Anthracycline Biosynthesis in *Streptomyces galilaeus*

Isao Fujii and Yutaka Ebizuka*

Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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I. Introduction

Anthracycline antibiotics are glycosides of anthracyclinone of which the basic structure is a 7,8,9,10-tetrahydro-5,12-naphthacenequinone. In the 1950s, rhodomycins (**26**) were isolated from *Streptomyces purpurascens* by Brockmann and Bauer.[1] Rhodomycins (**26**) showed potent antimicrobial activity. Then, in 1959, cinerubins (**25**) were isolated from *S. antibioticus*[2] and showed activity against Gram-positive bacteria and certain fungi, cytotoxicity to hen fibroblast cultures, and inhibition of various murine sarcomas and carcinomas. However, high toxicity to mice and rats prevented their development as antitumor agents. Di Marco *et al.* discovered daunomycin (**2**, Figure 1), the first clinically effective anthracycline which is useful for the treatment of acute leukemias.[3] Adriamycin (**3**, Figure 1) (generic name doxorubicin), 14-hydroxydaunomycin, was then isolated from *S. peucetius* var. *caesius* in 1967 by Farmitalia.[4] Soon after, doxorubicin (**3**) was found to be less toxic and active against a much broader spectrum of tumors than daunomycin (**2**).[5] Studies showed its effectiveness against breast cancer, osteogenic sarcoma, cancers of the bladder, lung, thyroid, and ovary, Wilms' tumor, neuroblastoma, Hodgkin's diseases and other lymphomas and acute leukemias.[6,7] However, its side effects such as cardiotoxicity, alopecia, and stomatitis stimulated fur-

Isao Fujii was born in Toyama, Japan, in 1955. He received his B.S. in 1978, and Ph.D. in 1983 from Faculty of Pharmaceutical Sciences, The University of Tokyo, studying under the direction of Professor Ushio Sankawa. His graduate work centered on enzymology on fungal secoanthraquinone biosynthesis. Then, he carried out postdoctoral research at The Ohio State University, Department of Chemistry, with Professor Heinz G. Floss. After two and half years working on riboflavin biosynthesis and "hybrid" antibiotic projects there, he joined the laboratory of Professor Sankawa, Faculty of Pharmaceutical Sciences, The University of Tokyo, as an Assistant Professor. In 1988, he visited Professor C. Richard Hutchinson's laboratory at University of Wisconsin to learn molecular genetics. Since then, he has been studying biosynthesis of microbial polyketide compounds mainly at enzyme and gene levels. He is now working with Professor Yutaka Ebizuka as an Associate Professor.

Yutaka Ebizuka was born in Yokohama, Japan, in 1946. He graduated from The University of Tokyo in 1969 and received Ph.D. in 1974 from Faculty of Pharmaceutical Sciences, The University of Tokyo, under the supervision of Professor Ushio Sankawa. His Ph.D. work was mainly concerned with the biosynthesis of fungal athraquinonoids. After two years postdoctoral experience in Department of Chemistry and Department of Botany at University of British Columbia in Vancouver, he joined the Faculty of Pharmaceutical Sciences of the home University as an Assistant Professor. During this time he became interested in the enzymes involved in the biosynthesis of natural products and started to look into the enzymological aspects of natural product biosynthesis with Professor Sankawa. He has been Professor of Natural Product Chemistry since 1995, and his current research interests include the application of chemical, enzymological, and molecular biological techniques in the biosynthetic studies of natural products with pharmaceutical importance.

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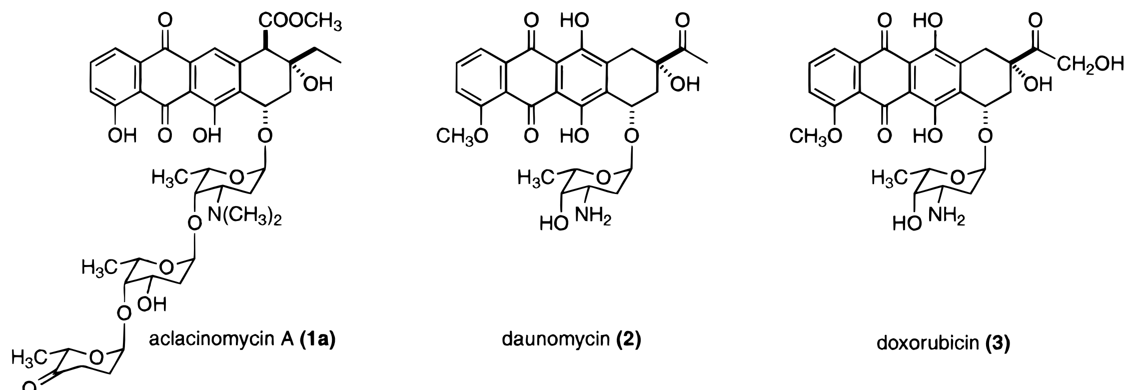


Figure 1. Structures of representative anthracycline antitumor antibiotics.

Table 1. Anthracyclinones

compounds	position of phenolic groups	ring A substitution R1	R2	R3	others
aklavinone (**4**)	4, 6	$-OH$	$-CH_2CH_3$	$-CO_2CH_3$	
bisanthydroaklavinone (**5**)	4, 6	$-OH$	$-CH_2CH_3$	$-CO_2CH_3$	7,8,10-dehydro 9-deoxy
10-decarbomethoxyaklavinone (**6**)	4, 6	$-OH$	$-CH_2CH_3$	$-H$	
7-deoxyaklavinone (**7**)	4, 6	$-H$	$-CH_2CH_3$	$-CO_2CH_3$	
2-hydroxyaklavinone (**8**)	2, 4, 6	$-OH$	$-CH_2CH_3$	$-CO_2CH_3$	
4-O-methylaklavinone (**9**)	6	$-OH$	$-CH_2CH_3$	$-CO_2CH_3$	4-OCH_3
13-mthylaklavinone (**10**)	4, 6	$-OH$	$-CH_2(CH_3)_2$	$-CO_2CH_3$	
adriamycinone (**11**)	6, 11	$-OH$	$-COCH_2OH$	$-H$	4-OCH_3
auramycinone (**12**)	4, 6	$-OH$	$-CH_3$	$-CO_2CH_3$	
α-citromycinone (**13**)	4, 11	$-OH$	$-CH_2CH_3$	$-CO_2CH_3$	
daunomycinone (**14**)	6, 11	$-OH$	$-COCH_3$	$-H$	4-OCH_3
ϵ-pyrromycinone (**16a**)	1, 4, 6	$-OH$	$-CH_2CH_3$	$-CO_2CH_3$	
ζ-pyrromycinone (**16b**)	1, 4, 6	$-H$	$-CH_2CH_3$	$-CO_2CH_3$	
η-pyrromycinone (**16c**)	1, 4, 6	$-H$	$-CH_2CH_3$	$-CO_2CH_3$	9-deoxy
1-deoxypyrromycinone (**17**)	4, 6	$-OH$	$-CH_2CH_3$	$-CO_2CH_3$	
α-rhodomycinone (**18a**)	4, 6, 11	$-OH$[a]	$-CH_2CH_3$	$-OH$	
β-rhodomycinone (**18b**)	4, 6, 11	$-OH$	$-CH_2CH_3$	$-OH$	
γ-rhodomycinone (**18c**)	4, 6, 11	$-H$	$-CH_2CH_3$	$-OH$	
δ-rhodomycinone (**18d**)	1, 4, 11	$-OH$	$-CH_2CH_3$	$-CO_2CH_3$	
ϵ-rhodomycinone (**18e**)	4, 6, 11	$-OH$	$-CH_2CH_3$	$-CO_2CH_3$	
10-deoxy β-rhodomycinone (**19**)	4, 6, 11	$-OH$	$-CH_2CH_3$	$-H$	
ϵ-isorhodomycinone (**20**)	1, 4, 6, 11	$-OH$	$-CH_2CH_3$	$-CO_2CH_3$	
sufurmycinone (**21**)	4, 6	$-OH$	$-CH_2COCH_3$	$-CO_2CH_3$	

[a] Epimer.

ther effort for the discovery of related anthracycline antibiotics. In 1975, the aclacinomycins A (**1a**, Figure 1) and B (**1b**) were isolated from *S. galilaeus* strain MA144-M1[8] and showed potent antileukemia activity and low cardiotoxicity. Aclacinomycins have been used in prescription especially in Japan and Asian countries. The aglycon of the aclacinomycins (**1**) is aklavinone (**4**), which is also a key intermediate in the formation of other anthracycline aglycons such as rhodomycinone (**18**) and daunomycinone (**14**) (Table 1).

Because of their clinical importance, intensive investigation has been carried out on the anthracycline antibiotics. Strain improvement by single spore selection and mutation increased the production level up to more than 10 times.[9,10] Mutant strains were also screened for selective production of the most active component. This type of mutant analysis led to the isolation of biosynthetically blocked mutants that accumulated key biosynthetic intermediates. Classical feeding experiments with ^{14}C- or ^{13}C-labeled acetates provided critical information on the biosynthesis of the anthracyclinone skeleton.

Recent progress in molecular genetics of streptomycetes has enabled us to analyze the genes responsible for anthracycline biosynthesis.[11−14] One of the key features of the molecular genetic approach has been the production of "hybrid" compounds by introduction of genes into heterologous hosts.[15] The cumulative information on polyketide synthase genes suggests the possibility of more precise structural control of novel aglycon production in the near future.[16]

This review focuses on the biosynthesis of the anthracycline antibiotics in *Streptomyces galilaeus*, giving a historical overview as well as covering recent progress on molecular genetic analysis.

Table 2. Anthracyclines Produced by *S. galilaeus* and Other Streptomycetes

rhodosamine (RN) 2-deoxyfucose (dF) L-cinerulose A (C) cinerulose B (CB)

daunosamine (DN) rhodinose (Rh) aculose (Ac) rednose (Rd)

compound	aglycon	sugar	representative producer
aclacinomycin A (**1a**)	aklavinone (**4**)	7-RN-dF-C	*S. galilaeus*
aclacinomycin B (**1b**)	aklavinone (**4**)	7-RN-dF-CB	*S. galilaeus*
aclacinomycin Y (**1c**)	aklavinone (**4**)	7-RN-dF-Ac	*S. galilaeus*
aclacinomycin X (**1d**)	aklavinone (**4**)	7-RN-dF-Rd	*S. galilaeus*
aklavin (**22**)	aklavinone (**4**)	7-RN	*S. galilaeus*
auramycin A (**23a**)	auramycinone (**12**)	7-RN-dF-C	*S. galilaeus*
auramycin B (**23b**)	auramycinone (**12**)	7-RN-dF-CB	*S. galilaeus*
sulfurmycin A (**24a**)	sulfurmycinone (**21**)	7-RN-dF-C	*S. galilaeus*
sulfurmycin B (**24b**)	sulfurmycinone (**21**)	7-RN-dF-CB	*S. galilaeus*
cinerubin A (**25a**)	ϵ-pyrromycione (**16a**)	7-RN-dF-C	*S. antibiotics*
cinerubin B (**25b**)	ϵ-pyrromycione (**16a**)	7-RN-dF-CB	*S. antibiotics*
daunomycin (**2**)	daunomycinone (**14**)	7-DN	*S. peucetius*
doxorubicin (**3**)	adriamycinone (**11**)	7-DN	*S. peucetius* var. *caesius*
β-rhodomycin I (**26a**)	β-rhodomycinone (**18b**)	7-RN	*S. purpurascens*
β-rhodomycin II (**26b**)	β-rhodomycinone (**18b**)	7,10-RN	*S. purpurascens*

II. Anthracyclines and Anthracyclinones Isolated from S. galilaeus

There have been several *S. galilaeus* strains used for biosynthetic studies on anthracyclines. In 1975, the Institute of Microbial Chemistry and Sanraku-Ocean Co. (now, Mercian Co.) groups in Japan isolated aclacinomycins A (**1a**) and B (**1b**) from *Streptomyces* sp. No. MA144-M1[8] which was later classified as *S. galilaeus* (ATCC 31133).[17] The strain was isolated from a soil sample collected in Kami-osaki, Tokyo, Japan. After intensive analysis, more than 21 anthracycline compounds were isolated from the strain as shown in Table 2. Their aglycon structures were determined to be aklavinone (**4**), bisanhydoaklavinone (**5**), 7-deoxyaklavinone (**7**), ϵ-pyrromycinone (**16a**), and 1-deoxypyrromycinone (**17**).[18] Nippon Roche group of Japan then obtained *S. galilaeus* OBB-111 (ATCC 31533) and found that the strain produced aclacinomycins (**1a,b**) as the major components along with auramycins (**23**) and sulfurmycins (**24**),[19] a production pattern very similar to that of *S. galilaeus* (ATCC 31133). They also isolated *S. galilaeus* OBB-731 (ATCC 31615) which produced aclacinomycins (**1a,b**). A German group led by Eckardt later isolated *S. galilaeus* F 198 from a soil sample collected on the Isle of Hidensee, Germany.[20] This strain produces a mixture of anthracyclinones [ϵ-, ζ-, and η-pyrromycinones (**16a−c**)] and several ϵ-pyrromycinone glycosides, the major component being cinerubin A (**25a**).

The aglycons of anthracyclines produced by *S. galilaeus* vary by the nature of the side chains at C-9. For example, compounds with an ethyl group are represented by aklavinone (**4**) and pyrromycinone (**16**). Others have acetonyl, isopropyl, and methyl groups at this position, as exemplified by sulfurmycinone (**21**), 13-methylaklavinone (**10**) and auramycinone (**12**), respectively. These variations are assumed to be the result of starter unit selection in polyketide biosynthesis. Introduction of hydroxy group(s) in the aklavinone skeleton results in structural variations of the aglycons.

The saccharides found in anthracycline antibiotics produced by *S. galilaeus* are listed in Table 2. Aclacinomycins (**1**) possess rhodosamine (RN) as the first sugar and 2-deoxyfucose (dF) as the second one. Variation in the third sugar gives aclacinomycin A (**1a**) with L-cinerulose A (C), aclacinomycin B (**1b**) with cinerulose B (CB), aclacinomycin Y (**1c**) with aculose (Ac), and aclacinomycin X (**1d**) with rednose (Rd). Other minor anthracyclines produced by *S. galilaeus* have a similar glycosidation pattern. Aklavin (**22**) is a rhodosaminyl aklavinone, which is a probable monosugar intermediate of aclacinomycin biosynthesis.

III. Aglycon Biosynthesis

A. Aklavinone as a Key Intermediate of Anthracycline Aglycons

By examining the structures of the anthracycline aglycons, it is readily appreciated that the pyrromycinones (**16**) and rhodomycinones (**18**) come from hydroxylation and further decarbomethoxylation of aklavinone (**4**). Yoshimoto *et al.* carried out a feeding experiment with [9-^{14}C]- and [16-^{14}C]-labeled aklavinone prepared by feeding [1-^{14}C]propionate and [*methyl*-^{14}C]methionine to a mutant strain 3AR-33 of *S. galilaeus* which accumulates aklavinone (**4**).[21] These labeled aklavinones were fed to a baumycin (4′-substituted daunomycins)-negative mutant strain 1U-222 of *Streptomyces coeruleorubidus* ME130-A4.

Figure 2. Conversion of [^{14}C]aklavinone (**4**) to daunomycinone (**14**). Dots indicate the positions of the ^{14}C-labeled carbons.

Figure 3. The labeling pattern established by the incorporations of sodium [1-^{13}C]- and [2-^{13}C]acetate into daunomycinone (**14**) and [1-^{13}C]-, [2-^{13}C], and [^{18}O$_2$]acetate into aklavinone (**4**).

Figure 4. Structures of anthracyclinone and anthraquinone compounds (**27**−**30**) isolated from *S. galilaeus* strain J-14 by Vaněk *et al.*

After 40 h of incubation, the resultant anthracyclinones had been labeled, demonstrating a conversion of [9-^{14}C]aklavinone to daunomycinone (**14**) and ε-rhodomycinone (**18e**). On the other hand, label from [16-^{14}C]aklavinone was only incorporated into ε-rhodomycinone (**18e**) and not into daunomycinone (**14**), thus establishing that aklavinone (**4**) is a common intermediate in the biosynthesis of daunomycinone (**14**) via ε-rhodomycinone (**18e**) and its 10-decarbomethoxylated intermediate (Figure 2).

B. Origin of Carbons and Oxygens

Paulick *et al.* carried out feeding experiments with [^{13}C]acetates in *S. peucetius*, and the resultant labeling pattern in daunomycinone (**14**) established the folding pattern of the polyketomethylene intermediate of the daunomycinone (**14**) precursor[22,23] (Figure 3). From these results, it is reasonable to assume that aklavinone (**4**) biosynthesis in *S. galilaeus* is similar to that in *S. peucetius*. In fact, feeding of ^{13}C-labeled acetate to *S. galilaeus* 3AR-33 indicated the expected pattern of acetate labeling in aklavinone (**4**).[24]

Vaněk *et al.* have isolated several anthracyclinones such as compounds **27**−**30** from *S. galilaeus* J-14, a strain which lacks glycosidation capacity[25] (Figure 4). If compounds like **27**−**29** serve as intermediates of aklavinone (**4**) biosynthesis, oxygen atoms located at C-7 and C-9 should come from molecular oxygen, possibly introduced by oxygenases, rather than directly from the carboxyl oxygen atoms of acetate. To answer this question, Wakui *et al.* fed [1-^{13}C,^{18}O$_2$]acetate to *S. galilaeus* 3AR-33 and analyzed the incorporation of ^{18}O by ^{13}C NMR spectroscopy, looking for ^{18}O-induced α-shifts in the labeled ^{13}C signals, indicative of intact ^{13}C−^{18}O bonds (Wakui *et al.*, unpublished data). The results clearly indicated that all the oxygen atoms of aklavinone (**4**), except the C-12 carbonyl oxygen, came from acetate. Intriguingly, even the oxygen at C-9 which comes from the propionate starter unit were labeled, though intact retention of the ^{13}C−^{18}O bond was about 16%, far lower than other positions (44−60%) (Figure 3). Indeed, the retention at C-9 of oxygen from [1-^{13}C,^{18}O$_2$]propionate was confirmed directly by feeding experiments. The low incorporation of oxygen from acetate at the C-9 hydroxy group indicated a secondary incorporation of acetate via propionate through TCA cycle. This incorporation of ^{18}O atoms from acetate into the oxygens of the aklavinone (**4**) nucleus, except at C-12, excluded the involvement of compounds **27**−**29** isolated by Vaněk *et al.* as true intermediates in the biosynthesis of aklavinone (**4**). These compounds might be shunt products or nonspecifically dehydrated derailment compounds from the central biosynthetic pathway.

C. Mutation Analysis

The investigation of precursors obtained through mutationally interrupted biosynthesis has been an effective procedure for establishing metabolic sequences. In the course of such studies with *S. galilaeus*, several mutant strains were isolated following treatment of wild-type organisms with UV irradiation or chemical mutagens, leading to the production of anthraquinones in addition to anthracyclinones. In the study of the genetic improvement of aclacinomycin (**1**) production in *S. galilaeus* MA144-M1 by NTG and UV treatment, a variety of blocked mutants were obtained.[9] Of these, mutant KE-303 was isolated as an anthracycline nonproducing mutant which retained glycosidation activity like a wild-

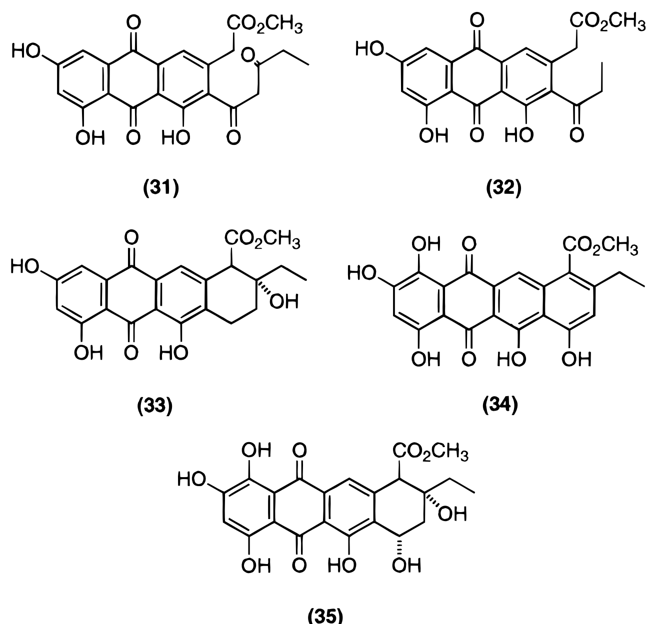


Figure 5. Structures of anthracyclinone and anthraquinone compounds (**31**−**35**) isolated from *S. galilaeus* mutant strain ANR-58.

type strain. In the aglycon-producing strains, 3AR-33 and ANR-58 were found to produce aklavinone (**4**) and 2-hydroxyaklavinone (**8**), respectively. Recently, mutation experiments were carried out by a Finnish group who isolated and characterized blocked mutants with similar product profiles.[26]

From strain ANR-58, the Mercian group isolated anthraquinones **31** and **32** and anthracyclinones **33**−**35**, as shown in Figure 5.[27] These compounds could not be converted to their glycosides when fed to glycosidation active mutant KE-303, suggesting that these aglycons were not true intermediates in the biosynthesis of aclacinomycins (**1**) and related antibiotics. However, 2-hydroxyaklavinone (**8**), the main product of strain ANR-58, was converted into 2-hydroxyaclacinomycin (**46**), but not to aclacinomycin (**1**).

Aklanonic acid (**37**) is an anthracyclinone-related anthraquinone derivative that was initially detected in cultures of an unidentified *Streptomyces* species by Eckardt *et al.*[28] Feeding experiments with anthracycline-negative mutants of different *Streptomyces* strains revealed that aklanonic acid (**37**) played an important role as an early intermediate in the biosynthetic pathway to anthracyclines.[29−31] *Streptomyces galilaeus* strain F 198 was isolated by Eckardt and was found to produce aklavinone (**4**) type anthracyclines. By treatment with NTG, one mutant strain was obtained which produced the orange-yellow pigment, aklanonic acid (**37**).[20] Another nonpigment-producing *S. galilaeus* mutant was able to convert exogeneously fed aklanonic acid (**37**) into cinerubin (**25**) and related anthracyclines.[30] Thus, it was concluded that aklanonic acid (**37**) is an essential early intermediate involved in the biosynthesis of anthracycline aglycons.

Eckardt's group has also isolated aklaviketone (**39**) from mutant strain S 383 of *S. galilaeus*.[32] This compound has the characteristic ring A structure and was considered to be the direct precursor of aklavinone (**4**). In fact, aklaviketone (**39**) was converted to cinerubin A (**25a**) when fed to *S. galilaeus* S 727, an anthracycline-negative mutant.[33] The conversion of aklaviketone (**39**) to aklavinone (**4**) is assumed to be catalyzed by a specific ketoreductase. Thus, the scheme of aklavinone (**4**) biosynthesis is considered as follows (Figure 6): First, a decaketide with a propionate starter unit cyclizes to form the aklanonic acid anthrone (**36**), which is then oxidized to aklanonic acid (**37**). Esterification of the carboxylic acid and cyclization to form the fourth ring gives aklaviketone (**39**). It is at this stage that the specific reductase catalyzes the formation of aklavinone (**4**). This pathway is considered to be common to the biosynthesis of most anthracycline antibiotics including daunomycin (**2**), doxorubicin (**3**), and aclacinomycin (**1**). Cell-free studies with a daunomycin (**2**)-producing *Streptomyces* strain C5 and *S. peucetius* by Strohl's group have identified the presence of aklanonic acid O-methyltransferase, aklaviketone reductase, and aklavinone C-11 hydroxylase. They have also detected O-methyltransferase and aklaviketone reductase activities but not aklavinone C-11 hydroxylase in the cell-free extract of *S. galilaeus*.[34]

Additionally, Strohl *et al.* have identified the genes in *Streptomyces* strain C5 responsible for aklanonic acid O-methyltransferase, the cyclase to form aklaviketone (**39**),[35] and aklaviketone reductase.[36] Similar genes should exist in anthracycline-producing streptomycete chromosomes.

IV. Aklavinone Biosynthesis Gene Cluster in S. galilaeus

A. Cloning

Molecular genetic studies of polyketide biosynthesis in streptomycetes have led to the isolation of the entire set of genes required for the biosynthesis of actinorhodin,[37] tetracenomycin,[38] and others.[16] All these genes form clusters of structural, self-resistance, and regulatory genes, and the products of the early genes of polyketide biosynthesis show high sequence homology.[39] Strohl *et al.* have demonstrated that the *actIII* gene for actinorhodin PKS reductase[40] complemented the mutation in *S. galilaeus* ANR-58.[41] The *actIII* transformant of ANR-58 produced aklavinone (**4**) instead of its normal metabolite 2-hydroxyaklavinone (**8**). This interchangeability of PKS gene components between *S. galilaeus* and *S. coelicolor* indicated a high sequence homology for reductase genes between these two streptomycetes.

Tsukamoto *et al.*[42] carried out Southern blot analysis of *S. galilaeus* genomic DNA and detected three bands (4.1, 3.4, and 2.5 kb) which hybridized to the *actIII* probe when genomic DNA of *S. galilaeus* was digested with *Bam*HI. Thus, for the cloning of PKS genes of *S. galilaeus*, the *actIII* gene was used as a screening probe. From the genomic DNA library, two types of clones were obtained. One contained a 2.5 kb *Bam*HI fragment and the other a 3.4 kb fragment. Also, clones which weakly hybridized with the *actIII* probe were obtained that contained a 4.1 kb fragment. Of these, the clone containing the 3.4 kb fragment and the fragment itself hybridized with the *actI* probe. The subclone named pAKD11 that con-

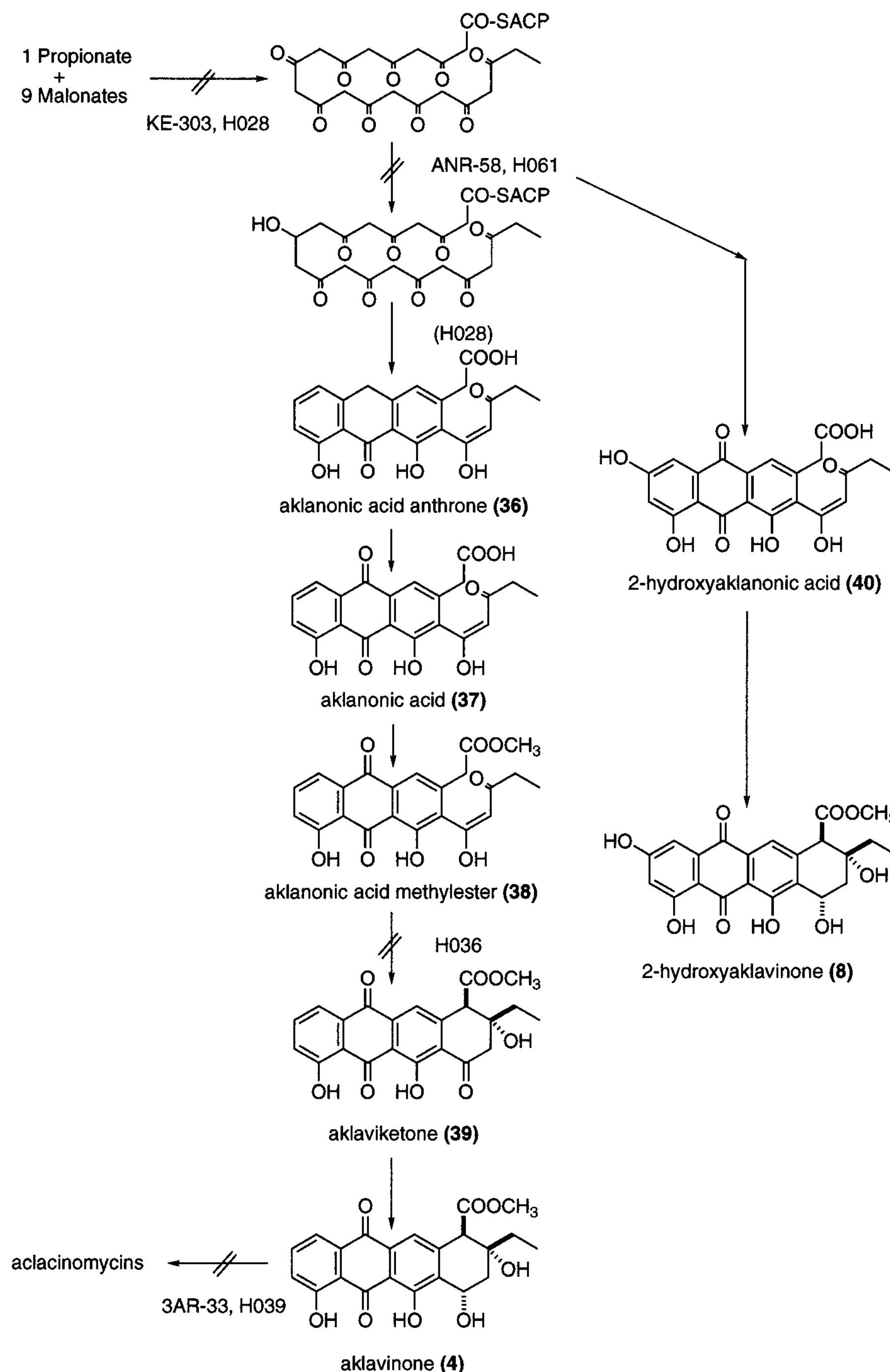


Figure 6. Biosynthetic pathway of aklavinone (**4**) and aclacinomycins in *S. galilaeus*. The proposed blocks of mutants (KE-303, ANR-58, and 3AR-33 by Yoshimoto *et al.*;[9] H028, H036, H039, and H061 by Ylihonko *et al.*[26]) are shown.

tained this 3.4 kb *Bam*HI fragment complemented the production of aklavinone (**4**) in ANR-58 and also aclacinomycin (**1**) production in *S. galilaeus* mutant KE-303, results which confirmed that the cloned gene is involved in aklavinone (**4**) biosynthesis in *S. galilaeus* (Figure 7).

B. Open Reading Frame Analysis

Sequencing and open reading frame (ORF) analysis of the cloned gene from *S. galilaeus* indicated the presence of open reading frames most typical of bacterial type II PKS genes.[12] In common with all cloned bacterial PKS genes, a linear sequence of β-ketoacyl synthase (KS), *aknB*, and chain length factor (CLF),[43,44] *aknC*, genes were identified. Following them, a small ORF acyl carrier protein (ACP), *aknD*, gene was found. However, just upstream of the KS gene, a small ORF (*aknX*) was identified in the same transcriptional direction. In the opposite direction to these ORFs, a β-ketoreductase (KR), *aknA*, gene and cyclase, *aknE*, gene were identified by homology analysis.[45] The 3.4 kb *Bam*HI fragment contained complete *aknA*, *aknX*, and *aknB* genes. From the results of complementation, AknA reductase is responsible for reduction of the keto group at the ninth carbon from the carboxyl terminus of the assembled polyketide. In other words, mutant ANR-58 is defective in the *aknA* gene. This result was

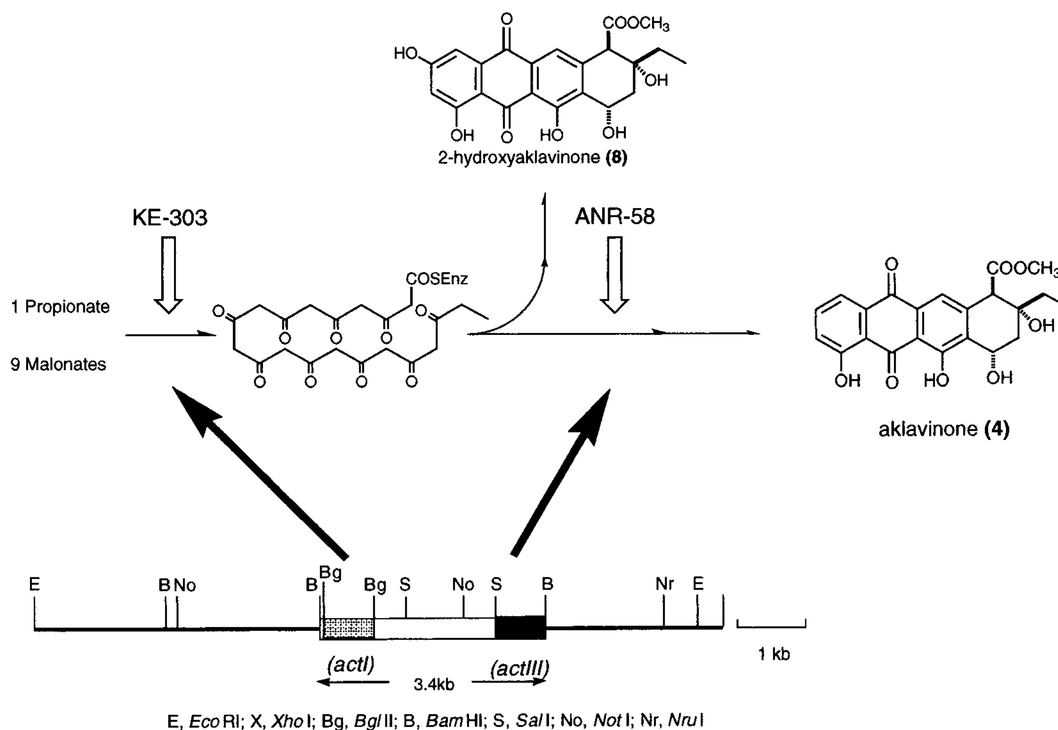


Figure 7. Restriction map of the cloned gene from *S. galilaes* and complementation of mutation in *S. galilaues* mutant strains ANR-58 and KE-303 by the 3.4 kb *Bam*HI fragment. Hatched bar indicates the *actI*-hybridizing region and black bar indicates the *actIII*-hybridizing region.

further confirmed by cloning and sequence analysis of the *aknA* locus of the ANR-58 mutant, revealing the existence of a 5-bp deletion to cause a frame shift.[46] KE-303 is a mutant strain unable to produce anthracycline pigments. Thus, the strain has mutation(s) in anthracyclinone-forming PKS genes, possibly the KS or the CLF and/or in their regulatory regions. The existence of the complete KS ORF but only an incomplete CLF in the 3.4 kb *Bam*HI region strongly indicated the presence of a mutation in the *aknB* KS region. It was found that the cloned *aknB* gene of KE-303 has a point mutation causing a frame shift (Chung *et al.*, in preparation). The fact that each of these two mutants actually has a mutation in the cloned region and that their mutation can be complemented by the cloned genes unambiguously established that the *akn* genes are genes of aklavinone (**4**) biosynthesis in *S. galilaeus*.

C. Comparison with Other Anthracycline Biosynthesis Gene Clusters

As detailed in the previous section, aklavinone (**4**) is regarded as the common intermediate of the anthracycline aglycons. Thus, the aklavinone (**4**)-forming PKS genes of anthracycline-producing streptomycetes, if not identical, should show high homology. During the preparation of this review, three other anthracycline PKS genes have been reported from *Streptomyces* strain C5[47] and *S. peucetius*,[48] both of which produce daunomycin (**2**) and doxorubicin (**3**), and from *S. nogalater*[49] which is a producer of nogalamycin. The PKS regions which might be necessary for the elaboration of aglycon, or at least the earliest stable intermediate aklanonic acid, are schematically shown in Figure 8.

The *dau* PKS[47] and *dps* PKS[48] gene constructs are very similar, as expected. The characteristic feature of these PKS gene clusters is the location of the ACP genes which were found separated quite far away from the KS genes. The ACP genes usually are located just after the CLF gene to generate a minimal PKS gene cluster (KS-CLF-ACP) such as typified by the *act* gene cluster.[50] The ACPs of both *akn* and *sno* are also located in the typical position just after the CLFs. An interesting feature of the *dau* and *dps* gene clusters is the additional existence of a KS like gene just downstream of the CLF and a possible propionyl transferase gene following in the same transcriptional direction. No such additional KS and propionyl transferase genes have been identified in either the *akn* and *sno* gene clusters to date. The *sno* gene cluster[49] is different from the *akn* cluster in some respects. The *sno* gene cluster has an activation gene and a methyltransferase gene situated between the KS and KR genes which have not been identified in the *akn* gene cluster. A second point of difference is the location of the oxygenase genes. In the *akn* cluster, *aknX* is located just upstream of the *aknB* (KS) gene in the same transcriptional direction, but the *snoB* gene is located between the activator and the methyltransferase genes. The second KS gene and the propionyl transferase gene are present only in the *dau* and *dps* gene clusters, but have not been identified in *akn* and *sno* gene clusters. In *S. galilaeus*, starter unit specificity seems to be rather low, as analogues are found in its anthracycline products like sulfurmycins (**24**) and auramycins (**23**) suggesting a starter group flexibility.[19] Thus, the propionyl transferase and the second KS may be responsible for loading of the starter propionyl group onto the first KS.

The small ORF, designated as *aknX* and located before the KS, shows homology to the *tcmH* gene of tetracenomycin biosynthesis. A similar small ORF

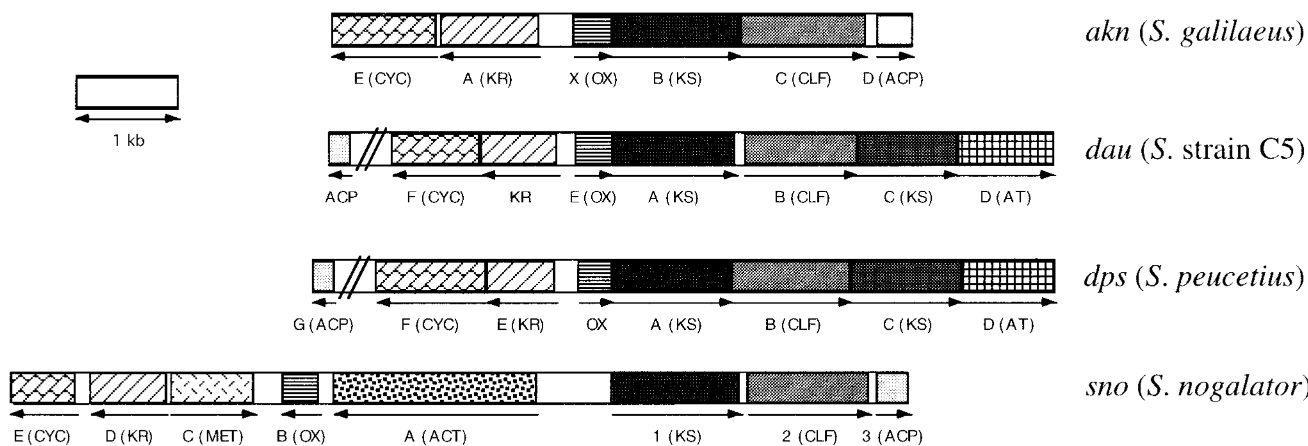


Figure 8. Organization of biosynthetic gene clusters for anthracycline antibiotics. ACP, CLF, KR, and KS are defined in the text; the other abbreviations are as follows: ACT, activation; CYC, cyclase; MT, methyltransferase; OX, oxidation. Gene clusters *akn*, *dau*, *dps*, and *sno* are from *S. galilaeus*, *Streptomyces* strain C5, *S. peucetius*, and *S. nogalater*, respectively.

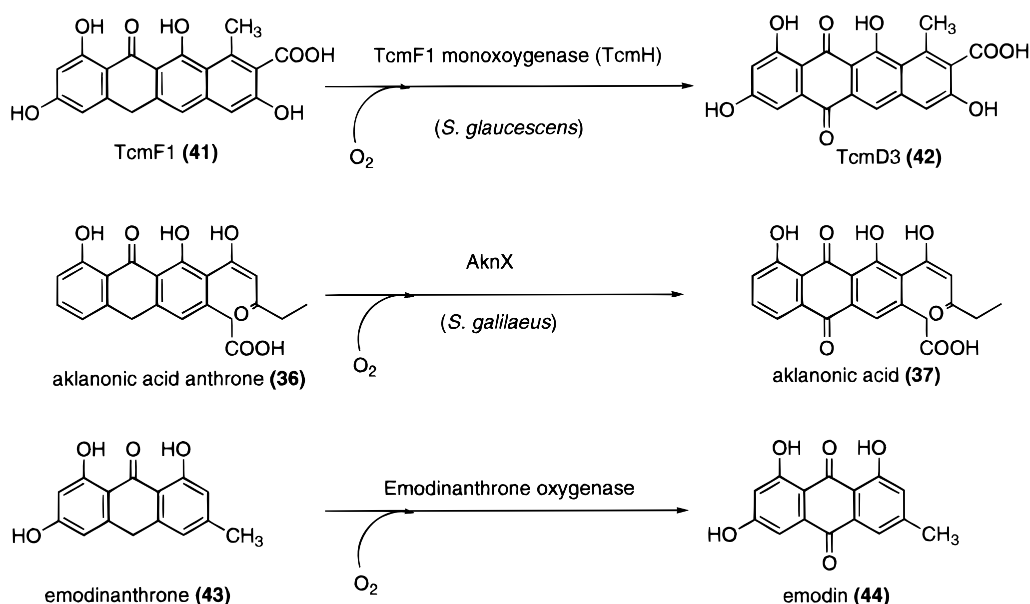


Figure 9. Reaction scheme of anthrone oxygenases.

was found in the *dau*, *dps*, and *sno* clusters. The TcmH protein was identified as an oxygenase by expression in *S. glaucescens* and shown to catalyze the oxidation of TcmF1 (**41**) to TcmD3 (**42**).[51] Thus, the AknX protein was assumed to be the aklanonic acid anthrone monooxygenase (Figure 9).

In the study of fungal emodinanthrone oxygenase, Fujii *et al.* detected an emodinanthrone (**43**)-oxidizing activity in *S. galilaeus*.[52] To identify its actual function, the AknX protein was expressed in *E. coli* using the T7 RNA polymerase-based pET system.[53] The first few codons of AknX were changed to favorable codons in *E. coli* by PCR mutagenesis because of the GC bias of streptomycete genes. Without this modification, no expression of the AknX protein was detected. The highly expressed AknX protein was easily purified by an affinity column, taking advantage of a His-tag introduced in its C-terminal. The purified protein showed emodinanthrone oxygenase activity, converting emodinanthrone (**43**) to anthraquinone emodin (**44**) (Figure 9). This clearly indicated that the *aknX* gene codes for the protein responsible for the oxygenation of anthrone to anthraquinone in *S. galilaeus*, aklanonic acid anthrone (**36**) to aklanonic acid (**37**) (Chung *et al.*, in preparation).

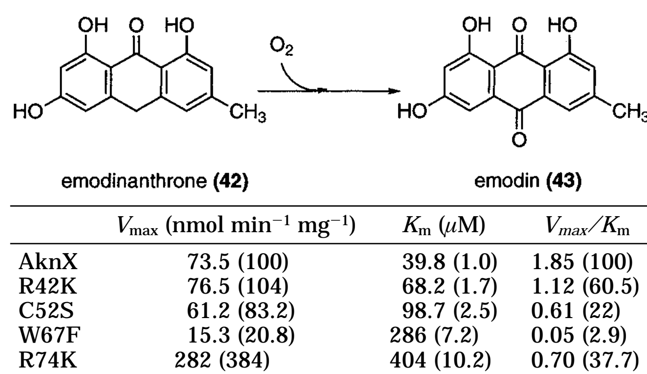
Shen and Hutchinson have purified the TcmH oxygenase which was found to be a homotrimer. They did not detect any prosthetic group and proposed a protein radical as the key reaction intermediate for this reaction.[51] The purified AknX enzyme also appeared to be a homotrimer and no prosthetic group has been detected so far. In Figure 10, alignment of these possible anthrone oxygenases is shown. The presence of several highly conserved amino acids was observed.

Chung *et al.* prepared mutant AknX proteins at several conserved amino acids and analyzed their activity as shown in Table 3. This result indicated that the Trp-67 serves as a key amino acid residue in this oxygenation (Chung *et al.*, in preparation). In some enzymes, tryptophan is the residue where the radical is formed.[54] Sankawa and his co-workers proposed the involvement of non-heme ferric iron as a cofactor in the fungal emodinanthrone oxygenase reaction.[55] However, further analysis is necessary to

TcmH	MATISP ---SPDLFTL VNVFGVAPEK QRELRDHLVQV TEDLIRHMPGF VSATF----- ---HL	52
Dps-orf8	MPQPEP NDAGSGSVTF VNRFTLSGSA E-DFEAAFAET AEFLCRR-PGF RWHALLVPAD TGPGS	61
DauA-orfE	VEQRCLWPEP NDAGSGSVTF VNRFTLSGSA E-DFEAAFAET AEFLCRR-PGF RWHVLLAP-- TGSGS	63
AknX	MTDHEP GTEGADAVTF VNTFTVHAEP E-VFEKEFART SEFMARQ-PGF VRHTL----- --CRH	54
Consensus	........eP ...g...vTf VN.Ft..... e..fe..fa.t .Ef..R..PGF ..h.l.....	65
TcmH	SRDGEQVVNY AQWRSEADFR AMHADPRLQP HFDYCRSVSR PKPIF----- -CEVTHSFGA TSPEGA--*	112
Dps-orf8	ADARPQYVNI AVWDDEASFR AAVAHPEFPA HAAALRALST SEPTLYRHRQ IRVAPDVPAV SGPGGRTT*	129
DauA-orfE	ADVRPQYVNI AVWDDEASFR AAVAHPQFPA HAAVLRALST SEPTLYRSRQ IRVAPGAPAM SRPEGRTT*	131
AknX	AERPGQYVNV AEWRDLASFR AAVSHDDFRP HAGALRALSE SRPELYLVRL RREGAPGLDG PASEGEEI*	123
Consensus	a....QyVN. A.W.d.AsFR Aav.h..f.. Ha..lRalS. s.P.ly.... .r........ ....G...*	134

Figure 10. Alignment of deduced amino acid sequences of anthrone oxygenases. TcmH is TcmF1 monooxygenase from *S. glacescens*. AknX, Dps-orf8, and Dau-orfE are possible aklanonic acid anthrone oxygenases from *S. galilaeus*, *Streptomyces* strain C5, and *S. peucetius*, respectively.

Table 3. Kinetic Analysis of Mutated AknXs



	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)	V_{max}/K_m
AknX	73.5 (100)	39.8 (1.0)	1.85 (100)
R42K	76.5 (104)	68.2 (1.7)	1.12 (60.5)
C52S	61.2 (83.2)	98.7 (2.5)	0.61 (22)
W67F	15.3 (20.8)	286 (7.2)	0.05 (2.9)
R74K	282 (384)	404 (10.2)	0.70 (37.7)

reveal the detailed mechanism of aklanonic acid anthrone oxygenase.

V. *S. galilaeus* as a Host for Anthracycline Production

A. Glycosidation

Anthracyclinones themselves are biologically inactive and glycosidation with one to five sugar units at the C-7 and/or C-10 positions is necessary for their antimicrobial and antitumor activity. The presence of an amino group in the aglycon or of an amino sugar is also another prerequisite for biological activity. For example, glycosides consisting of only a neutral sugar are all found to be biologically inactive. Aclacinomycin A (**1a**), one of the most active anthracyclines produced by *S. galilaeus*, carries rhodosamine (RN)-deoxyfucose (dF)-cinerulose (C) at the C-7 position of aklavinone (**4**) nucleus. Other active glycosides, daunomycin (**2**) and doxorubicin (**3**), contain one daunosamine (DN) at the same position.

The mutant strain *S. galilaeus* KE-303 is an anthracycline nonproducing mutant. Molecular genetic analysis established that the strain has a mutation in the *aknB* (KS) gene, as discussed in section IV.B. However, this strain was very active in glycosidation of exogeneously administrated anthracyclinones. Almost all anthracyclinones except daunomycinone (**14**), 13-deoxydaunomycinone (**15**), and adriamycinone (**11**) were converted to their corresponding glycosides,[56] such of β-, γ-, and ϵ-rhodomycinones (**18b,c,e**), ϵ-isorhodomycinone (**20**), and ϵ-pyrromycinone (**16a**).[57] In addition, chemically derived unnatural aglycons such as 10-decarbomethoxyaklavinone (**6**) and 4-*O*-methylaklavinone (**9**) were also converted to their corresponding aclacinomycins in KE-303. γ-Rhodomycinone (**18c**), which possesses a hydroxy group at C-10 but not at C-7, was converted to the *O*-glycoside at the C-10 position. Thus, this result indicated that *S. galilaeus* has the ability of glycosyltransfer not only to the C-7 hydroxy group but also to the C-10 hydroxy group.

Interestingly, a new anthracycline, compound (**45**), was obtained by microbial glycosidation of α-citromycinone (**13**) in KE-303.[58] The structure was identified as 10-*O*-(cinerulosyl-2-deoxyfucosyl-rhodosaminyl)-α-citromycinone (**45**). In this case, the hydroxy group at C-10 was the preferred glycosidation site over C-7. A structural comparison between the natural aglycon aklavinone (**4**) and α-citromycinone (**13**) indicated that the first glycosidation enzyme recognizes the partial structure of the C-5 carbonyl, C-6 hydroxyl, and C-7 hydroxyl functionalities because compound (**45**) possesses the analogous partial structural features of the C-10 hydroxyl, C-11 hydroxyl, and C-12 carbonyl groups (Figure 11). This was also true in the case of glycosidation of γ-rhodomycinone (**18c**) which possesses C-10 hydroxyl, C-11 hydroxyl and C-12 carbonyl groups but lacks a C-7 hydroxy group, as mentioned above. In all cases, glycosidation with this mutant produces anthracyclinone trisaccharides with fundamentally the same sugar chain as that of aclacinomycin (**1**).

2-Hydroxyaklavinone (**8**) is produced by a blocked mutant ANR-58.[59] This anthracycline could be converted by KE-303 to the glycoside 2-hydroxyaclacinomycin (**46**), which was found to have an improved antitumor activity[60] (Figure 11). For the efficient production of this new aclacinomycin, protoplast fusion of blocked mutants ANR-58 and KE-303 was

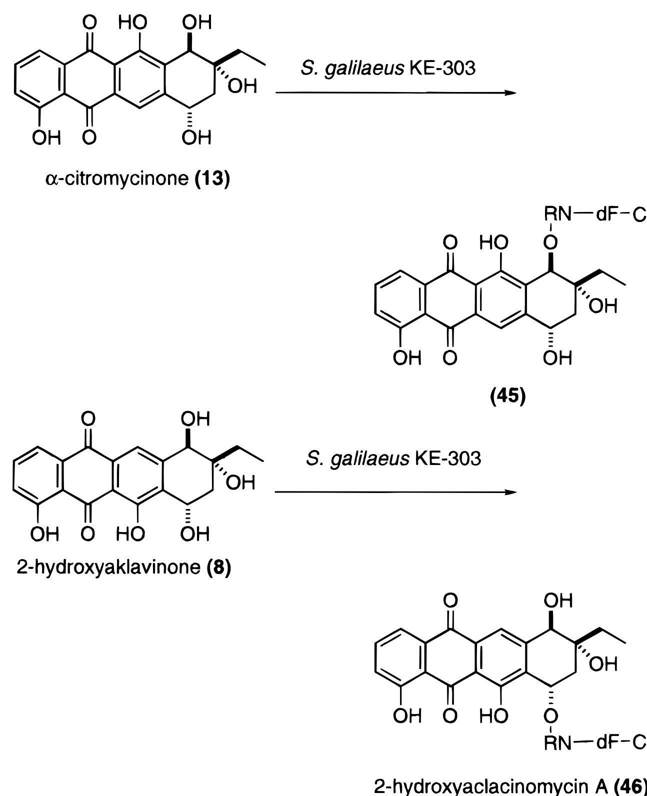


Figure 11. Conversion of anthracyclinones, α-citromycinone, and 2-hydroxyaklavinone into their glycosides by *S. galilaeus* mutant strain KE-303.

attempted to obtain a recombinant strain having the ability to produce this compound.[61]

KE-303 was also examined for its ability to further glycosidate anthracycline monosaccharides.[62] It was anticipated that the glycosidation would be expanded to monosugar anthracyclines to yield the corresponding anthracycline trisaccharides. However, the glycosidation of anthracycline monosaccharides with amino sugars resulted in the production of new disaccharides substituted with either rhodinose (Rh) or 2-deoxyfucose (dF) at C′-4′. These results suggested that the presence of an aminosugar residue affects the biosynthetic formation of the trisaccharide residue on the aglycon, although the type of aminosugar had no effect on disaccharide formation and the glycosidation pattern.

B. Expression of Heterologous Biosynthesis Genes

The functional expression of heterologous streptomycete biosynthesis genes was first exemplified by Hopwood *et al.* and resulted in the production of "hybrid" antibiotics after the introduction of actinorhodin biosynthesis genes into heterologous hosts.[15] Soon after, *act* genes were introduced into *S. galilaeus* and their functional analysis was carried out in this host bacterium.[41] The introduction of the *actIII* locus containing the β-ketoreductase into *S. galilaeus* ANR-58, which normally produces 2-hydroxyaklavinone (**8**), caused production of aklavinone (**4**), suggesting *act* reductase can function in *S. galilaeus* to reduce the C-9 carbonyl of the polyketomethylene intermediate. The product of the ANR-58 transformant with *actI*-ORF1,2 was desoxyerythrolaccin (**48**), but aloesaponarin II (**47**) was produced in the wild-type *S. galilaeus* MA144-M1 transformant with the same loci. The structural difference between these compounds is a single hydroxyl group at the C-3 position of anthraquinone. Aloesaponarin II (**47**) lacks the hydroxyl group because of reduction by the *actIII* product in ANR-58 or *S. galilaeus akn* reductase which acts in concert with the *actI*-ORF1,2 products before aromatization. These results clearly indicated the interchangeability of these reductases.

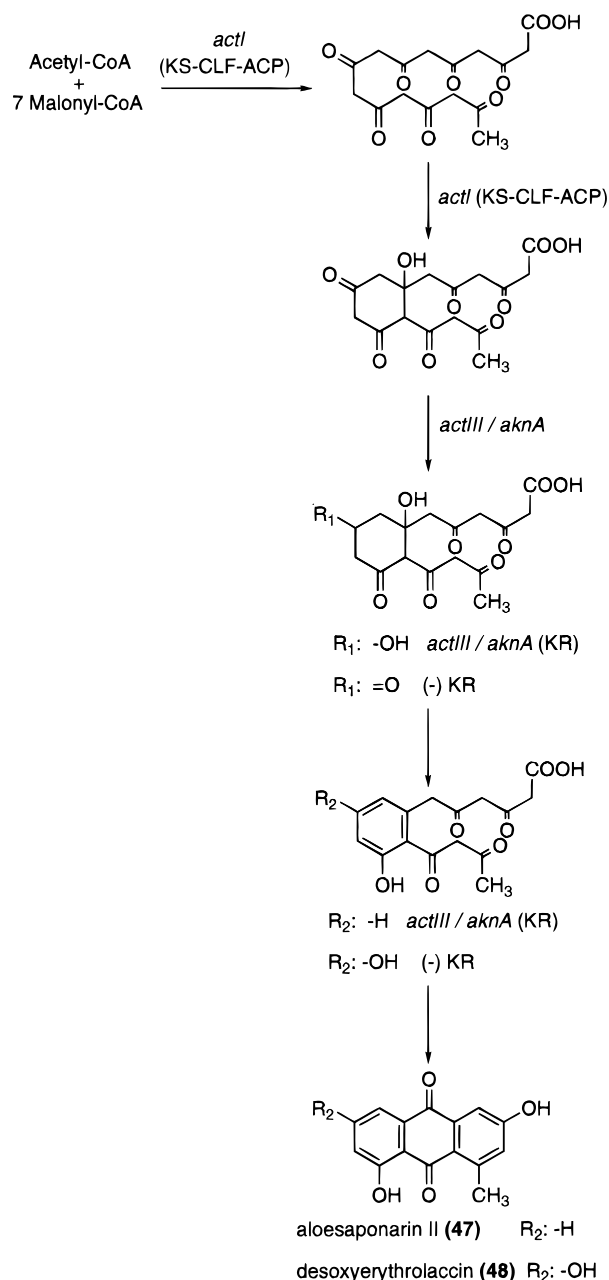


Figure 12. Biosynthetic pathway of aloesaponarin II (**47**) and desoxyerythrolaccin (**48**) in *S. galilaeus* transformants harboring plasmids containing *act* genes of *S. ceolicolor*.

When the *actI*, *III*, *IV*, and *VII* loci were introduced into wild-type *S. galilaeus* or its mutant ANR-58, both transformants produced aloesaponarin II (**47**) as a major product. Recent analysis of *act* genes showed that the KS, CLF, and ACP genes are located in the *actI* locus. The *actVII* and *actIV* are the genes coding for aromatase and cyclase, respectively.[63] All of these genes could be expressed in the heterologous

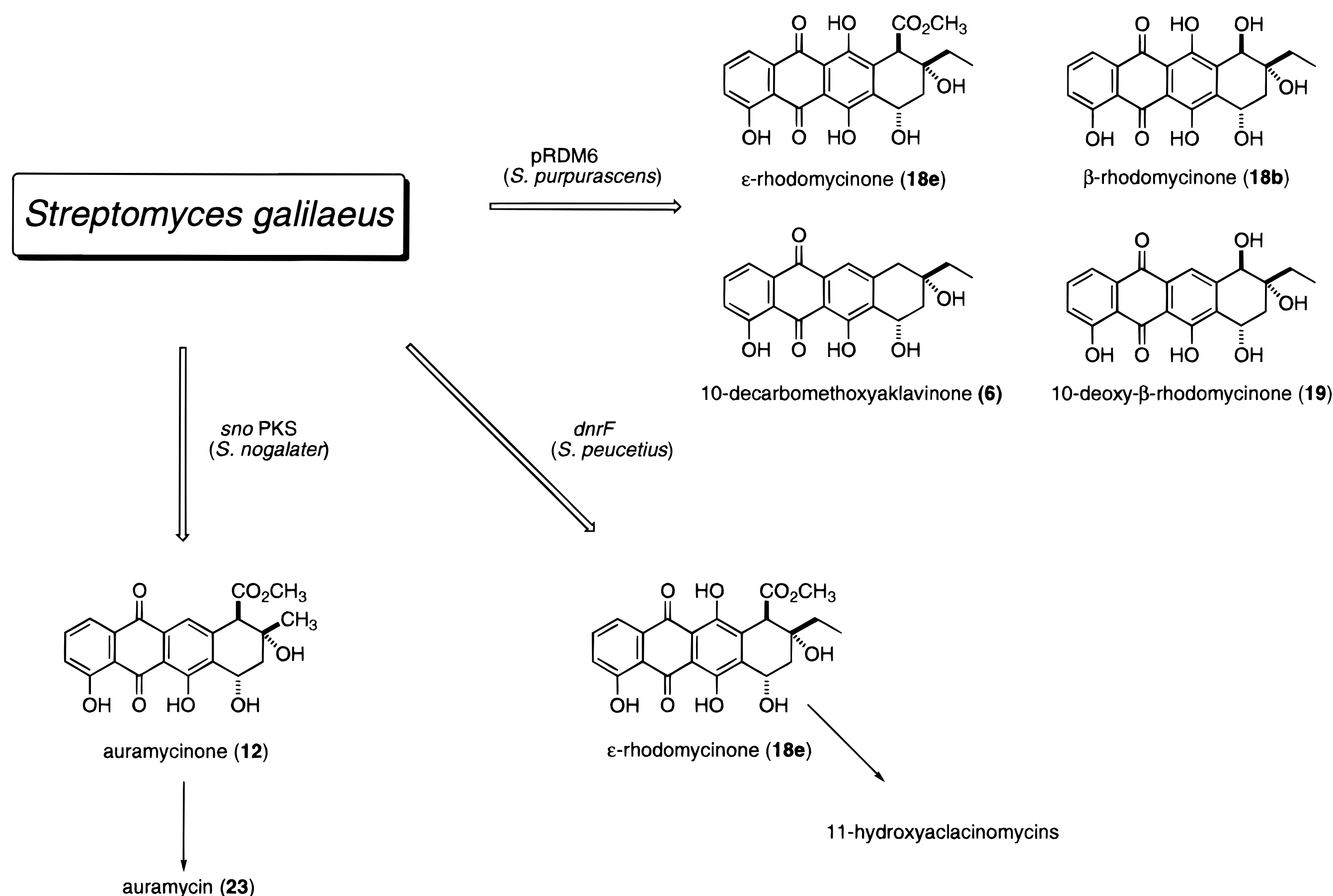


Figure 13. Production of anthracyclinones and anthracyclines by heterologous expression of anthracycline biosynthetic genes in *S. galilaeus*. *S. galilaeus* transformant harboring pRDM6 of which insert is derived from *S. purpurascens* produced ϵ-rhodomycinone (**18e**), β-rhodomycinone (**18b**), 10-decarbomethoxyaklavinone (**6**), and 10-deoxy β-rhodomycinone (**19**). *S. galilaeus* transformant harboring *dnrF* gene of *S. peucetius* produced 11-hydroxyaclacinomycins possibly via ϵ-rhodomycinone (**18e**). *S. galilaeus* transformant harboring *sno* PKS genes of *S. nogalater* produced auramycinone (**12**) and its glycoside auramycin (**23**).

host *S. galilaeus* and work as they do in *S. coelicolor*, suggesting a potential role for *S. galilaeus* as a host for hybrid antibiotic production (Figure 12).

Streptomyces purpurascens produces anthracycline antibiotics such as β-rhodomycins (**26**). From a genomic library of *S. purpurascens* DNA, several clones were obtained by hybridization with the homologous *actI* probe. When introduced by transformation, one of these clones (pRDM6) generated new anthracyclines not found in the host.[64] The structures of these novel aglycons were determined to be β- and ϵ-rhodomycinones (**18b,e**), 10-decarbomethoxyaklavinone (**6**), and 10-deoxy-β-rhodomycinone (**19**) (Figure 13). Considering the structural differences among these aglycons and aklavinone (**4**), a major anthracycline aglycon of *S. galilaeus*, it is assumed that pRDM6 contained the genes for the 11-hydroxylase of aklavinone (**4**) and the enzymes catalyzing demethylation and decarboxylation of ϵ-rhodomycinone (**18e**). The new aglycon, 10-decarbomethoxyaklavinone (**6**) and 10-deoxy-β-rhodomycinone (**18b**) should be produced from aklavinone (**4**) by a demethylase and a decarboxylase. The location of the 11-hydroxylase gene was determined by deletion of the cloned gene and product analysis of their transformants.

Lee and his co-workers cloned the *dnrF* gene which codes for aklavinone 11-hydroxylase in *S. peucetius* subsp. *caesius*, a doxorubicin (**3**) producing strain.[65] As an application for hybrid biosynthesis, the *dnrF* gene was introduced into *S. galilaeus*.[66] The transformant produced reddish metabolites and their structures were determined to be 11-hydroxyaclacinomycin A, 11-hydroxyaclacinomycin B, and 11-hydroxyaklavin. Also, a new anthracycline, named 11-hydroxyaclacinomycin X (3‴-amino-11-hydroxyaclacinomycin Y) was isolated (Figure 13). These results indicated not only that the *dnrF* gene was stable and expressed in *S. galilaeus* to introduce a hydroxyl group at C-11 of aklavinone (**4**) but also that the host strain produced 2‴-aminoaclacinomycin Y (aclacinomycin X) (**1d**). The production of aclacinomycin X (**1d**) in the wild-type strain of *S. galilaeus* was later confirmed by the same group.[67]

Nogalamycin is an anthracycline antibiotic produced by *S. nogalater*.[68-70] This compound differs from aclacinomycin (**4**) in its glycosylation profile as well as in the aglycon moiety. The aglycon of nogalamycin is derived from 10 acetate units,[71] instead of one propionate starter and nine acetates in aklavinone (**4**), and the stereochemistry at C-9 is different. Auramycinone (**12**) is also formed from 10 acetates in a similar manner to nogalamycin. But in this case, the configuration at C-9 is the same as that of aklavinone (**4**). Ylihonko *et al.* have cloned the PKS gene cluster from the nogalamycin producing *S. nogalater* and identified the component ORFs.[49] The gene cluster was found to be quite similar to that

for aklavinone biosynthesis from *S. galilaeus*, *S.* strain C5, and *S. peucetius* as discussed in section IV.C. When the minimal PKS genes with their own promoter were introduced into a *S. galilaeus* mutant which was unable to produce anthracyclines, the transformant had the capacity to produce auramycinone (**12**), although the main product was still aklavinone (**4**)[72] (Figure 13). The structural difference between aklavinone (**4**) and auramycinone (**12**) is at C-9, suggesting different polyketide starter units. Thus, the minimal PKS of *S. nogalater* can determine the starter unit in the heterologous host *S. galilaeus*. The fact that aklavinone (**4**) was the main product of the transformant may suggest that the *S. nogalater* PKS could work with the propionate loading machinery of the host *S. galilaeus* as the starter unit to produce aklavinone (**4**).

VI. Concluding Remarks

Because of their clinical importance, many aspects of the anthracycline antibiotics have been intensively studied, such as their mode of action, chemical synthesis, modifications, screening for new derivatives and biosynthesis. In this review, we have summarized the biosynthesis of the anthracyclines produced by *S. galilaeus*, focusing on the aclacinomycins and the aglycon aklavinone, by reviewing the available information to date.

In addition to classical mutation analysis, recent progress in molecular genetics has revealed a significant portion of the gene structure and function of gene products for the assembly of aklavinone from propionate and malonate. In the very near future, further analysis and molecular genetic manipulations of not only anthracycline biosynthetic genes but also microbial polyketide biosynthetic genes should enable us to design and modify the biosynthetic genes for the production of "designed antibiotics".

The sugar moieties of the anthracycline antibiotics have been found to play a crucial role in their antitumor and antimicrobial activities. Biosynthetic information on the deoxy and amino sugars in anthracycline-producing streptomycetes is limited, however. Thus, more detailed enzymological and molecular genetic analyses on sugar biosynthesis, sugar chain formation, and glycosidation of aglycons are prerequisites to the development of novel biologically active compounds.

S. galilaeus was found to have a high capacity to accept foreign compounds as substrates for further modification as exemplified by the glycosidation of various aglycons. Also, the fact that heterologous streptomycete genes like *act* genes can function in this host suggests the potential utility of this bacterium as a host for the expression of genetically modified genes for the production of novel hybrid antibiotics.

VII. Abbreviations

ACP acyl carrier protein
CLF chain length factor
KR β-ketoreductase
KS β-ketoacyl synthase
NMR nuclear magnetic resonance
NTG *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
ORF open reading frame
PCR polymerase chain reaction
PKS polyketide synthase

VIII. Acknowledgments

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