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Received: April 1, 2003 [Z 610]

Conserved Amino Acid Residues Correlating With Ketoreductase Stereospecificity in Modular Polyketide Synthases

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KEYWORDS:

biosynthesis · ketoreductases · polyketides · sequences · stereospecificity

The polyketide group of natural products includes antibiotics, immunosuppressants, anticancer drugs and other valuable bioactive compounds. The biosynthesis of these compounds is an area of considerable interest. Polyketide synthases (PKSs) resemble fatty acid synthases (FASs) and are composed of several different enzymes that assemble carbon chains.[1] An acyltransferase (AT) loads a dicarboxylic acid extender unit onto an acyl carrier protein (ACP), where it undergoes condensation with a starter acyl unit in a reaction catalysed by a ketosynthase (KS). The product is a β-ketoacyl intermediate that remains thioester-linked to the ACP. The β-ketone group may then be processed by ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains to yield an alcohol, enoyl or methylene group before the extended chain migrates to a KS for another cycle of extension. When the final polyketide chain length is reached, termination occurs, usually accompanied by cyclisation.[1]

FASs generally use acetyl starter and malonyl extender units. As a group, PKSs use a wide range of organic acids as starter units, whereas the extenders may be malonyl, methylmalonyl, ethylmalonyl or methoxymalonyl units. The diversity of polyketide structures results in part from combinatorial assembly of different starter and extender units, together with varying degrees of processing of β-ketone groups. Chiral centres are introduced by the use of branched extender units and by the reduction of β-ketone groups to alcohols. The introduction of chirality increases the potential diversity further.

The PKSs that assemble complex polyketides contain a module for every cycle of chain extension. Each module contains the requisite enzymatic domains, which are covalently linked in the order, KS-AT-reduction domain loop-ACP. Three main types of reduction domain loop exist. These contain KR, DH-KR or DH-ER-KR domains and determine the extent of β-ketone group processing in each cycle.[1]

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

 © 2003 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim DOI: 10.1002/cbic.200300581

The modular nature of complex PKSs was first recognised with the sequencing of the erythromycin PKS, 6 deoxyerythronolide B synthase (DEBS).[2, 3] Although several other complex PKSs have been sequenced since that time,[4] the most detailed current views have come from studies on DEBS and engineered derivatives of this multienzyme system. These findings are generally assumed to apply to other modular PKSs. The AT domains of DEBS load (2*S*)-methylmalonyl groups onto the ACP domains[5] and condensation occurs with inversion of stereochemistry at the carbon atom derived from C2 of the extender unit,[6] so that a (2*R*)-2-methyl-3-ketoacyl thioester is generated initially. With some modules, the initial condensation product is epimerised to give a (2*S*)-2-methyl-3-ketoacyl chain before β-ketone group processing and/or transfer to the next module.[6, 7] Domain exchange experiments have shown that KR domains determine the stereochemical outcome of the ketoreduction reaction.[8] Some KRs may have to discriminate between (2*R*)- or (2*S*)-2-methyl-3-ketoacyl substrates and could therefore have a role in fixing the methyl stereochemistry at C2.

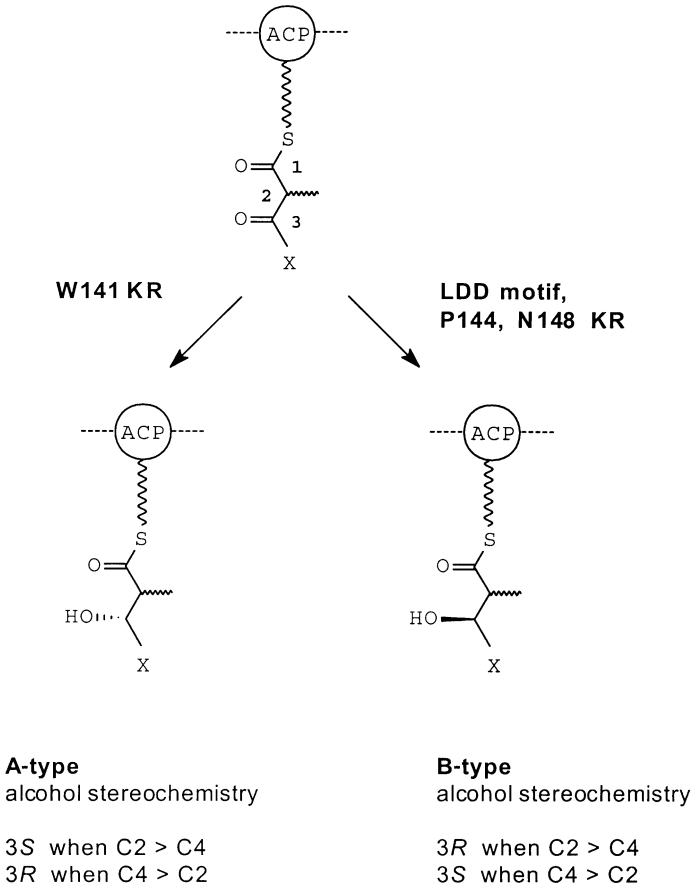
It is still unclear exactly how modular PKSs control alcohol stereochemistry in nascent oligoketide chains. This paper describes an analysis of KR sequences carried out in an attempt to highlight features that might be important for stereospecificity. Sequence analyses of 17 modular PKSs have been reviewed.[4, 9] At least 68 KR domain sequences are available for which the stereochemical outcome of the ketoreduction can be inferred from biosynthetic diagrams in original sequence papers (cited in ref. [4]), or from molecular models of absolute structures.[10–12] To avoid ambiguity, the two types of alcohol stereochemistry will be described as A and B (Scheme 1). KR domains were divided into groups according to whether they generate A- or B-type alcohol stereochemistry. A- and B-type KR sequences were aligned to allow identification of residues characteristic of each group.

Very few consistent differences were found between A- and B-type KRs. The motif LDD from residue 93 to 95 was found in the B group but not the A group (Scheme 2). In the B group, D95 is invariant and D94 is replaced by E in two cases, whereas L93 is less strictly conserved. None of the A-type KRs have a hydrophobic residue followed by two acidic residues in the 93 – 95 region.

The region from residue 141 to 148 also differs between the two groups. In the B group, residues 144 and 148 are typically P and N. Members of the A group have W at position 141 with the exception of RIF KR5 and RIF KR7 (Scheme 2). These KRs are unusual in that there is an M residue at position 142 instead of the G residue which is invariant in all other KR domains. W141 is absent from all of the B group KRs except for the KR1 domains of the PKSs for the 16-membered macrolides niddamycin and tylosin. NID KR1 and TYL KR1 have N at position 143, a feature typical of some B-type but not of A-type KRs.

The LDD motif and residues P144 and N148 correlate well with the formation of B-type alcohol stereochemistry. The absence of these residues and the presence of W141 correlates with A-type alcohol stereochemistry.

The KR alignments provide no clues about how KRs might select (2*R*)- or (2*S*)-2-methyl-3-ketoacyl substrates. Of the A-type KRs, 22 come from modules that incorporate branched extender units. Of these, seven can be predicted to epimerise (and 15 not to epimerise) C2 of the extended chain. However, no obvious residues or motifs have yet been found that are characteristic of either subgroup. This is also true of the 10 B-type KRs from modules that incorporate propionate extender units. Six of these KRs are in epimerising modules and four are in nonepimerising modules.



Scheme 1. *Correlation of KR domain motifs with alcohol stereochemistry. X = the remainder of the nascent oligoketide chain. The A-type alcohol stereochemistry is 3S when C2 has a higher priority than C4 and 3R when these priorities are reversed. The B-type alcohol stereochemistry is 3R when C2 has priority and 3S when C4 has priority. The correlation is not affected by the presence or absence of substituents at C2, or asymmetry introduced by substituents at this position.*

The 17 PKSs that were studied also contain 71 KRs for which the stereochemical outcome of the ketoreduction is hidden by subsequent dehydration, or dehydration and enoyl reduction. These domains have the LDD motif and PN motifs characteristic of B-type KRs (not shown, the alignments are shown in Figure 5 of the Supporting Information). This suggests that the preferred substrate for a PKS DH domain is a B-type 3-hydroxyacyl chain, as is present in fatty acid biosynthesis. This preference would be important for PKS engineering, since it means that inactivation of a DH domain should result in a hydroxy group of known stereochemistry.

W141, P144 and N148 are located close to active site residues within KR domains. Reid et al.[13] have recently shown that S136 and Y149 are important for the KR activity of KR6 of DEBS.

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88 - 103 region

A type			B type		
DEBSKR2	n	HAAGLPQQVAINDMDE	DEBSKR1	e	HAAAT**LDD**GTVDTLTG
DEBSKR5	n	HAAGVSTSTPLDDLTE	OLEKR1	e	HAAGA**LDD**GMTTVLTP
DEBSKR6	n	HTAGVPESRPLHEIGE	PIKKR1	e	HTAGA**LDD**GIVDTLTA
OLEKR2	n	HAAGVPQSTPLAEISV	TYLKR1	n	HTAGI**LDD**AVIDTLSP
OLEKR5	n	HAAGVPQSRPLAEISV	NIDKR1		HAAGV**LDD**GVLDGLTP
OLEKR6	n	HAAGVPQSTPLAEISV	RAPSKR2		HTAGV**VDD**GVVESLTV
PIKKR5	n	HTAGAPGGDPLDVTGP	RAPSKR5		HTAGV**VDD**GVVESLSA
TYLKR6	n	HTAGTPHSAEFTALDE	RAPSKR11		HTAGV**VDD**GVVESLTV
TYLKR7		HAAGVPQVTPLSRTSP	RAPSKR12		HTAGV**LDD**GVVESLTA
NIDKR6	n	HATGTPQTTPLTHLTP	FK506KR2	e	HMAGIV**DD**AVVATMRP
NIDKR7		HATGTPQTTPLTHLTP	FK506KR8		HTAAT**LDD**GILHALTP
AVEKR5		HTAGVNLFAPVSETDA	FK520KR2	e	HMAGIV**DD**AAVTTMRP
RIFKR5	n	HTAGVIDAGVIETLDR	FK520KR3		HTAAV**LDD**GVLTALTP
RIFKR7	e	HTAGVFDAGVTGALTR	FK520KR8		HTAAT**LDD**GILHALTP
SORKR4		HASGIEHHAPLDATSF	AVEKR1	n	HTAGI**LDD**ATLTNLTP
SORKR6	n	HAGGIEPHAPLAATSM	AVEKR4		HTAGI**LDD**ATLTNLTP
SORKR7		HAGGIGRHAPLAATSL	AVEKR7	n	HAAGV**LDD**ATIASLTP
EPOKR6	n	HAAGVFPVRPLAETDE	RIFKR6	e	HTAGVFE**D**GVIEALTP
MXAKR2	e	HAAALLTESNLENMDL	RIFKR8	n	HTAGV**LDD**GVIGTLTP
MXAKR5		HAAGVSTLVSLDTMDE	SORKR1		HTAGAR**DD**GLIGDMTP
MTAKR6	n	HAAGVSTHRSWQETDP	EPOKR2		HAAGL**LDD**GLLAHQDA
PIMKR6		HAAGVGQAAALADTPL	EPOKR8		HAAGLV**DD**GLLMQQTP
PIMKR7	e	HTAVTIELAPLDETTL	PIMKR1		HAAGV**LDD**GVLDALTP
NYSKR1	e	HAAAAIELSALADTTV	PIMKR8		HTAGTV**DD**GTLDTLTP
NYSKR2	n	HSAGVADGDARAADLT	PIMKR10		HTAGVI**DD**GVLDSLTP
NYSKR10		HTAGVGHYGPLDTLST	NYSKR12		HTAGVV**ED**HVVDALTP
NYSKR11	e	HTAATIELHTLDATTL	NYSKR14		HTAGV**LDD**GVLDRLTP
AMPHKR1	e	HTAAVIELAALADTTV	NYSKR16		HTAGV**LDD**GVLTGLTP
AMPHKR2	n	HSAGVAH**DD**APVADLT	NYSKR17		HTAGV**LDD**ATVGTLTP
AMPHKR10		HTAGVGHYPGLDALTP	NYSKR18		HTAGV**LDD**GVIASLTP
AMPHKR11	e	HTAATIELHTLDATTV	AMPHKR12		HTAGVV**DD**HVVDELTP
			AMPHKR14		HAAGV**LDD**GVIDRLTP
			AMPHKR15		HTAGV**LDD**GIFPSLTP
			AMPHKR17		HTAGV**LDD**ATIGTLTA
			AMPHKR18		HTAGV**LDD**ALTGSLTP

134 - 149 region

A type			B type		
DEBSKR2	n	FSSGAGV**W**GSARQGAY	DEBSKR1	e	FSSFASAFGA**P**GLGGY
DEBSKR5	n	FSSNAGV**W**GSPGLASY	OLEKR1	e	FSSISATLGI**P**GQ**A**NY
DEBSKR6	n	FSSGAGV**W**GSANLGAY	PIKKR1	e	FSSVSSTLGI**P**GQ**G**NY
OLEKR2	n	FSSNAGV**W**GSGGQAVY	TYLKR1	n	FSSVTGT**W**GNAGQGAY
OLEKR5	n	FSSNAGV**W**GSGGQAVY	NIDKR1		FSSIVGV**W**GNGGQATY
OLEKR6	n	FSSNAGV**W**GSGGQAVY	RAPSKR2		YSSAAGVLGSAGQ**A**NY
PIKKR5	n	YSSNAGV**W**GSGSQGVY	RAPSKR5		YSSAAGVMAGAGQ**G**NY
TYLKR6	n	FSSGAAV**W**GSGGQTAY	RAPSKR1		YSSAAGVLGSAGQ**G**NY
TYLKR7		YSSGAGV**W**GSAGQGAY	RAPSKR12		YSSAAGVMGGGGQ**G**NY
NIDKR6	n	FSSAAAT**W**GSSGQSLY	FK506KR2	e	YSSISATFGGAGQ**A**NY
NIDKR7		FSSAAAT**W**GSSGQSLY	FK506KR8		YSSAAAVLGS**P**GQ**G**NY
AVEKR5		FSSGAGA**W**GSGNQCAY	FK520KR2	e	YSSISATFGGAGQ**A**NY
RIFKR5	n	YSSVSAVFMGAGSGSY	FK520KR3		YSSAAGVLGS**P**GQ**G**NY
RIFKR7	e	YSSASSIFMGAGSGGY	FK520KR8		YSSAAAVLGS**P**GQ**G**NY
SORKR4		FSSGAAV**W**GGGQQGGY	AVEKR1	n	YSSAAATFGA**P**GQ**A**NY
SORKR6	n	FSSGAVV**W**GGGQQGGY	AVEKR4		YSSAAATFGA**P**GQ**A**NY
SORKR7		FSSIAGV**W**GGGQQAGY	AVEKR7	n	FSSAAGILGSAGQ**G**NY
EPOKR6	n	FSSGAAV**W**GGKGQGAY	RIFKR6	e	FSSVAGVMGGGGQGPY
MXAKR2	e	FSSTSTL**W**GASGLAHY	RIFKR8	n	FSSGSGVFGS**P**GQ**G**NY
MXAKR5		FSSGSAV**W**GSARMAHY	SORKR1		YASLSGVLGN**P**GQ**A**NY
MTAKR6	n	FSSASAV**W**GAQGMAAY	EPOKR2		FSSASGVFGSIGQGSY
PIMKR6		VSSIAGV**W**GSAGQSAY	EPOKR8		YASAAGLFGS**P**GQ**G**NY
PIMKR7	e	YSSTAGM**W**GSGAHAAY	PIMKR1		FSSIVGVLGNAGQ**A**NY
NYSKR1	e	YSSTAGM**W**GSGVHAAY	PIMKR8		FSSVAGTLGA**P**GQ**G**NY
NYSKR2	n	FSSGAAV**W**GSGGQPGY	PIMKR10		FSSSSGAVGNAGQ**A**NY
NYSKR10		FGSIAGV**W**GSGNQSAY	NYSKR12		FSSTAGVLGAAGQ**G**NY
NYSKR11	e	YSSTAGM**W**GSGAHAAY	NYSKR14		FSSASAAVGN**P**GQ**A**NY
AMPHKR1	e	YSSTAGM**W**GSGVHAAY	NYSKR16		FSSVAGAVGN**P**GQ**A**GY
AMPHKR2	n	FSSGAAV**W**GSGGQPGY	NYSKR17		YSSVAGVTGG**P**GQ**G**NY
AMPHKR10		FGSIAGV**W**GSGDQSAY	NYSKR18		YSSVSGVLGS**P**GQ**G**NY
AMPHKR11	e	YSSTAGM**W**GSGAHAAY	AMPHKR12		FSSTAGLIGAAGQ**G**NY
			AMPHKR14		FSSASSAVGN**P**GQ**A**NY
			AMPHKR15		YSSTAGVMGS**P**GQ**A**NY
			AMPHKR17		YSSVAGVTGG**P**GQ**G**NY
			AMPHKR18		YSSISGVIGG**P**GQ**A**NY

Replacement of Y149 with F abolished activity. This suggests that Y149 donates a proton to the β-carbonyl oxygen atom during the course of the reduction. Reid et al. also observed independently that D95 is conserved in all B-type KRs and in KRs preceded by DH domains. However, the presence of D95 in AMPH KR2, an A-type KR, indicates that this residue alone is not absolutely diagnostic of KR domain stereospecificity. Scheme 2 shows that the LDD motif is the strongest indicator, whilst the 141, 144 and 148 positions should also be useful for predicting alcohol stereochemistry from KR sequences.

Experiments with DEBS KR1, KR2, KR5 and KR6 indicate that both A- and B-type KRs use the 4-*pro-S* hydride ion of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH).[14, 15] Two models have been proposed to explain how almost identical KR domains achieve different stereochemical outcomes.[13, 14] Reid et al.[13] propose that the relative positions of the nicotinamide ring and Y149 are fixed, so that β-ketoacyl chains must enter the KR active sites from opposite directions for the two types of alcohol stereochemistry to be generated. Herein, a third model (Model 1, Scheme 3) is suggested as another possibility that also satisfies these constraints. According to the double-helical structure proposed for PKSs,[16] in a dimeric module the orientation of one KR domain is rotated 180° with respect to that of the other. It has previously been assumed that β-ketoacyl ACPs always interact with the KR on the same polypeptide chain. However, interaction with the KR on the opposing subunit would reverse the direction of the ketoacyl chain in the active site and, as a consequence, the eventual stereochemical outcome. The LDD motif, P144 and N148 might favour interaction of a β-ketoacyl-ACP chain with the KR on one polypeptide, whereas W141 might exclude the chain from this KR but allow binding to the KR on the other polypeptide.

Experiments with heterodimers reconstituted from mutant FAS and PKS polypeptides led to the unexpected finding that the KS and AT domains can

Scheme 2. *Alignment of the residue 88 – 103 and 134 – 148 regions of various A- and B-type KR domains. These regions are easily recognisable within complete KR sequences. The alignments reported by Donadio and Katz[18] were used to assign Residue 1 of the KR domain. The residues of the LDD, PN and W motifs are shown in bold type. The PKSs and sequence accession numbers are: DEBS, erythromycin, L07626, X62569; OLE, oleandomycin, AF220951; PIK, pikromycin, AF079138; TYL, tylosin, U78289; NID, niddamycin, AF016585; RAPS, rapamycin, X86780; FK506, AF082100, y10438; FK520, AF235504; AVE, avermectin, AB032367; RIF, rifamycin, AF040570; SOR, soraphen, U24241; EPO, epothilone, AF217189; MXA, myxalamid, AF319998; MTA, mxyathiazole, AF188287; PIM, pimaricin, AJ278573; NYS, nystatin, AF263912; AMPH, amphotericin, AF357202. KR numbers refer to the module in which the domain appears. KRs from modules thought to epimerise the C2 atom of a 2-alkyl-3-ketoacyl intermediate are marked e, those from nonepimerising modules are marked n.*

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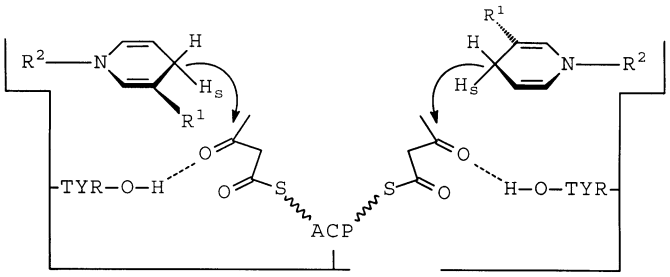
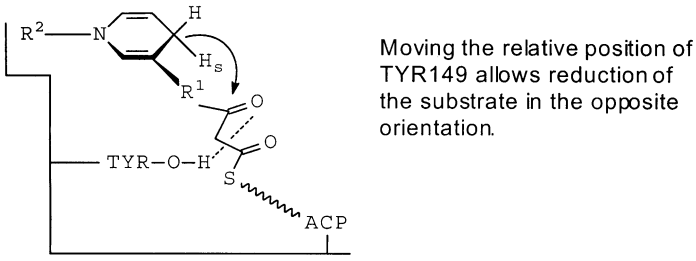
Model 1
Residues at positions 93–95, 141, 144 and 148 allow binding to one or other KR



Model 2
Residues at positions 93–95, 141, 144 and 148 cause changes in the active site.



Scheme 3. Models to explain how key residues might influence stereospecificity. R^1 = carboxamide group of nicotinamide ring; R^2 = remainder of NADPH. Model 1: The active sites of the two KR domains are related by rotational symmetry. A β-ketoacylchain attached to an ACP enters the two KR active sites in different orientations, from the carboxamide side or the opposite side in this depiction. The stereochemical outcomes are different because different faces of the β-ketone group are presented to the nicotinamide ring. The key residues favour binding to one KR and prevent binding to the other. Model 2: The amino acid residues at the key positions bring about subtle changes in the active site. This allows reduction with different substrate orientations. No substituents are shown at C2 of the acyl intermediate.

cooperate with the ACP of either subunit.[17] There has previously been no reason to suspect interactions between ACPs and KRs on opposite multienzyme polypeptides. The complementation approach could be used to investigate whether or not these interactions can occur. Model 1 in Scheme 3 predicts that only one type of KR should be functional in KR^-ACP^+/KR^+ACP^- PKS heterodimers.

In the model proposed by McPherson et al.,[14] it is implicit that the relative positions of the nicotinamide ring of NADPH and Y149 are different in the two types of KR, so that reduction can occur with two different substrate orientations. The LDD motif, P144, N148 and W141 might adjust the active site in this way, as outlined in Model 2 in Scheme 3. Whatever their precise roles, the amino acids at these positions appear to be useful indicators of KR stereospecificity.

Finally, interactions across a subunit interface could also account for the stereochemistry of enoyl reductions. Where incorporation of a branched extender unit is followed by complete β-ketone processing, interaction of the α,β-unsaturated acyl-ACP with one or other of the ER domains could determine the final alkyl stereochemistry at C2. Only a few amino acid sequence differences might be required to switch the outcome. Since ER domains occur infrequently in PKS modules, relatively few sequences are available. However, ER–ACP interactions could also be investigated in complementation studies.

Experimental Section

Sequences of PKSs were extracted from the EMBL database. Biosynthetic diagrams in sequence papers could be used to assign KR domain stereospecificity as A- or B-type for the erythromycin, rapamycin, FK520, avermectin, myxalamid, and myxathiazole PKSs. For all other KR domains, models of absolute structures were used. The ClustalW programme (available at http://www.ebi.ac.uk) was used to align A- and B-type KR sequences.

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Received: February 20, 2003 [Z581]

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