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MECHANISTIC INSIGHTS INTO THE BIOSYNTHESIS OF POLYKETIDE ANTIBIOTICS

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To

ISHTIAK

ABSTRACT

Anthracyclines are a group of aromatic polyketide compounds with significant medical importance due to their antineoplastic properties. Doxorubicin and daunorubicin, members of this family are among the two most commonly used anticancer drugs. These compounds exhibit severe side effects like cardiotoxicity and multi-drug resistance. A promising approach towards the production of modified anthracyclines with improved toxicity profiles appears to be combinatorial biosynthesis, including the redesign of biosynthetic enzymes; however, structural and mechanistic information of the biosynthetic enzymes is necessary for the redesigning approach.

The main focus of this thesis is the structural and functional studies of several anthracycline biosynthetic enzymes. The crystal structures of two polyketide cyclases, SnoaL and AknH were determined to 1.35Å and 1.9Å respectively. These enzymes share very similar α+β structural folds and catalyze a novel type of intramolecular aldol condensation reaction using acid/base chemistry. Moreover, comparison of the cyclase structures, followed by site-directed mutagenesis provided insights into the structural basis of stereoselectivity of products in AknH.

Several enzymes from aromatic polyketide biosynthetic pathways, including SnoaL/AknH provide illustrative examples of divergent evolution. The related enzymes usually share the same fold but contain different catalytic machineries to catalyze diverse reactions.

AknOx is a FAD-dependent oxidoreductase which is involved in the sugar modification in aclacinomycins and catalyzes two consecutive oxidation reactions. The structure of AknOx to 1.65Å resolution was obtained from a pseudomerohedrally twinned crystal by MAD. AknOx crystals show an unusual multi-domain twinning with six twin domains. Investigation of the active site and mutagenesis of the proposed residues revealed a unique feature in AknOx compared to other flavoenzymes. The enzyme contains a dual active site with two different sets of catalytic residues for catalyzing two consecutive reactions.

LIST OF PUBLICATIONS

1. Sultana A, Kallio P, Jansson A, Niemi J, Mäntsälä P and Schneider G. Crystallization and preliminary crystallographic data of SnoaL, a polyketide cyclase in nogalamycin biosynthesis. Acta Crystallographica Section D; 2004; 60(6); 1118-1120.

2. Sultana A, Kallio P, Jansson A, Wang JS, Niemi J, Mäntsälä P, and Schneider G. Structure of the polyketide cyclase SnoaL reveals a novel mechanism for enzymatic aldol condensation. The EMBO Journal; 2004; 23(9); 1911-1921.

3. Kallio P, Sultana A, Niemi J, Mäntsälä P, and Schneider G. Crystal structure of the polyketide cyclase AknH with bound substrate and product analogue: implications for catalytic mechanism and product stereoselectivity. Journal of Molecular Biology; 2006; 357(1), 210-220.

4. Sultana, A., Alexeev, I., Kursula, I., Mäntsälä, P., Niemi, J., and Schneider, G. Structure determination by multiwavelength anomalous diffraction of aclacinomycin oxidoreductase: indications of multi-domain pseudomerohedral twinning. Accepted in Acta Crystallographica Section D on the October, 2006.

5. Alexeev, I., Sultana, A, Mäntsälä, P., Niemi, J., and Schneider, G. (Alexeev, I and Sultana, A are joint first authors). Crystal structure of aclacinomycin oxidoreductase: an unusual flavin enzyme with a dual active site. In manuscript, 2006.

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LIST OF ABBREVIATIONS

SnoaL	Nogalonic acid methyl ester cyclase
AknH	Aklanonic acid methyl ester cyclase
AknOx	Aclacinomycin oxidoreductase
TcmI	Tetracenomycin F2 cyclase
PCMH	P-cresol methylhydroxylase
GOOX	Glucooligosaccharide oxidase
NAME	Nogalonic acid methyl ester
Nog	Nogalamycinone
AKV	Aklavinone
AAME	Aklanonic acid methyl ester
AclN	Aclacinomycin N
AclA	Aclacinomycin A
AclY	Aclacinomycin Y
DbrA/T/B	11-deoxy-β-rhodomycin A/T/B
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
MAD	Multiple-wavelength anomalous dispersion
SIRAS	Single isomorphous replacement with anomalous scattering
SAD	Single-wavelength anomalous dispersion
MIRAS	Multiple isomorphous replacement with anomalous scattering
SIR	Single isomorphous replacement
MIR	Multiple isomorphous replacement
NMR	Nuclear magnetic resonance
PEG	Polyethylene glycol
EDTA	Ethylenediaminetetraacetic acid
DNA	Deoxyribonucleic acid
R_{sym}	$R_{sym} = \Sigma_{hkl} \Sigma \mid Ii - \langle I \rangle \mid / \Sigma_{hkl} \Sigma i \langle I \rangle$, where Ii is the intensity measurement for a reflection and $\langle I \rangle$ is the mean value for this reflection.
R_{work}	$\Sigma \|F_{obs}\| - \|F_{calc}\|/\Sigma\|F_{obs}\|$; $\|F_{obs}\|$ is the observed and $\|F_{calc}\|$ is the calculated structure factor amplitudes
R_{free}	The same as R_{work} which is calculated for the set of reflections that is not used during the refinement

1 Introduction:

1.1 Antibiotics: definition and history

Antibiotics are natural or synthetic substances with antimicrobial properties. These compounds are used as bactericidal or bacteriostatic agents to treat different types of bacterial infections. The name 'antibiotic' is derived from Greek word "anti-bios", which means "against life" and the concept of antibiotic was first introduced in 1889 (Drews, 1999; Vuillemin, 1893). In 1949, Selman A. Waksman described antibiotics as "products of the metabolisms of microorganism, with molecular weight of less than 2000 Dalton" (Waksman, 1955). However, this definition was extended later due to the addition of many synthetic or semisynthetic substances with antibacterial properties in the list of conventional antibiotics.

The German scientist E. von Freudenreich isolated a product termed as pyocyanase from *Bacillus pyocyaneus* in 1888, which showed antibacterial properties against a multitude of disease-causing bacteria. Pyocyanase was the first natural antibiotic but it could not be used clinically due to its high toxicity and instability. In 1928, Alexander Fleming discovered penicillin which was isolated and purified in larger quantity and used for clinical trials by Howard Florey, Ernst Chain, and Norman Heatley in 1940. In the 1930s, René Dubos isolated gramicidin, the first clinically tested antibiotic agent from soil bacteria (Dubos and Hotchkiss, 1941 ; Hotchkiss and Dubos, 1940; Van Epps and Dubos, 2006). He also isolated tyrothricin from the same source, which was used against skin infections (Dubos and Hotchkiss, 1941 ; Hare, 1954; Hotchkiss and Dubos, 1940).

1.2 Polyketide antibiotics are secondary metabolites

Polyketide antibiotics belong to the class of secondary metabolites which are produced in the microorganisms at slow growth rate and their presence is not required for the growth of the producing organisms. The polyketides exhibit different types of activities including antibacterial (erythromycin, rifamycin B), anticancer (doxorubicin), anticholesterol (lovastatin) and immunosuppressant (rapamycin) (Strohl *et al*., 1997). They are mainly produced by actinomycetes, specifically the *Streptomyces* (figure 1) a group of filamentous bacteria grown from soil.
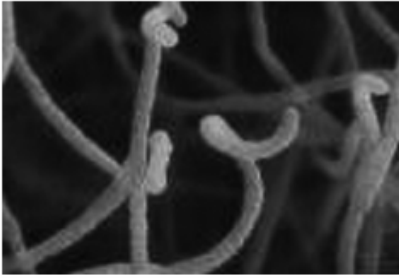
Figure 1 : *Streptomyces* mycelium

Polyketides are synthesized by the successive condensation of different kinds of building units such as acetate, propionate, malonate or butyrate under the control of a multi-enzyme complex, Polyketide synthase (PKS). The resulting linear chain formed after several cycles of reactions carries unreduced keto groups attached to the β-carbons. The occurrence of these keto groups at many of the alternate carbon atoms gives rise to the name 'polyketide' for this class of compounds (Hopwood and Sherman, 1990).

Based on their diversity in structure and function, polyketides can be divided into three overall classes (Shen, 2003). The type I polyketides or complex polyketides are a group of non-aromatized compounds synthesized by type I PKS system, a large multifunctional protein complex carrying all the active sites required for polyketide carbon chain assembly (Katz, 1997). Some examples of most well-known complex polyketides include erythromycin and rapamycin (figure 2).
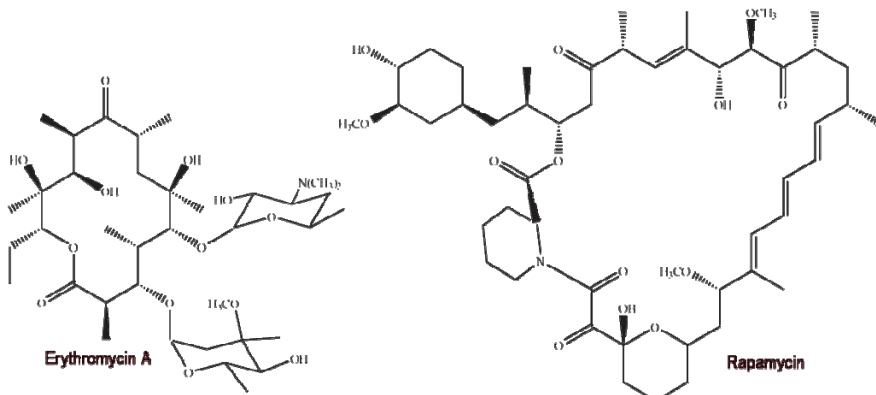
Figure 2: Structures of complex polyketide compounds

The type II iterative polyketide compounds, also known as aromatic polyketides, are mainly produced through the condensation of acetate groups, with the exception of the starter unit reactions, through several reaction steps catalyzed by type II PKS. During

the biosynthesis of aromatic polyketides the acyl chain with desired length undergoes spontaneous or programmed folding to produce the aromatic structure (Katz, 1997). A number of common examples of aromatic polyketides are shown in figure 3.

Finally, the type III polyketides include chalcones and stilbenes in plants and polyhydroxy phenols in bacteria. Chalcone synthase and Stilbene synthase are comparatively small proteins with a single polypeptide chain and are involved in the biosynthesis of tetraketide intermediates followed by cyclization to produce the final products. (Austin *et al*., 2004a; Austin *et al*., 2004b; Ferrer *et al*., 1999; Funa *et al*., 1999)
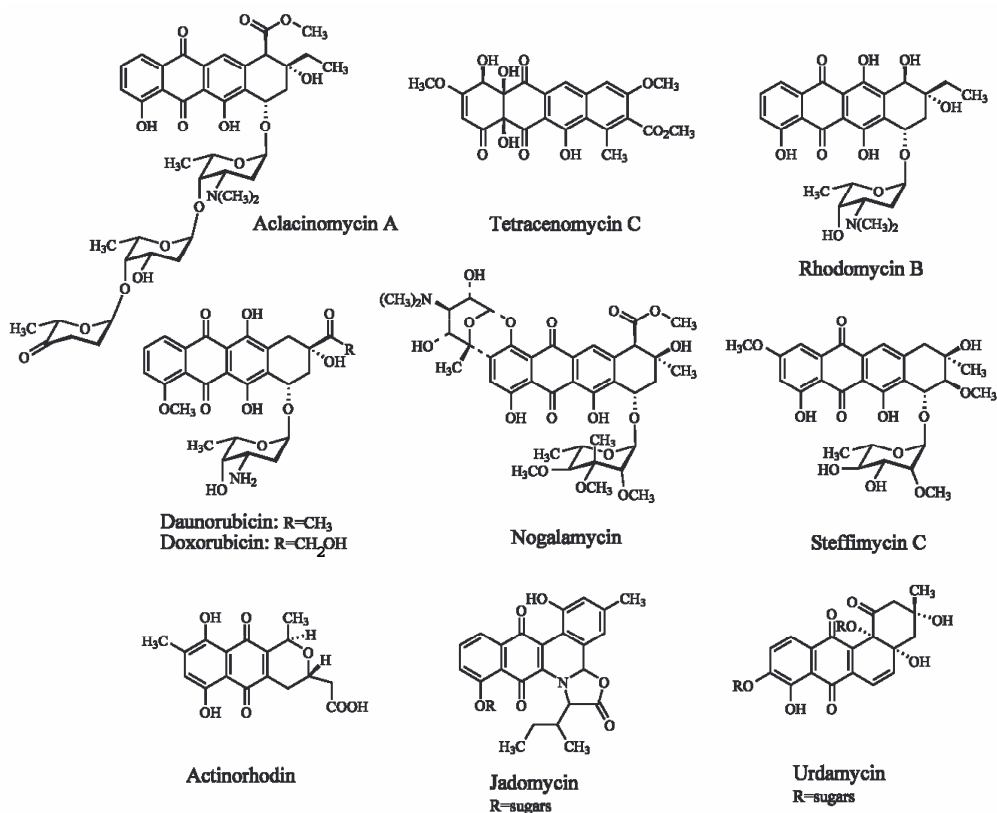
Figure 3: Structures of some common aromatic polyketides.

1.3 Anthracyclines

1.3.1 Historical background and overall structure

In the 1950's, H. Brockman and his co-workers identified the "yellow-red or red, optically active dyes" which was named as anthracyclinone due to its resemblance to the anthraquinone chromophore (Brockmann, 1963; Brockmann and Bauer, 1950).

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Since then, "anthracycline" has been used to refer to microbial products that contain an anthracyclinone moiety, typically as a glycoside (Brockmann, 1963). The basic scaffold of anthracycline is a 7,8,9,10-tetrahydro-5,12-napthacenequinone structure. The structural diversity is generated by different modifications of the tetracyclic polyketide core, 'aglycone' as well as differences in the glycosylation steps (figure 4). The different substitutions on the polyketide core are mainly due to the reactions catalyzed by tailoring enzymes which include hydroxylation, reduction, methylation and glycosylation (Niemi *et al*., 2002; Schneider, 2005; Strohl *et al*., 1989). The three-dimensional structures of several anthracyclines like Daunorubicin (Angiuli *et al*., 1971), Nogalamycin (Arora, 1983) and aclacinomycin A and B (Yang and Wang, 1994) have been determined by X-ray crystallography or NMR.

| Position	Substituents
R1	H, OH, glycoside
R2	H, OH, glycoside
R4	OH, OCH$_3$
R6	H, OH
R7	H, OH, glycoside
R9	CH$_3$,CH$_2$-CH$_3$,COCH$_3$,COCH$_2$OH, CHOHCH$_3$,CHOHCH$_2$OH, CH$_2$COCH$_3$
R10	H, OH COOCH$_3$, glycoside
R11	H, OH
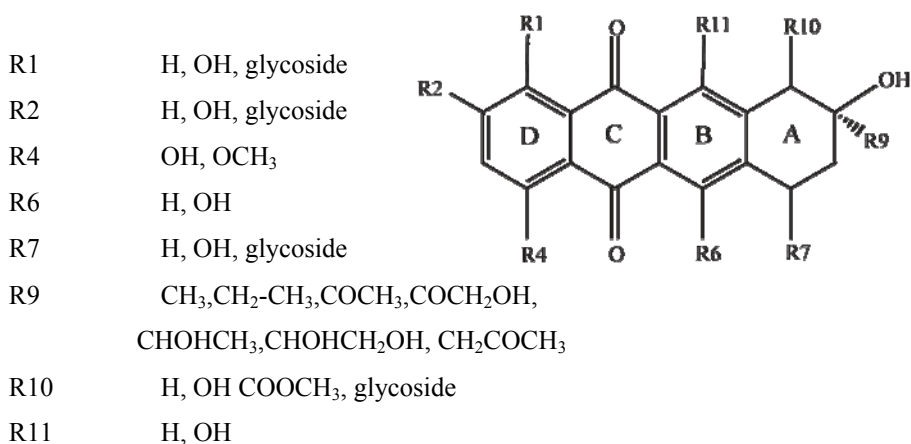
Figure 4: The general structure of the aglycone moiety for most commonly used anthracyclines.Common substituents are listed to the left

The antibiotic properties of the *Streptomyces* organisms were first discovered in 1939 from soil specimen. (Brockmann and Bauer, 1950). Rhodomycin was the first anthracycline compound identified by H. Brockman (Brockmann, 1963). The discovery of Daunorubicin was made independently by the laboratories at Farmitalia, where it was named as daunomycin (Grein *et al*., 1963) and at Rhône-Poulenc with the name rubidomycin (Dubost *et al*., 1963). Another important anthracycline, Doxorubicin (Adriamycin) was first isolated from *Streptomyces peucetius* in 1967 by Farmitalia (Arcamone *et al*., 1969a; Arcamone *et al*., 1969b; Di Marco *et al*., 1969). Nogalamycin, which differed structurally from other anthracyclines (Wiley *et al*., 1977b; Wiley *et al*., 1968) was first discovered by Bhuyan and Dietz in 1965 from *Streptomyces nogalater* (Bhuyan and Dietz, 1965) and studied extensively for anticancer properties by the Upjohn Company (Neil *et al*., 1979). The organism had an

odor in culture of black walnuts and the name of the strains was derived from *nogal*, Spanish for walnut and *ater*, Latin for black or dark. Nogalamycin was evaluated as an anticancer agent 20 years ago, but was discarded as being too toxic for clinical use (McGovren *et al*., 1984; Weiss *et al*., 1986). However, several derivatives of nogalamycin have been shown to have improved activity (Wiley *et al*., 1977b). Aclacinomycins are second generation class II anthracycline antibiotics and the isolation of Aclacinomycin A and B was carried out by Sanraku-Ocean Co. and the Institute of Microbial chemistry in Tokyo (Oki *et al*., 1975). The class II anthracyclines are characterized by their higher ability to inhibit of nucleolar RNA syntheses which is mainly caused due to the presence of a carboxymethyl group at the C10 position of the aglycone and a di- or trisaccharide moiety in class II compounds (Crooke *et al*., 1978; DuVernay *et al*., 1979a; DuVernay *et al*., 1979b) (figure 3). The trisaccharide aclacinomycin was used for clinical trial in 1978 (Umezawa, 1978a; Umezawa, 1978b) and showed significantly lower cardiotoxicity compared to doxorubicin (Oki, 1988). Since then, a large number of anthracycline compounds have been characterized and described in the literature (Kelly *et al*., 1977; Lown, 1993; Strohl *et al*., 1997)

1.3.2 Anthracyclines as anticancer drugs

Anthracyclines are widely used in clinical practice because of their anticancer properties. Doxorubicin is mainly used in the treatment of different kinds of solid tumors (Hortobagyi, 1997; Minotti *et al*., 2004) while daunorubicin and aclacinomycin are used for treating acute leukemia (Fujii and Ebizuka, 1997; Oki *et al*., 1975; Warrell, 1986; Wiernik and Dutcher, 1992). These compounds exhibit their effect against the cancer cells through several mechanisms.

Most of the antitumor activities of anthracyclines are caused by their strong but non-covalent intercalation with DNA (Brana *et al*., 2001; Lown, 1993; Reinert, 1983). The 3D structures of daunorubicin, nogalamycin as well as aclacinomycin A and B in complex with DNA show that the aglycone moiety of these compounds is intercalated with DNA mainly through van-der Waals interactions as well as some direct or solvent mediated hydrogen bonds. The sugar moieties also affect the binding of molecules with the DNA (Arora, 1985b; Liaw *et al*., 1989; Moore *et al*., 1989; Quigley *et al*., 1980; Wang *et al*., 1987; Williams *et al*., 1990; Yang and Wang, 1994) by binding to the minor groove (Pilch *et al*., 1997).

Inhibition of the topoisomerase II by anthracyclines plays a major role in controlling the cell growth of the cancer cells. Some anthracycline compounds like doxorubicin, daunorubicin, epirubicin act as topoisomerase II poisons which stabilize the anthracycline-DNA-topoisomerase II ternary complex and inhibits the enzyme from resealing the DNA breakage (Capranico *et al*., 1990; Jensen *et al*., 1993; Minotti *et al*., 2004; Sorensen *et al*., 1992; Tewey *et al*., 1984). The sugar moieties can play an important role in forming the topoisomerase II-DNA-drug complex, where the flexible

sugar parts can interact with the topoisomerase II, thus facilitating the complex formation (Pilch *et al*., 1997). However, several other anthracyclines like aclacinomycins belong to a group of the topoisomerase II inhibitors which interferes with the binding of DNA to topoisomerase II and thus interferes the DNA cleavage by topoisomerase II (Larsen *et al*., 2003; Sorensen *et al*., 1992).

Anthracyclines are capable of generating free radicals which can cause direct injury to the cell membranes through a process known as lipid peroxidation (Chichuk *et al*., 1999) as well as inactivation of receptors and membrane bound enzymes (Horenstein *et al*., 2000; Straghan *et al*., 1996). Moreover, the generation of free radicals is also the main cause of the cardiotoxicity, which will be discussed in the following chapter. Doxorubicin and many other genotoxic agents activate p53-DNA binding and p53 is suggested to interact with topoisomerase II and inhibits its ligase function, eventually amplifying the amount of formation of irreversible strand break (Cowell *et al*., 2000; Dunkern *et al*., 2003; Minotti *et al*., 2004).

An alternative target for anthracycline activity was demonstrated by their properties to inhibit DNA helicase activity, reported by several laboratories (Bachur *et al*., 1993; Bachur *et al*., 1998; Bachur *et al*., 1992; Gewirtz, 1999; Skladanowski and Konopa, 1994a; Skladanowski and Konopa, 1994b). This work has been further supported by Tuteja *et al* describing inhibition of both unwinding and ATPase activities of purified human helicase II (Tuteja *et al*., 1997).

1.3.3 Cardiotoxicity and Multiple-drug resistance (MDR)

The anthracyclines exhibit severe side effects like cardiotoxicity. It has been described as the most serious side effect (Mordente *et al*., 2001; Wojtacki, 1998; Wojtacki *et al*., 2000) resulting from a combination of free radical production by the anthracycline metabolites and an unusual sensitivity of the mammalian heart to oxidative stress (Horenstein *et al*., 2000; Jain, 2000).

The effectiveness of chemotherapy by anthracycline compounds is often limited by the multi drug resistance (MDR) which occurs as a result of resistant tumor recurrence after initially effective chemotherapy (Priebe and Perez-Soler, 1993). Most of the commonly used anthracyclines like doxorubicin or daunorubicin are involved in MDR which results in ineffective prolonged treatment.

1.4 Biosynthesis of Anthracyclines

1.4.1 Gene clusters from different *Streptomyces* strains

In 2002, the complete genome of the actinomycete *S. coelicolor* with the overall size of 8.6Mbp was sequenced. It contained about 8000 protein coding sequences and over 20 of the gene clusters (4.5% of the whole genome) were predicted to encode biosynthetic

enzymes for secondary metabolites (Bentley *et al*., 2002). In another study, the genome sequenced from *S. avermitilis* contained 6% of the total genome responsible for secondary metabolite biosynthesis (Ikeda *et al*., 2003).

Hopwood and Malpartida in 1985 performed the first successful cloning of an entire aromatic polyketide biosynthetic gene cluster to produce actinorhodin (Hopwood *et al*., 1985). During the last 20 years, a large number of anthracycline biosynthetic genes were isolated from different types of *Streptomyces* gene clusters. The biosynthetic gene clusters of five common anthracyclines are listed in Table 1 which will be discussed in more detail in the following part.

The gene clusters for producing doxorubicin and daunorubicin are most extensively studied because of their wide-spread use as anticancer agents. The *dnr* biosynthetic genes were first cloned from *S. peucetius* in 1989 (Otten *et al*., 1990; Stutzman-Engwall and Hutchinson, 1989), sequenced and most of the genes were characterized (Hutchinson, 1997). At the same time, William Strohl with his co-workers cloned, sequenced and characterized most of the *dau* biosynthetic genes from *Streptomyces sp. C5* (Dickens *et al*., 1995; Strohl *et al*., 1997; Ye *et al*., 1994). The genes in the *dau/dnr* clusters from *S. peucetius* and *Streptomyces sp. C5* showed sequence identities of about 93%. A mutant of S. *peucetius*; *S. peucetius* subsp. *Caesius* ATCC27952, a producer of doxorubicin was isolated and some of the genes of this strain were characterized (Stutzman-Engwall and Hutchinson, 1989).

Rhodomycins, the first discovered anthracyclines are produced in *S. purpurascens* (*rdm* gene cluster). Because of the major similarity of these *rdm* genes with the *dau/dnr* cluster, they have been used extensively in combinatorial biosynthesis (Niemi and Mantsala, 1995; Niemi *et al*., 1994).

Aclacinomycins are produced from several *Streptomyces galilaeus* strains and the published biosynthetic studies on aclacinomycins have been mainly performed with the *S. galilaeus* strain MA144-M1 (ATCC 31133) (Oki *et al*., 1975). The organism produced a new water-insoluble pigment antibiotic complex named aclacinomycins, which was extracted and separated into thirteen yellow and seven red coloured components. All of them belong to the aklavinone and ε- pyrromycinone group of anthracyclines and aclacinomycin A and B were produced preferentially (Oki *et al*., 1979a; Oki *et al*., 1979b). Several of the biosynthetic genes with the gene prefix *akn* were cloned and characterized (Fujii and Ebizuka, 1997; Raty *et al*., 2002; Raty *et al*., 2000).

Nogalamycin (*sno* gene cluster) is produced by *Streptomyces nogalater* strain, ATCC2745 and the entire *sno* biosynthetic gene cluster has been cloned and sequenced (Torkkell *et al*., 2001; Torkkell *et al*., 1997; Ylihonko *et al*., 1996b). Nogalamycin is in many ways unusual compared to other common anthracyclines. Nogalamycin biosynthesis starts from an acetate starter unit instead of the propionate group (Wiley *et al*., 1978), starter unit for doxorubicin/daunorubicin biosynthesis.

Table 1: Biosynthetic gene clusters from different *Streptomyces* species upto the sugar modification steps are listed below. An extended list of genes with regulatory properties and unknown functionality are shown in Niemi *et al* (Niemi *et al*., 2002). The gene clusters encoding the enzymes studied in this thesis are marked as **bold**. The data of this table is mainly derived from the review by Jarmo Niemi (Niemi *et al*., 2002)

Biosynthetic function		*Sno*	*akn*	*dau/dnr*	*Rdm*	function
Aglycone biosynthesis	1	*snoa1*	*aknB*	*dpsA*		Min PKS KS-α
	2	*snoa2*	*aknC*	*dpsB*		Min PKS KS-β CLF
	3	*snoa3*	*aknD*	*dpsG*		ACP for minPKS
	4		*aknE2*	*dpsC*		propionate starter unit
	5		*aknF*	*dpsD*		ACP acytranferase
	6	*snoaD*	*aknA*	*dpsE*	*rdmJ*	ketoreductase
	7	*snoaE*	*aknE1*	*dpsF*	*rdmK*	aromatase
	8	*snoaM*	*aknW*	*dpsY*		cyclase
	9	*snoaB*	*aknX*	*dnrG/dauG*		oxygenase
Post-Postketide biosynthesis	10	*snoaC*	*aknG*	*dnrC/dauC*		methyltranferase
	11	**snoaL**	**aknH**	*dnrD/dauD*	*rdmA*	cyclase
	12	*snoaF*	*aknU*	*dnrE/dauE*	*rdmL*	ketoreductase
Aglycone modification	13			*dnrF/dauF*	*rdmE*	C11 hydroxylase
	14			*dnrP/dauP*	*rdmC*	C16 methylesterase
	15			*dnrK/dauK*		4-O-methyltransferase
	16			*doxA*		P450 monooxygenase
	17				*rdmB*	C10 hydroxylase
	18	*snoaL2*	*aclR*			C1-hydroxylase
Deoxy-sugar biosynthesis	19	*snogJ*	*aknY*	*dnmL*		TDP-glucosesynthase
	20	*snogK*	*aknR*	*dnmM*		4,6-dehydratase
	21	*snogH*	*aknN*	*dnmT*		2,3-dehydratase
	22	*snogI*	*aknZ*	*dnmJ*		transaminase
	23	*snogF*	*aknL*	*dnmU*		3,5-epimerase
	24	*snogG/ snogC*	*aknM/ aclM*	*dnmV*		4-ketoreductase
	25	*snogA/ snogX*	*aknX2 /aclP*		*rdmD*	aminomethylase
	26		*aknQ*		*rdmF*	3-ketoreductase
	27		*aknP*		*rdmI*	3-dehydratase
	28	*snogG2*				C-methyltransferase
	29	*snogY*				O-methyltransferase
	30	*snogL*				O-methyltransferase
	31	*snogM*				O-methyltransferase
Glycosyl transfer	32	*snogE/ snogZ*	*aknS*	*dnmS*	*rdmH*	glycosyltransferase
	33	*snogD*	*aknK*	*dnrH/dauH*		glycosyltransferase
Sugar modification	34		**aknOx**			oxidoreductase

Because of the difference in the starter unit, nogalamycin contains a methyl group at the C-9 position instead of an ethyl group (observed in doxorubicin or daunorubicin). Furthermore, the stereochemistry at the C-9 position is also opposite to that observed in most other anthracyclines (Arora, 1983; Torkkell *et al*., 2000; Torkkell *et al*., 2001). Nogalamycin contains an unusual amino sugar moiety, nogalamine, which is attached to C1 and C2 instead of C7 of the aglycone moiety (figure 3).

1.4.2 Biosynthesis of the polyketide core, aglycone

The biosynthesis of the polyketide core starts from one propionate (the starter unit) and nine malonates which are the primary precursors of the anthracyclinone part for most of the common anthracyclines (doxorubicin, daunorubicin, aclacinomycin), determined by isotope labeling experiments (Kitamura *et al*., 1981; Paulick *et al*., 1976). However, biosynthesis of some anthracyclines requires a different starter unit than propionate. For instance, nogalamycin and steffimycin biosynthesis starts with an acetate unit (Wiley *et al*., 1978). Two genes [*dps*C (*akn*E2) and *dps*D (*akn*F) in table 1] from the anthracycline biosynthesis gene cluster have been shown to act as a 'fidelity factor' which plays an important role in selecting the starter unit as propionate (Bao *et al*., 1999a; Bao *et al*., 1999b; Grimm *et al*., 1994; Rajgarhia *et al*., 2001). It has been shown that the PKS in *S. peucetius* accepts both acetate and propionate in the absence of these genes but only propionate in their presence. This observation is also supported by the fact that the nogalamycin biosynthetic pathway uses acetate as the starter group and both the genes are absent in the *sno* gene cluster.

1.4.2.1 Formation of the β-ketoacyl chain by minPKS

The biosynthesis of the polyketide core is initiated by a sequence of reactions catalyzed by the minimal polyketide synthase II (PKS), the enzyme complex which catalyzes repeated Claisen condensations between acyl thioesters to build up the initial polyketide chain (Dreier and Khosla, 2000; Hutchinson, 1997; Katz and Donadio, 1993; Khosla *et al*., 1999; McDaniel *et al*., 1993; Strohl *et al*., 1997). The minimal PKS mainly consists of four different proteins, an acyl carrier protein with pantetheinyl arm (ACP), a malonyl-CoA:ACP malonyltransferase (MAT), as well as, a heterodimeric complex of ketosynthase (KS) and chain length factor (CLF) (Dreier and Khosla, 2000; Fujii and Ebizuka, 1997; Hutchinson, 1997).

In the first step of the synthesis of the ketoacyl chain, ACP is malonylated by MAT and the malonate is attached to ACP through the pantetheinyl arm. The polyketide chain synthesis is then initiated when a malonyl-ACP encounters an unoccupied KS-CLF heterodimer (figure 5a). Decarboxylation of the malonyl group followed by the transfer of the resulting acetyl group to the active site of ketosynthase leads to priming of the synthase by an acetate group. The elongation step continues with a certain number of

extension cycles with malonyl units until full-length poly-β-ketoacyl chain is synthesized (figure 5b).

The termination of the keto-acylchain synthesis is promoted by the release of the KS-CLF heterodimer from ACP-acylchain part (figure 5c) which is followed by the concomitant cyclization of the released chain (Dreier and Khosla, 2000).

During the last few years, several three dimensional structures of minPKS enzymes were determined mainly from the actinorhodin biosynthetic pathway which provided specific information about the structural and mechanistic features of these enzymes.

The structure of ACP was determined from several aromatic polyketide biosynthetic pathways (Crump *et al.*, 1997; Findlow *et al.*, 2003; Li *et al.*, 2003). The crystal structure of MAT from *Streptomyces coelicolor* contained a large domain with α/β hydrolase fold and a smaller domain with a ferredoxin-like fold. The enzyme uses a Ser-His catalytic pair for transferring the malonyl unit (Keatinge-Clay *et al.*, 2003). A crucial step towards understanding the mechanism of the biosynthesis of the ketoacyl

A.
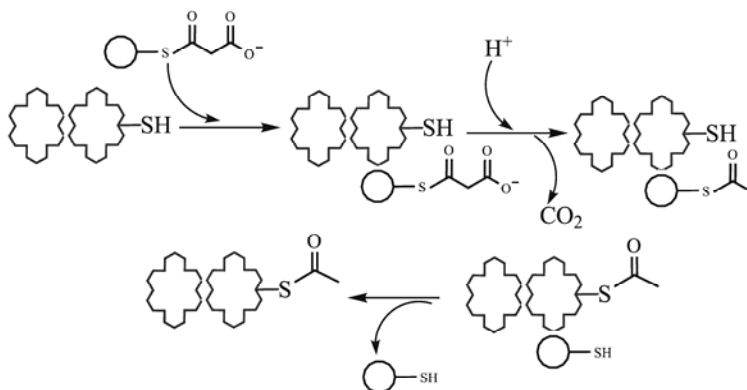
B.
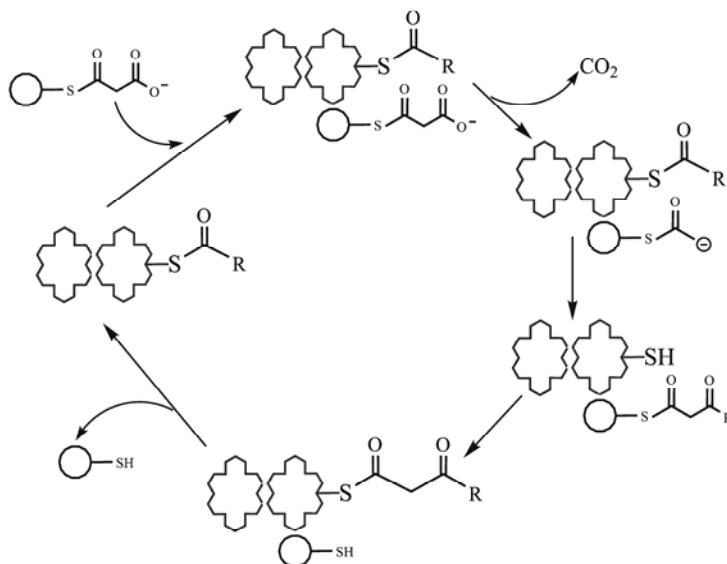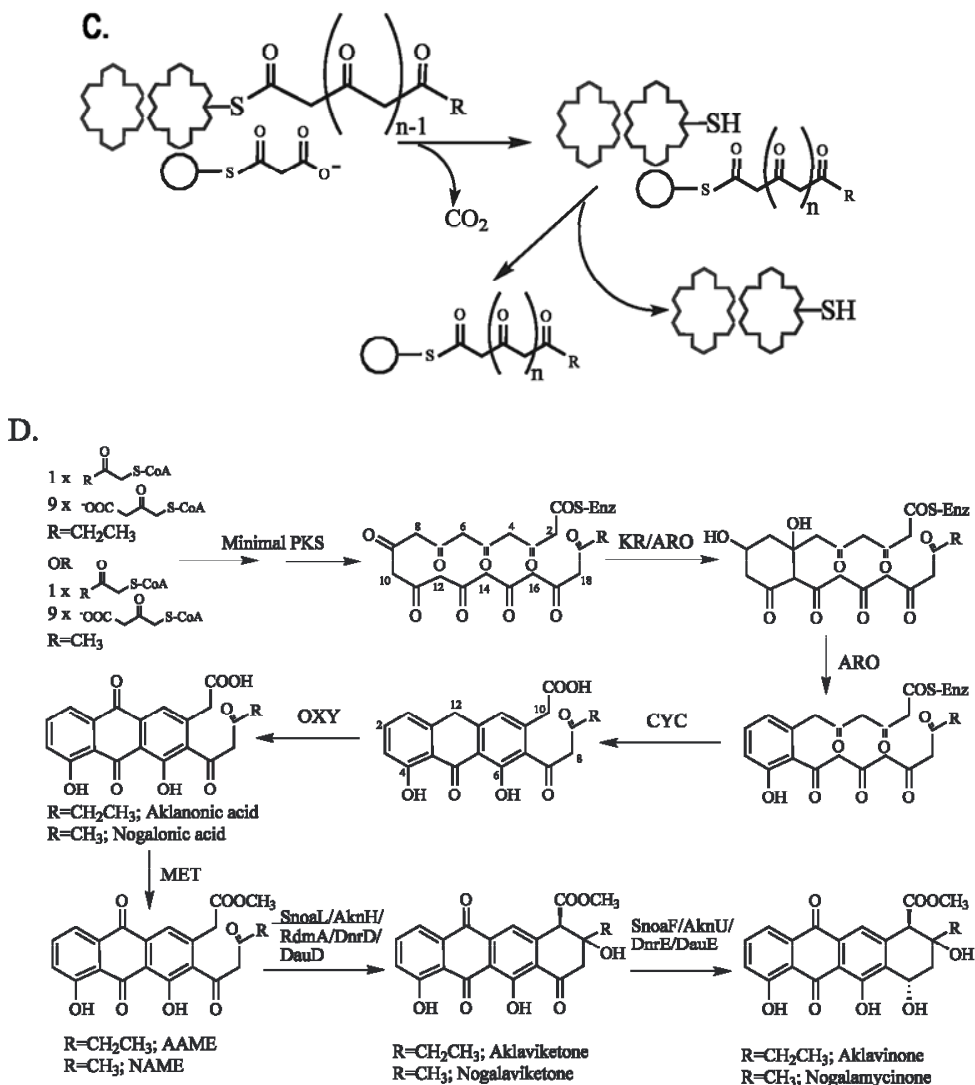
Figure 5: The biosynthesis of the polyketide core. The biosynthesis catalyzed by PKS is shown as (a) initiation of the polyketide chain biosynthesis; (b) elongation of the chain and (c) the termination of the reaction cycle after the polyketide chain with target length is reached. The KS-CLF dimer is shown as the hexameric shape and the ACP is shown as the circle. This figure is adapted from Dreier and Khosla (Dreier and Khosla, 2000). (d) Subsequent minPKS and post-PKS reaction steps are shown which lead to the formation of the aglycone moiety.

chain was revealed from the structural investigation of the KS-CLF heterodimer. The structures of KS and CLF showed a typical thiolase fold (Keatinge-Clay *et al*., 2004). The structural and functional analysis of the KS-CLF dimer confirms the role of KS which mainly catalyzes the chain initiation and elongation steps. At the interface the dimer contains an amphipathic channel which allows maintaining the linear form of the chain during elongation and the desired length of the acyl chain is mainly controlled by the architecture of the channel (Keatinge-Clay *et al*., 2004) (figure 6a).

1.4.2.2. Aglycone biosynthesis by additional minPKS and post-PKS enzymes

In order to process the nascent chain to form specific polyaromatic compounds, additional PKS subunits are required, which include a ketoreductase (KR), aromatase (ARO) and cyclase (CYC). Ketoreductase reduces the keto group at the C9 of the β-ketoacyl chain, which is numbered as C2 after the aglycone moiety is formed (figure 1 in paper 2). The first aromatic ring is formed by an aromatase (ARO), which is followed by the cyclization of the second and third aromatic ring catalyzed by a cyclase (CYC) (Hautala *et al*., 2003; Hopwood, 1997; Khosla *et al*., 1999). Oxygenation at C-12 produces aklanonic acid/nogalonic acid, which is the end product of the PKS pathway and the first isolable intermediate (Kantola *et al*., 2000; Strohl *et al*., 1997; Vetrivel and Dharmalingam, 2001; Wagner *et al*., 1984) (figure 5d).

A   **B**

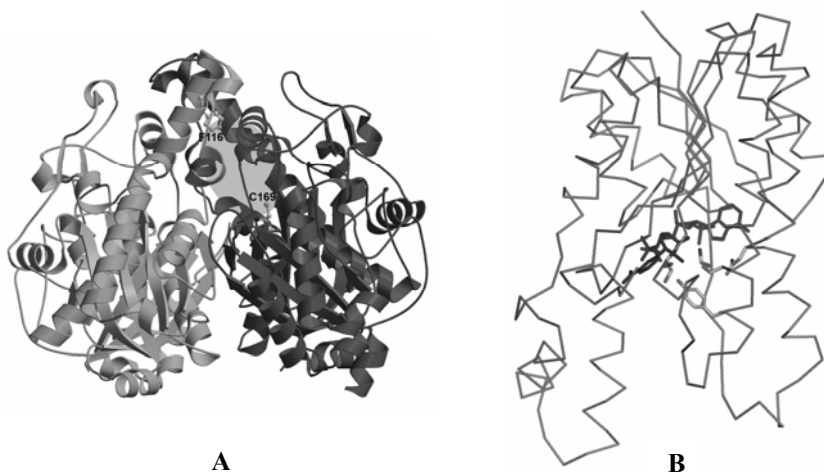Figure 6: Structures of enzymes from minPKS and additional PKS.
    (a) The heterodimer of the KS-CLF. The amphipathic tunnel is marked
    by a grey-shaped ellipsoid. This figure is adopted from Keatinge-Clay *et al*.
    (Keatinge-Clay *et al*., 2004).
    (b) The crystal structure of actIII KR. The bound NADPH and active site
        tetrad are shown in dark and light grey respectively.

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The structure analysis of actIII, a type II polyketide ketoreductase from *Streptomyces coelicolor* confirmed that the enzyme belongs to the short chain reductase (SDR) superfamily (Hadfield *et al*., 2004; Jornvall *et al*., 1995; Korman *et al*., 2004; Oppermann *et al*., 2003) (figure 6b). The ketoreductases from different aromatic polyketide biosynthetic pathways show quite high sequence identity of 39-80% suggesting that they share the same fold. The catalytic site of act KR is comprised of an Asn114-Ser144-Tyr157-Lys161 tetrad, conserved in this family suggesting a similar catalytic mechanism for this class of enzymes (Korman *et al*., 2004). The structure of a priming ketosynthase, ZhuH from the biosynthetic pathway of the polyketide R1128 was studied in complex with the priming substrate acetyl-CoA. The overall thiolase fold of the structure (Pan *et al*., 2002) was similar to that observed in many other ketosynthases (Davies *et al*., 2000; Ferrer *et al*., 1999; Huang *et al*., 1998; Keatinge-Clay *et al*., 2004; Moche *et al*., 2001; Olsen *et al*., 1999). The reactions catalyzed by ketoreductase/aromatase/cyclase in the biosynthetic pathway of actinorhodin are followed by a monooxygenase, ActVA-Orf6 (Sciara *et al*., 2003) which exhibits 22-25% sequence identity with AknX or SnoaB (Table 1) from anthracycline biosynthesis. Details about the structure and catalytic properties of ActVA-orf6 will be discussed in the chapter 2.1.9.

The post PKS reactions (figure 5d) start with a methyltransferase (MET), which converts the tricyclic acid to the corresponding methyl ester. This reaction is a prerequisite for the aldol condensation closing the fourth ring catalyzed by a cyclase. The first asymmetric center present in the anthracyclinone is produced during this reaction. The *sno* cyclase (SnoaL) produces the 9S, 10R configuration whereas the *akn/dau/dnr* cyclases, AknH, DauD and DnrD produce 9R 10R isomer (Kendrew *et al*., 1999; Torkkell *et al*., 2000). Two of the cyclases, SnoaL and AknH will be discussed in detail in the following chapters. The aglycone biosynthesis is completed by the ketoreductase which produces the aklavinone/nogalamycinone with 7S configuration at C7.

1.4.3 Modification of the aglycone moiety

The structural diversity of the aglycone is generated by modification reactions which are carried out by different tailoring enzymes. Most of the post modification of the aglycone core requires a glycosylated substrate (Fujii and Ebizuka, 1997) with a few exceptions. One of these is the hydroxylation at the C1 position of the unglycosylated aglycone core of nogalamycin, nogalamycinone (Torkkell *et al*., 2001) catalyzed by the C1-hydroxylase, Snoal2 (figure 7-left). This enzyme showed no sequence or structural similarity to either FAD or P450 monooxygenases, which are usual hydroxylases in antibiotic biosynthetic pathways (Beinker *et al*., 2006; Niemi *et al*., 2002). Similar hydroxylation at the C1 position has been observed in *S. galilaeus*, most likely carried out by AclR, a homolog to SnoaL (Beinker *et al*., 2006; Oki, 1984; Strohl *et al*., 1997).

In the biosynthetic pathway of rhodomycin, aklavinone is converted to ε-rhodomycinone by another hydroxylase RdmE or Aklavinone-11-hydroxylase (figure 7-right) which is a FAD dependent enzyme (Hong *et al.*, 1994; Niemi *et al.*, 2002; Niemi *et al.*, 1999).
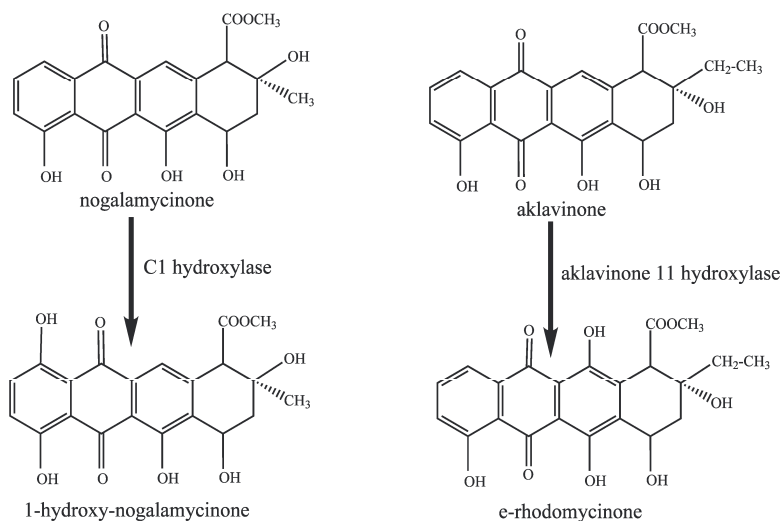
Figure 7: Some of the modification reactions of the unglycosylated aglycone are shown. Nogalamycinone converted to 1-hydroxy-nogalamycinone is shown in the left reaction. To the right, the reaction shows the 11-hydroxylation of aklavinone producing ε-rhodomycinone. The reaction to the right is FAD dependent whereas that to the left is not.

The modification of the glycosylated aglycone starts by the action of a methylesterase that is encoded by the genes *dau/dnr*P or *rdm*C (figure 8). These enzymes catalyze the removal of the methoxy group at the C15 position of the aglycone moieties. The free carboxylic acid is relatively unstable and decarboxylation occurs spontaneously in aqueous solution (Tanaka *et al.*, 1983). The crystal structure of RdmC (Jansson *et al.*, 2003b) contained two domains and the larger catalytic domain has a α/β hydrolase fold, typical for esterases, lipases and hydrolases (Ollis *et al.*, 1992). This enzyme catalyzes a hydroxylation reaction via a Ser-His-Asp catalytic-triad (Jansson *et al.*, 2003b). *dnrK/dauK* encodes for an O-methyltransferase (Dickens *et al.*, 1995; Madduri and Hutchinson, 1995a; Miyamoto *et al.*, 2000; Niemi *et al.*, 2002) which produces the O-methyl functionality in doxorubicin/daunorubicin biosynthesis (figure 8). A homologous gene, *rdm*B is present in the corresponding position in the *rdm* gene cluster. RdmB acts as a hydroxylase and catalyzes the addition of the hydroxyl group at the C10 position of 15-demethoxyaclacinomycin (Niemi *et al.*, 2002; Wang *et al.*, 2000). DnrK/DauK and RdmB share 55% sequence homology (Dickens *et al.*, 1995; Madduri *et al.*, 1993) but RdmB showed no methyltransferase activity either in the original strain *S. purpurascens* or in the recombinant RdmB producing strains like
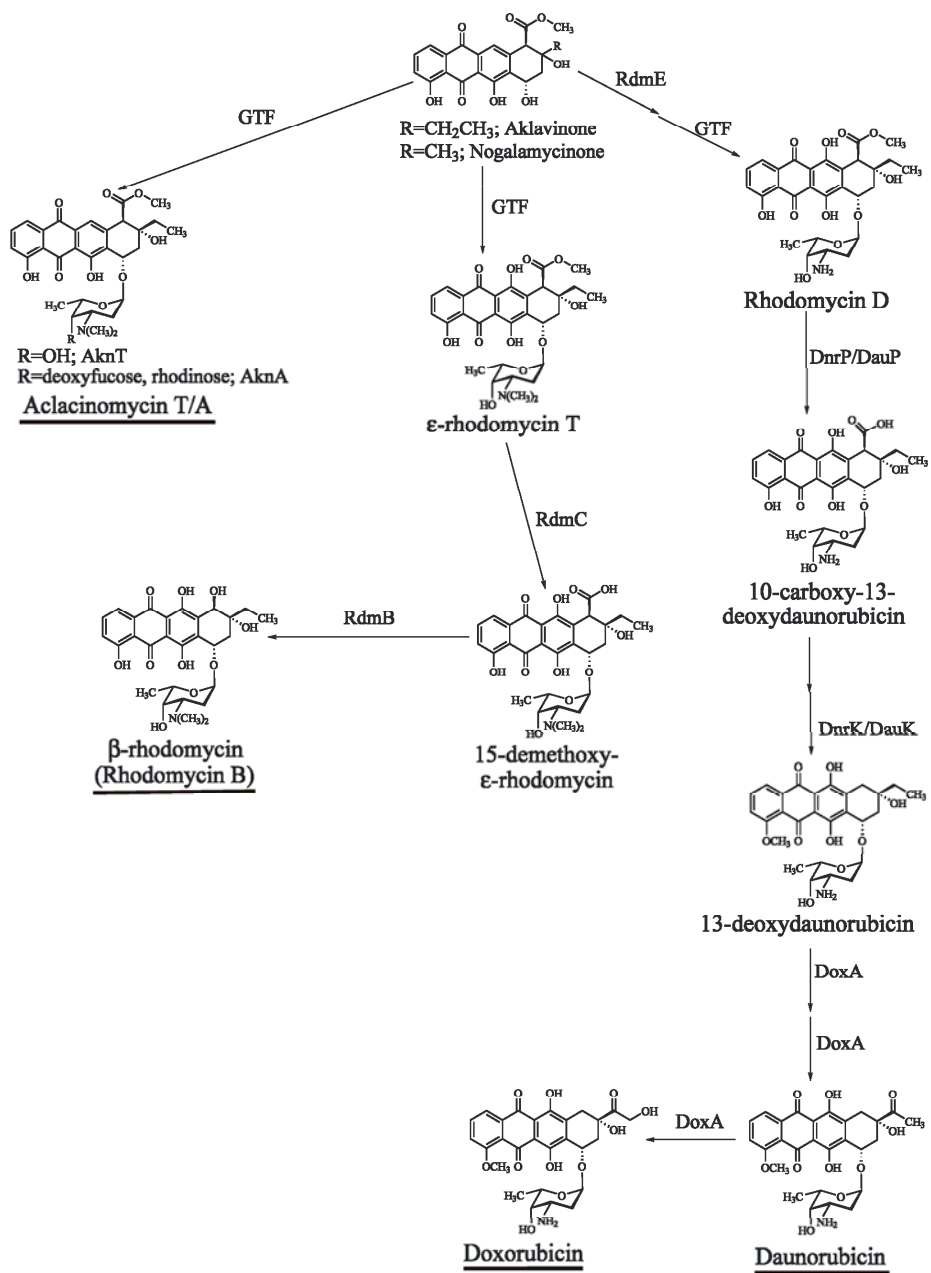
Figure 8: The modifications of postglycosylated aglycones are shown sequentially from different *Streptomyces* species: the pathway located at the left downstream from the aklavinone/nogalamycinone describes the aclacinomycin production in *S. galilaeus*; the middle part shows the β-rhodomycin production from *S. purpurascens*; the part to the right shows the reaction for doxorubicin and daunorubicin production in *S. peucetius* and *S. sp C5*. The final products from different pathways are underlined.

S. galilaeus (Wang *et al*., 2000). The crystal structures of RdmB and DnrK as binary and ternary complexes have been determined recently which showed very similar structural folds for these two enzymes. However, the structural and functional investigation of RdmB clearly revealed a particularly striking example of divergent evolution in an enzyme family (Jansson *et al*., 2005; Jansson *et al*., 2004; Jansson *et al*., 2003a). The last three reactions in doxorubicin biosynthesis are catalyzed by the gene product of *dox*A which is a cytochrome P450 monooxygenase. DoxA catalyzes the oxidation of the 13-positon to a hydroxyl and eventually to a keto group, as well as converting the 14-position into a hydroxyl functionality (Niemi *et al*., 2002; Walczak *et al*., 1999) (figure 8).

1.4.4: Glycosylation: biosynthesis and tranfer of the sugar moieties

Anthracycline compounds contain different sugar moieties which are usually attached to the C7 or C10 position (also C1 and C2 in case of nogalamycin) of the aglycone core (Lown, 1993). These sugars are attached by the tailoring enzymes of the biosynthetic pathways. Glycosylation of anthracyclines is required for antimicrobial and antitumor activity, specifically the presence of an amino group in the polyketide core or in the sugar moiety is important for the biological activity of anthracyclines (Fujii and Ebizuka, 1997).

Glucose is the source of the deoxysugar moieties found in anthracyclines. The sugar units are mainly synthesized as thymidine diphospho (dTDP) derivatives (Niemi *et al*., 2002). A number of genes are involved in the biosynthesis of these glycosyl units and most of them are cloned and characterized (Niemi *et al*., 2002; Trefzer *et al*., 1999) (Table 1).The biosynthesis process starts with the reactions catalyzed by dTDP-glucose thymidylyltransferase and two dehydratases which produce a common intermediate (dTDP-4, 5-ketoglucose) (Draeger *et al*., 1999) for all the amino sugars and the neutral sugar for aclacinomycin and rhodomycin biosynthetic pathways (figure 9). The three dimensional structure of dTDP-glucose 4, 6 dehydratase from *S. venezuelae* was determined in 2004 (Allard *et al*., 2004) and this enzyme is homologous to the 4, 6 dehydratase involved in sugar biosynthesis for anthracycline compounds (Niemi *et al*., 2002). Different amino (with primary or secondary amine) and neutral sugars (no amino group) are synthesized from the intermediate, dTDP-4, 5-ketoglucose by the action of several enzymes, like epimerases, ketoreductases, aminotransferases, and methylases (in aclacinomycin and rhodomycin biosynthesis) (Niemi *et al*., 2002).

The neutral sugar, nogalose, is however synthesized from an earlier intermediate (dTDP-4-keto-6-deoxyglucose) and three methyltransferases are involved for the O-methylation in nogalose (Niemi *et al*., 2002; Torkkell *et al*., 2001) (figure 9).
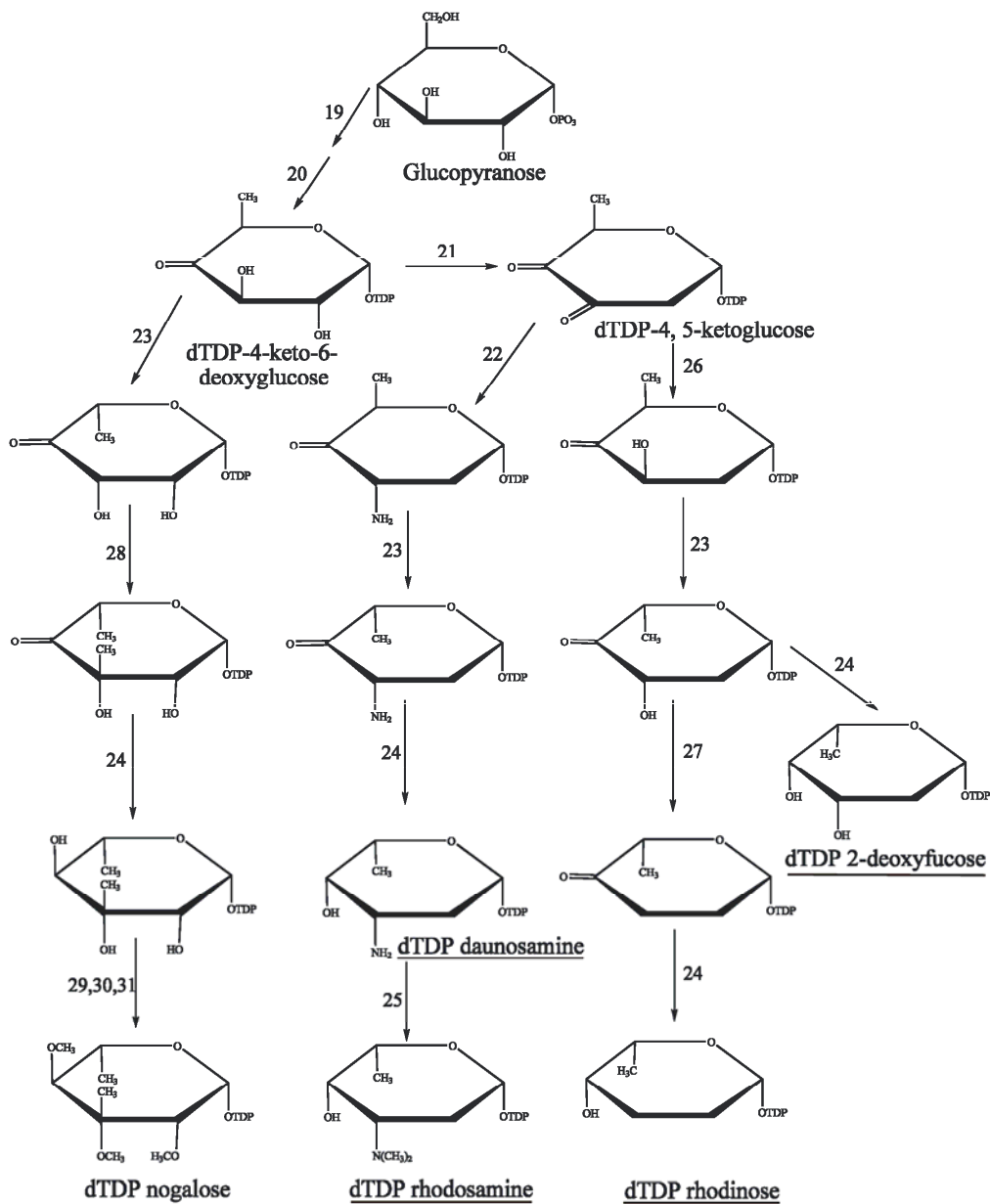
Figure 9: Biosynthesis of amino sugar and neutral sugar moieties in different anthracycline biosynthetic pathways. The biosynthetic enzymes are numbered and listed according to Table 1. The final sugar moieties for different anthracyclines are underlined.

The dTDP-sugar is attached to the aglycone or a previously attached sugar unit by a glycosyltransferase. Two glycosyl transferases (GTF) are present in the *dau/dnr* and *akn* clusters (Olano *et al*., 1999; Raty *et al*., 2000; Scotti and Hutchinson, 1996), whereas the *sno* cluster contains three GTF (Oki, 1977; Olano *et al*., 1999; Scotti and Hutchinson, 1996; Torkkell *et al*., 2001; Torkkell *et al*., 1997) (Table 1). TDP-daunosamine and rhodosamine are the sugar derivatives attached to the C-7 of the aglycone (Oki, 1977; Olano *et al*., 1999; Scotti and Hutchinson, 1996) during doxorubicin/daunorubicin and rhodomycin biosynthetic pathway respectively. Aclacinomycins contain a trisaccharide moiety attached at the same position as in doxorubicin /daunorubicin and the first triglycoside product (aclN) contains a rhodosamine, a deoxyfucose and a rhodinose (Oki *et al*., 1975; Ylihonko *et al*., 1994). Nogalamycin has a different glycosylation profile considering the presence of an amino sugar, nogalamine connected to the oxygen atoms at C1 and C2 of the aglycone by an unusual carbon-carbon bond (Torkkell *et al*., 2001) as well as a neutral sugar, nogalose to the C-7 position of the aglycone (Niemi *et al*., 2002).

In the aclacinomycin biosynthetic pathway, the postglycosylation modification is carried out by an oxidoreductase (AknOx) which modifies the terminal sugar of the trisaccharide moiety from rhodinose to cinerulose A and then to L-aculose (Madduri and Hutchinson, 1995b; Yoshimoto *et al*., 1979).

1.5 Approaches for the production of new and modified anthracyclines

Anthracycline antibiotics have a wide range of clinical and commercial significance because of their antineoplastic properties. However, their use in the treatment of cancer exhibits severe side effects. The compounds mainly cause cardiotoxicity by generating free radicals (Mordente *et al*., 2001). Moreover, limitation for using anthracyclines in clinical practice is also caused by the emerging resistance to these anti-cancer drugs which ultimately leads to treatment for a prolonged time (Priebe and Perez-Soler, 1993). Several chemical and genetic approaches have been used in order to produce new and modified anthracyclines which share the same activity as the parent compound at a more efficient level, with less toxicity, absence of cross resistance or activity against tumor cells insensitive to the parent drugs (Wadler *et al*., 1986). Some of the most commonly used approaches are described as follows.

1.5.1 Production of new and semisynthetic derivatives

During last 20 years, more than 1000 different anthracycline analogues have been obtained in several ways, such as isolating from soil samples or rational synthesis. A number of these compounds have been successfully used for chemotherapy (Weiss *et*

al., 1986). Carminomycin was isolated from the mycelia of a new actinomycete species, *Actinomadura carminata* in 1980s (Debusscher *et al*., 1985; Gause *et al*., 1974; Monneret, 2001) and used as one of the commercially available chemotherapeutic drugs. In another approach to produce new compounds, the parent *Streptomyces* organisms were subjected to various techniques for inducing mutations and genetic code modifications. Doxorubicin is a good example of this approach, because it is produced from a mutant strain of the daunorubicin producing *Streptomyces* (Ghione, 1975).
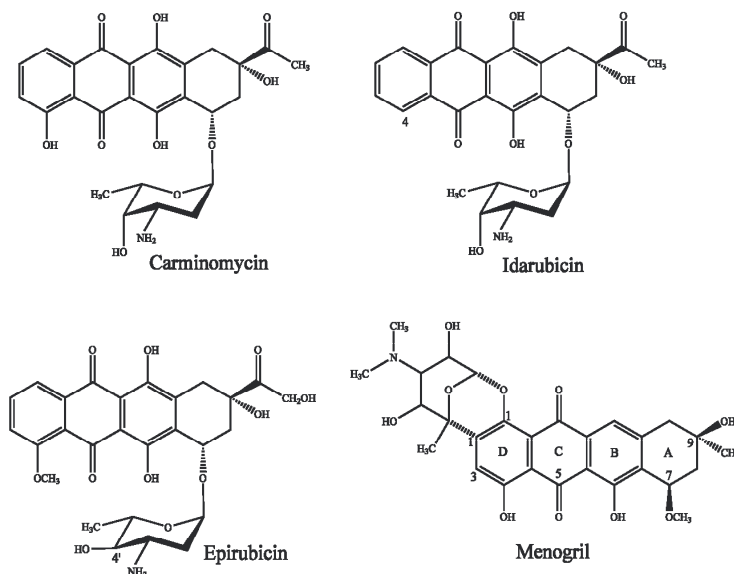
Figure 10: Structure of several semisynthetic derivatives of anthracycline compounds.

A number of structurally modified compounds are also produced based on the chemical modification of the most commonly used anthracyclines, doxorubicin, daunorubicin or nogalamycin (Cassinelli *et al*., 1984; Ferrari *et al*., 1984; Muggia and Green, 1991). Epirubicin (4' epidoxorubicin) (Cassinelli *et al*., 1984; Ferrari *et al*., 1984; Wadler *et al*., 1986) and Idarubicin (4-demethoxydaunorubicin) (Arcamone *et al*., 1976; Cersosimo, 1992) (figure 10) are some of the clinically useful semisynthetic derivatives, which exhibit increased antitumor efficiency compared to their parent compounds with less cardiotoxicity. Menogaril, a derivative of nogalamycin (the amino sugar is attached to the ring A instead of ring D) (Wiley *et al*., 1977a) (figure 10) showed high cytotoxic effect on the leukemia cell-line with reduced side effects (Dorr *et al*., 1986; Schrijvers *et al*., 2002) and proceeded upto phase II clinical trial (McGovren *et al*., 1984; Obasaju *et al*., 2001).

1.5.2 Combinatorial biosynthesis of hybrid antibiotics

Combinatorial biosynthesis of 'hybrid antibiotics' is a process which involves interchanging secondary metabolism genes between antibiotic-producing organisms to create an unnatural or 'mixed' gene combination. This approach has been studied extensively for the anthracycline biosynthesis during the last two decades (Hopwood, 1993; Hopwood *et al.*, 1985; Strohl and Connors, 1992). Hybrid antibiotics are the result of the combinatorial biosynthesis using genes from two or more antibiotic producing strains that produce structurally related antibiotics.

These compounds usually combine the structural features from more than one antibiotic that are not produced by the same organism (Hopwood, 1981; Hopwood *et al.*, 1985).The production of hybrid antibiotics was first reported by Hopwood *et al* in 1985. Actinorhodin biosynthetic genes from *Streptomyces coelicolor* A3(2) were transferred into the medermycin producer *Streptomyces sp.* AM-7161 resulting in the production of mederrhodins A and B (Hopwood *et al.*, 1985; Omura *et al.*, 1986).

In efforts to produce hybrid metabolites, the early biosynthetic genes for actinorhodin biosynthesis were cloned into different anthracycline producing *Streptomyces* strains which resulted into the production of a new compound aloesaponarin II (Strohl and Connors, 1992).

The tailoring enzymes of the anthracycline biosynthetic pathways were also targeted for the combinatorial biosynthesis approach. *Streptomyces galilaeus* has been selected as an ideal host for this study because aklavinone, the aglycone moiety from this strain is a precursor of aglycones in most of the anthracycline biosynthetic pathways (Eckardt and Wagner, 1988; Niemi *et al.*, 1994). Therefore, this compound is acceptable as a substrate for the aglycone modifying enzymes from other biosynthetic gene clusters. The production of hybrid antibiotics by using the tailoring enzymes were first applied by transforming the gene cluster for the late acting enzymes from *Streptomyces purpurascens* to *Streptomyces galilaeus* resulting in some aclacinomycin-type products with a modified rhodomycin-type aglycone and glycosylation profile determined by the host, *S. galilaeus.* The glycosides of 10-demethoxycarbonylaklavinone and 11-deoxy-β-rhodomycinone were isolated as novel hybrids and three glycosides (DbrA/DbrT/DbrB) of the latter aglycone showed cytotoxic activity against L1210 mouse leukemia cells (Jansson *et al.*, 2004; Niemi *et al.*, 1994).

In another attempt to produce hybrid compounds, the early biosynthetic genes from *Streptomyces nogalater* were introduced into the host strain of *S. lividans* TK24 and the resulting products exhibited the structural features that could be associated with either parent strain (Kunnari *et al.*, 1999; Ylihonko *et al.*, 1996a). A successful example of the hybrid approach was the transformation of the *S. galilaeus* ATCC31133 with a plasmid containing the aklavinone-11-hydroxylase gene (*dnrF*) from *S. peucetius* which resulted in a derivative of aclacinomycin A hydroxylated at the position C11(Hwang *et al.*, 1995; Kim *et al.*, 1996).

Redesigning of the biosynthetic enzymes is another approach which was considered important for producing modified compounds. It has been shown that bacterial aromatic PKSs can be engineered to synthesize rationally modified aromatic polyketide (Hutchinson and Colombo, 1999; Lee *et al*., 2005b; Metsa-Ketela *et al*., 2003; Shen *et al*., 1999; Tang *et al*., 2004). Recently, a non-acetyl primed analogue of aklanonic acid was produced by an engineered bimodular aromatic polyketide synthase (Lee *et al*., 2005b).

1.6. Aim of the thesis

The extensive use of anthracyclines in oncology makes them clinically very important. In order to produce modified anthracyclines with improved toxicological activities, precise knowledge about the mechanism of the biosynthetic enzymes is necessary. Investigation of the three dimensional structure can provide specific insights about the catalytic properties and substrate specificity of the enzymes which can be used for further approaches for redesigning the biosynthetic enzymes in the near future.

The aim of this thesis is focused mainly on structural studies of several biosynthetic enzymes by X-ray crystallography. The structural investigation of the enzymes provided necessary information for understanding their catalytic properties and mechanism of action. The first part of the thesis describes the structural and functional features of two cyclases from two different *Streptomyces* species. The following part is focused on the crystal structure and functional aspects of an oxidoreductase from the aclacinomycin biosynthetic pathway.

2 Results and Disscussion

2.1 The polyketide cyclases: SnoaL and AknH : (Paper 1, 2 and 3)

SnoaL (Raty *et al*., 2002; Torkkell *et al*., 2000) and AknH (Fujii and Ebizuka, 1997; Kantola *et al*., 2000; Raty *et al*., 2002) are members of a polyketide cyclase family which are involved in the last ring-closure step of the tetracyclic ring system of the polyketide core in nogalamycin (*S. nogalater*) and aclacinomycin (*S. galilaeus*) biosynthetic pathways respectively (figure 1 in paper 2). SnoaL converts nogalonic acid methyl ester (NAME) to nogalaviketone while AknH uses aklanonic acid methyl ester (AAME) as substrate and produces aklaviketone. These enzymes consist of 144 amino acid residues with a molecular weight of 20 KDa and share 67% sequence identity with each other as well as >60% identity with other corresponding polyketide cyclases from doxorubicin (DnrD) (Kendrew *et al*., 1999), daunorubicin (DauD) (Dickens *et al*., 1995) and rhodomycin (RdmA) (Niemi and Mantsala, 1995) biosynthetic pathways. However, the products have differences in their C-9 stereochemistry and these fourth-ring closing polyketide cyclases are responsible for this stereoselectivity of the products (Kendrew *et al*., 1999). SnoaL is unique in this class of polyketide cyclases because the product of the SnoaL catalyzed reaction has a 9S,10R stereochemistry whereas AknH as well as most other characterized members of this polyketide cyclase family produces 9R,10R stereochemistry of the product (Torkkell *et al*., 2000). However, besides nogalamycin, steffimycin is also known to have 9S configuration at the C9 position (Arora, 1985a) and recently the biosynthetic gene cluster of steffimycin has been characterized (Gullon *et al*., 2006).

Understanding of these polyketide cyclases in the biosynthesis of aromatic polyketide biosynthesis is important for the rational designing of novel compounds because the timing and regio-or stereoselectivity of cyclization is a significant determinant of the final natural products. However, before the work described in this thesis, not much was known about the functional properties of these polyketide cyclases, i.e. their catalytic properties as well as determinants of stereo-or regiospecificity during the cyclization reactions.

2.1.1 Crystallization and structure determination of cyclases

Crystallization trials of SnoaL with the substrate NAME and data collection statistics were described in paper 1. For phase determination, single isomorphous replacement with anomalous scattering (SIRAS) based on a uranium derivative of SnoaL was used. Information about the position of the heavy atoms was obtained by inspecting the difference Patterson map and two uranium ions were found, bound to the side chains of Glu6 and Asp30 in SnoaL. The electron density map to 1.87Å resolution was used for

automatic model building (Perrakis *et al*., 1999) which allowed building an almost complete (139 out of 144 amino acids) protein model. The diffraction data from the high-pH crystal form to 1.35Å resolution was used during the refinement process (paper 2).

Two mutants of SnoaL (Asp121Asn and Gln105Ala) were crystallized in different conditions compared to the native one. Crystals were obtained in a PEG/ION screen which uses 20% PEG3350 as precipitant with different salts. Conditions with 0.2M tri potassium citrate monohydrate and 0.2M lithium chloride anhydrous resulted in crystals from Asp121Asn and Gln105Ala mutants respectively. Diffraction data were collected from these crystals to 1.63Å (Asp121Asn) and 1.9Å resolution (Gln105Ala) respectively after flash-freezing with 25%PEG550 cryo-solution. Details about the data collection statistics are listed in Table 2.

Table 2 : Data collection statistics of two SnoaL mutants (Asp121Asn and Gln105Ala) and the SnoaL-nogalamycinone (Nog) complex.

Parameters	Asp121Asn	Gln105Ala	SnoaL-Nog
Beam line	I-711	I-711	I-711
Wavelength (Å)	1.094	1.094	1.094
Resolution (Å)	27.19-1.63	29.32-1.9	22.72-1.8
Space group	I222	$P2_12_12$	I222
Unit cell parameters (Å)			
a	64.94	64.09	69.24
b	71.93	68.45	72.17
c	68.98	72.61	65.37
No. of observations	113286	161522	112501
No. of unique reflections	19725	23282	15492
R_{sym} (%)[a]	4.1 (13.4)	5.5(19.9)	6.3(10.9)
Completeness (%)	96.3(96.3)	99.9(100)	99.8(99.8)
Mean I/σ (I)	15.4 (10.9)	21.5(8.0)	27.5(14.9)
Wilson B factor ($Å^2$)	16.31	19.56	16.11

In an attempt to understand the substrate specificity of SnoaL, it was co-crystallized with nogalamycinone (Nog) (figure 16), which is the aglycone moiety from the nogalamycine biosynthetic pathway. The crystal was flash-frozen with 25% ethylene glycol and a dataset was collected to 1.8Å resolution (Statistics are given in Table 2). The structural features of this complex are discussed in the chapter 2.1.6.

The structure of mutants and the SnoaL-Nog complex were determined by molecular replacement using the structure of SnoaL-NAME complex as the template. Details about the refinement statistics are shown in Table 3.

Table 3 : Data refinement statistics of SnoaL mutants and SnoaL-nogalamycinone complex.

Parameters	SnoaL Asp121Asn	SnoaL Gln105Ala	SnoaL-Nog
Resolution (Å)	24.89-1.63	29.32-1.9	49.93-1.8
R_{work} (%)	17.8	18.7	18.8
R_{free} (%)	20.6	23.5	22.8
Protein atoms	1203	2382	1189
Ligand atoms	0	75	29
Water molecules	193	293	142
B factor ($Å^2$)			
Protein atoms	14.43	19.94	15.29
Ligand atoms	0.0	38.72	27.11
Solvent	32.0	35.73	25.31
Deviations from ideals (r.m.s.d.)			
Bond length (Å)	0.012	0.011	0.011
Bond Angle (°)	1.38	1.34	1.36
Ramachandan Plot (%)			
Most favoured region	89.2	87.3	87.4
Additional allowed region	9.2	11.0	11.0
Generously allowed region	0.8	0.8	0.8
Disallowed region	0.8	0.8	0.8

AknH can also use NAME, the substrate of SnoaL but produces auraviketone with C9-R stereochemistry (Kantola *et al.*, 2000). AknH was therefore co-crystallized with NAME and aklavinone and the datasets were collected to 2.1Å and 1.9Å resolution respectively. The structure of the AknH-NAME complex was determined by molecular replacement using the SnoaL structure as template and the AknH-NAME structure was further used to obtain the structure of the AknH-aklavinone complex (paper 3).

2.1.2 The tertiary and quaternary structure of SnoaL

The monomer of SnoaL belongs to the α+β class of structures. The core of the monomer contains a central five-stranded mixed β sheet. Besides B1, the N-terminal part (residue ranges 2-26 and 34-59) consists of four helices, two α-helices and two 3_{10} helices. The curved central β-sheet and three N-terminal helices (H1, H2 and H4) form a barrel-like structure (figure 11). The polypeptide chain followed by B5 folds into one C-terminel α-helix (H5) which is a part of the α+β barrel and is extended with a long loop region.
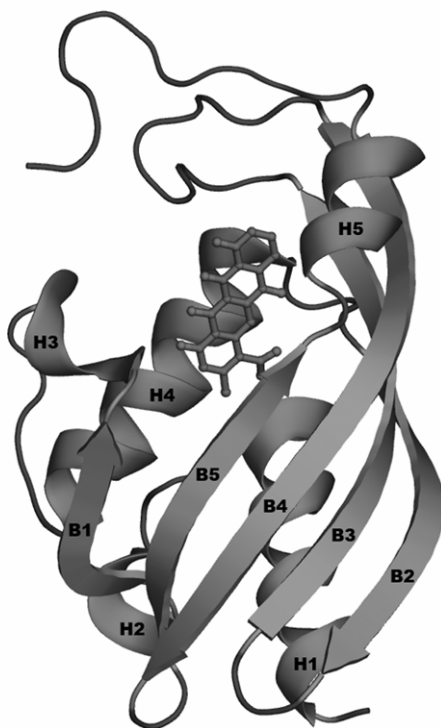

Figure 11: The tertiary structure of SnoaL. The bound

ligand is shown as ball-stick representation

The quaternary structure of SnoaL is a tetramer both in crystal-packing and in solution. In the crystal, the tetramer is formed as a dimer of dimers. Two monomers form a tight-interface dimer and are related by one of the crystallographic two-fold symmetry axis. Two of these dimers form another dimer interface along another two-fold axis, thus generating the tetramer (figure 5 in paper 2).

The formation of the dimer also allows complete burial of the active sites with the bound ligand totally inaccessible to the solvent. Therefore, this feature would strongly suggest a conformational change during the binding or release of the substrate/product. A loop region between β3 and β4 and the C-terminal loop region (figure 11) was suggested as the most probable sites for conformational change during ligand binding.

2.1.3 Ligand binding features and active site

SnoaL was co-crystallized with the substrate NAME, but the electron density map clearly showed the presence of a closed ring compound which indicated that cyclization of the substrate took place during the crystallization. The ring A of the compound was also observed in a planar conformation with the absence of the hydroxyl group, which resembles a product derivative bound in SnoaL, formed upon the elimination of water from nogalaviketone (figure 16). This red-coloured dehydrated product analogue was observed previously during the reaction studies with DnrD (polyketide cyclase from doxorubicin biosynthetic pathway), which is a homolog of SnoaL (Kendrew *et al*., 1999; Kunnari *et al*., 1999). The colour of the SnoaL crystals being dark-pink instead of the expected yellow (colour of NAME/nogalaviketone) further indicates the presence of the dehydrated compound.

In the structure, the ligand was bound in the interior of the α+β barrel, that mainly consists of hydrophobic residues. The protein-ligand interactions in SnoaL are mainly dominated by van der Waals and hydrophobic interactions (figure 7 in paper 2). H-bonding interactions with the product derivative include only one direct interaction with Gln105 and two water-mediated H-bonds to Thr128 and His39. Two residues from the second monomer of the tight-interface dimer also participate in ligand binding through water mediated H-bonding bridges (figure 7 in paper 2).

The side chain of Asp121, invariant in this class of polyketide cyclases, was located in the vicinity of the C9 carbon of the ring A and was proposed as the main catalytic residue. Asp121 was H-bonded with Asn33 which might be involved in maintaining the specific conformation of Asp121 during catalysis. This proposal was further emphasized by modeling nogalaviketone in the structure which clearly showed Asp121 being ideally positioned to form H-bonding interaction with the C9 hydroxyl group (figure 12).
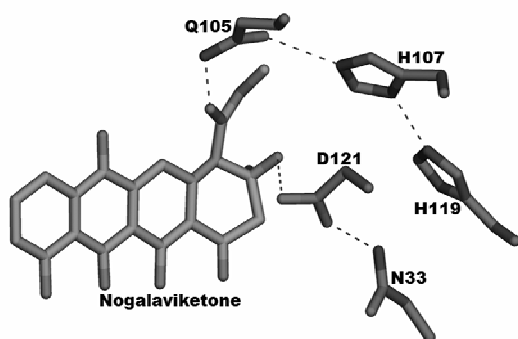
Figure 12: The modeled nogalaviketone, product of SnoaL is modeled in the SnoaL active site. The product is shown is grey and the active site residues are shown in black.

Gln105 was also considered as an important residue in the active site due to its direct interaction with the bound ligand. The H-bonding bridges observed among Gln105-His107-His119 might be necessary to stabilize the specific conformation of Gln105 (figure 12).

2.1.4 Structural features of the SnoaL mutants:

The structure of the SnoaL Asp121Asn mutant was determined to 1.63Å resolution. The overall structural fold and the active site conformation was the same compared to the native structure. Although this mutant was co-crystallized with NAME, no density for the bound ligand was observed in the active site (figure 13), indicating a lower affinity of the mutant for the substrate/product.

Figure 13: Electron density of the active site SnoaL Asp121Asn mutant.

The structure of the Gln105Ala mutant to 1.9Å resolution contains two molecules in the asymmetric unit, which were arranged as a dimer. The quaternary structure of Gln105Ala is a tetramer as observed in the wild-type SnoaL and it is formed by generating the symmetry-related molecules in the crystal packing. The dimer in the

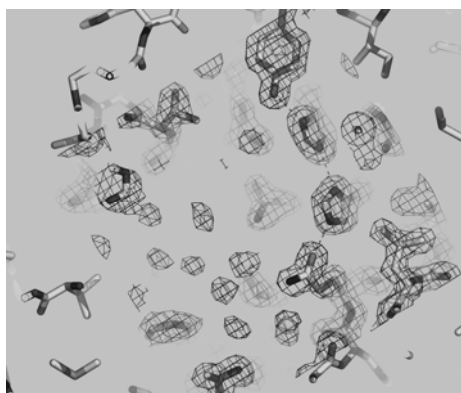asymmetric unit resembles the tight-interface dimer part of the tetramer. The ligand was bound as the dehydrated product derivative in both molecules. However, investigation of the ligand binding site in these two molecules showed significant differences in the loop region including residue 38-41. In molecule A, His39 was H-bonded directly to the O21 atom of the ligand. It is noteworthy that His39 was involved in ligand binding in the wild-type structure through water-mediated H-bridges rather than direct binding (figure 14a). Due to the conformational changes in the molecule B, His39 is pointing in the opposite direction from the ligand and the space between the loop region and ligand is filled by a PEG molecule from the crystallization condition (figure 14b) which was H-bonded to the O22 of the ligand. The altered conformation of the His39 in molecule B and the presence of the PEG can be described as a crystal artifact.

A   B

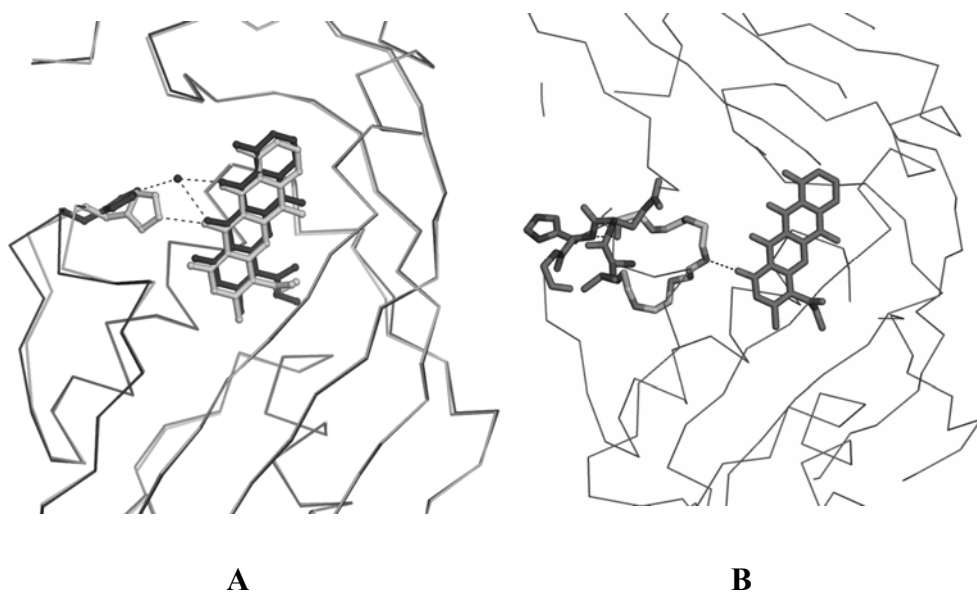Figure 14a and 14b: The ligand binding site of SnoaL Gln105Ala mutant in two molecules in the asymmetric unit. The native SnoaL (black) superimposed on molecule A of SnoaL Gln105Ala mutant (grey) is shown in 14a. The ligand and His39 are shown with same colour representation. Figure 14b shows the ligand binding site in molecule B. His39 as well as ligand are shown in dark grey whereas the PEG molecule is shown in light grey.

2.1.5 AknH-NAME complex

The overall fold of the AknH structure is very similar to that of SnoaL. The only structural differences observed were in two loop regions which are the loop comprising residues 40-43 between H3 and H4 and the C-terminal loop (figure 4 in paper 3). The quaternary structure of AknH is a tetramer with the same arrangement as reported for the SnoaL tetramer (figure 3b in paper 3). The electron density for the bound ligand represented the dehydrated product analog as observed in SnoaL which was mainly bound through hydrophobic interactions as well as three water mediated H-bonding bridges (figure 15). However, comparison with the SnoaL ligand binding site reveals a completely different orientation of the bound ligand in AknH in the same hydrophobic pocket (figure 4 in paper 3). Ring A is located far away from the proposed active site residues Asp121, Gln105, His107 (figure 15) which were crucial for catalysis according to the mutagenesis of these residues (Table 1 in paper 3) Therefore, the ligand binding mode in the AknH-NAME complex appears not catalytically competent and is considered as an artifact after the ring closure/dehydration step.
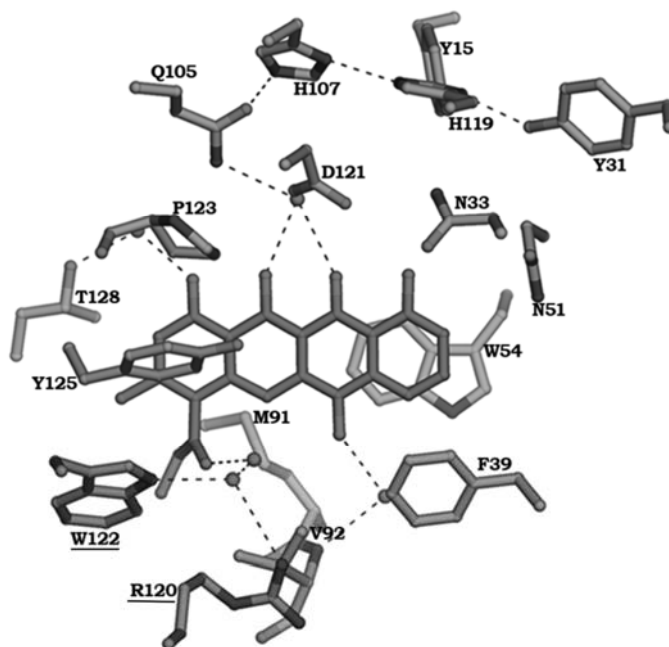
Figure 15: Ligand binding site of the SnoaL-NAME complex.
The residues from the adjacent monomer are underlined.

2.1.6 AknH-AKV (aklavinone) complex

In an attempt to study the active site of AknH more extensively, as well as, to entrap a compound with the C9 hydroxyl group, the structure of AknH in complex with aklavinone (AKV) was determined. Aklavinone is the aglycone moiety from *Streptomyces galilaeus* which has a hydroxyl group at the C7 (figure 16).

(1) Nogalaviketone

(2) Aklaviketone

(3) Nogalamycinone

(4) Aklavinone
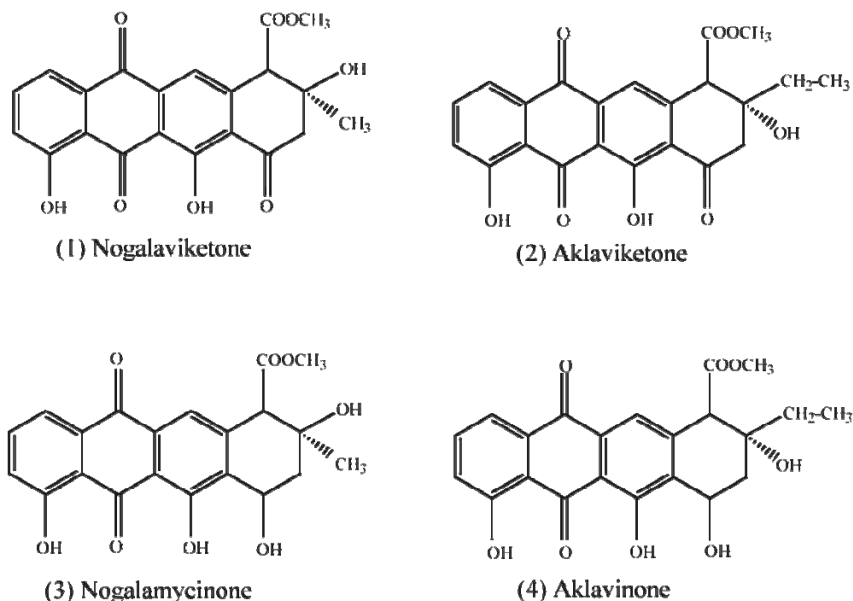
Figure 16: Nogalaviketone and Aklaviketone are the two products of the SnoaL and AknH catalyzed reaction respectively. These compounds could not be trapped in the SnoaL/AknH structures due to the formation of the dehydrated analogue by the elimination of water from the C9 position. Therefore, the aglycones, nogalamycinone and aklavinone were used for co-crystallization with SnoaL and AknH respectively in order to obtain a bound ligand with the C9 hydroxyl group. The reduced OH group at the C7 of the aglycones prevents the non-enzymatic loss of the C9-OH group.

This modification will prevent the aromatization after dehydration and thus disfavours the non-enzymatic loss of the hydroxyl group at the C9. Surprisingly, the bound ligand represented an open ring compound, formed due to the reversal of the catalytic reaction (figure 17). Notably in this case, the ligand orientation was similar to that observed in the SnoaL complex (Figure 4 in paper 3). The H-bonding patterns for ligand binding
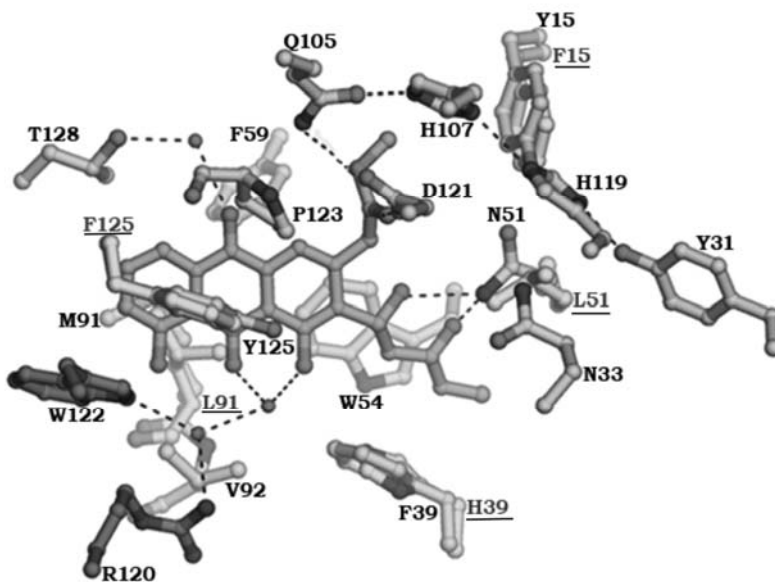
Figure 17: The ligand binding site in AknH-AKV complex. The amino acids
from the second monomer of the dimer are shown in black. The residues that
are different in SnoaL are superimposed on the corresponding residues in
AknH are underlined.

resemble the H-bonding interactions observed in the SnoaL-NAME complex which
includes similar direct H-bonding with Gln105 and several water-mediated interactions
with Thr128 as well as two residues from another monomer of the tetramer (figure 17).
The catalytically important residues (Asp121, Gln105) as well as most other polar
residues were positioned in the vicinity of the atoms that form the ring A which further
suggested this ligand orientation to be favourable for catalysis.
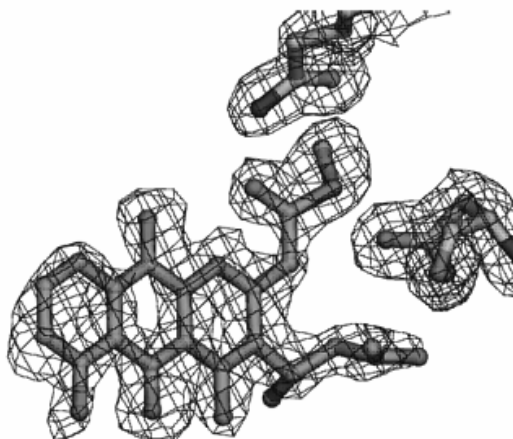
Figure 18: The electron density of the
open ring species bound in the SnoaL-
nogalamycinone complex

The corresponding complex of SnoaL with nogalamycinone (Nog) (figure 16) also showed the bound ligand as ring A opened (figure 18) as observed in the AknH-AKV complex.

2.1.7 The proposed reaction mechanism for the polyketide cyclases

The type of reaction catalyzed by SnoaL or AknH is an intramolecular aldol condensation. This class of reaction involves condensation of two carbonyl compounds with exquisite control of the stereochemistry of the product. Therefore, aldol condensation has been considered as an important reaction in synthetic chemistry (Cooper *et al*., 1996). The crucial step in this type of reaction is the abstraction of a proton from the C-H group followed by the formation of an enolate/carbanion intermediate.

2.1.7.1 Aldol condensation in different aldolases

The aldolases are present in most living organisms (Gefflaut *et al*., 1995) and are widely known for their potential in the field of biotransformation and synthetic organic chemistry like the synthesis of novel antibiotics (Barbas *et al*., 1997; Wagner *et al*., 1995). They have been classified into two classes based on the catalytic mechanism (Rutter, 1964).

The class I aldolases contain a catalytic lysine residue that forms a Schiff base with the substrate carbonyl carbon in order to stabilize the intermediate (figure 19a) (Gefflaut *et al*., 1995). The structures of several class I aldolases have been determined previously which include human and *E.coli* transaldolases (Jia *et al*., 1996; Thorell *et al*., 2000), fructose-6-phosphate aldolase (Thorell *et al*., 2002) and fructose 1,6-bisphosphate aldolases from different sources like human muscle (Dalby *et al*., 1999; Gamblin *et al*., 1991), rabbit muscle (Sygusch and Beaudry, 1984), *Drosophila melanogaster* (Hester *et al*., 1991) and Archaea (Lorentzen *et al*., 2003). The class II aldolases, also known as metalloaldolases are found mostly in lower eukaryotes and bacteria. They require a divalent metal ion usually zinc or magnesium, which is coordinated to the substrate during catalysis (Morse and Horecker, 1968). This active center zinc ion acts as a Lewis acid and polarizes the carbonyl bond of the ketose substrate (Hall *et al*., 1999) (figure 19b). The first structure of a class II aldolase, L-fuculose-1,5-bisphosphate aldolase was published in 1996 (Dreyer and Schulz, 1996) which was followed by the structure of *E.Coli* type II fructose 1,6-bisphosphate aldolase published in the same year (Blom *et al*., 1996; Cooper *et al*., 1996). These two enzymes use a zinc cofactor for stabilizing the intermediate (Dreyer and Schulz, 1996; Hall *et al*., 1999). However, 2-dehydro-3-deoxy-galactose aldolase is another class II aldolase which uses a magnesium ion as cofactor. The crystal structure of this enzyme in holo/apo form suggested a novel mechanism compared to other class II aldolases which involves a

phosphate group acting as a proton acceptor for generating the carbanion intermediate and a water molecule connected to a histidine residue as a proton donor/acceptor in the second step of the reaction (Izard and Blackwell, 2000).

C.
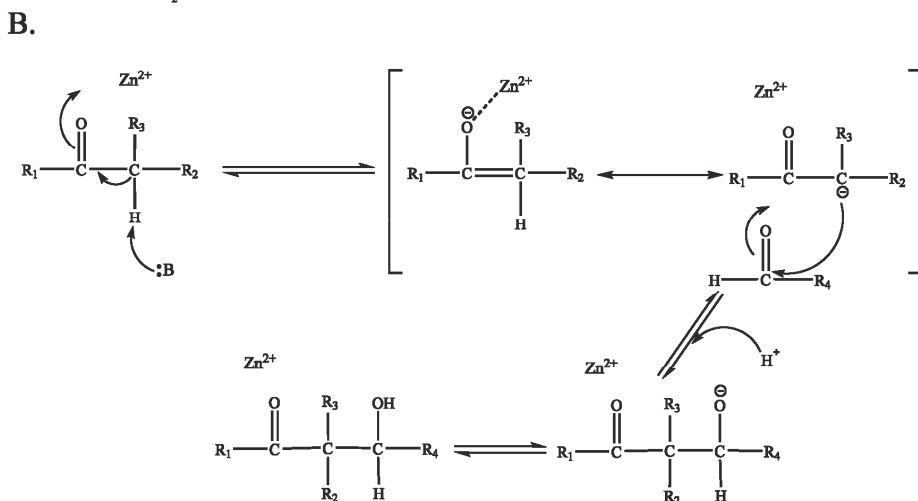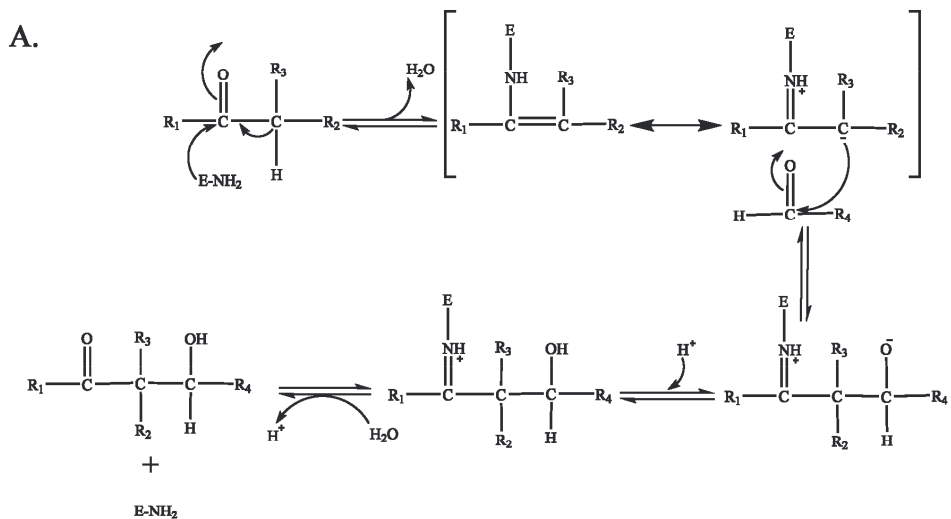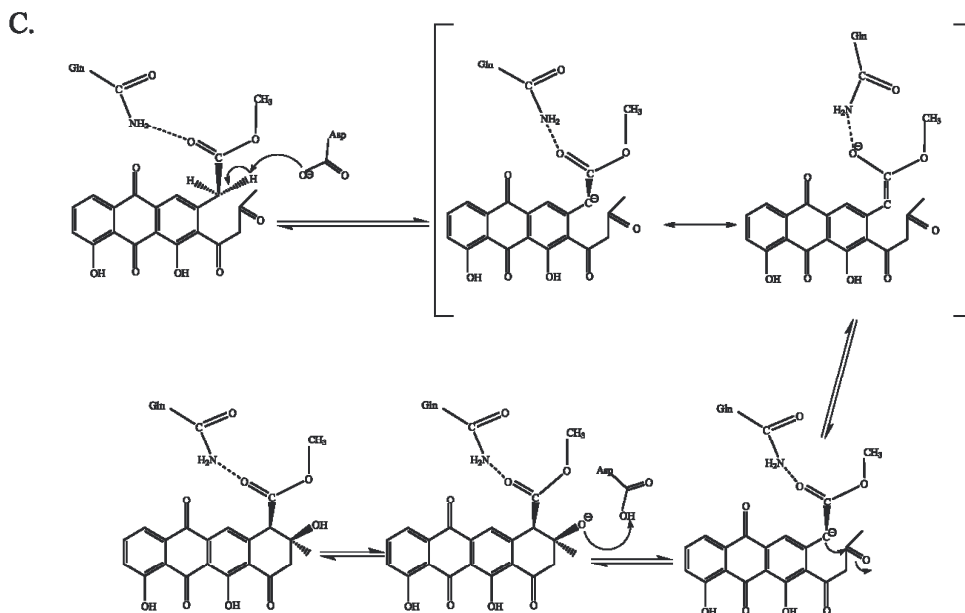
Figure 19: Different types of aldol condensation reactions. (A) Class I aldolases through Schiff's base formation; (B) Class II aldolases via metal dependent mechanism and (C) the mechanism through acid-base chemistry in SnoaL

2.1.7.2 Novel reaction mechanism proposed for SnoaL

The structural investigation of SnoaL excludes the possibility of the aldol condensation mechanism through the Schiff's base formation due to the absence of any lysine residue in the active site. Moreover, no electron density was observed close to the bound ligand which can represent a metal ion. The activity of SnoaL was also not affected by the use of metal chelating agent (EDTA), which indicated that SnoaL does not use the metal dependent mechanism either. Studies of the SnoaL homolog, DnrD, also previously suggested that these polyketide cyclases are neither metal dependent nor produce Schiff base intermediates (Kendrew *et al*., 1999). Therefore, the structural analysis and mutagenesis of the active site residues revealed a novel aldol condensation mechanism for SnoaL which follows simple acid/base chemistry.

Mutation of Asp121 resulted in complete inactive enzyme which confirmed the key role of this residue in catalysis. During the first step of the reaction, Asp121 abstracts a proton from the C10 position of the substrate (figure 19c). There are several possibilities for stabilizing the resulting enolate intermediate. The active site feature of SnoaL suggested the direct H-bonding of Gln105 with the C14 carbonyl oxygen of the substrate as a possible way to stabilize the intermediate. However, mutation of Gln105 leading to partial enzyme activity (Table 2 in paper 2) indicated that this residue might be involved in ligand binding but is not necessary for catalysis. The resulting enolate

can be stabilized by the delocalization of the electrons over the π system of neighboring aromatic ring system which also facilitates the proton abstraction by the catalytic base by lowering the pK$_a$ of the C-H proton compared to an aliphatic substituent. The reaction is continued by the nucleophilic attack of the carbanion intermediate onto the carbonyl carbon at C9 and the negative charge on the C9 oxygen is then stabilized by a proton transfer from Asp121 (figure 19c).

The proposed mechanism and the role of Asp121 as both proton donor and acceptor further support the stereochemical outcome of the SnoaL product. The production of the 9S isomer at the C9 position would require the nucleophilic attack from the *re* face of the aldehyde which will result the alcoholate anion to point towards the Asp121 for the subsequent protonation. On the other hand, the presence of only hydrophobic residues on the *si* face of the aldehyde omits the possibility of producing the 9R isomer of the product due to the fact that there is no residue which can act as a proton donor during the last step.

The positioning of catalytic residues (Asp121, Gln105) in the AknH-AKV complex and the mutation studies (Table 1 in paper 3) suggested a similar aspartic-acid driven catalytic mechanism of AknH as observed in SnoaL. However, one significant difference in the mechanism of AknH requires that the attack of the enolate occurs from the *si* face of the C9 carbonyl group, which is necessary for the 9R stereochemistry of the product (figure 7 in paper 3). Thus, the role of Asp121 as the proton donor in SnoaL will not hold in the case of AknH because the alcoholate anion will point away from the side chain of Asp121. One possible option for the protonation of the alcoholate anion is a crystallographically identified water molecule adjacent to Tyr15 in the model of AknH-product complex (figure 7 in paper 3).

2.1.8 Product stereoselectivity of the polyketide cyclases

The structural determinants in SnoaL and AknH which can be responsible for the stereoselectivity of the corresponding products were suggested by comparing the active sites of these two cyclases. The comparison showed two polar residues within a 6Å radius of the C9 atom which are different in these two enzymes. Tyr/Phe15 and Asn/Leu51 in AknH/SnoaL (figure 17) are positioned in the proximity of ring A of the aglycone and were subjected to single and double mutation studies in order to examine their role in the stereoselectivity of the product. Single mutation of Tyr15Phe in AknH showed 20% of the products with 9S stereochemistry while the double mutation of both Tyr15Phe and Asn51Leu in AknH showed a racemic mixture of the products (50% with 9S stereochemistry). Therefore, mutagenesis of these residues clearly indicated that Asn51 and Tyr15 are responsible for the stereoselectivity of the product in AknH.

The structure of AknH and SnoaL also supports the role of these two residues in the stereochemical outcome of the reaction. The 9R stereochemistry of the product in AknH is favoured by the direct and water-mediated interactions with Asn51 and Tyr15

respectively in the model of AknH-product complex (figure 7 in paper 3). These two residues are replaced by the Leu51 and Phe15 in SnoaL which creates a hydrophobic environment and favours the methyl group pointing towards them instead of the hydroxyl group, thus resulting in the 9S configuration in the product (figure 7 in paper 3). It is noteworthy that the two hydrophobic residues, Leu51 and Phe15 are only observed in SnoaL, which are replaced by two polar residues (Asn51 and Tyr15) in AknH and all other polyketide cyclases that form 9R conformation of the product. Therefore, from the structural point of view, it is also evident that these two polar residues in AknH play a vital role in the stereoselectivity of products in AknH.

2.1.9 Examples of divergent evolution towards different chemistry

SnoaL2 and AclR are two homologous hydroxylases from *Streptomyces nogalater* and *Streptomyces galileus* involved in nogalamycin and cinerubin biosynthesis respectively. There is genetic evidence that these enzymes are involved in the hydroxylation of C1 of the polyketide core, thus producing a 1-hydroxylated aglycone moiety (Oki, 1984; Torkkell *et al.*, 2001). They share weak sequence identity (approximately 25%) with the members of the polyketide cyclase family such as, SnoaL (Raty *et al.*, 2002; Torkkell *et al.*, 2000), AknH (Raty *et al.*, 2002) or DnrD (Kendrew *et al.*, 1999) and do not show any similarities to flavin or metal-dependent hydroxylases.

A   **B**
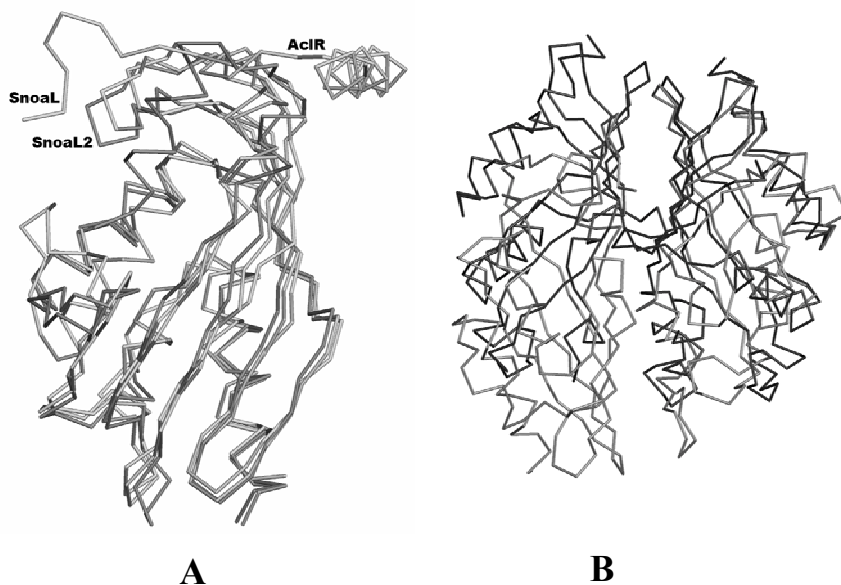
Figure 20: Superimposition of (a) the monomers of SnoaL, AclR and SnoaL2 and (b) the AB dimer of SnoaL tetramer (grey) and dimer of SnoaL2 (black).

The overall structural fold of SnoaL2 and AclR is a α+β barrel fold (Beinker *et al.*, 2006) which is very similar to the overall fold observed in SnoaL or AknH. Structural superimposition of SnoaL on SnoaL2 or AclR gave an r.m.s.d value of 2.35Å for the Cα atoms which further reflects overall structural similarity (figure 20a). The moderate degree of sequence similarity and the preservation of the fold between these cyclases and hydroxylases suggest an evolutionary relationship among them. However, the quaternary structures of Snoal2/AclR and SnoaL/AknH were considerably different. SnoaL/AknH were tetramers, described as a dimer of dimers (paper 2) whereas SnoaL2/AclR were arranged as a dimer. The packing of the dimer in the cyclases and hydroxylases were also significantly different (figure 20b). It thus appears that these enzymes have evolved from a common protein scaffold by divergent evolution, but they catalyze completely different reactions like aldol condensation (cyclases) and hydroxylation, an illustrative example of how chemical diversity evolves in one metabolic pathway within an evolutionary related enzyme family.

The tetracenomycin F2 cyclase (TcmI) is a type II polyketide cyclase which catalyzes the ring closure step at the fourth ring of the polyketide core during the biosynthesis of tetracenomycin C from *Streptomyces glaucescens* (Shen and Hutchinson, 1993). This enzyme does not show any sequence similarity to the SnoaL-type cyclases. The overall fold of TcmI is completely different than that observed for SnoaL/AknH and shows a ferredoxin-like fold (Thompson *et al.*, 2004) (figure 21a). However, TcmI also catalyzes the reaction via an intramolecular aldol condensation reaction and it was

A   **B**
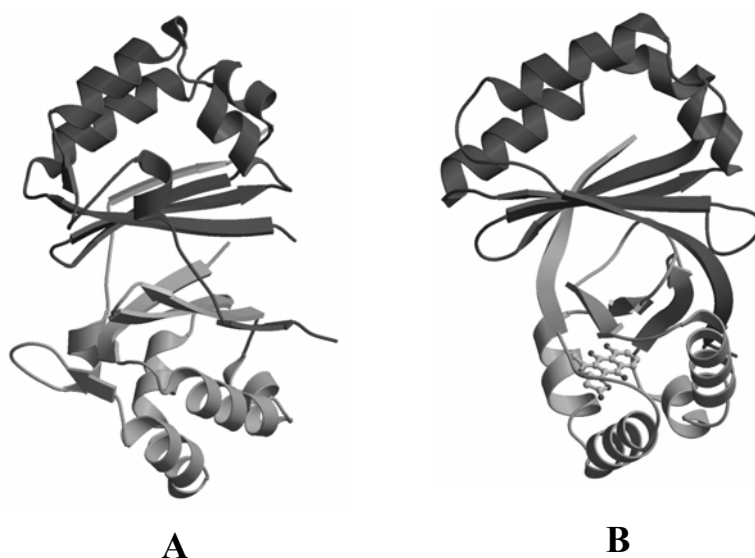
Figure 21: The dimers of TcmI (A) and ActVA-orf6 (B).

reported from the biochemical studies that this enzyme does not use any co-factor (Shen and Hutchinson, 1993). Moreover, the proposed active site of TcmI does not contain any lysine which might be important for the Schiff's base formation (Thompson *et al*., 2004). TcmI also is another example of divergent evolution due to the structural similarity to the monooxygenase ActVA-Orf6 from the actinorhodin biosynthetic pathways. ActVA-Orf6 catalyzes the oxygenation in the polyketide ring without any cofactor or metal ions (Kendrew *et al*., 1997) and the overall fold belongs to the ferredoxin-like fold as observed in TcmI (Sciara *et al*., 2003) (figure 21b). The overall similarity in the structures of TcmI and ActVA-Orf6 suggests an evolutionary relationship between these two enzymes, albeit it is barely detectable at the sequence level (Schneider, 2005).

2.2 Aclacinomycin oxidoreductase (AknOx): Paper 4& 5

Aclacinomycin oxidoreductase (AknOx) is a flavoenzyme, which is involved in the modification of the terminal sugar moiety in the aclacinomycin biosynthetic pathway. It was first isolated and purified from the *Streptomyces galilaeus* strain MA144-M1 in 1979 (Yoshimoto *et al.*, 1979). This enzyme catalyzes two consecutive oxidation-reduction reactions using FAD as cofactor and converts AclN to AclA and then eventually the latter compound to AclY (figure 22). AclA is a well-known anticancer drug and is being used commercially (Raty *et al.*, 2002), while AclY has been shown to have higher antimicrobial activity compared to AclA (Yoshimoto *et al.*, 1979). During the AknOx catalyzed reactions, molecular oxygen is required in order to re-oxidize FAD.

In the first step of the reaction, AknOx converts the rhodinose moiety of AclN to cinerulose A in AclA by oxidizing the hydroxyl group to a keto group. In the next step, cinerulose A is converted to L-aculose of AclY by a dehydrogenation reaction which forms a double bond between two carbon atoms in L-aculose (figure 22). AknOx consists of 502 amino acid residues with a molecular weight of 54.8KDa. This enzyme is synthesized as a pre-protein which contains 43 additional residues at the N-terminus responsible for the extracellular secretion.
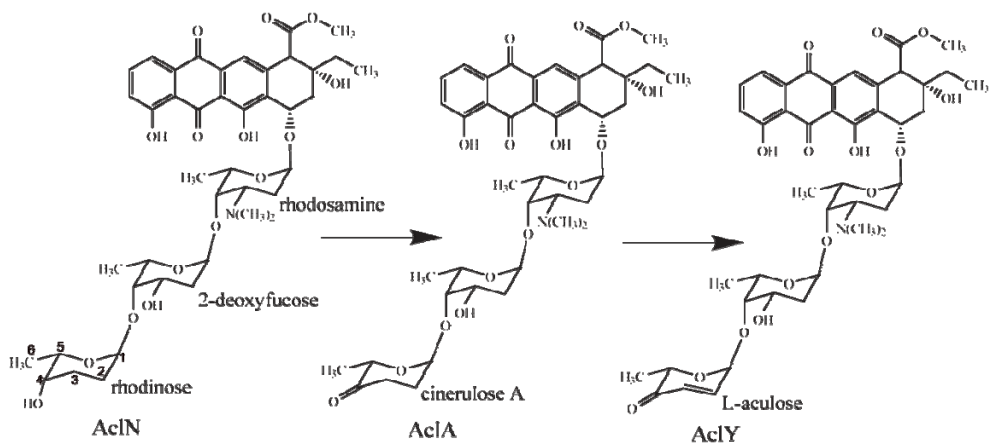

Figure 22: The two consecutive reactions catalyzed by AknOx

2.2.1 Crystallization and structure determination of AknOx

Crystallization and data collection from native and selenomethionine (SeMet) substituted AknOx has been described in paper 4. The phase problem was solved by MAD (Multiple-wavelength Anomalous Dispersion) with the three datasets collected from the SeMet crystal and the model building was performed in the 2.7Å experimental density map. Phase combination was used during manual building in order to obtain the missing part of the structure (paper 4).

The analysis of intensity statistics for all collected datasets suggested twinning in AknOx crystals. Therefore, the refinement with twin protocol in SHELXL (Sheldrick and Schneider, 1997) was performed by using the 2.0Å and 1.65Å native datasets consecutively. Details about the overall refinement process are described in paper 4 and the present statistics are shown in the Table 1 in paper 5.

2.2.2 Twinning in AknOx crystals

2.2.2.1 Twinning: Definition and classification

Twinning can be defined as a crystal growth anomaly which occurs in a crystalline specimen containing two or more crystalline domains related by a special orientation. This orientation is related to the crystal lattice without belonging to the symmetry operations of the crystal point group (Koch, 1992). Twinning has been recognized and classified by several early crystallographers such as Donnay and Friedel (Donnay and Donnay, 1974; Friedel, 1926). Different types of twinning have been described according to whether the separate lattices coincide in fewer than three dimensions, in all three dimensions or in approximately three dimensions. According to Giacovazzo and Donay, there are two families (Donnay and Donnay, 1974; Giacovazzo, 1992):

i.   Quasi-twin lattice symmetry (QTLS) where two or more twin lattices do not completely overlap in three dimensions. If only two lattice axes can be aligned, these twins are called non-merohedral or epitaxial twinning. Crystals with this type of twinning produce multiple diffraction spots which are easily recognizable by the inspection of the three-dimensional diffraction pattern showing distinct interpenetrating reciprocal lattices (Chandra *et al*., 1999).

ii. Twin-lattice symmetry (TLS) or merohedral twinning is generated by perfect overlapping of the lattices in three dimensions from several twin domains. This type of twinning can not be interpreted from the diffraction images because the reciprocal lattices of each domain completely overlap and create one single diffraction spot (Chandra *et al*., 1999). Merohedral twins reflect the possibility of different non-equivalent orientations of crystals within a lattice of symmetry higher than the crystal point group symmetry. Therefore, this type of twinning usually occurs in the trigonal, tetragonal, hexagonal or cubic systems, with

lattices always having the apparent higher symmetry of the system (Barends *et al.*, 2005; Dauter, 2003). Hemihedral and tetartohedral twinning are the two subgroups of merohedral twinning where the diffraction pattern is a superimposition of the pattern from two or four twin domains respectively (Barends *et al.*, 2005; Chandra *et al.*, 1999; Dauter, 2003; Liang *et al.*, 1996; Lietzke *et al.*, 1996; Rudolph *et al.*, 2003; Yeates, 1997; Yeates and Fam, 1999).

If the overlapping profile of the lattices from different twin domains takes place approximately in three dimensions, this phenomenon gives rise to another kind of twinning, termed as pseudomerohedral twinning (Yeates, 1997). Usually, this type of twinning is the result of the unit-cell parameters being close to fulfilling that for higher symmetry (Yang *et al.*, 2000). Pseudomerohedral twinning can occur in monoclinic and orthorhombic space group due to the presence of fortuitous unit cell parameters. For instance, a=b in an orthorhombic cell can mimic tetragonal space group (Frazao *et al.*, 1999). In a monoclinic system, several conditions for emulating an orthorhombic lattice exist 1) a=c (Ban *et al.*, 1999; Ito *et al.*, 1995; Yang *et al.*, 2000) , 2) β angle close to 90° (Barends and Dijkstra, 2003; Larsen *et al.*, 2002) or 3) in case the cell parameters satisfy the condition, *c cosβ = -a/2* (Declercq and Evrard, 2001; Rudolph *et al.*, 2004). However, cell parameters of a=c and β close to 120° in the monoclinic system can emulate a hexagonal metric. This kind of pseudomerohedral twinning is termed as 'Drilling' (german for tripling) (Sheldrick and Schneider, 1997) and contain three twin components related by two twinning fractions.

In order to deal with twin crystals, it is necessary to identify i) the twin law which is the description of the orientation of the different twin domains relative to each other and ii) the twinning fraction, α which can be defined as the fractional volume contributed by each twin component.

Crystal twinning has been considered as one of the main obstacles for successful structure determination for proteins. However, a number of structures have been determined from twinned crystals using different methods of experimental phasing such as MAD or MIR (Multiple Isomorphous Replacement). Lists of several protein structures determined by experimental phasing from pseudomerohedrally and merohedrally twinned crystals are listed in Table 4a and 4b respectively.

Table 4a: Examples of proteins structures determined from pseudomeroherally twinned crystals by experimental phasing.

Protein	Exp.Method	True Space group	special unit cell geometry	PDB ID	reference
50S ribosomal subunit	MIRAS/SAD	P2$_1$	a=c	1c04	(Ban *et al*., 1999)
gpD	MAD	P2$_1$	a=c	1c5e	(Yang *et al*., 2000)
Peroxiredoxin 5	SIR	P21	ccosβ=-a/2	1h4o	(Declercq and Evrard, 2001)
PlgR	MIR	P2$_1$	ccosβ=-a/2	1xed	(Hamburger *et al*., 2004)

PlgR= polymeric immunoglobulin like receptor
gpD= bacteriophage lambda head protein D

Table 4a: Examples of proteins structures determined from meroherally twinned crystals by experimental phasing.

Protein	Exp.Met.	Space group	PDB ID	reference
Interleukin -1β	MAD	P4$_3$	f42w	(Rudolph *et al*., 2003)
MltA	MAD	P3$_1$	2ae0	(Barends *et al*., 2005)
Rna1p	MIRAS	I4$_1$	2ca6	(Hillig and Renault, 2006)
DAOCS	MIR	R3	1dcs	(Rees and Lipscomb, 1980; Valegard *et al*., 1998)
Carboxypeptidase A	MIR	P3$_2$	4cpa	(Rees and Lipscomb, 1980) (Rees and Lipscomb, 1982)
Hydroxylamine oxidoreductase	MIR	P6$_3$	1fgj	(Igarashi *et al*., 1997)

MltA= lytic transglycosylase A
DAOCS= deacetoxycephalosporin C synthase

2.2.2.2 Twinning analysis in AknOx crystals: <u>Paper 4</u>

The diffraction datasets from native crystals were reduced to the space group $P2_1$ with the cell dimensions of a=c and β angle being very close to 120° (Table 1 in paper 4). Several twinning tests clearly suggested pseudomerohedral twinning in these crystals. However, the cell parameters of AknOx suggested several possibilities for pseudomerohedral twinning which includes 1) two equal cell dimensions, a=c can emulate an orthothorhombic space group or 2) β close to 120° in addition to a=c will give rise to a hexagonal metric.

The refinement of the AknOx model using either two-domain or three-domain twinning operator gives comparable Rfree values (24.2% and 26.5%). The investigation of the crystal packing and the refinement statistics suggests the presence of multi-domain twinning with six twin domains which is a combination of two twinning operators perpendicular to each other, i.e. a perfect two-fold twinning operator along the diagonal between a and c and a three-fold operator almost parallel to the crystallographic b axis.

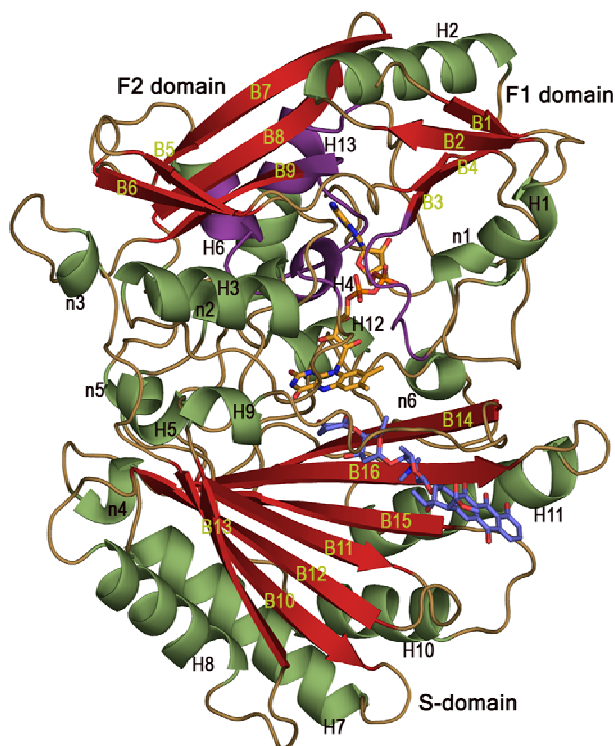## 2.2.3 The overall fold of AknOx: <u>Paper 5</u>

Figure 23: The structure of AknOx is shown with the bound FAD and ligand. The FAD and ligand is coloured in yellow and blue respectively. The conserved FAD binding motifs are coloured in purple.

The structure of AknOx has two distinct domains for binding FAD and the ligand. The FAD binding domain (F-domain) is further divided into two α+ β subdomains; F1 and F2 (figure 23). These two subdomains pack against each other to accommodate the FAD. The F1 part contains a 4-stranded mixed β-sheet flanked by two α-helices and the F2 subdomain folds into a five stranded antiparallel β-sheet which packs on one side against 4 α-helices and one 3_{10} helix.

The substrate binding domain (S-domain) in AknOx contains a large central seven stranded β-sheet flanked by four α-helices on one side (figure 23). The ligand binding pocket extends from the protein surface towards the interior of the domain. The flavin and ligand binding domains are connected by two long loop regions L1 and L2 containing residues 210-226 and 449-471 respectively. L1 loop also contains a 3_{10} helix with residues 218-221.

2.2.4 AknOx belongs to the PCMH superfamily

The structure of AknOx belongs to the PCMH (p-cresol methylhydroxylase) superfamily according to the structural comparison analysis. Other members of this family are listed in Table 2 in paper 5. The family name is derived from the chain fold of p-cresol methylhydroxylase, the first enzyme with reported three-dimensional structure (Mathews *et al.*, 1991).

2.2.4.1 The conserved F-domain and FAD binding features in the PCMH superfamily

The proteins from the PCMH superfamily showed a conserved F-domain fold that includes two α+β subdomains. The binding pocket for the FAD is also very similar for all family members. The FAD is accommodated between the two subdomains and the conserved extended architecture between two subdomains allows adopting the elongated conformation of FAD (figure 23) (Dym and Eisenberg, 2001). FAD interacts with the protein by several H-bonding and van der Waals contacts and there are four different sequence motifs typical in this superfamliy for binding the co-factor. The motif 1 is termed as the p-loop which includes the residues 66-72 in AknOx and interacts with the phosphate group of FAD (Aravind *et al.*, 2004). Residues in motif 2 (residues 134-139 on helix 4 in AknOx) interact with the ribityl and phosphate parts of FAD. The third motif contains the residue 188-203 in AknOx and residues from this motif (Gly197 and Val203) are involved in binding the adenine part. The last motif is placed at the C-terminal part of the F-domain (residues 482-488 in helix 13) and is involved in the structural stabilization of this domain by several H-bonding and hydrophobic interactions (Lee *et al.*, 2005a) (figure 23).

2.2.4.2 Diversity in the structure of the substrate binding domain is observed in the PCMH superfamily

The substrate binding domain of the members of PCMH superfamily showed similar structural fold but significant variation in architecture of the substrate binding site, required for accommodating different substrates ranging from a small lactate to bulky cholesterol, long chain oligosaccharides and polyketide compounds (Cunane *et al*., 2005; Huang *et al*., 2005). Both AknOx and glucooligosaccharide oxidase (GOOX) possess an open, accessible ligand binding pocket for binding oligosaccharides compared to the closed conformation observed in PCMH (Cunane *et al*., 2005) or vanillyl-alcohol oxidase (VAO) (Fraaije *et al*., 1999; Fraaije *et al*., 2000). The closed conformation limits the size of the ligand binding pocket and applies the "size-exclusion mechanism" for binding small ligands (Huang *et al*., 2005). Another example of structural diversity in the S-domain related to the substrate specificity is observed as the major structural difference in the loop regions in AknOx and GOOX at the entrance of the substrate binding pocket. GOOX contains a long loop of 15 residues which is replaced in AknOx by a shorter loop of only seven amino acids (figure S2 in paper 5). The specific shape of the loop in AknOx is required because one residue of this loop (Phe339) is involved in the recognition and binding of the polyketide core of the AknOx substrate (figure S2 in paper 5).

2.2.5 FAD binding site in AknOx

The isoalloxazine ring of FAD is bound at the interface of the F- and S-domains, while the ADP-ribosyl part is located between two subdomains of the flavin binding domain. The ribityl part of FAD is H-bonded with main chain and side chain atoms of several residues (figure 3a in paper 5) whereas the phosphate group is predominantly H-bonded with the main chain residues from a loop region (p-loop) including residues 66-70. The phosphate group is also connected with the side chain of R66 via two water molecules. The ribose and adenine part of FAD is involved in three direct H-bonding interactions with the main chain and side chain atoms and has one-water mediated interaction with the backbone of Val65 (figure 3a in paper 5).

2.2.6 Covalent flavinylation in AknOx and other flavoproteins

Covalent attachments between protein molecule and flavin have been recognized for about 40 years following the work of Singer and co-workers (Kearney and Singer, 1955a; Kearney and Singer, 1955b; Singer *et al*., 1956) on mammalian succinate dehydrogenase. Since then a number of flavoproteins has been identified which interact covalently with FAD or FMN.

2.2.6.1 Categories of covalent flavinylation observed in different flavoenzymes

According to Mewies and his co-workers (Mewies *et al*., 1998), the covalent flavoproteins were divided generally into two categories. These two groups of protein contain covalently bound flavin through the 8α methyl group and the C6 atom of the isoalloxazine ring respectively. The former category can be subdivided based on the type of amino acid residues that interacts with the flavin. These subgroups with different interacting residues (figure 24) are described briefly as follows:

1) 8α-N1-(or N3)-Histidyl-FAD: This group of proteins is involved in covalent linkage with the 8α-methyl group of FAD through the N1 or N3 atom of the histidine residue. The structure of cholesterol oxidase (Kenney *et al*., 1979), cytokinin dehydrogenase (Malito *et al*., 2004) shows the N1-histidyl interaction while those of 6-hydroxy-D-nicotine-oxidase (Brandsch *et al*., 1987; Bruhmuller and Decker, 1973; Koetter and Schulz, 2005), succinate dehydrogenase (Walker and Singer, 1970) and vanillyl-alcohol oxidase (VAO) (de Jong *et al*., 1992; Fraaije *et al*., 1999) are the examples of N3-histidyl covalent binding with the FAD

2) 8α-O-tyrosyl-FAD: Covalent linkage between the hydroxyl group of tyrosine residue with the FAD is observed in several enzymes like p-cresol methylhydroxylase (McIntire *et al*., 1981) and 4-ethylphenol methyl hydroxylase (Reeve *et al*., 1989).

3) 8α-S-cysteinyl-FAD: The sulfur atom of a cysteine residue in several proteins is involved in the covalent binding with FAD. One example of this type of linkage is the structure of monoamine oxidase (Li *et al*., 2006; Nagy and Salach, 1981).

4) 6-S-cysteinyl FMN: The covalent bonding between the C6 of FMN and cysteine from the protein has been observed in the triethylamine dehydrogenase and dimethylamine dehydrogenase (Boyd *et al*., 1992; Kenney *et al*., 1978; Yang *et al*., 1995).

Recently, a novel form of covalent linkage was identified in the structure of glucooligosaccharide oxidase (GOOX) (Huang *et al*., 2005) which showed a bi-covalently bound FAD (figure 24) through its 8α-methyl group and C6 position interacting with a histidine and cysteine residue respectively, thus forming a 6-S-cysteinyl-8α-N1-histidyl-FAD.
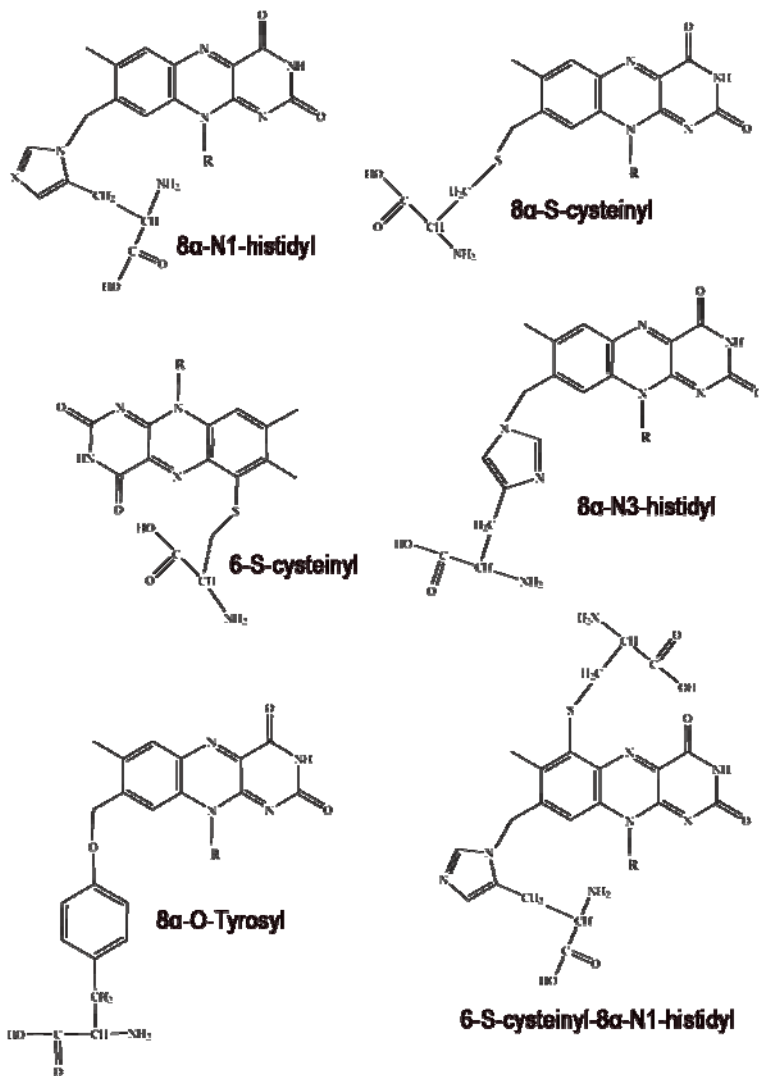
Figure 24: Different types of covalent flavinylation observed in flavoenzymes

2.2.6.2 The flavinylation observed in AknOx and PCMH superfamily

The FAD in AknOx is bound via a bi-covalent mode where the N1 of His70 and the thiol group of Cys130 are involved in covalent interactions with the 8α-methyl group and C6 of the FAD respectively (figure 3a in paper 5). This type of double covalent attachment is not that common so far and only observed in the structure of GOOX previously (Huang *et al.*, 2005). However, based on mass-spectrometry analysis, S-reticuline oxidase, also known as Berberine Bridge Enzyme (BBE), has been reported

to have a similar bi-covalent enzyme-FAD interaction (Winkler *et al.*, 2006). Mutation studies of His70 in GOOX and His422 in VAO suggested a role of these residues in maintaining the redox potential of FAD.

The enzymes from the PCMH superfamily share a similar conformation and binding features for FAD but it can be bound either covalently or non-covalently to the protein. Most of the members interact with FAD through covalent bonding with the exception of lactate dehydrogenase which exhibits non-covalent interaction with the co-factor (Dym *et al.*, 2000). The types of covalent flavinylation can also differ significantly among the members of this superfamily. AknOx and GOOX show a bi-covalent flavinylation that involves covalent linkage of FAD with histidine and cysteine residues. The other members mostly use the 8α-methyl group of the isoalloxazine ring for cross-linking with the N1 or N3 atom of histidine (Coulombe *et al.*, 2001; Koetter and Schulz, 2005; Malito *et al.*, 2004) or tyrosine residues (Mathews *et al.*, 1991).

2.2.7 Ligand binding features and active site

Clear electron density for the bound ligand was observed in only one molecule in the asymmetric unit which represented the product AclY whereas AclA was used in the crystallization trials, indicating that the reaction has proceeded during crystallization process. The polyketide core of the ligand was bound at the protein surface of the ligand binding domain while the trisaccharide part was extended into a ligand binding pocket towards the active site (figure 23). The ligand is predominantly bound by several hydrophobic interactions which include the stacking interactions of the polyketide core with Phe339 and Trp372 as well as several phenylalanines and one tryptophan residues involved in hydrophobic contact with the trisaccharide moiety (figure 25). H-bonds are formed by Thr408 with the tertiary amine of rhodosamine and hydroxyl group of ring A of the polyketide core.

There are three tyrosine residues (Tyr450, Tyr144 and Tyr378) positioned in the vicinity of the L-aculose moiety and proposed as active site residues of AknOx (figure 25). The side chain of Tyr450 interacts through direct H-bonding with the keto group of the terminal sugar moiety. Moreover, Ser376 and Glu374 together with Tyr378 form a putative catalytic triad which can be involved in the reaction (figure 25). The N5 nitrogen of the isoalloxazine ring is also located close (ranging 3.7-3.9Å) to the C2, C3 and C4 atoms of the L-aculose moiety which can have important catalytic implications.
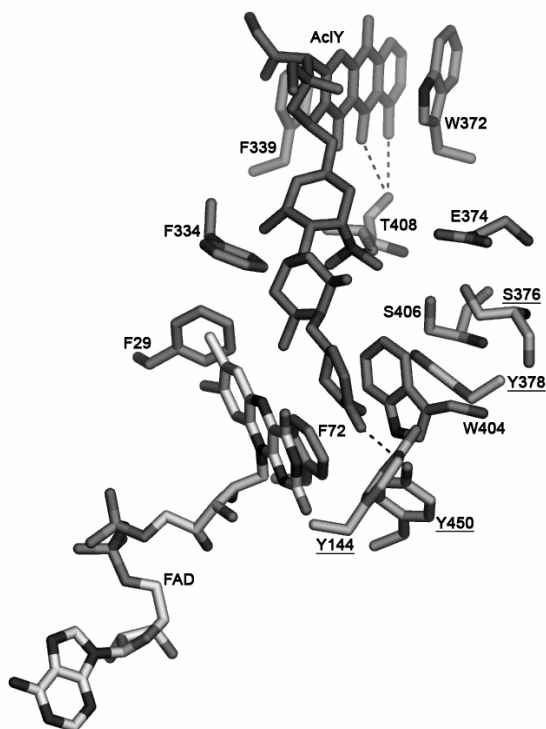
Figure 25: The ligand binding site and active site of AknOx. The AclY is coloured as black and labelled. The active site residues are underlined.

2.2.8 Catalytic mechanism of AknOx

AknOx is considered as a unique oxidoreductase due to the fact that it catalyzes two consecutive reactions with different chemistry leading towards the biosynthesis of aclacinomycin Y. The mechanism of the reaction catalyzed by AknOx comprises of two half-reactions; the reductive half reactions involve the oxidation of the hydroxyl group/dehydrogenation, while the oxidative half-reactions are performed by the molecular oxygen to obtain the oxidized FAD.

The first step of the reaction is the conversion of rhodinose to cinerulose A (figure 22) which involves the oxidation of the hydroxyl group at the C4 to a keto group. Structural investigation and mutagenesis of the active site residues (Tyr450 and Tyr144) suggested that Y450 is crucial for catalysis during this step of reaction. Tyr450 is in H-bonding distance from the C4 hydroxyl group of AclN, the substrate for the first reaction which was modelled in the AknOx structure. Mutation of this tyrosine to phenylalanine seriously impaired the enzyme activity (Table 3 in paper 5) which suggested that this residue may facilitate the reaction by assisting transfer of the proton from the C4 hydroxyl group (figure 26a). The proton can eventually be transferred to the side chain of Tyr144 which is placed in the vicinity of the Tyr450. Thus, Tyr144 can play a role in the proton transfer system which is confirmed by the significantly

decreased enzymatic activity in the Tyr144Phe mutant. Proton abstraction is followed by the hydride transfer from the C4 atom to the N5 of the isoalloxazine ring (figure 26a).

The second reaction catalyzed by AknOx is a dehydrogenation where a double bond is formed between C2 and C3 after the abstraction of formally two hydrogen atoms. This step converts cinerulose A to L-aculose (figure 22). Structural inspection of the AknOx-AclY complex and the modeled AknOx-AclA complex revealed that Tyr378 could act as a catalytic base for abstracting a proton from the C3 carbon of cinerulose A. The proton can be transferred through a proton relay system involving Ser376 and Glu374. The reaction continues by hydride transfer from the C2 to N5 of FAD leading to the formation of double bond in L-aculose (figure 26b). Total loss of activity due to the mutation of Tyr378 into phenylalanine confirmed its key role in catalysis. However, the requirement of Glu374 for proton transfer is not crucial because of the unaffected enzymatic activity in the Glu374Ala mutant compared to the wild-type AknOx. The decreased activity observed for the Ser376Ala mutant can be explained either as less efficient transfer of the proton to the solvent channel or necessity of the H-bonding interaction between Tyr378 and Ser376 for optimal positioning of the Tyr378 side chain.
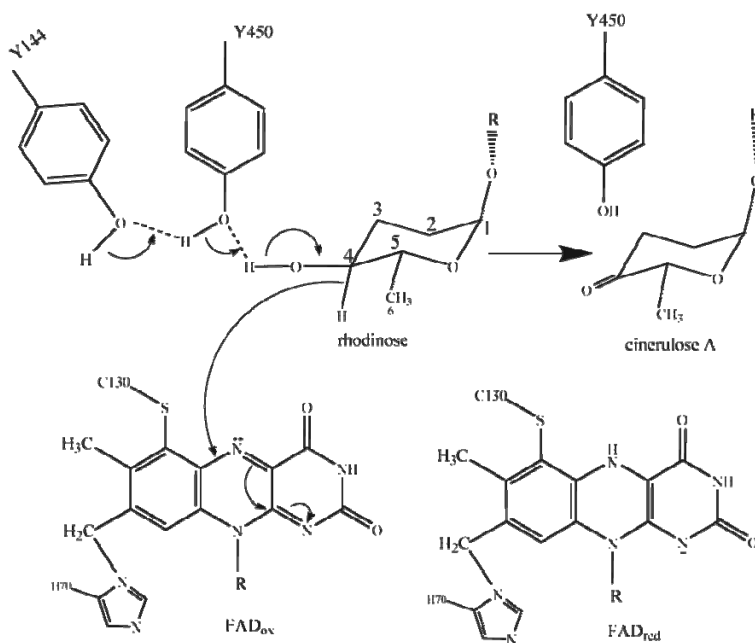
Figure 26a: The mechanism of first reaction converting rhodinose to cinerulose A.
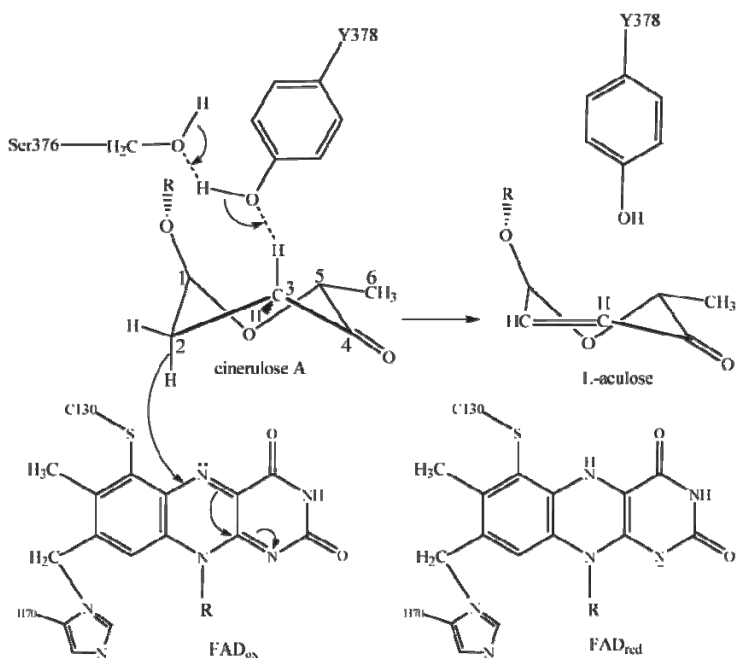
Figure 26b: The mechanism of second reaction from cinerulose A to L-aculose.

The oxidative half-reaction takes place after each cycle of reductive half-reaction by re-oxidizing the FAD by molecular oxygen. However, it is not clear whether AclA leaves the active site before the first oxidative half-reaction. The AknOx model shows an open channel at the interface of the flavin and ligand binding domains extending from the surface upto the isoalloxazine ring, which can be a possible way for molecular oxygen to enter the active site without dissociating the AclA (figure 7 in paper 5).

2.2.9 Comparison of catalytic properties in the PCMH superfamily

The proteins from the PCMH superfamily bind the substrate at the *si* face of the isoalloxazine ring of FAD which is typical for this class of proteins. Detailed comparison of active sites of AknOx, GOOX and PCMH revealed that these enzymes use a functionally conserved tyrosine residue (Tyr450, Tyr429 and Tyr473 respectively) for catalysis (figure 8 in paper 5). Tyr450 in AknOx corresponds to the position of Tyr429 in GOOX. Tyr450 in AknOx is involved in proton abstraction from the hydroxyl group of rhodinose, whereas Tyr429 in GOOX was proposed as the main catalytic residue that initiates the reaction. Asp355 in GOOX is responsible for proton transfer from Tyr429 via a connecting water molecule (Huang *et al*., 2005) and this

residue structurally corresponds to Tyr378 in AknOx. The position of Tyr378 being distant from the hydroxyl group of C4 in rhodinose and from Tyr450 omits the possibility of its role in proton abstraction from hydroxyl group or proton transfer from Tyr450. The mutation experiments further confirmed that Tyr378 is not involved in this particular reaction (Table 3 in paper 5). However, AknOx is unique from GOOX and PCMH due to the fact that it catalyzes two consecutive reactions; an oxidation reaction of the hydroxyl group in the same manner as GOOX followed by a dehydrogenation reaction that forms a C-C double bond in the same sugar moiety. Tyr378 is positioned ideally to initiate the second reaction in AknOx which is also confirmed by the Tyr378Phe mutant. Therefore, two individual tyrosine-mediated reactions in the same active site catalyzed by AknOx makes this enzyme distinctive among the other related members of this superfamily.

3. Conclusions

This thesis was mainly focused on the structural enzymology and mechanistic details of two different enzymes from the anthracycline biosynthetic pathways. The structural features were analyzed based on the three-dimensional structure of the proteins in complex with different substrate/product compounds which provided the necessary insights to pinpoint the active sites. In combination with functional data, the thesis also provides knowledge about the mechanism of reaction for these enzymes that could be useful for rational redesign of the biosynthetic enzymes for producing improved anthracycline compounds.

3.1 SnoaL and AknH

The ligand binding features in both SnoaL and AknH revealed that the ligand is pre-dominantly bound by hydrophobic residues rather than H-bonding. The substrate specificity is also mainly determined by the architecture of the ligand binding pocket rather than specific H-bonds, which explains the fact that these enzymes can accept a broad range of substrates with different degrees of aglycone modification.

The studies of SnoaL revealed a novel mechanism of intermolecular aldol condensation reaction which follows an aspartic acid driven acid/base chemistry and not via the formation of a Schiff's base or metal dependent mechanism for stabilizing the enol(ate) intermediate as observed in aldolases previously. In SnoaL, the intermediate is stabilized by the delocalization of electrons over the π system of the aromatic polyketide ring.

Two residues in AknH were identified to play a role in the stereoselectivity of the products. Site-directed mutagenesis confirmed that Tyr15 and Leu51 are the two residues, responsible for the stereoselectivity of product in AknH.

Comparison of the structures of SnoaL/AknH and some other biosynthetic enzymes showed explicatory examples of divergent evolution observed within aromatic polyketide biosynthetic enzymes. The two hydroxylases, Snoal2/AclR from different anthracycline biosynthetic pathways share very similar fold with the polyketide cyclases SnoaL/AknH, but show different catalytic properties (Beinker *et al*., 2006).

3.2 AknOx

The structure of AknOx was determined from a multi-domain pseudomerohedral twinned crystal by MAD. AknOx is a flavoenzyme and binds FAD in a bi-covalent manner through histidine and cysteine residues. This enzyme catalyzes two consecutive reactions: an oxidation of a hydroxyl group followed by a dehydrogenation. Investigation of the structure and mutagenesis study of the proposed residues revealed two distinct sets of catalytic residues in the same active site that can catalyze these two consecutive reactions, a dual active-site feature which is unique among the flavoenzymes.

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