Avermectin Biosynthesis

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1. Introduction

The prevention and treatment of endo- and ectoparasitic infections of livestock are important issues in animal husbandry and several antiparasitic compounds have been discovered to date. In addition to antibiotics, anthelmintic compounds, isolated from microbial metabolites are currently available. Of the numerous microbial products described to date, relatively few possess anthelmintic activity, and only amino glycoside antibiotics such as, hygromycin B, destomycin A, and paromomycin, have actually been used as antiparasitic drugs: the anthelmintic market is dominated by synthetic compounds. The discovery of avermectins has had a tremendous impact on veterinary health because the compounds have potent antiparasitic and broad-spectrum activity against nematode and arthropod parasites. Although similar in structure to antibacterial macrolides and antifungal polyenemacrolides, avermectins lack antibacterial and antifungal activities. High-affinity avermectin-binding sites identified in membranes from *Caenorhabditis elegans* have been shown to be associated with physiologically relevant drug target in nematodes.[1] Avermectins are capable of modulating γ-aminobutyric acid-gated chloride channels in vertebrate neurons.[2,3] Electrophysiological findings generated by injection of *C. elegans* mRNA into *Xenopus laevis* oocytes have indicated that avermectins act on glutamate-gated chloride channels in nematodes.[4,5] Furthermore, studies of avermectin-binding protein by using photoaffinity labeled avermectin, an azidoavermectin analog (4″α-(4-azidosalicylamido-ϵ-caproylamido-β-analylamido)-4″-deoxyavermectin B1a), revealed that azido avermectin analogs bound specifically and with high affinity to *C. elegans* membranes and, upon photoactivation, became covalently linked to three *C. elegans* polypeptides of 53, 47, and 8 kDa.[6] A single major polypeptide of about 47 kDa was also found in the membranes prepared from *Drosophila melanogaster* heads.[6] These proteins are believed to be associated with avermectin-sensitive chloride channels present in the neuromuscular system of *C. elegans* and *D. melanogaster*. On the other hand, azido avermectin analogs did not bind to rat brain membranes, suggesting that avermectins act selectively on nematode and insect receptors.[6]

"Ivermectin", a hydrogenated product of avermectin B1, has been used as an antiparasitic agent since 1981, and as an agricultural pesticide and an antiparasitic agent since 1985. The efficacy of ivermectin against nematode and arthropod parasites is unprecedented in potency and breadth of spectrum. Ivermectin has been used for livestock farming and health care of companion animals. The efficacy of ivermectin in human onchocerciasis has made it a promising candidate for the control of one of the most insidious and intractable of all tropical diseases.[7] Recently, ivermectin has been found to be effective against human disease strongyloidiasis in Okinawa Prefecture, Japan.[8]

Avermectins are a series of 16-membered macrocyclic lactones produced by *Streptomyces avermitilis* which was isolated from a soil sample collected in Shizuoka Prefecture, Japan, in 1978. They are pentacyclic polyketide-derived compounds linked to a disaccharide of the methylated deoxysugar oleandrose. Eight major compounds result from structural differences at three positions, C5, C22−23, and C26 (Figure 1).[9−11] Among these components, the B1 fraction has the most effective antiparasitic activity.[12] Because the industrial separation of B1a and B1b in the B1 fraction is quite complicated, the commercial product contains more than 80% of B1a and less than 20% of B1b components. Although *S. avermitilis* produces eight avermectin compounds, only two, B1a and B1b, are available to the medical, veterinary, and agricultural fields. The microorganism produces

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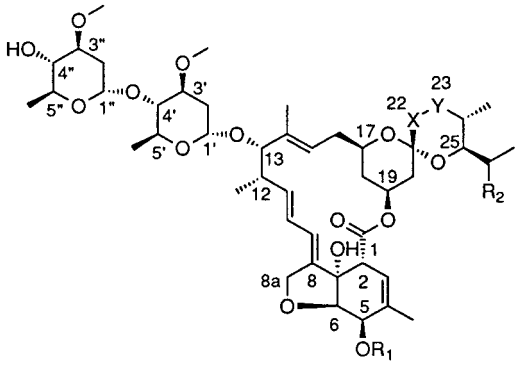
oligomycin and polyene in addition to avermectins. The former is a polyketide antibiotic and a specific inhibitor for the oxidative−phosphorylation system in mammalian cells.[13]

Avermectins have a good potency and broad spectrum against a variety of nematode and insect parasites combined with quite a low level of toxic side



		R_1	R_2	X-Y
Avermectin	A1a	CH_3	C_2H_5	CH=CH
	A1b	CH_3	CH_3	CH=CH
	A2a	CH_3	C_2H_5	CH_2-CH(OH)
	A2b	CH_3	CH_3	CH_2-CH(OH)
	B1a	H	C_2H_5	CH=CH
	B1b	H	CH_3	CH=CH
	B2a	H	C_2H_5	CH_2-CH(OH)
	B2b	H	CH_3	CH_2-CH(OH)
Ivermectin	B1a	H	C_2H_5	CH_2-CH_2
	B1b	H	CH_3	CH_2-CH_2

Figure 1. Structural formulae for avermectins and ivermectins. Both sugars are α-L-oleandrose. Ivermectin is chemically synthesized from avermectin B1 components (B1a and B1b) by selective hydrogenation and the commercial product of ivermectin consists of more than 80% of ivermectin B1a and less than 20% of ivermectin B1b.

effects against host organisms. However, there is a need for modified avermectins with enhanced potency, spectrum of activity, and extremely low levels of toxicity. Elucidation of the biochemistry and genetics of avermectin biosynthesis could lead to rational approaches for increasing the yield of avermectin, selective production of desired components, and production of novel avermectin derivatives through mutagenesis, and recombinant DNA technology.

In this review, we describe the current understanding of avermectin biosynthesis and the production of the specific component(s) of natural avermectins and novel derivatives by controlling the avermectin biosynthetic pathway, a process known as "engineered biosynthesis".

2. Biosynthesis

The biosynthetic pathway of avermectin has all but been elucidated.[14,15] The proposed pathway is based on evidence obtained from several studies, including incorporation of labeled precursors into the avermectins, identification of key intermediates produced by biosynthetically blocked mutants, the conversion of proposed intermediates to avermectins by biosynthetically blocked mutants or wild type-producing strains, and the *in vitro* measurement of enzymes involved in the biosynthesis of avermectins.

Avermectin biosynthesis can be classified into three stages as follows: the formation of the polyketide-

derived initial aglycon (6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycons), the modification of the initial aglycon to generate avermectin aglycons, and glycosylation of avermectin aglycons to generate avermectins. Labeling experiments have determined that the aglycon moiety of avermectins is derived from various fatty acids. After the presumptive aliphatic polyketide-derived precursor is lactonized to generate an initial aglycon, a series of modifications including cyclization, reduction and/or methylation occur to form the avermectin aglycons. In the terminal biosynthetic steps, *O*-glycosylation at C13 and C4′ is performed using dTDP-oleandrose to form avermectins.

2.1. Incorporation of Labeled Precursors

2.1.1. Biosynthesis of Initial Aglycon and Its Precursor

Cane *et al.*[16] determined the lactone to be formed by the condensation of fatty acids by feeding ^{13}C-labeled compounds. Studies with single-labeled precursors, [1-^{13}C]acetate, [1-^{13}C]propionate, [2-^{13}C]-acetate, and [3-^{13}C]propionate, double-labeled precursors, [1,2-^{13}C]acetate and [2,3-^{13}C]propionate, and radioisotope-labeled precursors [1-^{14}C]acetate, [2-^{14}C]acetate, [1-^{14}C]propionate, and [2-^{14}C]propionate, have established that the avermectin aglycons are formed by head to tail assembly of seven acetate and five propionate units.[16−18] The 2-methylbutyryl residue (C25−28) of the "a" components and the isobutyryl residue (C25−27) of the "b" components were not labeled by either acetate or propionate. They were determined to arise by catabolism of L-isoleucine or L-valine via a branched-chain 2-oxo acid dehydrogenase (*bkd*) pathway that gives the 2-methylbutyryl residue in "a" components or isobutyryl residue in "b" components, respectively.[17,19] This finding was supported by the efficient incorporation of L-[U-^{14}C]-isoleucine and its keto acid, [U-^{14}C]2-oxo-3-methylvalerate, into the "a" components and L-[3,4-^{3}H]-valine and its keto acid, [3,4-^{3}H]2-oxoisovalerate, into the "b" components.[20] Furthermore, C25 of avermectin B1a was highly enriched by [1-^{13}C] 2-methylbutyrate and that of avermectin B1b by [1-^{13}C] isobutyrate.[21] Interestingly, a mutant strain which lacks the activity of branched-chain 2-oxo acid dehydrogenase, produces natural avermectins in culture supplemented with 2-methylbutyrate (*S*(+)-2-methylbutyrate) or isobutyrate, but not in that supplemented with L-isoleucine, L-valine, 2-oxo-3-methylvalerate, or 2-oxoisovalerate.[19] The properties of this mutant indicate that branched-chain 2-oxo acid dehydrogenase of *S. avermitilis* provides CoA esters, 2-methylbutyryl-CoA and isobutyryl-CoA, for the starter unit during avermectin aglycon biosynthesis. Efficient incorporation of 2-methylbutyrate and isobutyrate into the avermectin aglycon indicates that an acyl-CoA synthase, able to convert exogenous branched-chain fatty acids to their CoA esters, has to exist in *S. avermitilis*. Presumably, the acyl-CoA synthase also recognizes (*R*)-(−)-2-methylbutyrate since supplementation with this isomer yields isomeric avermectins unseen in the wild-type strain.[19] This reduced substrate specificity has been exploited to synthesize a variety of avermectins with novel C25 residues. The ability of the mutant to incorporate various α-branched-chain carboxylic acids into novel avermectins indicates that production of malonyl-CoA and methylmalonyl-CoA by the degradation of branched-chain 2-oxo fatty acids, in which branched-chain 2-oxo acid dehydrogenase catalyzes the initial step, is not absolutely essential for avermectin biosynthesis.

The origin of the oxygen atoms in the aglycon moiety was investigated by measuring the incorporation of [1-^{13}C, 1-^{18}O]acetate and [1-^{13}C, 1-^{18}O]propionate.[16] The oxygens at C1, C5, C7, C13, C17, C19, and C23 (for the "2" components) retained their isotope content. This finding was similar to that in nemadectin except that the oxygen at C13 is absent.[17] The oxygen between C21 and C25 would probably be derived from the 2-methylbutyryl or isobutyryl side chain, but the oxygen in the benzofuran between C6 and C8a could not arise by this route. Although the origin of the furan ring oxygen has not been determined in *S. avermitilis*, it is probably "molecular oxygen", because similar studies on nemadectin biosynthesis[22] have shown that the oxygen in the nemadectin benzofuran between C6 and C8a is derived from molecular oxygen.

The mechanism for closure of the five rings in the avermectin aglycons was determined by labeling experiments. The retention of the oxygen from fed precursors on C17 and C25 but not C21 implies that the spiroketal is probably formed by ketalization of a carbonyl at C21[16] (Figure 2). On the other hand, the retention of oxygen at C7 indicates that the cyclohexene ring is generated by an aldol condensation between C2 and C7[16] (Figure 3).

The results of incorporation of ^{13}C-labeled precursors and analysis of the resulting ^{13}C-labeled avermectin indicate that the avermectin aglycons are derived by head-to-tail condensation of various acyl groups which is similar to the biosynthesis of other polyketides.[23] The polyketide synthases (PKS) use the appropriate CoA ester as a primer and add acetate units from malonyl-CoA, propionate units from methylmalonyl-CoA or butyrate units from ethylmalonyl-CoA to assemble the polyketides. Avermectin aglycon is formed by addition, to the starter unit (2-methylbutyrate or isobutyrate), of 12 acyl condensations in the order P-A-A-A-A-P-P-A-P-A-P-A-P (P, propionyl; A, acetyl). The overall reaction catalyzed by the avermectin PKS is similar to that employed in the formation of long-chain fatty acid by fatty acid synthase (FAS). Following each condensation, the β-keto group is reduced to a hydroxyl group by a specific ketoreductase (KR) and the hydroxyl group is dehydrated to generate a double bond by a specific dehydratase (DH). Enoylreductase (ER), which converts the double bond to a single bond is not required during the formation of avermectin aglycon, because no fully saturated β-carbon chain is found in the avermectin aglycon. It seems that the ketoreduction and dehydration of each keto group takes place after each acyl condensation, as opposed to having an elongated poly-β-ketone chain synthesized by acyl condensation, which is subsequently reduced and dehydrated. This is consistent with the lack of conversion of "2" components to "1" components observed in feeding experiments.[24]

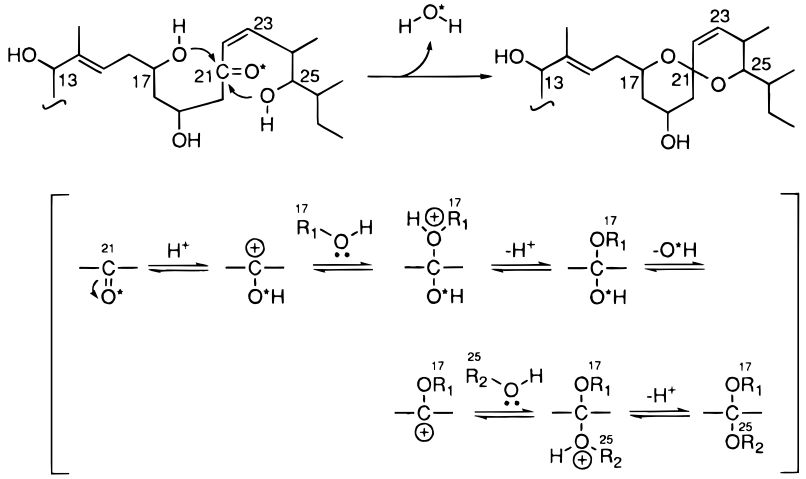


Figure 2. Spiroketal formation at C17−25. Oxygen at C21 carbonyl is released by ketalization of a carbonyl at C21 and two hydroxyl groups at C17 and C25.

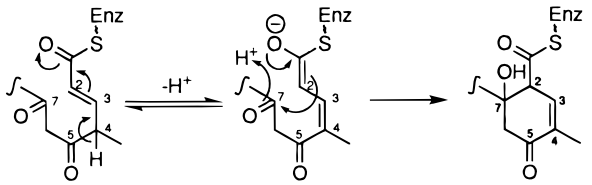


Figure 3. The formation of cyclohexene ring at C2−7. The ring formation is involves aldol condensation between C2 enoyl and C7 carbonyl.

2.1.2. Biosynthesis of Oleandrose and Other Molecules

A study with labeled glucose indicated that the label from [1-^{13}C]glucose and [U-^{13}C]glucose was highly incorporated into the oleandrose units.[18] This finding suggests that glucose is the direct precursor for oleandrose which is attached to avermectin aglycons. Since L-oleandrose is a 2,6-dideoxy-3-methylated hexose, the early steps of its biosynthesis presumably resemble those of rhamnose, in which dTDP-glucose is formed by dTDP-glucose synthase, and then converted to dTDP-4-oxo-6-deoxyglucose by dTDP-glucose-4,6-dehydratase. Furthermore, dTDP-oleandrose was found and purified from avermectin-nonproducing mutants and shown to be the immediate precursor of the oleandrose moiety.[25] The methoxy group of oleandrose at C3 has been confirmed to be derived from the methyl residue of L-methionine by labeling experiments,[24] and the methylation was found to be inhibited by an analogue of *S*-adenosyl-L-methionine, sinefungin, which is a potent inhibitor of methyltransferases. Similarly, a methyl group at aglycon position C5 of the "A" components was also derived from L-methionine by labeling experiments.[18] Furthermore, sinefungin inhibited not only both C3′ and C3″ *O*-methylations but also C5 *O*-methylation in avermectins.[26] Figure 4 summarizes the results obtained from the incorporation studies with labeled precursors.

2.2. Genetics of Avermectin Biosynthesis

Early approaches to the genetics of avermectin biosynthesis involved isolating and characterizing biosynthetically blocked mutants altered in avermectin biosynthesis, and mapping these mutations with other markers on the *S. avermitilis* genome[27,28] (Figure 5). The resulting genetic map of *S. avermitilis* revealed that the genes involved in avermectin biosynthesis formed a cluster on the chromosome as observed for other genes for antibiotic biosynthesis. Mutants that failed to produce avermectin and those

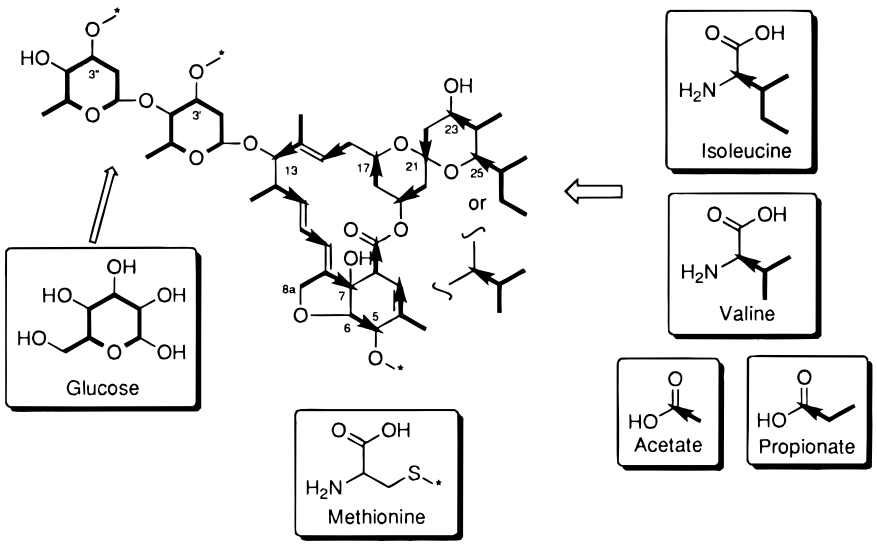


Figure 4. Incorporation of labeled precursors in the avermectins.

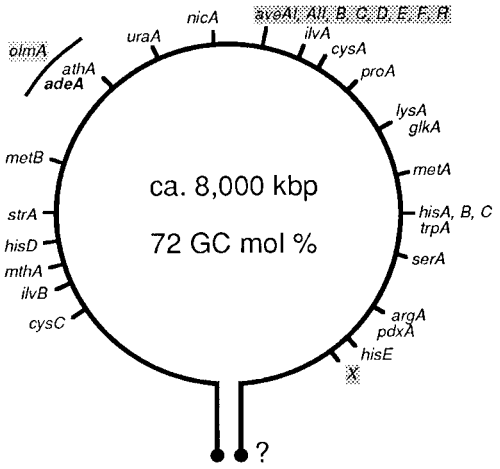


Figure 5. Chromosome map of *S. avermitilis*. The markers involving secondary metabolite production, *ave* and *olm*, indicate genes involved in avermectin or oligomycin biosynthesis, respectively. The distance between each marker is not precise.

that produced avermectin aglycons only have been designated as *aveA* and *aveB*, respectively.[27] After the characterization of these mutants was published, similar mutations were reported by other groups and designated as "*avr*".[29] The dual designation for the same genes is confusing, and we urge colleagues to adapt the original symbol, *ave*, for genes involved in avermectin biosynthesis.

The cloning of the gene cluster for avermectin biosynthesis has been reported independently by the Merck group[29,30] and us.[14,31] In both cases, the primary clones were obtained by shotgun cloning of a gene involved in the C5 *O*-methylation step by complementing a mutant defective in this step. There are clearly different TLC patterns between products from a mutant defective in the C5 *O*-methylation step (produces only "B" components) and the wild-type strain (produces "A" and "B" components). This difference was exploited to screen a genomic library of *S. avermitilis* DNA for complementation of the mutant defective in C5 *O*-methylation. The Merck group also used a mutant defective in glycosylation for similar screening. The DNA segments obtained were extended by genome walking using a cosmid library of *S. avermitilis* to obtain the entire gene cluster for avermectin biosynthesis. Each biosynthetic step was confirmed by complementation of mutants defective in each step of the pathway using cloned DNA segments, or by analysis of deletions in the avermectin gene cluster created using the DNA segments cloned. A series of chromosome walks gave a set of cosmid clones spanning 105 kbp. As shown in Figure 6, the gene cluster for avermectin biosynthesis was located on a 90 kbp segment by the above analysis.

Table 1. Characteristics of Mutants Affecting Avermectin Production

mutant class	fermentation products	mutation in
aveA [a]	none	PKS (polyketide synthase)
aveB [b]	aglycons	glycosylation
aveC	"2" components	C22,C23-dehydration (?)
aveD	"B" components	C5 *O*-methylation
aveE	6,8a-seco-6,8a-deoxy derivatives	C6,8a furan ring closure
aveF	5-oxoavermectins	C5 keto reduction
aveR	none	regulatory region
X	"a" components	?

[a] *aveAI* and *aveAII*. [b] The mutations are classified into seven classes.

2.2.1. Mutants Altered in Avermectin Biosynthesis

Several kinds of biosynthetically blocked mutants defective in the biosynthesis of the eight closely related avermectin components produced by the wild-type strain have been isolated after mutagenesis by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. These mutants were classified into three types as follows: mutations in the early stage of biosynthesis, which means the formation of the initial aglycons (6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycons), mutations in the middle stage of biosynthesis involved in the conversion of the initial aglycons to avermectin aglycons, and mutation in the late stage of biosynthesis (biosynthesis of oleandrose and glycosylation). Table 1 summarizes the characteristics of the *ave* mutants.[15] By analyses of products accumulated by the mutants and conversions of intermediates, the pathway for avermectin biosynthesis after formation of the initial aglycons was elucidated (Figure 7).

2.2.1.1 aveAI and aveAII (Involved in Aglycon Synthesis). This class of mutation prevents formation of the initial aglycons and all other avermectin compounds.[27] However, these mutants have the ability to convert 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycons or avermectin aglycons to natural avermectins.[24,27] Most of the class *aveAI* and *aveAII* mutants are presumed to be defective in avermectin PKS.

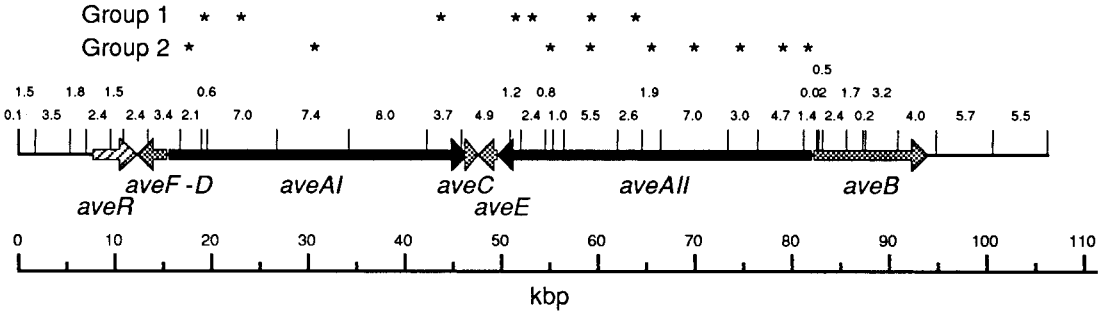


Figure 6. Physical and genetic map of the 110-kbp gene cluster for avermectin biosynthesis. The location of *Bam*HI sites is indicated by vertical lines above the map. The *Bam*HI fragments which form two related sequence groups of cross-hybridizing fragments are indicated by asterisks (*) above the map. The arrow indicates the transcriptional direction. The *aveC* or *aveE* belongs to a single transcription with *aveAI* or *aveAII*, respectively. The *aveD* and *aveF* form an operon.

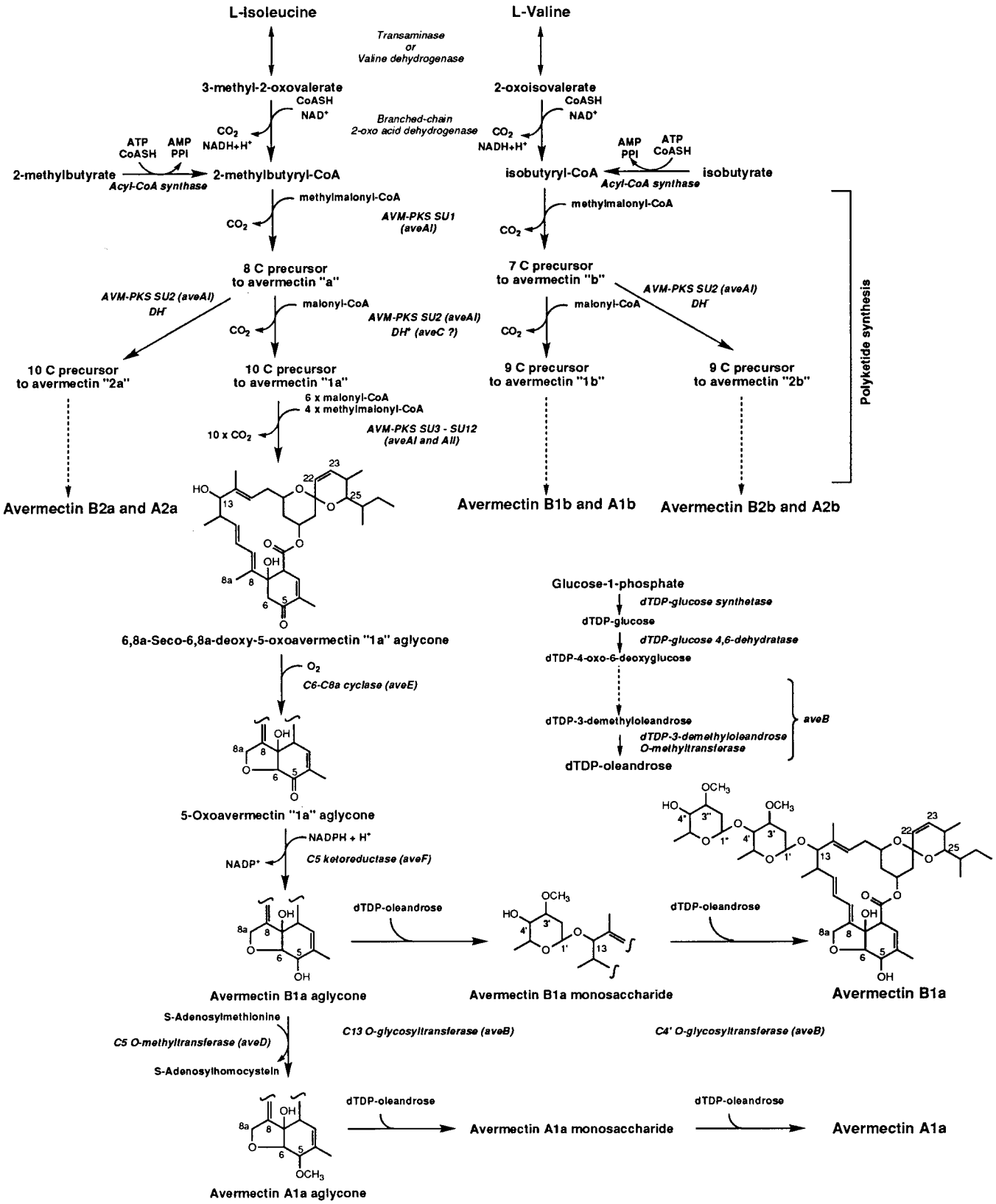


Figure 7. Pathway for the biosynthesis of avermectin A1a and B1a. The 12 SUs of avermectin PKS (AVM-PKS) act sequentially to add 12 acyl units (seven acetate and five propionate units). Until the formation of 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycon, which is designated "initial aglycon", is the initial stage of avermectin biosynthesis. The middle stage of biosynthesis that is post-polyketide modification is from *aveE* to *aveD*. The last stage is glycosylation.

Within the 90 kbp gene cluster for avermectin biosynthesis, the central 65 kbp segment was found to be required for aglycon biosynthesis by phenotypic analysis of strains containing deletion or insertion mutations in this region. Several cosmid clones containing segments of the *aveAI* and *aveAII* region cross-hybridized to various *Bam*HI fragment probes obtained from either region.[15,32,33] Southern hybridization analysis of chromosomal DNA from the wild-type and mutant strains found, in one cross-hybrid-

izing group, at least nine bands within the *aveAI* and *aveAII* region which cross-hybridized using a 1.9 kbp *Bam*HI fragment from the *aveAII* region as a probe; in another group seven cross-hybridizing bands were found using a 0.6 kbp *Bam*HI fragment from the *aveAI* region as a probe.[15] Interestingly, only one sequence, located within the 5.5 kbp *Bam*HI fragment in the *aveAII* region, cross-hybridized with both probes. A map of the gene cluster for avermectin biosynthesis showing the location of the identified genes and cross-hybridizing regions is shown in Figure 6.

A partial sequence analysis of the multiple cross-hybridizing regions within the gene cluster for avermectin biosynthesis indicated that these segments encode the avermectin PKS. The erythromycin PKS has been shown to consist of three large multifunctional polypeptides[34] which together contain six repeats of fatty acid synthase (FAS)-like functions, each of which is called a synthase unit (SU).[35,36] The DNA repeat encoding an SU is called a "module".[36] The minimum SU contains catalytic domains for β-ketosynthase (KS), acyltransferase (AT), β-ketoreductase (KR), and acyl carrier protein (ACP) to elongate β-hydroxyl carbon units. Some SUs contain additional domains for dehydratase (DH) and enoylreductase (ER), and the terminal SU contains an additional domain for thioesterase (TE), which catalyzes lactonization of the long aliphatic chain. The domains for KS, AT, KR, and ACP within the PKS polypeptides were identified by the presence of conserved active amino acid sequences. The DH and ER domains were imprecisely positioned in a region between the AT and KR domains and the TE domain was located in the C-terminus of the terminal SU.[35−37] The avermectin PKS genes are organized into two converging blocks of ORFs (Figure 8). From the results of limited sequencing analysis, a feature of the two regions, *aveAI* and *aveAII*, is that they encode large multifunctional polypeptides containing domains possessing putative FAS-like activities. The catalytic domains are generally arranged in the same relative order as those found in type I-FASs of mammalian and fungal PKSs. The avermectin PKS polypeptides have strong similarity to those of the erythromycin PKS. The size of the four avermectin PKS polypeptides would be 450, 700, 610, and 540 kDa, respectively, as determined by SDS-polyacrylamide gel electrophoresis of a crude cell-extract.[38] These values are consistent with those calculated on the basis of the encoding DNA sequences. The DNA sequence information, in which some ambiguities remain, and the measured size of the polypeptides indicate that the distribution of SUs consists of two in PKS-1, three in PKS-3 and PKS-4, and four in PKS-2. The 12 SUs correspond to the 12 cycles required for synthesis of the initial aglycon. Although the sequence data were not complete enough to identify all domains encoded by each module, a prediction of the domains to be found in each module was made using two assumptions. First, the domain order in each module is a subset of the order found in FAS.[39] Each avermectin PKS module would contain one of the following domain organizations: KS, AT, and ACP; KS, AT, KR, and ACP; or KS, AT, DH, KR, and ACP. The size of a module corresponds to the numbers of domains encoded by the module: the KS-AT-ACP set is less than 3.5 kbp, the KS-AT-KR-ACP set is about 4.5 kbp, the KS-AT-DH-KR-ACP set is 5.5 to 5.8 kbp.[40] Second, the first and last modules encode unique domains like the erythromycin PKS modules. The first module encodes the initial AT and ACP domains which function to load the starting acyl group on to the PKS and the last module encodes a final domain (TE) which releases the completed polyketide to form a lactone. In the avermectin PKS, all modules encode putative KS, AT, and ACP domains responsible for all of the acyl condensation processes but for a variable number of functions involved in the processing of the β-carbons. Modules 1, 4, 5, and 10 encode a KR domain only. Modules 3, 7, and 8 lack a KR. Modules 2, 6, 9, 11, and 12 carry KR and DH domains. Among these modules, some carry preserved sequences corresponding to KR or DH domains but these domains are dysfunctional due to a partial deletion or incorrect consensus amino acid sequence. When the PKS functions predicted to be required to synthesize the avermectin aglycon are compared to the domains identified in the avermectin PKS, it appears that two of the KR domains (modules 3 and 7), and one DH domain in module 10 are nonfunctional. Although module 3 contains a sequence corresponding to a KR domain, the size of the module (3.6 kbp) is smaller than that of other modules containing the KS-AT-KR-ACP set (4.8 kbp), perhaps due to a partial deletion of that domain. Furthermore, C21 is likely a carbonyl group because the spiroketal is probably formed by ketalization of a carbonyl at C21 (Figure 2) and two hydroxyl groups at C17 and C25. The β-hydroxy carbons at C17 and C25 are generated by KR domains of SU1 or SU5, respectively, during chain elongation by acyl condensation. The size of module 7 (4.8 kbp) is the same as that of the KS-AT-KR-ACP set (4.8 kbp), but the KR domain is nonfunctional because C7 must be a carbonyl residue in order to form the cyclohexene ring by aldol condensation between the C2 enoyl and the C7 carbonyl (Figure 3). The dysfunctional DH domain in module 10 is consistent with the retention of a hydroxyl group at C13. If the DH is functional, the hydroxyl group at C13 would be dehydrated to form an enoyl group which could not be glycosylated by dTDP-oleandrose. The DH domain of module 2 contains a partially conserved dehydratase consensus sequence with a few mismatched amino acids in the active site. This DH which corresponds to a C22−23 dehydration seems to have partial dehydratase activity because two intermediates that contain β-hydroxy or enoyl carbons at C22−23 are processed in subsequent acyl condensation. The size of module 1 which is responsible for the first acyl condensation is 6.6 kbp because it encodes initial AT and ACP starting domains. Since SU1 has initial AT and ACP domains and the AT domain is located near the N-terminus,[35−37] the SU functions in the first acyl condensation between 2-methylbutyryl-CoA (in "a" components) or isobutyryl-CoA (in "b" components), and methylmalonyl-CoA to form 8 or 7 carbon precursors, respectively. Accordingly, the first acyl condensation determines

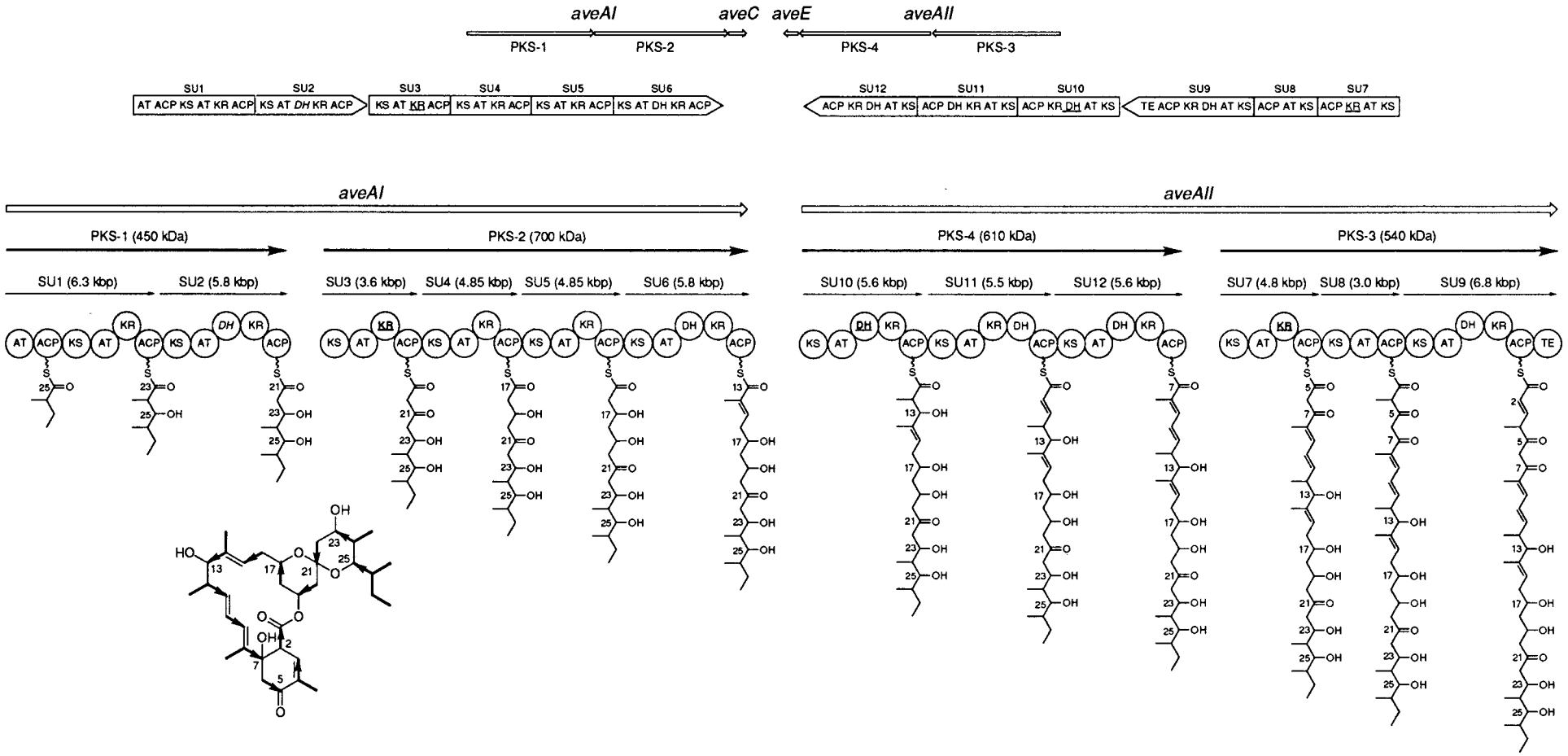


Figure 8. Model for 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycon formation and predicted domain structure of the avermectin PKS. Putative enzymatic activity abbreviations: ACP, acyl carrier protein; AT, acyltransferase; DH, dehydratase; KR, β-ketoreductase; KS, β-ketoacyl-ACP synthase; TE, thioesterase. The reaction order from SU10 to SU9 encoded in *aveAII* is drawn in the direction opposite to the gene order on the genome. Underlined symbols in SU3, SU7, and SU10 indicate nonfunctional domains. Italic symbol in SU2 indicates that the domain has a partial activity.

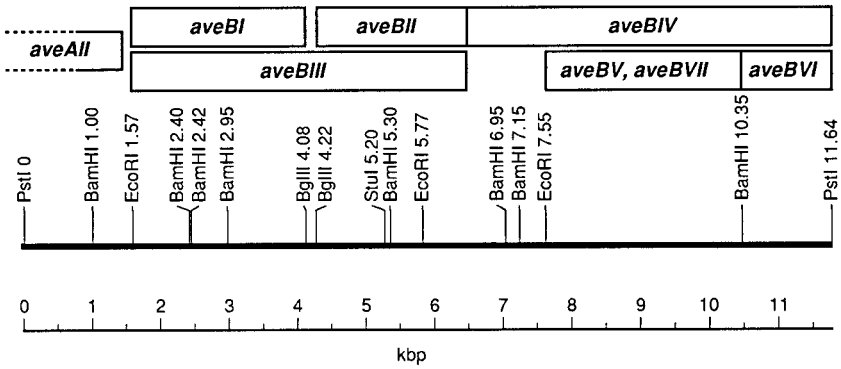


Figure 9. Organization of *aveB* cluster involving biosynthesis of dTDP-oleandrose and glycosyltransferase. Boxes indicates the complementation fragment of each mutant. The *aveBVII* encodes dTDP-demethyloleandrose C3 *O*-methyltransferase.

the components synthesized. Module 9 with the size of 6.8 kbp encodes an extra domain, thioesterase (TE), and is responsible for the last acyl condensation and the release of the completed polyketide from the PKS. As shown in Figure 8, each of the 12 modules encoding multifunctional SUs can be assigned to a specific step in the synthesis of the avermectin aglycon. A comparison of the module organization between the PKS genes of avermectin and erythromycin biosyntheses reveals some differences. The erythromycin PKS genes comprise a cluster of six modules and are transcribed in the same direction.[35,36] The order of the six modules in the chromosome is the same as the order in which the six SUs function to biosynthesize 6-deoxyerythronolide B.[36] On the other hand, the PKS genes for avermectin biosynthesis are organized into two convergently transcribed clusters of six modules. Each of the three polypeptides constituting the erythromycin PKS contains two SUs. According to the limited DNA sequence information from the PKS region and the size of the four proteins detected, the avermectins PKS proteins appear to contain two, three, or four SUs. PKS-1 with two SUs and PKS-2 with four SUs contain modules 1 and 2, and 3−6, respectively, whereas PKS-3 and -4 with three SUs contain modules 7−9, and modules 10−12, respectively. The conservation of amino acid and DNA sequences of the catalytic domains comprising the modules explains the cross-hybridizing segments identified by Southern analysis of the *aveAI* and *aveAII* regions.

2.2.1.2. aveB (Synthesis of dTDP-Oleandrose and Attachment of Oleandrose Moiety to Aglycon). The *aveB* mutants produce avermectin aglycons, indicating that the mutation affected glycosylation.[27,42] Several kinds of mutants involving transfer of the oleandrose component to the aglycon or synthesis of dTDP-oleandrose were isolated.[33,43,44] These glycosylation defective mutants are classified into seven complementation classes as shown in Figure 9. Six of them that fail to produce avermectins yield avermectin aglycons "A" and "B". The other class mutant (*aveBVII*) that is defective in 3′- and 3″-*O*-methylation produces demethylavermectins[45] (Figure 10). When the demethylavermectins were fed to the wild-type strain, two hydroxyl groups at C3′ and C3″ in the disaccharide moiety were not methylated, suggesting that the methylation of demethyloleandrose takes place before the sugar moiety is attached to the avermectin aglycons.[45] However, in other cases of

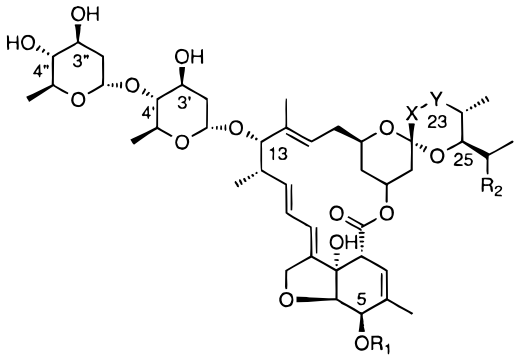


Figure 10. Structures of demethylavermectins produced by *aveBV* mutants. Variations at C5, C22−23, and C26 give rise to eight components: combinations of A ($R_1 = CH_3$), B ($R_1 = H$), 1 (X−Y = CH=CH), 2 (X−Y = CH_2CHOH), a ($R_2 = C_2H_5$) and b ($R_2 = CH_3$).

macrolide biosynthesis, *O*-methylation of deoxyhexose, mycarose in erythromycin (*eryG*) [46] or demethylmycinose in tylosin (*tylF*),[47] occurs after the sugar moiety is attached to the aglycons. Avermectin aglycon glycosyltransferase was detected in the cell-free extract and it used dTDP-oleandrose to catalyze the stepwise addition of oleandrose to avermectin aglycons to form the mono- and disaccharides.[25] The glycosyltransferase has a relatively lower substrate specificity because mono- or disaccharide derivatives of 6,8a-seco-6,8a-deoxy derivatives were detected from *aveE* mutants.

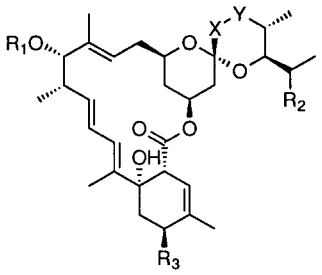
An 11 kbp subclone from the right end of the gene cluster for avermectin biosynthesis restored normal avermectin biosynthesis for all the mutants defective in glycosylation of avermectin or *O*-methylation at C3 of the oleandrose moiety[41] (Figure 9). In contrast, the genes involved in the glycosylation of macrolides erythromycin or tylosin do not form a cluster in a single region of the *ery*[48] or *tyl*[49] genes. The DNA sequencing of a 9 kbp fragment of the 11 kbp subclone showed that at least eight ORFs were present.[44] These eight ORFs would be sufficient to encode all the functions necessary for the biosynthesis of dTDP-oleandrose and its linkage to avermectin aglycons. The first of the eight ORFs which is adjacent to *aveAII* contains a reductase signature sequence,[44] but its disruption by an insertion mutation did not affect biosynthesis of avermectins. The gene product of this ORF may not function in avermectin biosynthesis; if so only seven classes of mutants which are defective in the glycosylation and dTDP-oleandrose synthesis would be obtained.

2.2.1.3. aveC (Involved in C22−23-Dehydration). The *aveC* type of mutant produces "2" components of avermectins.[14,41] The chemical structures of the type "1" components of avermectins differs from those of "2" only in the bond between C22 and C23: "1" components have a double bond, while "2" components have a single bond and an additional hydroxyl group at C23. Avermectins A1a and B1a were not labeled by ^{14}C-labeled avermectin B2a aglycon, suggesting that the type "2" aglycon is not a precursor of the type "1" aglycon.[21] On the other hand, avermectin B2a and avermectin A2a aglycons were converted to avermectin A2a by *aveAI* or *aveAII* mutants[24,33] or the wild-type strain fed cerulenin, which inhibits the endogenous formation of aglycons. These results indicate that dehydration at C22−23 occurs before formation of the aglycons.

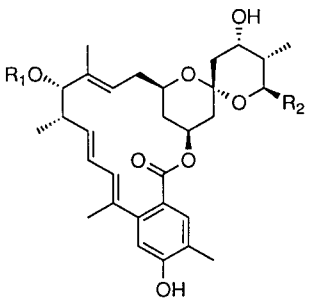
The complementation studies indicated that *aveC* mapped on the 4.9 kbp *Bam*HI fragment (Figure 6 map location 46−51 kbp) which is adjacent to *aveAI*. DNA sequencing of this region suggested that it is transcribed in the same direction as *aveAI*. Although mutation of *aveC* led to the production of type "2" components, there was no homology between the deduced amino acid sequence of *aveC* and a signature sequence in the active site of a putative dehydratase. Therefore the function of the gene product of *aveC* is not obvious. It may activate the DH activity of SU2 by associating with the PKS.

2.2.1.4. aveD (C5 O-Methylation: Avermectin B O-Methyltransferase). The *aveD* class of mutants produces "B" components of avermectins.[20,42] The mutants lack the enzyme activity of avermectin B *O*-methyltransferase, which catalyzes the conversion of "B" to "A" components by transferring the methyl residue of *S*-adenosyl-L-methionine to the C5 hydroxyl of the "B" components.[50] This enzyme, with a deduced molecular weight of about 64 kDa (we detected about 60 kDa by gel filtration) was purified and characterized. The substrate specificity for the avermectins, in order of decreasing activity, is as follows: B2a aglycon > B2a monosaccharide > B1a aglycon > B1a monosaccharide >B2a > B1a. The "2" components react faster than "1" components and there was virtually no activity detected with avermectin B1a as substrate.[50]

The *aveD* gene was first cloned from the gene cluster for avermectin biosynthesis by shotgun cloning into a mutant defective in C5 *O*-methylation. A primary clone with a large DNA insert was obtained by selecting complementation of the *aveD* mutation. After subcloning, *aveD* is located within a 3.4 kbp *Bam*HI (Figure 6 map location 14−17 kbp) at the left end of the avermectin gene cluster. Sequencing analysis indicates that the gene for *aveD* encodes a 30 kDa polypeptide which contains the signature sequence corresponding to an *S*-adenosyl-L-methionine binding domain. The methyltransferase forms a homodimer. The DNA segment containing the *aveD* region from the mutant genome contains a single-base transition in the coding region which causes an amino acid replacement. This amino acid replacement in the *aveD* product presumably causes a decrease in the catalytic activity.



R_1	R_2	R_3	X - Y
α-L-oleandrosyl	C_2H_5	OCH_3	CH_2-CH(OH)
α-L-oleandrosyl	C_2H_5	OH	CH_2-CH(OH)
α-L-oleandrosyl-α-L-oleandrosyl	C_2H_5	OH	CH_2-CH(OH)
H	CH_3	OCH_3	CH=CH
H	C_2H_5	=O	CH=CH
H	CH_3	=O	CH=CH
α-L-oleandrosyl-α-L-oleandrosyl	C_2H_5	OH	CH=CH
α-L-oleandrosyl	C_2H_5	OH	CH=CH
α-L-oleandrosyl	C_2H_5	=O	CH_2-CH(OH)
α-L-oleandrosyl	C_2H_5	=O	CH=CH
α-L-oleandrosyl	C_2H_5	OCH_3	CH=CH



R_1	R_2
H	CH_3
H	C_2H_5
α-L-oleandrosyl-α-L-oleandrosyl	C_2H_5

Figure 11. Structures of 6,8a-seco-6,8a-deoxy derivatives produced by *aveE* mutants. The aromatic ring at C2−7 is generated by dehydration of 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycons.

2.2.1.5. aveE (Involved in Furan Ring Closure at C6 to C8a). The *aveE* mutants produce 6,8a-seco-6,-8a-deoxy derivatives due to the mutation in the step of furan ring closure at C6 to C8a.[27,51,52] *aveE* mutants isolated by two groups, independently, produced several kinds of compounds consisting of avermectin aglycons, monosaccharides, and disaccharides lacking a furan ring (Figure 11). Furthermore, some of the compounds have a keto group at C5 position. Presumably, 6,8a-seco-6,8a-deoxy derivatives produced by the mutants would not be suitable substrates for glycosylation and C5 keto reduction. Feeding of 6,8a-seco-6,8a-deoxyavermectin aglycons to mutants with defective avermectin PKS[27] or the wild-type culture supplemented with cerulenin revealed that these aglycons are converted to monosaccharides and disaccharides lacking the furan ring at C6 to C8, but not to natural avermectins. Other aglycons such as, 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycons are converted to natural avermectins. These results indicate that the forma-

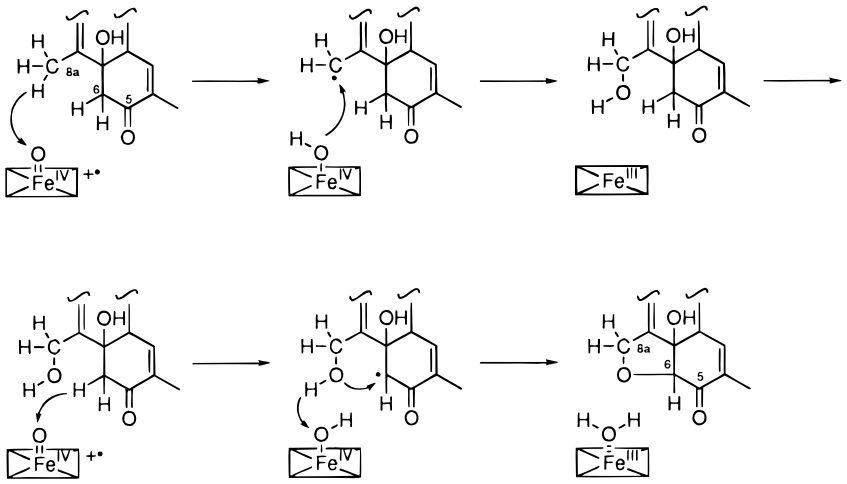


Figure 12. Proposed reaction mechanism of the formation of furan ring at C6 to C8a.

tion of the furan ring takes place after formation of the initial 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycons, and before the reduction of the keto group at C5. Thus, 6,8a-seco-6,8a-deoxyavermectins, their monosaccharides and aglycons, and 6,8a-seco-6,8a-deoxy-5-oxoavermectin monosaccharide are shunt products produced as a result of a mutation in the step of furan ring formation. Studies with the incorporation of labeled precursors [1-^{13}C,1-^{18}O]acetate and [1-^{13}C,1-^{18}O]propionate showed that the oxygen in the furan ring between C6 and C8a is derived from molecular oxygen,[16] suggesting that the gene product of *aveE* functions as a monooxygenase.

The complementation analysis using several cosmid clones and mutants defective in furan ring closure indicates that the *aveE* gene is located on the 4.9 kbp *Bam*HI fragment (Figure 6 map location 46−51 kbp) in the center of the gene cluster,[41,51] adjacent to *aveC*. Insertion and deletion analysis in this region suggests that a 2 kbp segment is required for formation of the furan ring.[51] Preliminary sequencing implied that *aveE*, which encodes about a 50 kDa polypeptide, may be on a single transcription unit with *aveAII*. Part of the deduced amino acid sequence in the C-terminal region resembles a consensus sequence in cytochrome P450. This result suggests that the furan ring closure is initiated by introducing an oxygen atom to the allylic methyl residue at C8a following which the furan ring is formed by an additional attacking oxygen (Figure 12). DNA segments containing *aveE* from the mutants contain a nonsense mutation which causes production of a truncated polypeptide.

2.2.1.6. aveF (C5 Keto Reduction: C5 Ketoreductase). Attempts to isolate mutants defective in C5 ketoreduction by mutagenesis by us and the Merck group have not been successful. The C5 ketoreductase activity which catalyzes the NADPH specific reduction of 5-oxoavermectins to avermectin "B" components has been demonstrated in cell-free extract of *S. avermitilis*.[53] This reductase is a dimer with an apparent size of 60 kDa and accepts both 5-oxoavermectins and 5-oxoavermectin aglycons as substrate. It is quite interesting that the C5 keto group is reduced by a monofunctional KR that is not within SU8 of the PKS. The formation of avermectin aglycons involves KRs which resemble type I- and II-FAS. Recently, we isolated *aveF* mutants by genetic engineering.[54] The mutants produce 5-oxoavermectins, but do not accumulate either aglycons or monosaccharides. From the results of biological conversions of the derivatives lacking a furan ring at C6 to C8a,[27] the reduction of the C5 keto group must occur after furan ring formation in avermectin biosynthesis.

The mutants defective in C5 keto reduction were obtained by introducing an insertion mutation in the promoter region of *aveD*.[54] Interestingly, the resulting mutants lack not only C5 *O*-methylation activity (*aveD*) but also C5 keto reduction (*aveF*), suggesting that both genes occupy a single transcription unit.[54] The time courses of expression of both enzyme activities for C5 *O*-methylation and C5 keto reduction and avermectin production were measured in a number of high-yielding strains of *S. avermitilis*. The maximum specific activities of these enzymes increased in direct proportion to the quantity of avermectin formed. The results support the hypothesis that genes coding for enzymes involved in the biosynthesis of the secondary metabolites are coordinately expressed and regulated. DNA sequencing analysis showed that *aveF* encodes a 32 kDa polypeptide containing a NAD(P)H-binding signature sequence. The deduced amino acid sequence is similar to that of a few KRs of type II-PKS but not to avermectin and erythromycin PKSs.

2.2.1.7. aveR (Involved in the Positive Regulation of Avermectin Biosynthesis). The *aveR* mutants showed the same phenotype as that of mutants with a defective avermectin PKS which do not produce any avermectins. The *aveR* mutants however did not have the ability to convert any of the intermediates generated after the formation of aglycons to natural avermectins.[29,31] This means that the mutations suppressed the expression of all identifiable steps in avermectin biosynthesis.[15,31] The pleiotropic phenotype of the mutants suggested that they lacked a positive regulatory function necessary to turn on the expression of all the genes involved in avermectin biosynthesis. From these results, the *aveR* mutants were defined as regulatory mutants in avermectin biosynthesis. Most avermectin-nonproducing mutants were defective in the avermectin PKS and a few of them were defective in this positive regulation

as determined by examining the ability to convert avermectin aglycons to avermectins. Furthermore, some mutants carrying transpositions of Tn*4560* are defective in avermectin biosynthesis and bioconversion of avermectin aglycons.[31] These transpositions occur on the left side of the gene cluster for avermectin biosynthesis and are accompanied by deletions in the region adjacent to the transposon.

Insertion and deletion analysis indicates that the regulatory region is located in about a 5 kbp segment on the left side of the avermectin cluster (Figure 6 map location 7−12 kbp). Insertion of a promoterless reporter gene supported that the transcriptional direction of *aveR* as opposite to that of the *aveD-aveF* operon. Although a few reports have described positive regulators of antibiotic biosynthesis,[55−58] the size of all these loci is somewhat smaller than that of *aveR* which is about 5 kbp long. It is likely that the regulator polypeptide is quite a large polypeptide.

2.2.1.8. X (Concerning Selective Incorporation of Branched-Chain Fatty Acids into Avermectin Aglycons). It has been shown that the substituent groups of the "a" and "b" components at C25 are derived from L-isoleucine and L-valine, respectively.[20,21] Mutants which accumulate avermectin "a" components but not "b" components have been isolated.[14,20] Since the biochemical and genetic characteristics of the mutants were unclear, the genotype was designed as *X*. It seemed that the mutants could incorporate L-isoleucine into the avermectin aglycon but not L-valine. Consequently, we examined the incorporation of L-isoleucine, L-valine, and corresponding keto acids, 3-methyl-2-oxovalerate and 2-oxoisovalerate provided through the deamination or transamination of L-isoleucine and L-valine, respectively, into the avermectin aglycon in both parent and mutant strains.[14,20] Both branched-chain amino and keto acids were efficiently incorporated into the avermectin aglycons in the wild-type strain. In the case of mutants, the incorporation of L-isoleucine and its keto acid was efficient, but that of L-valine and its keto acid was very poor. Inability to incorporate valine would explain the lack of production of avermectin "b" components by the mutants. Interestingly, mutants having selective incorporation of branched-chain fatty acids into avermectin aglycons possess the ability to decarboxylate both 2-oxo-3-methylvalerate and 2-oxoisovalerate which are derived from L-isoleucine or L-valine, respectively. These decarboxylation reactions are catalyzed by branched-chain 2-oxo acid dehydrogenase. This fact indicates that the selective incorporation of 2-methylbutyrate derived from 2-oxo-3-methylvalerate by decarboxylation is not due to diminished or changed activity of branched-chain 2-oxo acid dehydrogenase. The incorporation may be a result of altered catabolism of branched-chain fatty acids.[14]

3. Applications

In general, most microbial secondary metabolites, including antibiotics and other bioactive compounds, are produced as closely related complexes. Polyketide compounds which include macrolides, polyenes, and polyethers are typical examples of this. Since microorganisms produce many related components, the enzymes involved in the biosynthesis of these compounds may have lower substrate specificity and, therefore, an ability to accept related substrates. For example, the enzymes involved in C5 *O*-methylation and stepwise glycosylation of aglycons in avermectin biosynthesis can accept various intermediates as substrates for their catalytic reactions.

Elucidation of the biochemistry and genetics of secondary metabolite biosynthesis makes possible rational modification of biosynthetic pathways. This approach can suppress the production of unnecessary components by inactivation of the enzyme involved in the formation. Furthermore, the introduction of various combinations of mutations in the biosynthetic pathway might allow the production of unnatural components.

Although *S. avermitilis* produces eight related components of avermectins, only two (Figure 1), B1a and B1b, are used in the medical, veterinary, and agricultural field. The industrial-scale separation of these two components is difficult. The organism also produces an unwanted toxic compound, oligomycin. Thus, certain problems remain to be solved for the industrial production of avermectin B1a, the most effective of the components. An understanding of the biosynthetic pathway of avermectins could make it possible to eliminate undesirable products or to produce novel avermectins by selective inactivation of biosynthetic genes.

3.1. Engineering of Avermectin B1a and B2a Producers

We isolated several types of mutants affecting the production of avermectins. Among them, two mutants, K2021 and K2034[20] produced only specific components: avermectins A1a, A2a, B1a, and B2a in the case of K2021 and B1a, B1b, B2a, and B2b in the case of K2034. The strain K2034 produces "B" components alone. Accumulation of the "B" components in the mutant K2034 is due to lack of the activity of C5 *O*-methyltransferase (*aveD*) which catalyzes the conversion of "B" to "A" components. Another mutant K2021 produces four "a" components (*X*) because the incorporation of L-valine into the avermectin aglycon is markedly suppressed. Therefore, if the mutation in the C5 *O*-methylation step (*aveD*) could be introduced into strain K2021, or the *X* phenotype, which is involved in the selective incorporation of branched-chain fatty acids into avermectin aglycons, into strain K2034, the recombinants should produce only components B1a and B2a. In a study of genetic crossing and mapping in *S. avermitilis*, the recombination frequency was about 10^{-5} to 10^{-7} in mixed culture.[27] But the frequency was markedly increased to about 10^{-2} by a protoplast fusion in the presence of polyethylene glycol.[20] Moreover, although multiple crossovers in recombinants derived from the mixed culture of spores were rare, quadruple or more crossovers were detected in the recombinants derived from protoplast fusion. Therefore, recombinants derived from crosses between two closely linked loci on the chromosome might be recovered by protoplast fusion. To test this, an attempt was made to isolate recombinants possessing both phenotypes by protoplast fusion.[20] Eight puta-

tive recombinants of 192 colonies screened produced avermectins B1a and B2a. The recombination frequency of the crossing between auxotrophic markers was about 10^{-2} by the protoplast fusion, thus the putative recombinants possessing both selective producing phenotypes (*aveD* and *X*) were isolated efficiently. We have determined the locus of the *X* mutation. Genetic mapping revealed the *X* mutation to be distant from the location of the gene cluster for avermectin biosynthesis.[28] This suggests that one of the genes involved in the selective production of the specific components of avermectins affects the catabolism of branched-chain amino acids, rather directly affecting avermectin biosynthesis.

After we cloned the gene cluster for avermectin biosynthesis, a second attempt was made to obtain the above recombinants by a gene transplacement technique using a DNA segment cloned from the genome of strain K2034. Analysis of the *aveD* region of both the wild-type strain and K2034 indicated that a transition mutation occurred in the coding region of the mutant. The DNA segment derived from K2034 was introduced into the chromosome of strain K2021 through a double crossover recombination event. This procedure was extremely efficient for isolating recombinants which possessed the expected phenotype. The introduced *aveD* mutation did not affect the productivity of avermectins.

3.2. Engineering of Avermectin B2a Producers

In consideration of the proposed pathway of avermectin biosynthesis, it is estimated that introduction of at least three mutations into the avermectin biosynthetic pathway of the wild-type strain would be required to construct an avermectin B2a component producer. If three mutations, affecting C5 *O*-methylation (*aveD*), the selectivity of the incorporation of branched-chain fatty acids into the avermectin aglycons (*X*), and the dehydration between C22 and 23 residues (*aveC*), are introduced in the wild-type strain, the resulting mutant should produce only avermectin B2a. Since avermectin B2a can be chemically transformed to 22, 23-dihydroavermectin B1a, "Ivermectin B1a",[59] the most potent of the anthelmintic compounds,[60] a single component producer of avermectin B2a would be useful for industrial production of ivermectin B1a. The aglycon moiety of avermectin "a" components is derived from the condensation of seven acetate, five propionate and one 2-methylbutyrate units and is exclusively used for conversion to avermectin B2a.

A recombinant strain K2038,[20] a producer of avermectins B1a and B2a has already been constructed, by the protoplast fusion of two mutants, *aveD* and *X*, so the construction of an avermectin B2a producer might be accomplished by introduction of an *aveC* mutation (dehydration between C22 and 23) into the recombinant strain. A point mutation(s) in the *aveC* region on the chromosome of the recombinant strain K2038 was introduced using polymerase chain reaction (PCR). Although the efficiency of the mutations depends upon the length of DNA, number of amplification cycles and so on, about 2% of amplified DNAs contain a point mutation which is a transition or a transversion. A part of the region of the *aveC*-ORF was subcloned to minimize mutations in other regions, and then the segment was amplified by PCR using 4-fold excess concentrations of dCTP, dGTP, and dTTP to increase misincorporation of deoxynucleotides by *Taq* DNA polymerase during elongation. The amplified segments, some of which contain point mutation(s), were exchanged with the corresponding region in the chromosome by gene transplacement technique. Randomly chosen transformants which contained double crossover recombination events were screened to analyze their products by silica gel TLC. The resulting transplacement clone that was isolated from 144 transformants did produce avermectin B2a alone.[61] The DNA segment carrying the point mutation in the recombinant strain that produced avermectin B2a can be isolated from the genome. A more effective introduction of an *aveC* mutation into the wild-type strain or other mutants containing desirable mutations could be performed by using the DNA segment carrying the introduced *aveC* mutation.

3.3. Engineering of 5-Oxoavermectin Producers

Avermectin derivatives have been obtained by a variety of techniques, including mutagenesis,[62] biotransformation,[63] mutational biosynthesis,[64] and chemical modification.[65] The main purpose for the construction of such derivatives is to enhance the potency and spectrum and to lower the toxicity of the compound(s). Ivermectin, C22,C23-dihydroavermectin is a good example of a modification for enhancement of activity. Of these derivatives, 5-oxime derivatives of both avermectins and milbemycins increased their potency.[66] The 5-oxime derivatives are synthesized by a two-step chemical reaction[67] (Figure 13). Although oximation of the keto group at C5 of 5-oxoavermectins is a quantitative reaction, the yield of the selective oxidation of the hydroxyl group of avermectin "B" components by using active manganese oxide is low. The selective oxidation is the rate-limiting step for the preparation of 5-oximeavermectins. If the mutant strain produced 5-oxoavermectins directly, the 5-oxime derivatives would be easily and efficiently synthesized. During analysis of the region of *aveD* encoding C5 *O*-methyltransferase, the gene for the C5 keto reduction step was located in the 2.4 kbp *Bam*HI (Figure 6 map location 11 - 13 kbp) fragment adjacent to the 3.4 kbp *Bam*HI

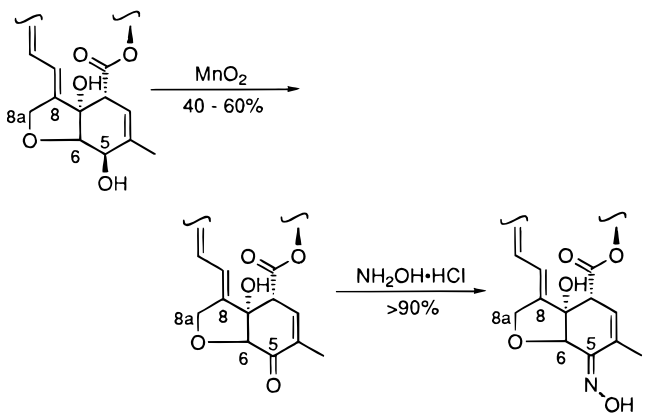


Figure 13. Synthetic steps for the preparation of 5-oximeavermectins.

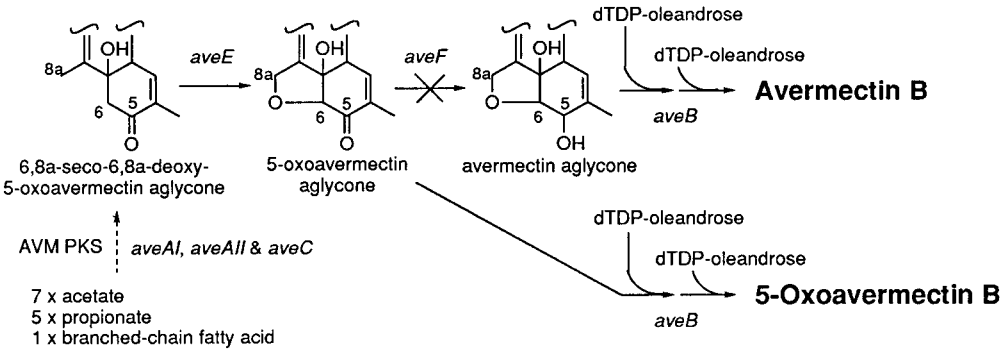
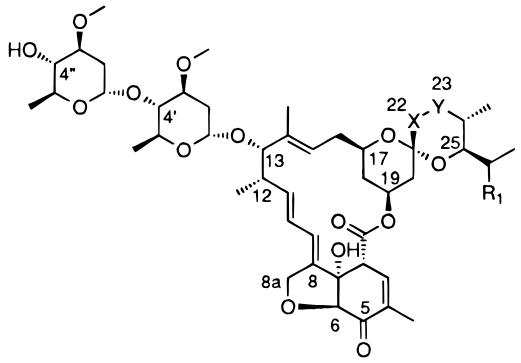


Figure 14. Biosynthetic pathway from 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycons to avermectin B and 5-oxoavermectin B.



		R_1	X-Y
5-Oxoavermectin	B1a	C_2H_5	CH=CH
	B1b	CH_3	CH=CH
	B2a	C_2H_5	CH_2-CH(OH)
	B2b	CH_3	CH_2-CH(OH)

Figure 15. Structures of 5-oxoavermectins produced by *aveF* mutants.

fragment containing *aveD* and a part of *aveAI*. The genes for C5 *O*-methylation (*aveD*) and C5 keto reduction (*aveF*) were found to be in the same operon. Since C5 *O*-methylation is not necessary for the production of 5-oxoavermectins, targeted inactivation of *aveD*−*aveF* transcription causes accumulation of 5-oxoavermectins (Figure 14). After an 8 bp insertion into the promoter region for *aveD*−*aveF*, the modified DNA segment carrying the insertion mutation was introduced into the corresponding region on the chromosome by a gene transplacement technique using a temperature-sensitive replication plasmid vector. After a double crossover recombination event occurs between the region of the *aveD*−*ave*F operon on the chromosome and that on the recombinant plasmid, the recombinant plasmid is removed by growing at high temperature. The products from mycelial extracts of transplacement clones containing the insertion mutation derived from wild-type strain are different from that of the wild-type strain on silica gel TLC and are identified as 5-oxoavermectins B1a, B1b, B2a, and B2b (Figure 15).[54] The inactivation of *aveD* and *aveF* by targeted gene disruption was also applied to the construction of a recombinant strain which produces 5-oxoavermectins B1a and B2a using a mutant strain K2021 (*X*) which possesses the mutation affecting the incorporation of branched-chain fatty acids into avermectin aglycons (accumulating "a" components). After introducing the mutation by targeted gene disruption, the transplacement clones expected to contain the insertion mutation at the promoter region of *aveD* and *aveF* operon derived from the mutant strain K2021 produced two components, 5-oxoavermectins B1a and B2a (Figure 11).[54]

3.4. Production of Novel Avermectin Derivatives by Mutational Biosynthesis

The SU1 module in the avermectin PKS contains the initial AT and ACP domains and functions during the first acyl condensation between 2-methylbutyryl-CoA or isobutyryl-CoA and methylmalonyl-CoA. The initial AT of SU1 has the ability to transfer the acyl residue of branched-chain acyl-CoA to ACP for initiation of condensation, but SU1 is unable to use the nonbranched acetyl-CoA and propionyl-CoA. It is likely that the initial AT accepts α-branched-chain acyl-CoA for acyl transfer.[68] Studies with labeled precursors indicate that 2-methylbutyryl-CoA and isobutyryl-CoA are derived from L-isoleucine and L-valine, respectively. In general, the branched-chain amino acids, isoleucine, valine, and leucine are transaminated or deaminated to form their branched-chain 2-oxo acids, then these 2-oxo acids are decarboxylated by branched-chain 2-oxo acid dehydrogenase to form branched-chain acyl-CoA. Therefore, it is suspected that the suppression of these amino acid conversions will cause a deficiency of branched-chain acyl-CoAs and reduce production of avermectins. This hypothesis was supported by isolation of a mutant strain which lacks functional branched-chain 2-oxo acid dehydrogenase activity. Since the mutant lacks the ability to form 2-methylbutyrate and isobutyrate from their 2-oxo acid precursors, it cannot produce avermectins unless supplied with these acids. The mutant is capable of making avermectins when supplied with either one of these acids because *S. avermitilis* has an acyl-CoA synthase to form acyl-CoA from these fatty acids. This strongly suggests that the branched-chain 2-oxo acid dehydrogenase functions uniquely in the wild-type strain to supply these essential natural avermectin precursors.

Interestingly, it has been shown that several analogues of the natural branched-chain fatty acids are taken up by a mutant with defective branched-chain 2-oxo acid dehydrogenase (*bkd*) and used to synthesize new avermectins in the absence of natural avermectin synthesis.[19,64] These results indicate that exogenously supplied short α-branched-chain fatty acids are readily taken up by *S. avermitilis*. The

acyl-CoA synthase is able to convert these fatty acids to their acyl-CoA derivatives, and the initial AT of SU1 is able to transfer these acyl-CoAs to the ACP of SU1. It seems that the acyl-CoA synthase and the initial AT of SU1 possess relatively low substrate specificity in their catalytic reaction. This property of the initial AT is obviously different from that of the other ATs of SUs because other ATs have quite high substrate specificity during elongation of the polyketide chain, and altered aglycons are not produced except in the C25-substituted components.

It is remarkable that a large number (>800) of carboxylic acids take part in the synthesis of avermectins with novel C25 substituents.[64] Eight-carbon acids appear to be the largest incorporated, while double and triple bonds as well as oxygen or sulfur atoms in the aliphatic chain are tolerated. An interesting finding is that C25 cyclopentyl avermectins are produced when cyclopentanecarboxylic acid or cyclopentanepropionic acid is fed to the culture.[64] This suggests that carboxylic acids lacking an α-branch can be incorporated presumably through metabolism by the enzymes of fatty acid β-oxidation as shown in Figure 16. Probably a series of enzymatic reactions similar to the catabolism of naturally occurring straight-chain fatty acids occurs as follows: (1) dehydrogenation to form cyclopentaneacrylic acid, (2) hydroxylation to yield β-hydroxy acid, (3) oxidation and cleavage to generate cyclopentanecarboxylic acid. The resulting cyclopentanecarboxylic acid is esterified to its CoA ester by acyl-CoA synthase and the CoA ester is used to synthesize C25 cyclopentyl avermectins by the avermectin PKSs. From the results of production of novel C25 substituted avermectins, only α-branched-chain acyl-CoAs would be recognized by the initial AT of SU1 because C25 isovaleryl avermectins in which the C25 substituent would be derived from L-leucine, are not produced.

Recently the genes for branched-chain 2-oxo acid dehydrogenase have been cloned from *S. avermitilis*.[69] Common branched-chain 2-oxo acid dehydrogenases are all composed of four polypeptides, but that of *S. avermitilis* appears to be composed of three polypeptides from the results of cloned DNA and deduced amino acid sequencing. Furthermore, the organism has at least two types of genes encoding the components of the branched-chain 2-oxo acid dehydrogenase complex, the *bkdABC* and *bkdFGH* gene clusters.[70] Although the inactivation of the *bkdABC* gene cluster did not cause obvious phenotypic changes, it has been found that the disruption of *bkdF* in the second cluster caused complete loss of avermectin production. The disruptants produced natural avermectin "a" when *S*-(+)-2-methylbutyrate was added to the culture, since the oxo acid could be formed by fatty acid degradation. The second cluster *bkdFGH* is obviously involved in providing an α-branched-chain fatty acid starter unit, either 2-methylbutyryl-CoA or isobutyryl-CoA, which is essential to initiate the synthesis of the avermectin polyketide backbone.[70]

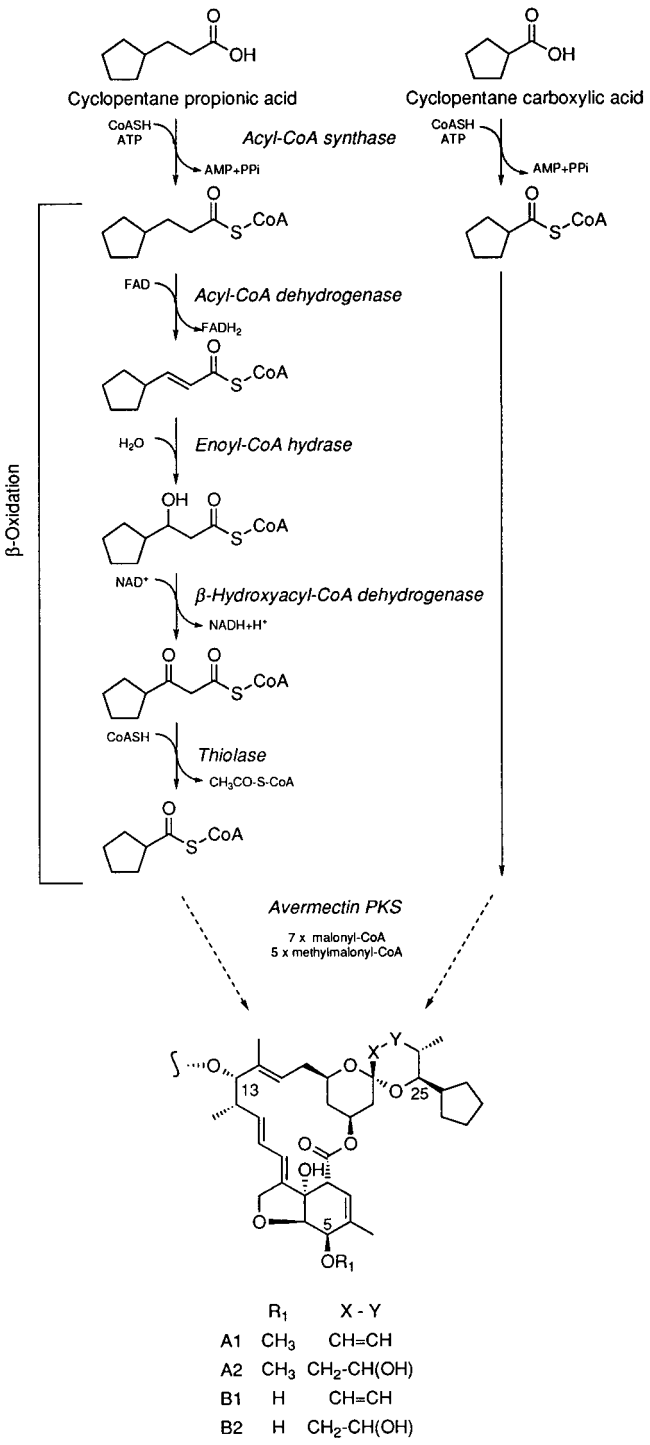
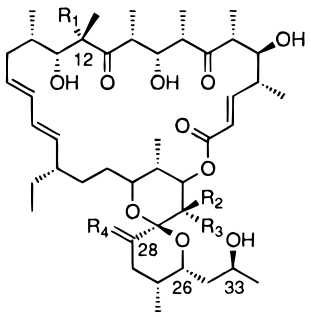


Figure 16. Proposed mechanism of incorporation of cyclopentanecarboxylic acid and cyclopentanepropionic acid into C25 cyclopentenylavermectins.

3.5. Suppression of the Accumulation of the Useless, Toxic Compound, Oligomycin, by Disruption of Genes for Oligomycin PKS

The wild-type strain of *S. avermitilis* produces not only avermectins but also polyketide-derived oligomycin and polyenes. Although the productivity of polyenes is quite low and the compound is easily removed from the mycelial extract, *S. avermitilis* produces significant amounts of oligomycin (Figure 17). The mutants, which produce specific component(s) of avermectins, are derived from the wild-type strain and still produce oligomycin. Oligomycin is a



	R_1	R_2	R_3	R_4
Oligomycin A	OH	CH_3	H	H_2
B	OH	CH_3	H	O
C	H	CH_3	H	H_2
D	OH	H	H	H_2
E	OH	CH_3	OH	O

Figure 17. Structure of oligomycin. Avermectin-producing *S. avermitilis* mainly produces oligomycin A.

toxic compound specifically inhibiting oxidative phosphorylation in mammalian cells, and therefore must be removed in the preparation of commercial products.

Oligomycin-nonproducing mutants have been isolated from the wild-type strain by induction of mutation with chemical mutagens or radiation. In this case, it is not easy to transfer this valuable phenotype to other useful variants which produce specific components or novel avermectins. Mutants caused by transposon insertion are easily cloned from the corresponding mutants by selection for the antibiotic resistance marker in the transposon. The transposon also facilitates transfer of the mutation to another strain by gene transplacement.

A transposon, Tn*4560*, one of the derivatives of Tn*4556* of neomycin-producing *S. fradiae*, was chosen for the transposon-induced mutagenesis because transpositions of Tn*4560* are randomly distributed in the genome of *S. avermitilis*.[31] About 0.1% of the transposon inserts caused auxotrophy or abolished antibiotic production. Among 2400 independent clones containing transpositions, two oligomycin-nonproducing mutants containing Tn*4560* insertions in the gene cluster for oligomycin biosynthesis were detected and these two mutants still produced avermectins. Transposon-induced avermectin-nonproducing mutants which produced abundant amounts of oligomycin were obtained at a frequency of about 10^{-3}. Two kinds of mutants in which the transposon was inserted in the regulatory (*aveR*) or PKS (only *aveAI*) region in the avermectin cluster, were classified.[31] This finding indicates that the two polyketides, avermectin and oligomycin, are synthesized by different PKS enzymes. Chromosomal DNA segments of oligomycin-nonproducing mutants carrying Tn*4560* were easily cloned by selection of the transposon-borne (viomycin phosphotransferase). DNA segments containing Tn*4560* insertions in oligomycin biosynthetic genes were subcloned into a plasmid with temperature-sensitive replication functions and transferred to other strains. The transplacement clones arising from recombination did not produce any oligomycin but were unchanged in production of avermectins.[31] For example, the transfer of the oligomycin-nonproducing phenotype was applied to two selective producers, K2038 (*aveD X*) producing avermectins B1a and B2a, and K2099 (*aveC aveD X*) producing avermectin B2a. The resulting transplacement clones produced the expected avermectin components but not oligomycin.[31,61] This obviates the need for separation of avermectin and oligomycin.

4. Conclusions and Prospects

A substantial amount of information on the biochemistry and genetics of avermectin biosynthesis has been obtained. The results reveals that avermectin biosynthesis involves an interaction of the primary and secondary metabolic pathways. The short-chain fatty acids that are building units of polyketide aglycons are synthesized from primary metabolic pathways. The biosynthesis of initial aglycons derived from polyketide, postpolyketide modification including furan ring closure, reduction, and C5 *O*-methylation, and dTDP-oleandrose biosynthesis and attachment are mediated by secondary metabolic pathways that result from *ave* gene-encoded enzymes. The biosynthesis of initial aglycons requires a PKS consisting of four multifunctional polypeptides with at least 57 distinct domains. The genes encoding the PKS polypeptides are arranged in the same relative order as that found in type I-FASs. The formation of avermectin aglycons also involves a monofunctional KR that is similar to type II-PKS. Almost all genes for avermectin biosynthesis have been characterized, but there has been little biochemical characterization of the gene products.

Rational approaches to selecting production of desired component(s) through genetic alteration of

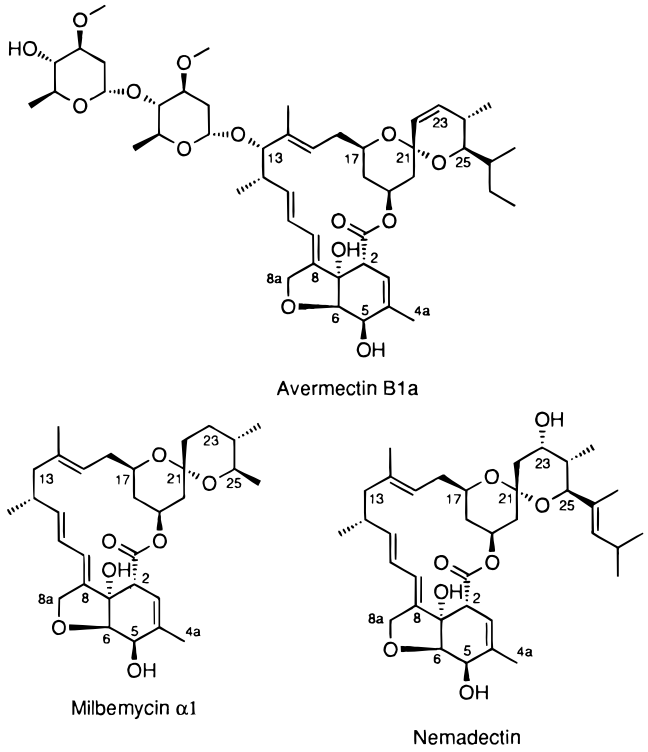


Figure 18. Major components of milbemycin, nemadectin, and avermectin families in which structures differ at C13, C22−23, and C25. The producing microorganisms accumulate minor components that differ at C4a, C5, C6−8a, C13, C22−23, and C25.

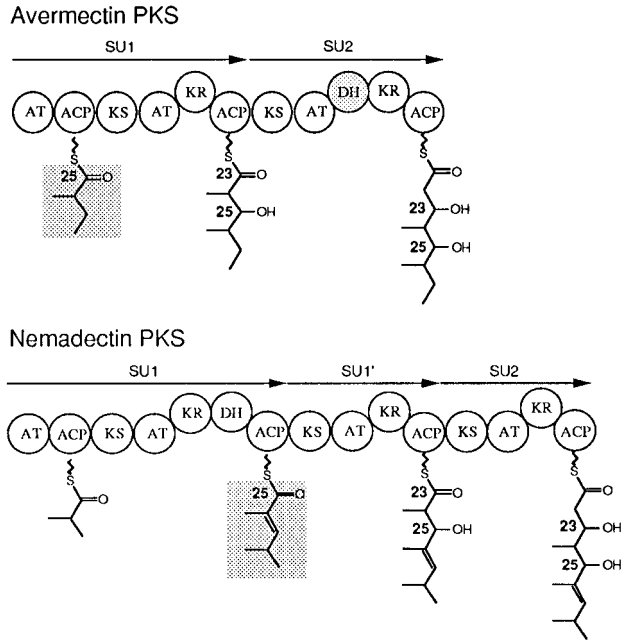


Figure 19. Comparison of the hypothetical reaction mechanism of SU1 and SU2 on avermectin PKS and nemadectin PKS.

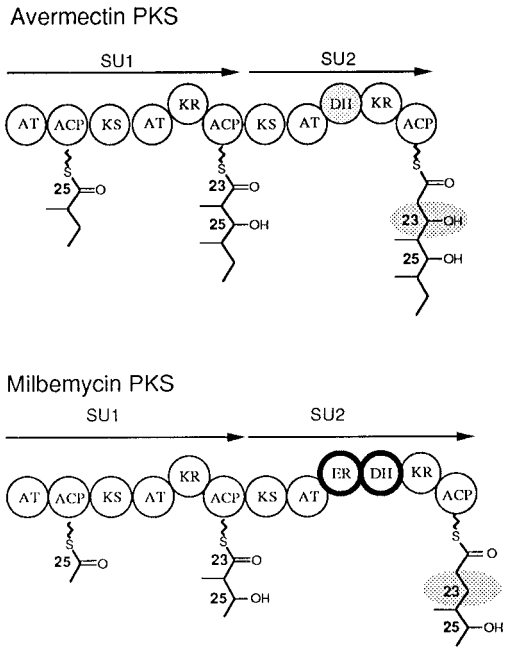


Figure 20. Comparison of the hypothetical reaction mechanism of SU1 and SU2 on avermectin PKS and milbemycin PKS.

the postpolyketide pathways by targeted gene disruption have been demonstrated. Such procedures can be applied to other valuable strains without affecting productivity and other phenotypes. Elegant work on the modification of the erythromycin PKS in *Saccharopolyspora erythraea* produced novel polyketides through genetic engineering of the PKS genes.[36] It would be extremely interesting to attempt the rational production of novel structures by genetic alteration of the avermectin PKSs. Although several aspects of avermectin polyketide synthesis await clarification, the basic rules of the programming are reasonably well understood. Reprogramming of polyketide synthesis will allow introduction of a

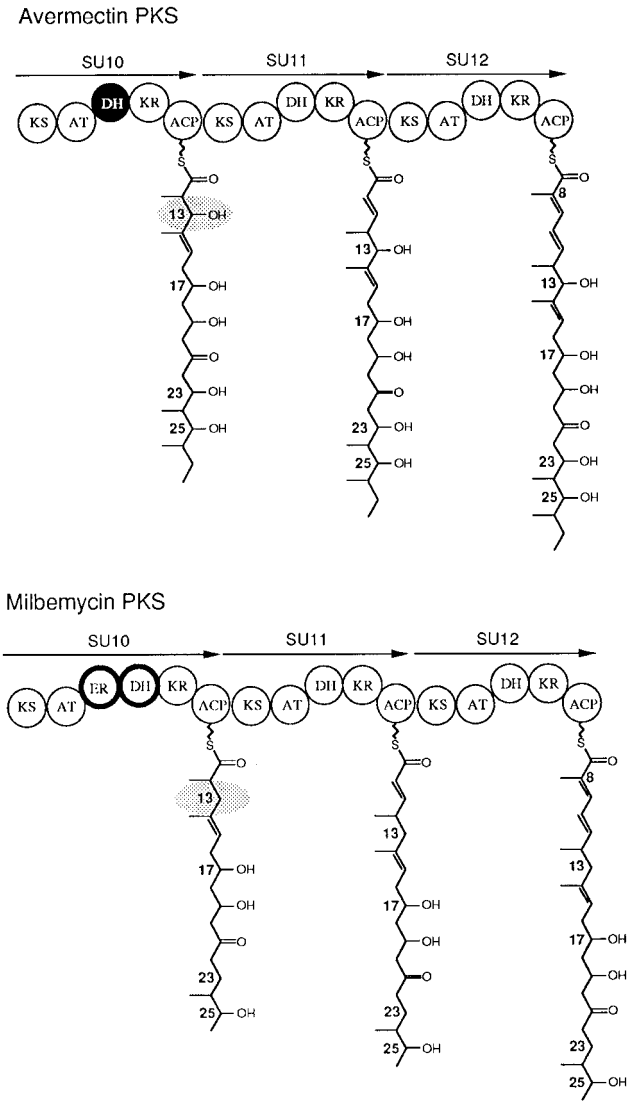


Figure 21. Comparison of the hypothetical reaction mechanism of SU10, SU11, and SU12 on avermectin PKS and milbemycin PKS.

number of possible modifications; for example, the ketone, hydroxyl, olefin, and fully saturated groups introduced during polyketide synthesis could be interchanged. Since the avermectin PKS genes and enzymes are large in size, genetic manipulation of the PKS genes to make small alterations in the PKS molecule might be complicated due to the role of inactivation of the enzyme. It should also be possible to produce novel compounds by exchanging a gene, encoding one of the avermectin PKS polypeptides with a PKS gene from a related pathway. The milbemycins[71−73] and nemadectins[74−77] in Figure 18 are also antiparasitic agents and are pentacyclic, 16-membered macrocyclic lactones related structurally to the avermectins and synthesized from condensation of acetate and propionate units by a similar polyketide pathways. They lack the disaccharide at C13 and differ from avermectins in the substituents at one or more of the following positions: 4a, 13, 22, 23, and 25. These differences depend on the composition of domains in the SUs: (1) The nemadectin PKS genes from *S. cyaneogriseus* have been cloned.[40,78] On the basis of the nemadectin structure, the initial polypeptide should contain an additional SU (Figure

19). The C25 substituent of avermectin is derived from 2-methylbutyrate or isobutyrate but that of nemadectin is from isobutyrate and propionate. (2) SU2 of the milbemycin PKS should contain fully active DH and additional ER domains to generate a fully saturated chain at C22−23 (Figure 20). The carbon chain at C22−23 of avermectin is enoyl or β-hydroxyl but that in milbemycins is alkane. (3) SU10 of the milbemycin PKS and nemadectin PKS should contain a functional DH and an additional ER to generate a fully saturated chain at C13−14 (Figure 21). For example, if the gene encoding the initial polypeptide in the avermectin PKS is exchanged with the corresponding milbemycin PKS gene in *S. avermitilis*, seven carbon precursors containing an alkane at C22−23 formed by SU1 and SU2 of milbemycin PKS-1 will be further processed by avermectin PKSs, and C25-methyl-C22,C23-dihydroavermectin will be produced. Other exchanges of PKS modules between these biosynthetic clusters could also produce novel components.

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