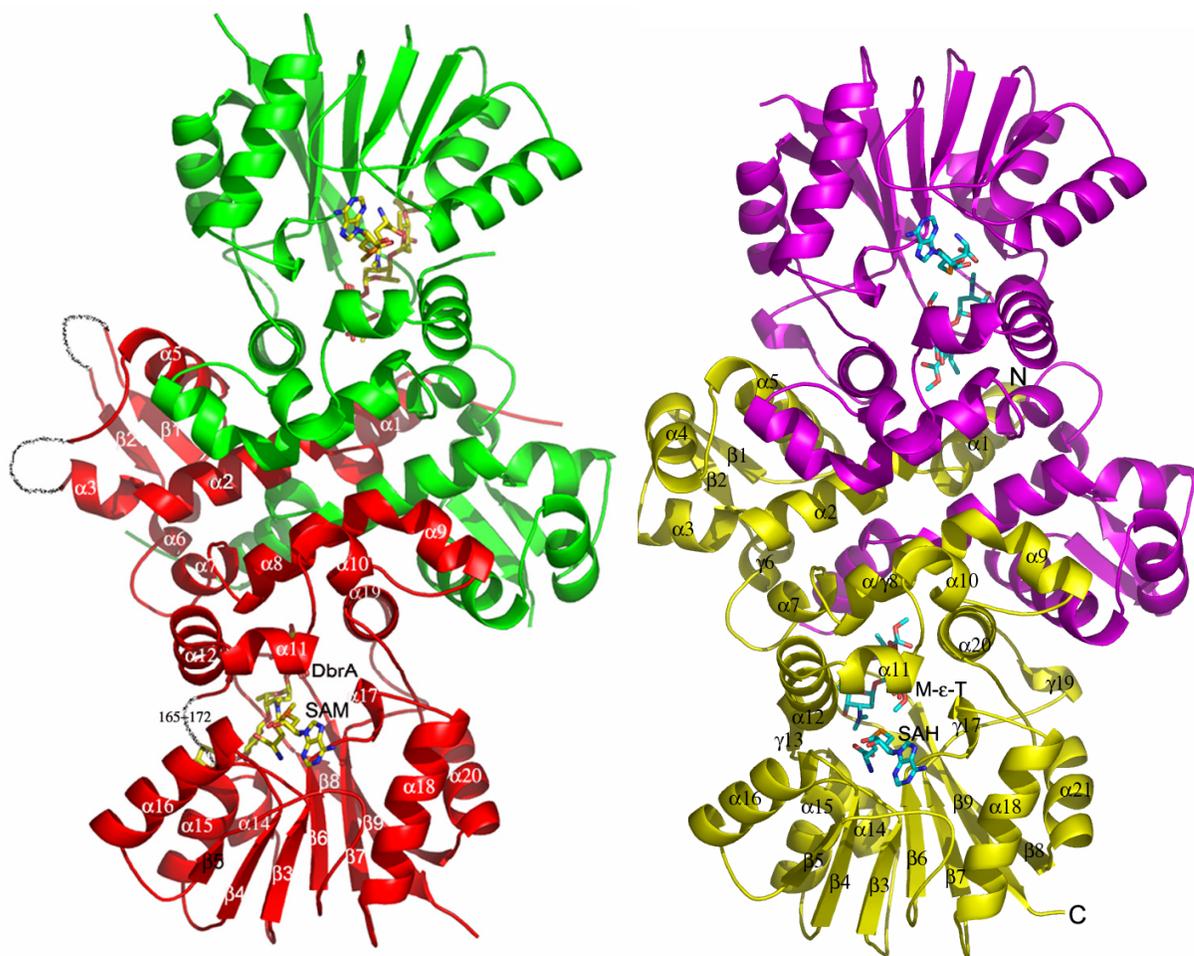
DnrK, consisting of 355 amino acids, is an anthracycline 4-O-methyltransferase that catalyses the last or second last step in daunorubicin biosynthesis, the methylation at the C-4 hydroxyl group of carminomycin, alternatively 13-deoxycarminomycin (figure 1.8) (146). It has been shown that DnrK also readily methylates the very same position in  $\epsilon$ -rhodomycin T ( $\epsilon$ -T) (figure 1.8 and fig 1, paper VI). The DnrK sequence contains the DLGGGXG motif for the binding site of SAM (146).

#### 3.6.1 Fold and substrate binding

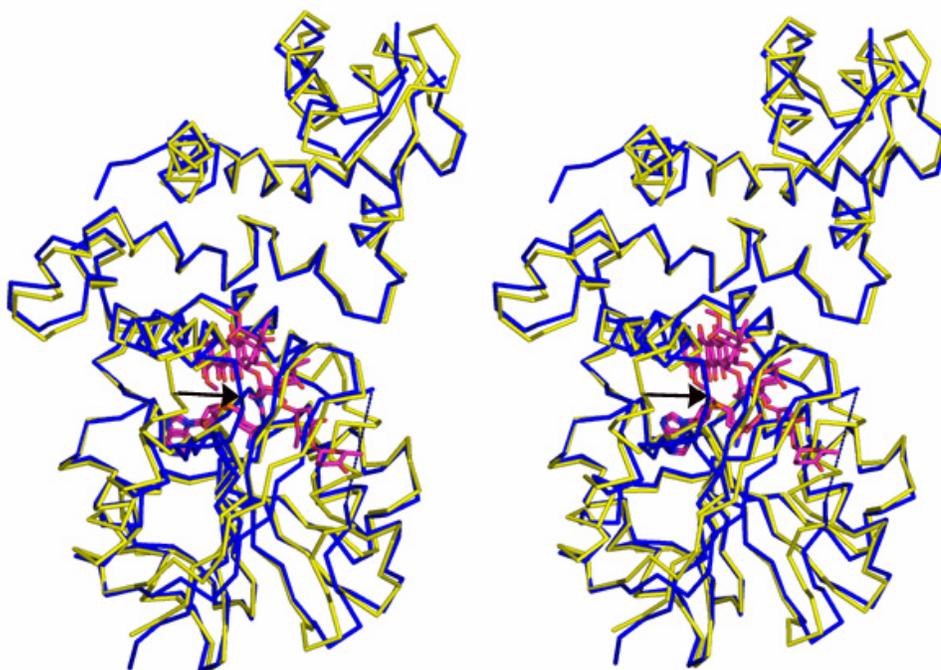
The structure of DnrK crystallized in the presence of SAM and the  $\epsilon$ -T substrate was solved in two space groups, C2 and P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>. The fold of DnrK very much resembles that of RdmB, the SAM dependent hydroxylase homologous to DnrK. The only difference in secondary structure elements between the two enzymes is the additional helix ( $\gamma$ 19) in DnrK, which is inserted between  $\beta$ 7 and  $\alpha$ 20 (figure 3.11 and figure 3, paper VI). Another noteworthy structural difference between RdmB and DnrK is loop 291-299 (287-294 in DnrK) that has moved closer to the substrate binding pocket in RdmB by 7 Å (figure 3.12).

When modeling SAM and  $\epsilon$ -T into the electron density map, positive density appears close to the 4-hydroxyl oxygen of the substrate and negative difference density is observed at the position of the methyl group of SAM (figure 2B, paper VI). This is indicating that the methyl transfer reaction has proceeded and the crystals thus contain SAH and the product 4-methoxy- $\epsilon$ -rhodomycin T (M- $\epsilon$ -T) (figure 2A, paper VI). Most of the interactions with the cofactor are conserved between DnrK and RdmB, with the exception of Arg152 that is hydrogen bonded to SAH in DnrK and replaced by an

alanine residue in RdmB (figure 3.13 and 3.14). Furthermore, the hydrogen bond of the carboxyl group of SAM/SAH to the side chain of Tyr171 in the binary complex RdmB/SAM is not conserved, because this tyrosine is substituted by a phenylalanine residue in DnrK. The binding of the product M- $\epsilon$ -T to the enzyme is dominated by hydrophobic interactions and only a few hydrogen bonds are formed (figure 3.14A). The oxygen atom O4 of M- $\epsilon$ -T forms a hydrogen bond to the side chain of Asn256, atom O10 interacts with the side chain of Arg302, and the side chain of Asn102 forms a hydrogen bond via a water molecule with atom O9 of the ligand. The sugar moiety forms two hydrogen bonds from the O4\* atom via a water molecule to the side chain of Asp162 and from N3\* to the main chain of Leu159 of the enzyme.



**Figure 3.11:** The dimers of RdmB (to the left) and DnrK (to the right). The secondary structure elements, the cofactors and products are marked. The missing residues in RdmB are marked by dotted lines.



**Figure 3.12:** Stereopicture of RdmB/SAM/DbrA in black superimposed on DnrK/SAH/M- $\epsilon$ -T in grey. The 7 Å movement of loop containing residues 291-299 in RdmB (corresponding to residues 287-294 in DnrK) is indicated with an arrow.

### 3.6.2 Methyltransferase reaction

Enzymes of the family of methyltransferases (MTase) catalyse the attack of a variety of nitrogen, oxygen, carbon and sulfur nucleophiles on the methyl group of SAM (figure 3.10C). DNA, RNA, proteins, polysaccharides, lipids and a range of small molecules are the major targets for methyltransferases. The degree of amino acids conservation is very low for these enzymes although they all contain the SAM-binding Rossmann-type fold. The structures most similar to DnrK are all members of the small-molecule MTases which include isoflavone O-methyltransferase (154), chalcone O-methyltransferase (154), yeco methyltransferase (155) and phenylethanolamine N-methyltransferase (156) as well as the odd member RdmB.

Based on the structure of DnrK/SAH/M- $\epsilon$ -T an  $S_N2$  mechanism is suggested which is consistent with previous proposals for this class of methyltransferases (157). The position and the orientation of the methyl group, the hydroxyl oxygen atom of  $\epsilon$ -T and the sulphur atom of SAM is in a almost linear arrangement as required for the transition state for a  $S_N2$  reaction (figure 3.15). The conserved residue Asn256 might have a key role by forming a hydrogen bond to the O4 oxygen atom of  $\epsilon$ -T, thus maintaining the proper orientation of the substrate. Deprotonation of the 4- hydroxyl group of  $\epsilon$ -T by a base would enhance the nucleophilicity of the hydroxyl group and acid/base catalysis has been suggested in the mechanisms of other methyltransferases. In DnrK there is

only one residue in the proximity of the 4-hydroxyl group that could act as a catalytic base, Tyr142 (figure 3.14B). However, replacement of this residue by tryptophan results in a mutant species with relatively high residual activity, which suggests that acid/base catalysis is not a major contributor to rate enhancement by DnrK. This contrasts the mechanism of DnrK to that of the related O-methyltransferases ChOMT and IOMT which use an active site histidine residue as a catalytic base in the reaction (154). DnrK thus appears to act as an entropic enzyme that utilizes proximity and orientation effects (158) as major means of rate enhancement, in a manner suggested for N-methyltransferases mRNA Cap guanine N7-MT (159) and glycine N-MT (160).

### **3.7 DNRK AND RDMB – AN EXAMPLE OF DIVERGENT EVOLUTION**

#### **3.7.1 Homologues in *Streptomyces***

There are over twenty amino acid sequences found in *Streptomyces* of known or hypothetical methyltransferases that show high (29-94%) sequence similarities to DnrK and RdmB. A blast search using either one of these two enzymes gives the same results among the top sixteen sequences (figure 3.13). Among these sequences are that of DauK from *S. sp* C5 (99) an O-methyltransferase from *S. globisporus* an O-methyltransferase from *Streptomyces carzinostaticus subsp. neocarzinostaticus* and a putative O-methyltransferase from *Streptomyces avermitilis MA-4680*. Among the enzymes that have been verified biochemically or genetically as methyltransferases, very few residues are conserved in the active site pocket (figure 3.13). In particular there are no prominent features in the sequences distinguishing the RdmB hydroxylase from all the methyltransferases (figure 3.13 and 3.14).

#### **3.7.2 Substrate specificity**

DnrK is quite tolerant to differences in the aglycone moiety and accepts a wide variety of substrates such as rhodomyin D, 10-carboxy-13-deoxycarminomycin, 13-deoxycarminomycin, 13-dihydrocarminomycin, carminomycin,  $\epsilon$ -T and AknT (99) (Jarmo Niemi, personal communication). Except for the natural substrates, carminomycin and 13-deoxycarminomycin, that both lack a C10 substituent, DnrK can accept substrates with larger substituents at this position which is possible as the size of the substrate pocket in the vicinity of the C10 position can accommodate larger substituents (figure 3.14B and fig 7, paper VI).

*S. peucetius* (DnrK) TAEPVVAARFQQIDALRRLIRLGLSHTPMVVRTAATLRVLDHLAGARTVKALAARTDTPEALLRLIRHLVAIGLLEEDAPG--EFVPTVEGELLADDH 99  
*S. sp. C5* (DauK) TAEPVVAARFQQIDALRRLIRLGLSHTPMVVRTAATLRVLDHLAGARTVKALAARTDTPEALLRLIRHLVAIGLLEEDAPG--EFAPTEVGLLADDH 99  
*S. purpurascens* (RdmB) SSSPGELEPTDQDLVLLKLNGLNVTMPALRVAATLRVLDHLAGADTLAGLADRTDHPQALSRLVRLTVVGLVGGKQGRPLRPTLRLGMLLADGH 102  
*S. globisporus* -----MLGDEDKAAELWSMANLGTMPAVRVAATLRADHITAGHTAGEIAEAAVHESLDRLLRLLYLVVRLVGLLDRDGLG--RYTLTLMGRPLCEDH 91  
*S. carzinostaticus* -----MG-KRAAHILGLRALADLTPMAVRVAATLRVLDHIAAGHTAEIASAAGAHADSLDRLLRHLVAVGLFTRDGGC--VYGLTEFGQLRDDH 89  
*S. avermitilis* TVESSEVMAKETTPRGGVMAAADLLTPMAVRVAATLRADHIAAGHTAEIALAEVAGADRALGRLLDHLVTAGVLSGTGFG--ADYLTAMGRHLCEGA 116  
*S. coelicolor* A3(2) -----MTTAQTAPPPMRLRELVFGAACAAALRAAARLGVADALGTPMVEDLAAAVKTEPKLRLRLALTCYGVFTEQ-RNG-TFAHTDMSRLREDD 94  
*S. avermitilis* -----MQLRELVFGAACAAAVRAAAKLGVDALGKPTAAEELAAVLGTEPRPRLRLRLALSCYGFPAQN-DDG-RFVHTSMSRLREDD 83  
*S. lavenderulae* (McmR) QTPENPQTAARAAAEETVNDILQGAWKARAIHVAELGVPELLEQGPRTATLAEATGAHEQTLRRLRLLATVGVFDDLGHDD-LFAQNALSAVLLPDP 103  
*S. alboniger* EHADPAEHAQFQAQERILLTWGYSSEILDLATRLDPLMGTGERAAAEALASLDDTPVATLRLRALAPALGLAEET-GAG-RPRLTPAGHRLRTDV 124  
*S. griseus* -----MATQALGTALRLGVFDRIGTGELRAEALAGSLGTHPRATLRLRALAGLQLLSEPEP-EPG-AFRTTAAGNVLRRADA 73  
*S. glaucescens* (TcmN) GQRTPASVLPTELHAQQLLLAASGRRLARIVHVLTELRIADLLADGPRHVAELAKETDHELSELVYVLSAASVGVFAEG-PVR-TFSATPLSDGLRTGN 248  
*S. avermitilis* -----PPFALLEMIMGVMSRAVHVVAELVAEALAEGLSAEELAGRVGADADALGRVLRLLASNGVFATR-ADG-TPELTPMADALRADH 114  
*S. griseus* TVTTPQIADPASAARTLLRLNTAYFQSKVLSAVELGLFALLAEPEATAETVFGRLGVRARPGADFLDALVGLGLLERK--DG-HYSNKPEAAAFVLPD 99  
*S. argillaceus* -----NTPAAAPSPA-DAREILQINVAYTRARVLTAMELGLFEYLSAGPTEAIEIHGRHLHSHLVRDWDALVGLGLLTD--GG-TYANTPRAEHFLVPG 96  
*S. collinus* (RubO) -----MAIRVAATLRGLSDHMAAGAVTETLAREADVDAIAGRLGLHVLNAGIYRRLPDG--AYEPTLDGALLRDDL 70

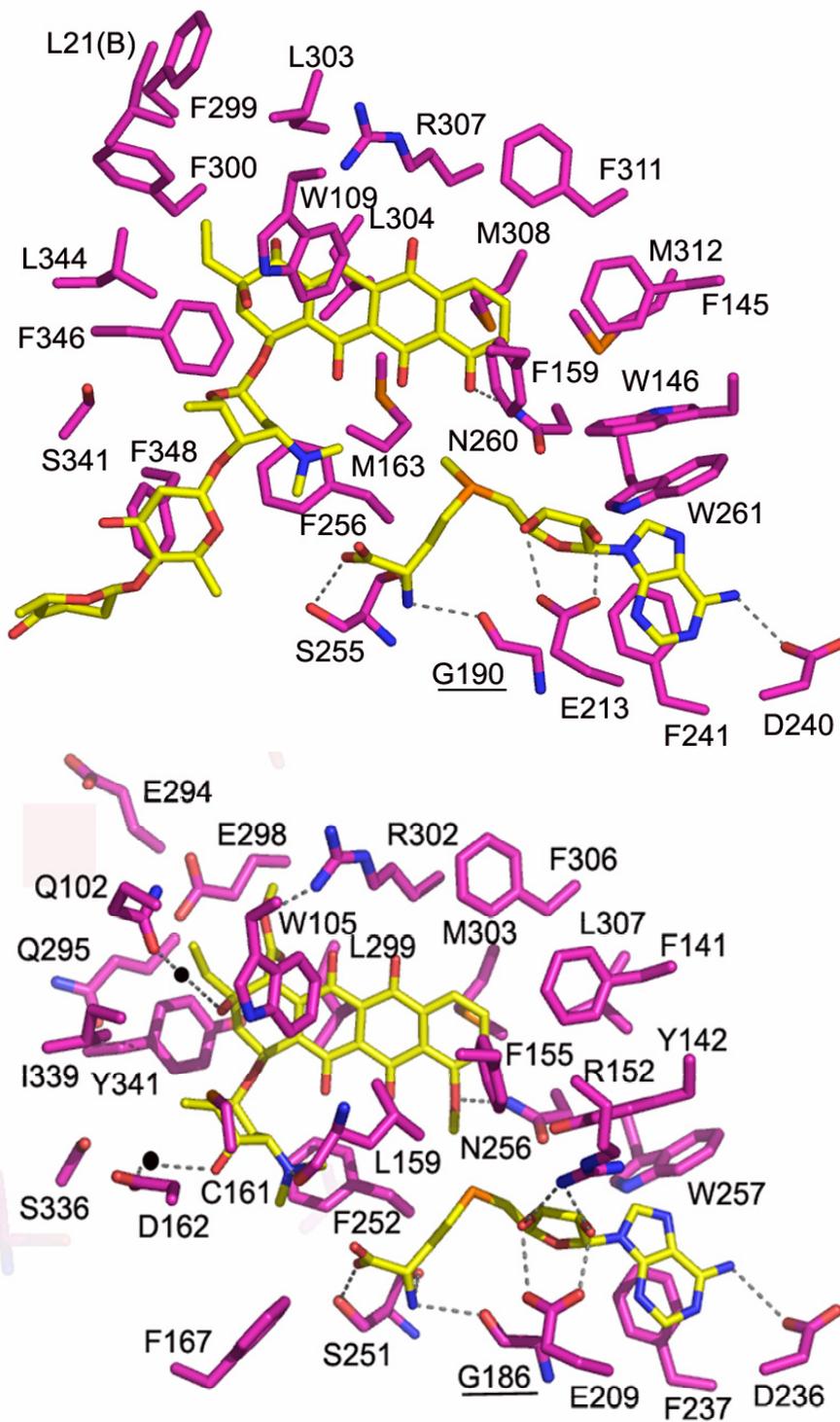
*S. peucetius* (DnrK) PAACRAMHDLTQAVARADISFTRLPAIRTGRPTYESIYKGP-----VEDLAGRPDLRASFDSLAACQDQV--AFADAPAAAYDWTNVRHVLVDCGG 189  
*S. sp. C5* (DauK) PAACRAMHDLTQAVARADISFTRLPEAIRSGRPTYESIYKGP-----VEDLAGRPDLRASFDSLAACQDQV--AFADAPAAAHWTNVRHVLVDCGG 189  
*S. purpurascens* (RdmB) PAQCRAHMDLNGAVSHADLAFTGLLDVVTRCPRPAYAGRYCRPE-----VEDLSADVALADSDALMSQDEDL--AYEAPADAYDWSAVRHLVDCGG 192  
*S. globisporus* PAQVRAHMDMEG-AGRGELSFVLLHSVRTGKAAPFLRYGRPE-----VEDLAEDPRRAESFNRLTQDQVAT--RPAVAVAGFDWASTGHVLDLGG 180  
*S. carzinostaticus* AAGKRAHMDMSAVGRGDLGFLVLAHSIRTGQPAYPVRYGTS-----VEDLGSDPVLSAEDTLMSHAELE--DYGTIAAAYDWAALGHVLDVDCGG 179  
*S. avermitilis* PEDMRALHDIEGALGHAEELVHLLHTVTRTEGAAPFQQYGVTE-----WDLSSDDGRAESFDLTMGARLTA--HSPAVAGYPWGTLRHVLDVDCGG 206  
*S. coelicolor* A3(2) PHSRNITLWCTEPTWTDA-WPRLDEAVRTGSAVNFVGLYKGP-----FTYLN-EDAPQSAEVENRAMTSSRQ--SAQDVAALLDLSASTSVADLGG 184  
*S. avermitilis* PHLRYSLWCTEPTWTDA-WPRLDEAVRSGSVFHELYKGP-----FDYLN-EDADHSAOVENRAMTSSRQ--SARDVAELLDLTVGVSTVADLGG 173  
*S. lavenderulae* (McmR) ASVYATDARFQAAPWHRA-WEQLTHSVRTGEASFPTWPTAE-----RSGSSPTDRPKARLENRAMGSVLT--BAGQVAAAYDFSGAATAVDLGG 194  
*S. alboniger* PDSLHAFVRRQMG-VFRQA-WSHFDHSIRTEGPAFPQVFTGTE-----FSLYS--ERPELSTSTSSMRBATMTSTALAKEEYDFSSYGTVDLGG 214  
*S. griseus* PGMVVAARMFTDPVLRG-WDLLEDSVRTGETTFTDVFDTG-----FGHLR--EHPLESAANEAMSQTRLTACTV--HHYDFGRFQRLVLDLGG 162  
*S. glaucescens* (TcmN) PDGVLPVWYNNMELTRP-YDEIMHSVRTGEPAFRRVCGSS-----FBELE--ANPEACQEFERFMAHNSR--RLVLDGLAQDQMERFSTIADLGG 337  
*S. ambofaciens* PMSMRPALLMGHPHWD-WSGFPEVTVTGEPALPKLRGMHA-----FBELE--KNAEYGVFEGGMSMSAS--EFGVIAAAYDFTRFVTVDLGG 203  
*S. griseus* APGLVGSIAQHARRHYRS-WASLTDLLRNEPADSPATAGDPKAVQLD-----HKDFENLNVRRMGMHDAFNTF--VHHLGRVCWDSRHRHTVLDVGA 196  
*S. argillaceus* HPHYIGAVLQHGVRHYEL-WNRFADALRDGKATSKGKITAG-----AVVEEQPLDRARFEDHMDTFNQF--VVLELTKALDWSRMTKTFVDVDCGG 185  
*S. collinus* (RubO) PQSGRDLRLDGPTRGEAAVFRLLDAVRSGEVPSYALYQGE-----WTVDESQDGLADGARRMAWRN--VVEPELLAQHWQSVRHLVDCGG 160

*S. peucetius* (DnrK) KGGFAAAIARRAPHVSATVLEPMAGTVDTARSYLKDEG-----LSDRVDDVVEGDFPEP-LPRKA-----246  
*S. sp. C5* (DauK) KGGFAAAIARRAPHVSATVLEPMAGTVDTARSYLKDEG-----LSDRVDDVVEGDFPEP-LPRKA-----246  
*S. purpurascens* (RdmB) NGGMLAAIARRAPHRLGTLVLELAGPAERARRFFADAG-----LADRVTVAEQDFPK-LPVTA-----249  
*S. globisporus* DGSLLTALLTACPSLRGTLVLEPEAVORAKESFAVSG-----LDRANAVAGSFFDA-LPAGA-----237  
*S. carzinostaticus* SGGLSALLTAHEDLSTVLDLQGPASAAHRFLDTG-----LSGRAQVVVGFDF-LPAGA-----236  
*S. avermitilis* DGTMLIAILQSHPLRGTVLDLQGPVRAEKAIAAAG-----LDHRADIAGSFFDA-LPAGA-----263  
*S. coelicolor* A3(2) QGHVVASLLEKYPAMRGTLLDLPFRVENADPRLEGGG-----LADRVRIVPDCREE-IPVRA-----242  
*S. avermitilis* QGHVVASLLEKHPDIRGTLVLEPRVVEKADPRLEGGG-----LAARARIVGDCREE-IPVEA-----231  
*S. lavenderulae* (McmR) RGSMLAAVLDAPFGLRGTLLRPPVAEEARELLTGRG-----LADRCEILPQDFET-IPDGA-----251  
*S. alboniger* DGSLLAAVLSAHPGVGVEVPEPEGARDAAATLDAAG-----VGERGRVETGDFET-VPGGG-----271  
*S. griseus* DGTLLASILRAHQEPRGLVLETAELQAAPRRLARG-----LDRVLTLETGDFET-APAGG-----219  
*S. glaucescens* (TcmN) DGNVLAQILRRHPHATGLLMLPFRVAASAGPVLEAAG-----VADRVTVLPQDFET-IPDGY-----395  
*S. ambofaciens* QGGLLAGILASSPATRGVLYPRVEENGAEEFLAAG-----VADRTERVAGLDFEV-PPGGA-----260  
*S. griseus* RGNVAARLVRTHCHLGGVPELPAVRPLFDEHMAAG-----TASQVGTACQDFDLPES-----253  
*S. argillaceus* RGNVAQQLVLDHDPKGSVPEVFGVEPLFDEHMAAG-----TTGRVDFVGGDFDLPALPA-----242  
*S. collinus* (RubO) SGDLAAVVDATPGARGTLLEAPAAAAERMPTEERTALVRAAVGVPDLPLKIESADMPAMDISARVADTFATRRVVFVAGAAAHMPTPGAFGASTGI 260

*S. peucetius* (DnrK) -DAIILSFVLLNMPDHDVAVRLLTRCAEAL-PPGRIILHERDDLHE-----NSFN-EQTELDRLMLVF-----LGGALRTREKWDGLAASAGL 327  
*S. sp. C5* (DauK) -DAIILSFVLLNMPDHDVAVRLLTRCAEAL-PPGRIILHERDDLHE-----NSFN-EQTELDRLMLVF-----LGGALRTREKWDGLAASAGL 327  
*S. purpurascens* (RdmB) -DVVLLSFVLLNMSDEDALTLRRCVRALE-PPGRIILVLDRAVDEG-----DGADRFSTLLDRLMLVF-----MGRVTRREVVLDAGSAGL 331  
*S. globisporus* -GAYVLSAVLHPDWDDEASVALRRCAEAAQ-QTGSVVFVLESTGSA-----GDAPHTGMDRLMLVF-----YGAKEERREEFELAGRAGL 315  
*S. carzinostaticus* -GGYVLSAVLHPDWDLSAVALRRCAEAAQ-SGGVVLVIEAVAGD-----BHAGTGMDRLMLVF-----FGKERSLAELGELAAQAGL 313  
*S. avermitilis* -DGYLLSILHNMDDASARILRRCADAQAQ-TTRGVLVVVDYFG-----DRTVQTEGDLRMLVF-----FGROHTLEQLAEAGTVGL 339  
*S. coelicolor* A3(2) -DYYVKNLIL-EWDDSDTARALRNVMAAG-PPGARVVVLENLVDD-----SPSMRFSTAMDRLMLVF-----VGGAKHTEKSLVTRMTEAGL 321  
*S. avermitilis* -DLYLKHVLLHPDWDVVRLLRRTATAMK-PPSRLLVLDLIDE-----RP-ASLFLVDFLLMLVF-----VGGAKHTEKSLVTRMTEAGL 310  
*S. lavenderulae* (McmR) -DYYLKHVLLHPDWDVVRLLRRTATAMK-PPSRLLVLDLIDE-----RP-ASLFLVDFLLMLVF-----VGGAKHTEKSLVTRMTEAGL 310  
*S. alboniger* -DLYVKSILHHPDWSADARSADLILRTRVRAAMP-AHARLLVVEVLLPPTV-----DSSAHPGLYLSDIYMLVF-----VGGRESERLDRLLSDYTF 354  
*S. griseus* -DLYLLNLSIHPDWDVVRG-HLRHRIDVPI-DHGSLLVIEFVPLPATV-----PADRFQNVYLSIDRMLVF-----VGGRETTADDFALACTAGF 301  
*S. glaucescens* (TcmN) -DAYLEKGVLRNMSDERAVTVLRRVREAIIGDDDLRLLIFQVMAPE-----ENEWDHAKLLDDMLVF-----VGGRETVLAEWRQLLEADF 476  
*S. ambofaciens* -DAYVLRKHLVDPWPEEQALQILRNVRAAIK-PPGRIILAEVMIPE-----QGDEPESKGLVDLMLVF-----VGGRETFQGYADLLARAGF 341  
*S. griseus* -DVVLLGHVLDHPWPAARELLRERVAAR-PPGRRVVYDAMLIDE-----RDDAVALLQSLNCSMLVF-----DGGSEYTVACRGYVEHAGF 334  
*S. argillaceus* -DVLVFGHVLHDPWPEESRKLRLQAFRALP-PGGTLLVYDAMLDD-----RSEAGAYLQSLVCSMLVF-----DGGSEYRVDRITAMAEETGF 322  
*S. collinus* (RubO) QDAYNLAWKLLVLSGTAGPGLLESYDAERREVAETVRQAMLRFAVREGKQYQVVAEELDDITMFGYCYVRSQAFAPAE-----SAPADVLEDPAHPSARPGA 360

*S. peucetius* (DnrK) VVEEVRQLPSPT-----HFDVLSLLVLAPAAATGA-----356  
*S. sp. C5* (DauK) VVEEVRQLPSPT-----HFDVLSLLVLAPAAATGA-----356  
*S. purpurascens* (RdmB) ALASERTSGSTT-----HFDVLSILEFTAVSEEAAPAAQASEALPAQE 374  
*S. globisporus* RVVAVHPAG-----HPSALIQMSAV 334  
*S. carzinostaticus* AVRAAHPIS-----VYVIVEMTAL 332  
*S. avermitilis* HTTSVTPAG-----FYSVVELRAGV 359  
*S. coelicolor* A3(2) VIDDIRPVN-----FVHFAFCTVPK 342  
*S. avermitilis* TVGEIRPVN-----AFHFAFCTVPA 331  
*S. lavenderulae* (McmR) RFRVSLPCG-----AGEVRIVEIRRA 351  
*S. alboniger* RTRVTRVTPG-----LHPSLEIAAPV 376  
*S. griseus* ACGA----- 305  
*S. glaucescens* (TcmN) DIVNTPS-----HFWITTELCRPV 494  
*S. ambofaciens* RLERVVET-----AAATLSLVEAVPV 361  
*S. griseus* RFDSEARVSE-----LQDRVLIAKSPS 357  
*S. argillaceus* TVEQVPLD-----MTHDRFLVARKPG 345  
*S. collinus* (RubO) RAPHLQLDCEGSPVVIDLYGDFEFTLVDEAAGRTWGAVEREAERLQVAL 410

**Fig 3.13:** Sequence alignment of sixteen putative methyltransferases from different *Streptomyces*. All conserved amino acids are marked with a star and all residues in the substrate binding pockets of DnrK and RdmB are labeled in black (see also figure 3:14).



**Figure 3.14:** **A.** The binding of M- $\epsilon$ -T and SAH to DnrK and **B.** The binding of DbrA and SAM to RdmB. The residue conserved between the methyltransferases as seen in figure 3:13 is underlined. Hydrogen bonds are shown in dotted lines and water molecules as circles. The substrates and cofactors are shown in grey and the residues surrounding them in black. The homologues of Cys161 and Phe167 in figure A are not included in figure B since they are in a part of the protein that is not visible in the electron density map of RdmB.

Moreover, the residues lining the pocket close to the C10 position (less than 10 Å) are generally more hydrophilic in DnrK than in RdmB. For example E294, Q295 and E298 in DnrK are equivalent to F299, F300 and L303 in RdmB (figure 13.4).

### 3.7.3 Sugar moieties

The composition and length of the sugar units attached to the aglycone is a limitation in the ability of the enzymes to accept different substrates. Previously it was suggested that DnrK (and DauK) would methylate solely sugars containing primary amines. Here it is shown that they can act on both tertiary amines as well as primary amines (paper VI). This is also consistent with the space available for both kinds of sugars in the binding pocket (figure 7, paper VI). Anthracycline glycosides with more than one sugar in length are, however, not substrates for DnrK. In the crystal structure of the ternary DnrK complex there is not space for triglycosides in the binding pocket due to the loop between  $\beta 8$  and  $\beta 9$  and  $\alpha 11$  which closes down over the active site (figure 7, paper VI). However, RdmB has been shown to accept both the monosugar (15-demethoxyanlacinomycin T) and trisugar (15-demethoxyanlacinomycin A) substrates and it is the flexibility of the local unfolding of  $\gamma 13$  and  $\alpha 14$  which allows for the sugars to protrude through the cavity into the solution (figure 3.11).

### 3.7.4 Evolution of novel mechanism through divergent evolution

There is a striking difference between the two enzymes: DnrK is a methyltransferase and RdmB a hydroxylase. The important question that has to be answered is then: Why?

#### 3.7.4.1 *Why is RdmB not a methyltransferase?*

The major difference in amino acid sequence around the methylation site is Tyr142 in DnrK that is replaced by a tryptophan in RdmB (figure 14A and B). This residue was first thought to be the base in the methylation reaction and its absence in RdmB would explain the lack of methyltransferase activity in this enzyme. Mutation of this tyrosine however did not lead to a significant decrease in activity by DnrK whereas a single exchange of Trp146 in RdmB to a tyrosine did not, on the other hand, convert RdmB to a methyltransferase. These observations made it necessary to rethink this proposal for the differences in activities.

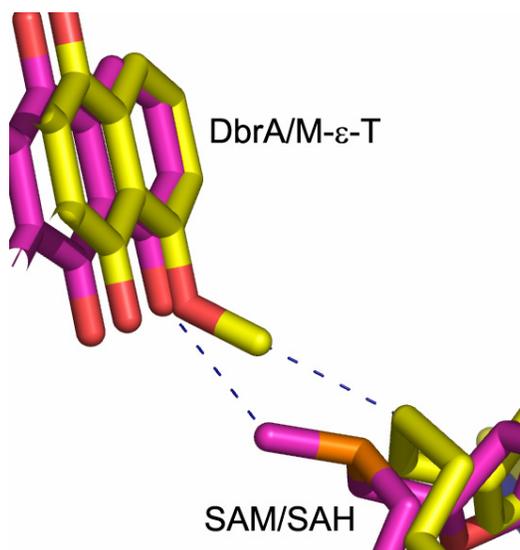
In a methyl transfer reaction using a  $S_N2$  mechanism, the methyl group has to be in line with the oxygen of the substrate as well as the sulphur atom of SAM for the reaction to proceed. This is the case in DnrK, where also a non-conserved arginine residue Arg152 (alanine in RdmB) could serve to help positioning of SAM. However, in RdmB the SAM moiety is shifted with the consequence that the methyl group of SAM points in a

direction not suitable for methyl transfer (fig 3.15). The SAM moiety is pushed away, probably by the involvement of neighboring residues, especially a phenylalanine, that also are shifted when compared to DnrK. These structural differences can be related to large changes between the enzymes in sequence and structure of the loop between  $\beta 7$  and  $\alpha 19$  comprising amino acids 288-296 (291-299 in DnrK). This loop is the only particular structural difference between RdmB and DnrK and in RdmB it has moved closer over the substrate binding pocket compared to in DnrK (fig 3.12).

#### 3.7.4.2 Why is RdmB a hydroxylase?

The unfavorable position of SAM relatively to the substrate in the structure of RdmB makes the methyltransferase reaction inefficient and it probably proceeds at a very slow rate, if at all (figure 3.15). Instead the SAM molecule gains another function, *i.e.* to assist in the hydroxylase reaction. The positive charge of the molecule seems to be the crucial feature since RdmB can use either SAM or sinefungin for the hydroxylation reaction but is inactive if using SAH (figure 3.10A and B).

If the substrate is carboxylated, a reactive decarboxylated intermediate is formed in the enzyme. This carbanion intermediate would most efficiently be protonated to get back



**Figure 3.15:** Comparison of the orientation of SAM/SAH in relation to DbrA/M-ε-T. SAM/DbrA is shown in black and SAH/M-ε-T in grey. The dotted lines are showing how well the cofactors are aligned suitable for methyltransfer relative to the O4 position of the substrate.

into a more favorable, lower energy state. The resonance structures of the aromatic substrate together with the positive charge of SAM however, stabilize the carbanionic state of the substrate sufficiently for hydroxylation, *i.e.* oxygen activation to occur. The residues close to the C10 position of the substrate are of a more hydrophobic character than their counterparts in DnrK. The hydrophobic pocket facilitates binding of oxygen sufficiently for formation of a caged-radical followed by the spin forbidden reaction. Together these features have led to a new function of SAM and thereby RdmB acting as a hydroxylase.

## 4 CONCLUSIONS

The crystallographic and mechanistic studies of the four enzymes explored in this thesis provide some general insights into the structural enzymology of the production of anthracyclines. The knowledge of the three dimensional structure of these enzymes has now given us a structural basis for the design of enzymes with novel activities, using either rational design of or structure-assisted directed molecular evolution.

### 4.1 SUBSTRATE SPECIFICITY AND BINDING

The enzymes involved in the biosynthesis of anthracyclines accept a rather broad range of substrates with different degrees of aglycone modifications and sugar lengths. The binding of the substrates in all structures is dominated by hydrophobic interactions and the specificity is controlled by shape of the pocket rather than through specific hydrogen bonds. This feature helps to explain the diversity of the different composition of anthracyclines that could be modified by the very same enzyme.

### 4.2 NOVEL MECHANISMS

#### 4.2.1 RdmB

RdmB is the first SAM dependent hydroxylase known, and the thesis provides the first mechanistic insights into a novel function of SAM in enzymatic reactions. SAM has long been known as a methyl group donor in methyltransferase reactions. However, DAPA synthase, an amino transferase involved in the biosynthesis of biotin, uses SAM as an amino donor, which is a unique feature for SAM (161, 162) (figure 3.10D). Recently a third function of SAM in reactions has come to light mainly from the contribution of Perry Frey and his collaborators where SAM plays a role through the intermediate formation of 5'-deoxyadenosyl radical (163) (figure 3.10E). RdmB thus belongs to a fourth class of enzymes, catalyzing a SAM dependent hydroxylation using SAM for a novel function not seen before. SAM plays its role by using the positively charged sulphur atom for stabilising the delocalisation of electrons in the aromatic substrate. This prevents a direct protonation of the substrate after the decarboxylation reaction and the stable carbanion formed is instead used for subsequent activation of oxygen.

### 4.2.2 SnoaL

SnoaL catalyses a novel form of intramolecular aldol condensation without Schiff base formation or metal ion to stabilize the enol(ate) intermediate. Instead the intermediate is stabilised by delocalisation of the electrons into the  $\pi$ -system of the substrate. The cyclisation of the last ring will lead to a specific stereochemistry.

Both these structures show the importance of the aromatic structure for the ability to distribute the electrons over the ring system and thereby decrease the level of energy needed for these reactions to take place.

## 4.3 DIVERGENT EVOLUTION

RdmB and DnrK are two related enzymes, sharing the same type of methyltransferase fold, but catalyzing different reactions in a similar metabolic context; DnrK a methyl transfer and RdmB a hydroxylation reaction in anthracycline biosynthesis. In spite of catalysing completely different chemistry, the two enzymes retained their original fold as well as the general choice of substrate. The structures of RdmB and DnrK are illustrative examples of divergent evolution in an enzyme fold family, leading towards new activity.

Understanding the relationship between protein sequence, structure and function has long been a major focus of biochemistry. This has been even more important in the era of “proteomics” where approximately 40% of the encoded proteins have unknown functions (164). Lots of efforts have been made in bioinformatics to elucidate function from sequence comparison, particular from structural genomics projects. Results from this thesis show that this is not always a viable way, but that additional information from genetic and biochemical data has to be added, in order to get reliable answers.

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**Am I really finished with my thesis now.....???**