REVIEW ARTICLE

Biosynthesis of Bioactive Microbial Metabolites and Its Application to the Structural Studies and Production of Hybrid Compounds

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Microorganisms produce a variety of secondary metabolites with interesting structural features and biological activities as we can not imagine. Some of them have occupied a weighty position as medicines and agricultural drugs and for animal health. Recent progress of isolation and purification techniques and apparatus such as NMR spectroscopy in addition to establishment of new screening methods for bioactive microbial metabolites have resulted in discovery of novel biologically active compounds[1]. Biosynthetic studies on secondary metabolites using compounds enriched with ^{13}C and other stable isotopic atoms followed by NMR spectroscopic analyses provided us not only biosynthetic information of the carbon skeleton but also enzymatic and stereochemical aspects for a C-C bond formation and its reconstruction. Especially, the labeling patterns of ^{13}C enriched compounds to polyketide skeletons which are typified by polycyclic aromatic and macrocyclic compounds, branched-chain fatty acid lactone, and their complexes inform us the regularity in C-C bond formation[2~4]. Furthermore, it suggests that the biosynthetic findings deduced from the feeding experiments of ^{13}C atom and its NMR spectrometry would be useful means for the structure elucidation of novel compounds[5]. Particularly, the approach of the enzymatic function in each condensation step in the chain-elongation process and molecular genetics to polyketide biosynthesis will occupy a dominant position in production of new compounds.

Present article is reviewed in centering around our hitherto biosynthetic studies on bioactive compounds, including the biosynthesis and stereochemistry of clinically useful macrolide antibiotics and the production of new hybrid macrolides by microbial conversion and of a novel polyketide by gene manupilation.

I. Biosynthesis of Bioactive Compounds from Microorganisms

During past 25 years, ŌMURA and his coworkers[6,7] have found many novel compounds with a variety of biological activities from microorganisms by introducing and constructing effective screening systems. Some of new compounds possess unique structural skeleton deriving from pathways via polyketide, shikimate, followed by condensation with segments from amino acid and or mevalonate pathways. In this item, biosynthetic pathways and its mechanisms involving a unique formation, cleavage, rearrangement of C-C bond and their enzymatic reactions, in addition to biosynthetic origin for the carbon skeleton via metabolic pathway consisting of lower organic acids, isoprenoid, shikimate, amino acids and their complex are described in exemplifying some of bioactive compounds, hitachimycin, okilactomycin, setomimycin, furaquinocin, vineomycin, asukamycin, reductiomycin, purpactin A, lactacystin, and pyripyropene.

A. Hitachimycin

Hitachimycin[8,9] is an antiprotozoal antibiotic isolated from the culture broth of actinomycete strain No. KM 4927. A novel 19-membered ring lactam skeleton for hitachimycin is substantially distinguishable with ansamycin antibiotics[10] in the point that the antibiotic contains no aromatic or quinoid nucleus in the ansa-chain moiety. The labeling pattern by [1-^{13}C]acetate of hitachimycin molecule indicated that the antibiotic consists of eight malonates, one methylmalonate and one phenylalanine[11], as illustrated in Fig. 1. A high level of enrichment at C-19 in the feeding experiment of

D,L-[1-^{13}C]phenylalanine suggests the conversion of phenylalanine to β-phenylalanine by aminomutase in a hitachimycin-producing strain and then its incorporation into a polyketide chain presumably as a starter unit. The occurrence of the intramolecular rearrangement of an amino group from α- to β-position was evidenced from the relative intensity with natural occurrence of the signal of an amide nitrogen atom in the ^{15}N NMR spectrum of hitachimycin enriched with DL[^{15}N]-α-phenylalanine. Although tyrosine aminomutase has been reported in the biosynthesis of a peptide antibiotic, edeine[12], the occurrence of phenylalanine aminomutase in a hitachimycin-producing strain may be the first finding in microbial secondary metabolites.

B. Okilactomycin

Okilactomycin[13,14] is an antitumor antibiotic produced by Streptomyces griseoflavus subsp. zamamiensis subsp. nov. The antibiotic possesses a unique 13-membered ring with the intra-ether bridge forming a tetrahydro-δ-pyrone ring with the exo-methylene at the β-position. The feeding experiments of [1-^{13}C]acetate, [1-^{13}C]propionate, and L-[methyl-^{13}C]methionine revealed that the carbon skeleton of okilactomycin is built up from four acetates, four propionates, one methionine, and three carbons at C-12, C-13, and C-14, as shown in Fig. 2. In general, it seems to be a common observation that occurence of C-methyl group on a polyketide chain in actinomycetes is derived biosynthetically from methyl of propionate, rather than from methionine. In this point, occurrence of the methyl group at C-11 deriving from methionine seems to be a rare case in a biosynthetic pattern of metabolites from actinomycetes. The biosynthetic origin of the remaining three carbons was deduced from the feeding experiment of 75% enriched D-[U-^{13}C$_6$]glucose. The observation of the ^{13}C-^{13}C coupling pattern among C-13, C-14 and C-15 implies that the unit arises from an intact glycerol unit derived via glucose[15]. This finding suggests that the C$_3$ unit in tetrocarcin A aglycone which contains a structurally

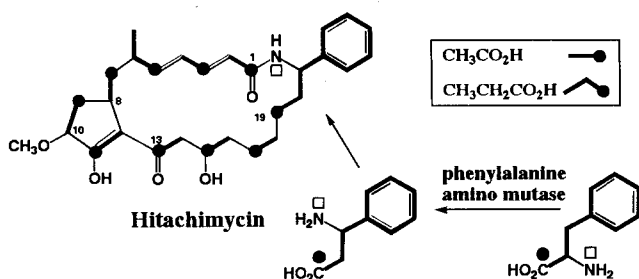Fig. 1. The biosynthetic building unit of hitachimycin.

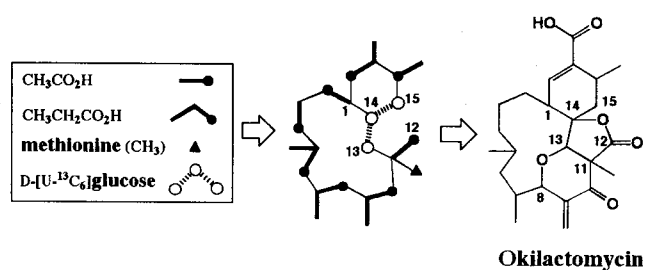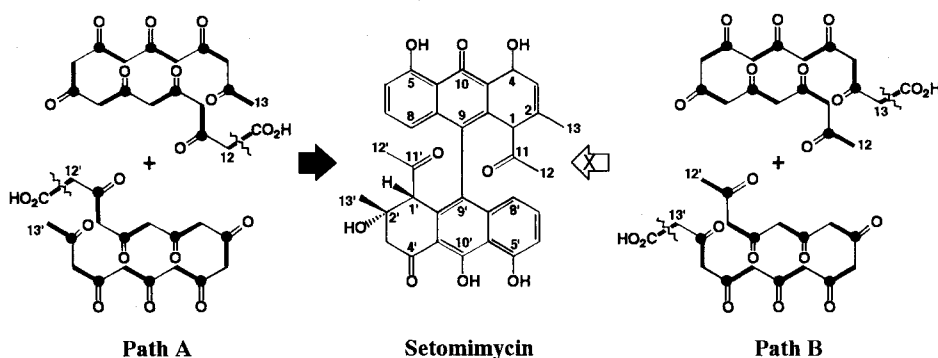Fig. 2. The biosynthetic building unit of okilactomycin.

Fig. 3. The biosynthetic pathway of setomimycin.

Path A   Setomimycin   Path B

similar unit as okilactomycin is also derived from glycerol as a precursor[16].

C. Setomimycin

Setomimycin[17] is an antibacterial and antitumor antibiotic which was isolated from the cultured broth of *Streptomyces pseudovenezuelae*. The structure of the antibiotic was determined to be a unique substituted 9,9'-bianthryl skeleton by means of various ^{13}C NMR techniques, including $^{13}C\{^1H\}$ selective decoupling, $^{13}C\{^1H\}$ selective population transfer, and $^{13}C\{^1H\}$ NOE experiments. Setomimycin appears to be constructed by the oxidative coupling of substituetd hydroanthracene monomer which may be derived from polyacetate in its carbon skeleton. Appearance of 16 enriched signals in the ^{13}C NMR spectrum of setomimycin labeled with sodium[1-^{13}C]acetate clearly indicated that the antibiotic is biosynthesized from two nonaketides in which each contains nine carbon atoms accompanied by the loss of each one of the carboxyl carbons *via* path **A** or **B**, as shown in Fig. 3. The site of decarboxylation in the nonaketide was evidenced from the appearance of two enriched singlet signals assignable to the methyl groups (C-12 and C-12') of acetate unit in the NMR spectrum of [$1,2$-$^{13}C_2$]acetate-labeled setomimycin. Appearance of triplet signals due to ^{13}C-^{13}C couplings for all carbons except for the above mentioned methyl groups implies that setomimycin is derived from two nonaketide metabolites *via* decarboxylation at the terminals, as demonstrated in path **A**[18].

D. Furaquinocin

Furaquinocins[19,20] are a novel antibiotic complex with a cytotoxic activity against HeLa S_3 cells, produced by *Streptomyces* sp. KO-3988. The antibiotic was predicted to consist of two biosynthetic units, a naphtho-

Fig. 4. The biosynthetic pathway of furaquinocin A.

1, 3, 6, 8-tetrahydroxynaphthalene

Furaquinocin A
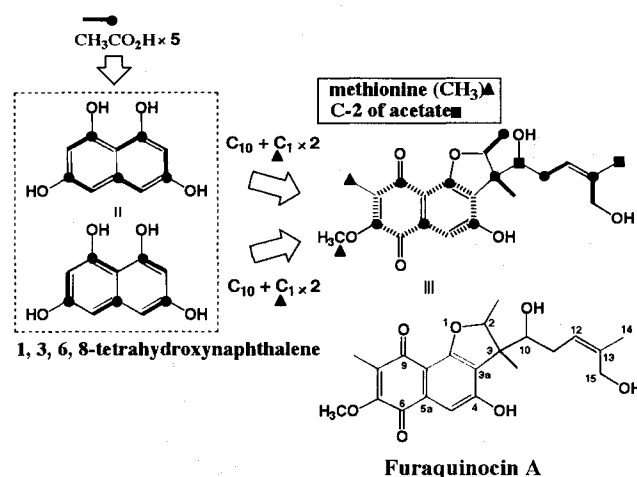
quinone chromophore and an isoprenoid-like side chain from the structural feature. The feeding of [$1,2$-$^{13}C_2$]-acetate exhibited the enrichment of twenty carbons in which eleven carbons derived from C-2 of acetate, as shown in Fig. 4. Each carbon in the naphthoquinone ring enriched with ^{13}C showed two kinds of coupling pattern with equal signal intensities. This means that the naphthoquinone ring may be formed by two different manners originated in acetate arrangement, which are also indicated by the 2D-INADEQUATE spectrum of [$1,2$-$^{13}C_2$]acetate labeled furaquinocin. The antibiotic is most likely produced through a synmetric intermediate, 1,3,6,8-tetrahydroxynaphthalene. On the other hand, the labeling pattern of [$1,2$-$^{13}C_2$]acetate for the C_{10} unit consisting of the dihydrofuran ring and the side chain indicates that the unit derives from mevalonate pathway from the appearance of singlet signal for C-10 and C-14 and eight additional satellite peaks for the other carbons of the side chain. The biosynthetic origin of the remaining

two methyl carbons (MeO-7 and Me-8) comes from a methyl of L-methionine, not from a methyl of propionate. The concurrent attachment of an isoprenoid side chain and a C_1 unit from methionine onto polyketide backbone is also interesting. Although a few isoprenoid antibiotics produced by actinomycetes have been reported, furaquinocins may be the first example involving a carbon-carbon bond between an inside position of the isoprenoid chain (C-3) and the polyketide nucleus (C-3a)[21].

E. Vineomycin

Vineomycins[22], isolated from the cultured broth of *Streptomyces matensis* subsp. *vineus* are a benzanthraquinone antibiotic with an antitumor activity against solid Sarcoma 180. The aglycone moieties of vineomycins A_1 and B_2 consist of ten acetate units from the labeling pattern of ^{13}C labeled acetate[23,24], as shown in Fig. 5. There are two possibilities for biosynthetic process on polyacetate elongation of the chromophore of both aglycones. As a plausible formation mechanism for the aglycones, it was thought that the chromophore of component A_1 would be formed first and then converted to that of B_2 by ring opening from the following labeling pattern of ^{13}C labeled acetate to the chromophore. The feeding experiment of [1,2-$^{13}C_2$]acetate to B_2 appeared 16 carbon signals flanked by satellite signals due to the ^{13}C-^{13}C splitting and the singlet signals assignable to the remaining three carbons, C-1, C-3′, and C-4′. Appearance of each singlet signal means cleavages at least two C-C bonds of acetate units during the formation of the chromophore of B_2. Since the C-4′ signal was enriched, whereas those at C-1 and C-3′ were not from the feeding experiment of [1-^{13}C]acetate, it was concluded that C-1 and C-3′ are derived from the C-2 carbon of acetate. This means that both carbons at C-1 and C-4′ originated from the same acetate molecule *via* pathway of a simple decaacetate for B_2. Therefore, the chromophore of B_2 would be derived from that of A_1 by the cleavage between C-12b and C-1 in A_1 *via* pathway A. Furthermore, the appearance of at least two ^{13}C-^{13}C coupling pairs, *i.e.*, between C-5 and C-6 and between C-11 and C-11a suggests that the mode of elongation of the decaacetate is the same as that of B_2. The site of decarboxylation was determined to be at C-2, because of appearance of only one enriched singlet signal for C-2. Vineomycin biosynthesis is confident to give the useful information for the structural characteristics of novel isotetracenone antibiotics such as kerriamycins[25] and urdamycins[26] which possess a methyl group at C-3 position and an

Fig. 5. The biosynthetic pathway of vineomycins A_1 and B_2.

Vineomycin A_1

Vineomycin B_2

Path A :

Path B :
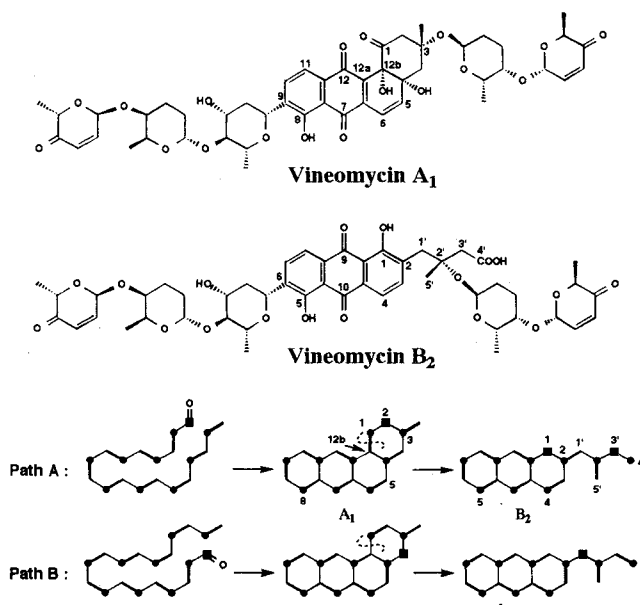
functional group of oxygen atom at C-1, C-7, C-8 and C-12 deriving biosynthetically from an oxygen of acetate.

F. Asukamycin

Asukamycin[27,28], a new antibacterial compound, was been isolated from the cultured broth of *Streptomyces nodosus* subsp. *asukaensis*. The antibiotic shows particularly anticoccidial activity in chickens. The structural and biosynthetic interestings are intensed to a central structural element a multifunctional m-C_7N unit which consists of a six-membered ring carrying one carbon and one nitrogen atom in a meta disposition and C_5N unit (2-amino-3-hydroxycyclopent-2-enone). The m-C_7N unit is most commonly quininoid as exemplified by rifamycin[29], pactamycin[30], geldanamycin[31], and ansamitocin[32]. The biosynthesis of the m-C_7N unit in these antibiotics have been demonstrated to proceed by a branch of the shikimate pathway, with 3-amino-5-hydroxybenzoic acid (AHBA) or 3-aminobenzoic acid (ABA) as proximate precursors[33]. The feeding experiment of AHBA and ABA which are potential precursors labeled with ^{13}C in the carboxyl group in addition to feeding of [1-^{13}C] and [1,2-$^{13}C_2$]acetate, indicated that either the unit is not derived from the shikimate pathway. To provide a new biochemical pathway, additional experiments were conducted with ^{13}C precursors such as acetate, succinate and glycerol[34], as shown in Fig. 6. The feeding of sodium [1-^{13}C]acetate clearly enriched positions C-6 and C-7 in the m-C_7N unit, suggestive of a "tail-to-tail". This arrangement was evidenced from

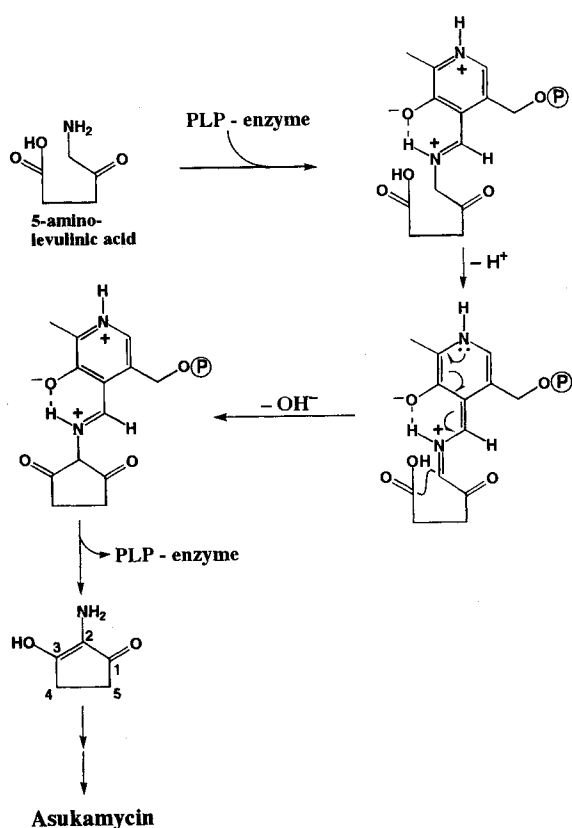Fig. 6. The biosynthetic building unit of asukamycin.
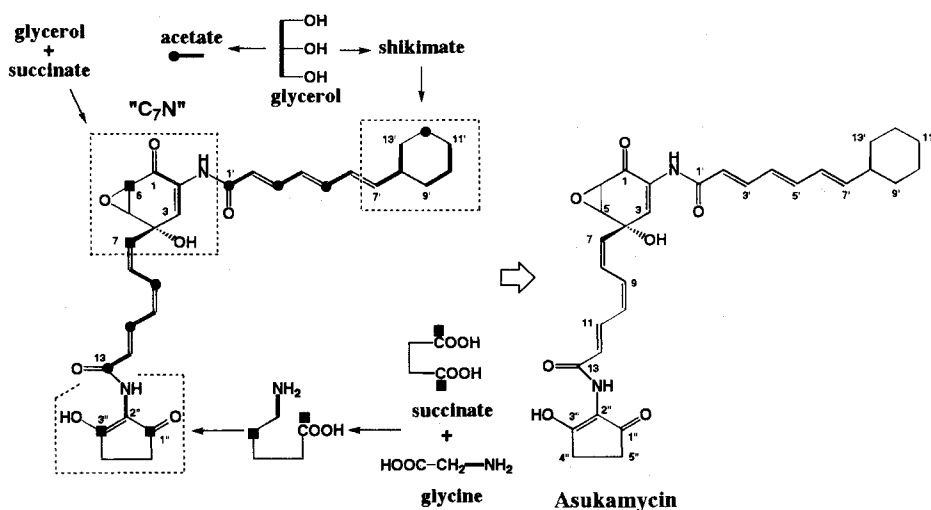
the enrichment and ^{13}C-^{13}C coupling between C-4/C-7 and C-5/C-6, suggesting that the four-carbon segment extending from C-7 and C-6 derives from TCA cycle intermediate. This speculation was confirmed by the feeding experiment of $[1,4^{-13}C_2]$succinate which gave enrichments only at C-6 and C-7. The feeding of $[U^{-13}C_3]$glycerol revealed the enrichment and coupling at C-4/C-7 and C-5/C-6 and intact incorporation into C-1 to C-3 of the m-C_7N unit in asukamycin and manumycin[35], suggesting that the m-C_7N moieties in both antibiotics represent a departure from the biosynthesis of most other type of m-C_7N unit encountered in nature. This unusual biosynthetic pathway derives a four-carbon unit closely related to succinate from the TCA cycle and a three-carbon unit from the triose pool. FLOSS et al.[36] have proposed succinyl-CoA and dihydroxyacetone (or its phosphate) as the most plausible precursors for assembly of m-C_7N units, as indicated in Fig. 7. The proposed biosynthetic process may involve two separate enzymatic systems, that are essential for the incorporation of the natural m-C_7N unit as a central starter unit. One of them, an amide synthetase, is responsible for the connection of the upper side chain to the C-2 amino group. The other activates the m-C_7N unit carboxyl group (CoA transferase) for the subsequent chain-extention process via the polyketide pathway. Beside the m-C_7N unit, the asukamycin and manumycin group antibiotics contain biosynthetically interesting structural moiety, C_5N unit which is found in other antibiotics, moenomycin[37], senacarcin A[38], virustomycin A[39], bafilomycin B_1[40], L-155,175[41] and reductiomycin[42]. The feeding experiments of ^{13}C labeled

Fig. 7. Proposed biosynthetic pathway for assembly of m-C_7N unit in asukamycin.
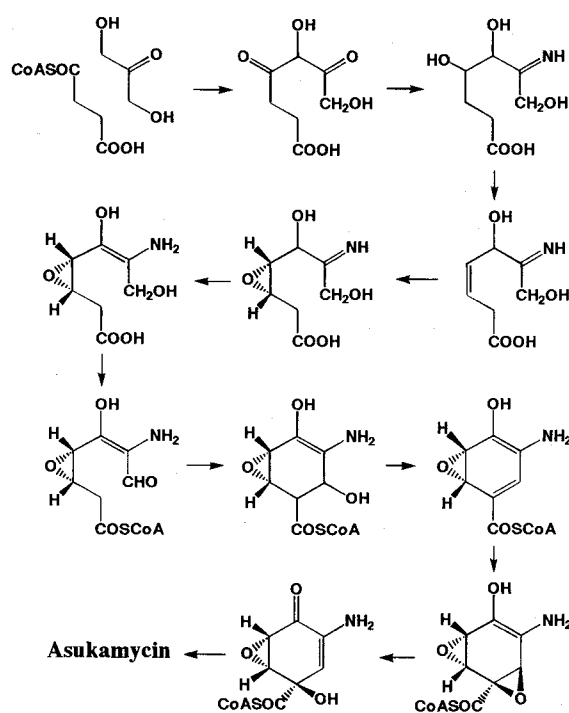
acetate, glycine, succinate, and 5-amino-levulinic acid clearly indicated that the C_5N unit was formed through a novel intramolecular cyclization of 5-amino-levulinic acid[43]. For its formation mechanism, it is speculated that intramolecular ring closure of 5-amino-levulinate is facilitated by formation of an α-carbanion equivalent, possibly through the intermediacy of a Schiff base with a pyridoxal phosphate cofactor, as shown in Fig. 8.

The evidence for the carbon source for the remaining functional units, two triene systems and cyclohexane ring containing C-7' in asukamycin, was obtained from the incorporation of ^{13}C labeled acetate and [U-^{13}C$_3$]glycerol. The formation of the cyclohexane ring and C-7' was confirmed by the shikimate-type labeling pattern, namely, two doubly coupled spin systems (C-7'/C-8'/C-9'
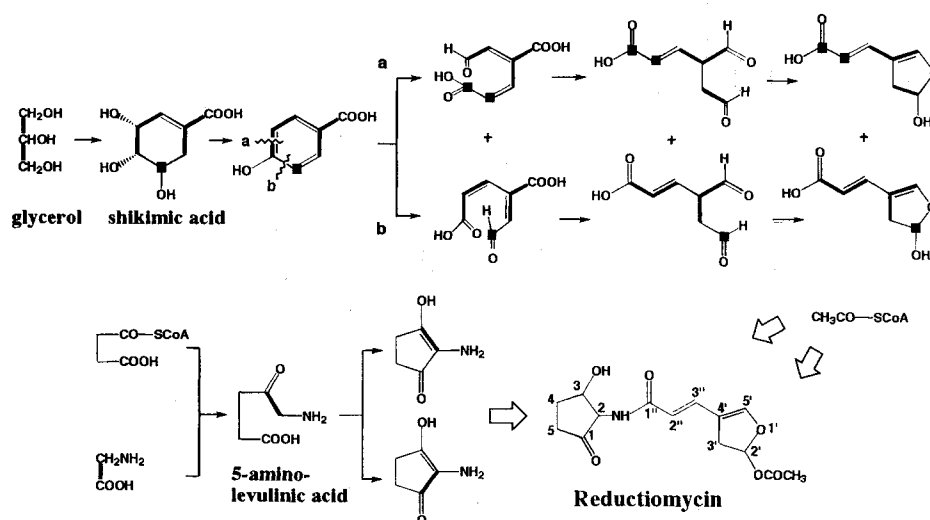
and C-11'/C-12'/C-13') and a single enriched, noncoupled signal for C-10', from [U-^{13}C$_3$]glycerol.

G. Reductiomycin

Reductiomycin[42,44], an antibiotic produced by *Streptomyces xanthochromogenus*, possesses antitumor and antibacterial activities. Reductiomycin consists of two structural units, C$_5$N unit which is found in asukamycin molecule and an acetoxydihydrofuran unit bearing an acrylic acid side chain, as shown in Fig. 9. The biosynthetic origin of the C$_5$N unit was unequivocally proven by feeding of [4,5-^{13}C$_2$] aminolevulinic acid, as described in asukamycin biosynthesis[45]. Regarding to the carbon source of the dihydrofuranylacrylic acid moiety, it was thought that the entire-nine carbon assembly, including the acetoxyl group, could be derived by a ring cleavage of phenylalanine or tyrosine, followed by a Baeyer-Villiger oxidation. This assumption on the origin of the remaining seven carbon atoms of the dihydrofuran moiety was elucidated from the labeling pattern of reductiomycin derived from [U-^{13}C]glycerol[45], in addition to the incorporation of [1,6-^{14}C]shikimate and L-[U-^{14}C]phenylalanine. The 2D-INADEQUATE spectrum clearly indicates the two possible arrangements of an intact three-carbon unit involing C-3' and C-4', *i.e.*, C-3'/C-4'/C-5' and C-3'/C-4'/C-3" for seven carbons labeled with ^{13}C in the dihydrofuran moiety, as shown in Fig. 9. The results obtained from the feeding experiment of [U-^{13}C]glycerol give rise to two species of reductiomycin, one showing the labeling pattern (a) and the other pattern (b). Coupling pattern (b) means ring cleavage between C-4 and C-5 of shikimate or a metabolite thereof. On the

Fig. 8. Formation mechanism of the 2-amino-3-hydroxy-cyclopent-2-enone moiety in asukamycin.

Fig. 9. The biosynthetic pathway of reductiomycin.

other hand, coupling pattern (a) reflects bond cleavage between C-3 and C-4. A high level of the incorporation of 4-hydroxy [7-^{13}C]benzoate to C-5' of reductiomycin indicated that 4-hydroxybenzoate or a closely related product, e.g., the corresponding aldehyde must be the substrate for the ring cleavage reaction leading to the formation of the dihydrofuran moiety. The hypothetical pathway and the formation mechanism of the dihydro-furanylacrylic moiety have been proposed by analyzing the incorporation pattern of 4-hydroxybenzoic acid and its aldehyde labeled with ^{13}C and deuterium[46].

H.　Purpactin

Purpactins[47,48] were found from the culture broth of *Penicillium purpurogenum* FO-608 as an inhibitor of acyl-CoA: cholesterol acyltransferase (ACAT) by an assay using rat liver microsomes as enzyme source. Purpactins are the first microbial ACAT inhibitors. Purpactin A which possesses a tricyclic skeleton and mevalonate moiety is structurally similar to penicillide from *Penicillium*, but the detailed biosynthetic pathway had not been clarified. Each feeding experiment of [1-^{13}C] and [2-^{13}C]acetates revealed the enrichment of seven carbons and the eight carbons, indicating that the tricyclic skeleton is derived from octaketide chain. Furthermore, six pairs of doublet signals (C-1/C-12a, C-3/C-4, C-4a/C-5, C-7a/C-8, C-9/C-9-Me, and C-11/C-11a), three pairs of signals made up each of two doublets (C-1/C-2 and C-12a, C-2/C-1 and C-3, and C-3/C-2 and

C-4), and two singlet signals (C-7 and C-10) were observed in the feeding of [1,2-^{13}C$_2$]acetate, as shown in Fig. 10. This labeling demonstrated clearly that the tricyclic skeleton is formed from two hypothetical benzophenone intermediates and is derived *via* decarbox-ylation of a single octaketide chain and oxidative cleavage at the **B** ring of a intermediate, an anthraquinone or anthrone. Two singlet carbon signals at C-7 and C-10 of purpactin A originate from the C-2 of acetate unit as a result of the decarboxylation and oxidative cleavage reactions, respectively. Appearance of three pairs of double-doublet carbons in purpactin A enriched with [1,2-^{13}C$_2$]acetate due to the rotation of the **A** ring after oxidative cleavage of the **B** ring[49]. A similar rotation has been reported from the biosynthesis of griseo-fulvin[50]. The biosynthetic origin for 3'-methylbutyl moiety is a mevalonate from the incorporation of D,L-[2-^{13}C]mevalonolactone at C-4' of purpactin A. Thus, purpactin B is the first isogrisan compound derived from a single octaketide chain and is nonenzymatically converted to purpactin A.

I.　Lactacystin

Lactacystin[51,52], a novel compound which induces differentiation of Neuro 2a cells, a mouse neuroblastoma cell line, possesses a unique γ-lactam skeleton containing hydroxyisobutyl and cysteinylthioester moieties. Three units consisting of L-leucine, L-cysteine and isobutyrate were speculated as its precursor for the carbon skeleton

Fig. 10.　The biosynthetic pathway of purpactin A.

Purpactin B

Purpactin A
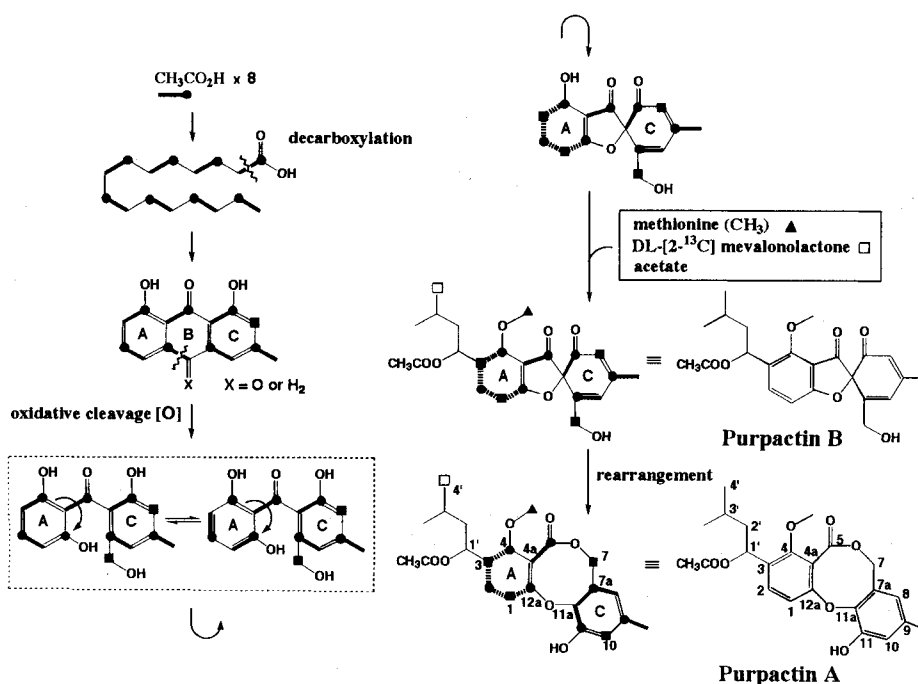
of lactacystin. The feeding of L-[2-^{13}C]leucine showed a very intense signal for C-5, indicating that C_6 segment (C-4, C-5, C-9, C-10, C-11, and C-12) is derived from L-leucine, as shown in Fig. 11. Labeling pattern of sodium[1-^{13}C]isobutyrate revealed equal levels of enrichment for C-1, C-4, C-8, and C-14. Especially, the incorporation of [1-^{13}C]isobutyrate to C-8 provided unequivocal evidence that the γ-lactam ring is formed by condensation of the C_6 unit arising from L-leucine and a Schiff base of methylmalonic semialdehyde in the presence of a pyridoxal phosphate cofactor, followed by intramolecular cyclization. Furthermore, enrichment at C-4 implies that the β-hydroxyleucine moiety is derived by condensation of 2-ketoisovalerate from valine with acetyl-CoA which was metabolized from [1-^{13}C]isobutyrate, followed by hydroxylation. Feeding of L,L-[1',1-^{13}C$_2$]cystine resulted in a very high enrichment at C-1, indicating that the C_3 unit (C-1, C-2 and C-3) is derived from L-cysteine itself formed by an enzymatic reduction of the labeled cystine. The feeding experiment of a new type of ^{13}C labeled L-leucine obtained by fermentation of a leucine-producing microorganism, using a mixture (1:2) of 98% [U-^{13}C$_6$]glucose and non-labeled glucose as a carbon sourse, was carried out for a stereospecific NMR assignment of the two diastereotopic methyl groups, C-11 and C-12 of lactacystin. The ^{13}C NMR spectrum of lactacystin obtained by feeding of ^{13}C labeled L-leucine exhibited satellite peaks, on intact coupling between C-10 and C-11 and between C-4 and C-5, respectively, and singlet peaks for C-9 and C-12.

This corresponds to the spectral pattern of the precursor, L-leucine; it clearly demonstrates that no racemization at C-10 has occurred in the formation of this segment from leucine. The retention of the configuration at C-10 during the formation of lactacystin was proved by incorporation pattern of D,L-[2-^{13}C, 4-^{2}H]leucine. Therefore, diastereotopic methyl groups, C-11 and C-12 are assignable as pro-R and pro-S, respectively[53,54]. Such feeding experiments in general are valuable means to understand the biosynthesis of secondary metabolites containing valine and leucine moieties.

J.   Pyripyropene

Pyripyropenes[55,56] are an inhibitor of acyl-CoA: cholesterol acyltransferase (ACAT) found from the culture broth of Aspergillus fumigatus FO-1289. The structure consisting of pyridine, α-pyrone and sesquiterpene moieties were evidenced by NMR spectral and X-ray crystallographic analyses for pyripyropenes A and B[57]. The feeding experiments of [1-^{13}C], [2-^{13}C], and [1,2-^{13}C$_2$]labeled acetate showed the incorporation of the intact eleven acetates and three C_1 units derived from C-2 of acetate to pyripyropene A, as shown in Fig. 12. The labeling pattern implies that pyripyropenes are constructed with sesquiterpene deriving from three mevalonates. This was supported by the enrichment of the three carbons, C-3, C-7 and C-11, in the feeding experiment of D,L-[2-^{13}C]mevalonolactone. When nicotinic acid was fed to the fermentation medium, the production of pyripyropene increased approximately two

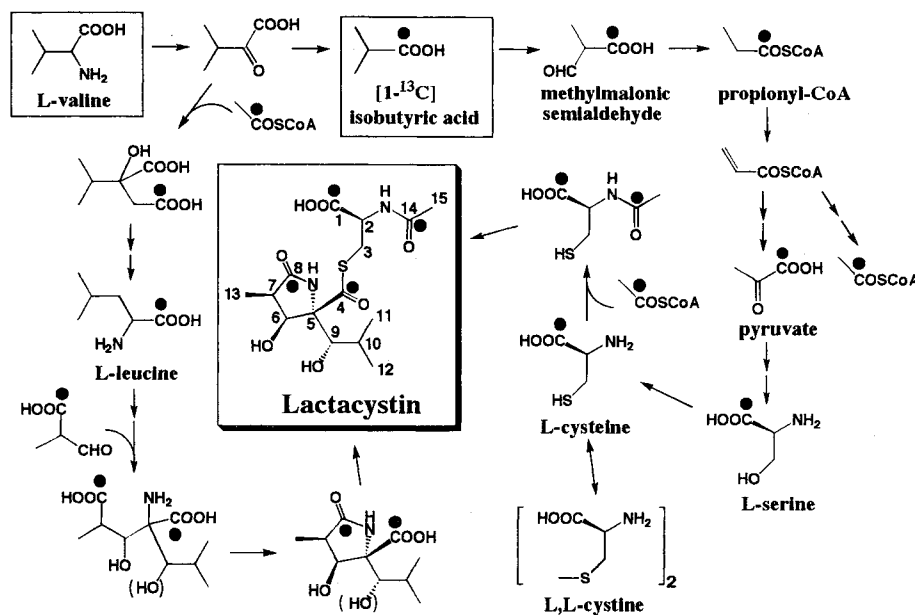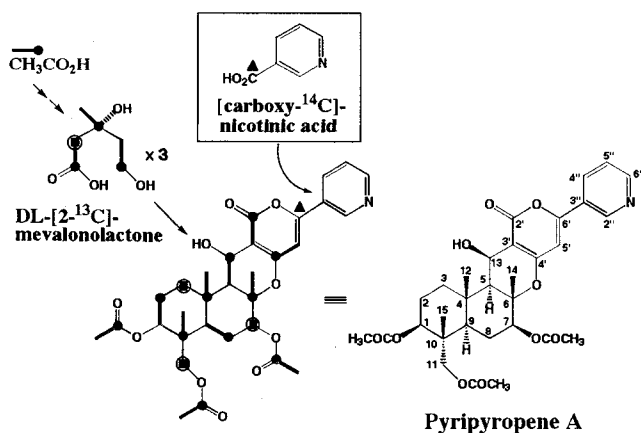Fig. 11.   Incorporation of [1-^{13}C]isobutyrate into lactacystin.

Fig. 12. The biosynthetic building unit of pyripyropene A.

Pyripyropene A

Fig. 13. The biosynthetic building unit of elasnin and conversion of the α-pyrone to γ-pyrone by methylation.

Elasnin

fold, suggesting that nicotinic acid was incorporated as a precursor to the molecule. This speculation was confirmed by the incorporation of [^{14}C]nicotinic acid followed by degradation of ^{14}C-labeled pyripyropene by Jones' oxidation and then treatment of CH_3ONa to nicotinic acid. This is the first finding that an intact nicotinic acid works as an acyl primer unit for oligoketide formation in fungal secondary metabolites[58]. For the biosynthesis of pyripyropene A, the following steps are proposed: 1) the pyridino-α-pyrone moiety is produced via condensation of a primer nicotinic acid with two acetates in a "head to tail" fashion, 2) an all farnesyl pyrophosphate is formed from three mevalonates, 3) the two parts are linked and cyclized to form the core skeleton, 4) then three acetyl residues from acetates are introduced into the skeleton to build up pyripyropene A.

II. Structure Elucidation on Polyketide Antibiotics by Biosynthetic Means

A polyketide skeleton in second microbial metabolites consists of an assembly of acetate, malonate, methyl-malonate, a methyl of methionine, and lower molecular organic acids such as succinate and butyrate. Especially, biosynthetic findings on typical polyketides such as tetracyclines, macrolides and polyethers from actinomycetes have provided us that the carbon skeletons are built up via each step of a condensation and chain elongation with a definite regularity. This implies that an incorporation pattern of ^{13}C enriched precursors would be useful means for not only the assignment of biosynthetic origin of the carbon skeleton, but also structure elucidation by NMR assignment for the C-C connectivity of polyketide skeleton[5]. The structure elucidation of polyketides by biosynthetic means using
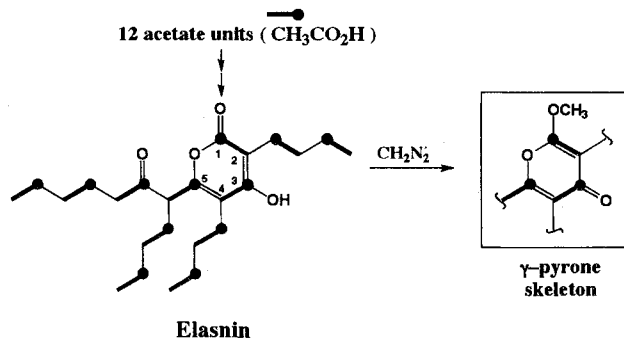
^{13}C labeled compounds, followed by NMR spectrometory is reviewed in giving examples of elasnin, herbimycin, irumamycin, phthoramycin, aurantinin, and xanthoquinodin which have been found by ŌMURA and his coworkers.

A. Elasnin

Elasnin[59] was isolated from the cultured broth of Streptomyces noboritoensis KM-2753 by screening program for inhibitor of human granulocyte elastase. Elasnin was assigned to have a highly alkylated 4-hydroxy-α-pyrone skeleton by chemical degradations and mass spectral analysis[60,61]. Feeding experiment of [1,2-^{13}C$_2$]acetate, in parallel with the structural studies provided that the carbon skeleton of elasnin was built up from four caproic acids, as shown in Fig. 13. Methylation of elasnin with diazomethane affords its monomethyl ether accompanied with an isomerization from α-pyrone to γ-pyrone. This isomerization was evidenced from appearance of satelite signals due to intramolecular acetate unit with ^{13}C-^{13}C coupling ($J_{1,2} =$ 76.3 Hz and $J_{3,4} = 61.0$ Hz in elasnin and $J_{1,2} = 85.4$ Hz and $J_{3,4} = 47.3$ Hz in its methyl ether), as indicated in Fig. 13. Elasnin is the first report which clarified the structural correlation of tautomeric isomers through the detection of ^{13}C-^{13}C coupling in the ^{13}C NMR spectrum of a polyketide enriched with a ^{13}C-labeled precursor biosynthetically.

B. Herbimycin

In the course of screening program for herbicidal substance, herbimycin[62] was isolated from Streptomyces hygroscopicus strain No. AM-3672. Later, UEHARA et al.[63], found that herbimycin possesses the activity in converting the transformed morphology of Rous sarcoma virus-infected rat kidney cells to the normal

morphology. In those days, the application of 100 MHz ^{1}H NMR spectroscopy to the structural elucidation of herbimycin led to the proposal of partial structures consisting of a benzoquinone nucleus **A** as the chromophore and three fragments **B** ~ **D**, but not the whole structure. The connectivity among three fragments in the ansa-chain was deduced from a high level of incorporation for four carbon signals at C-1, C-7, C-9 and C-13 by the feeding experiments of sodium[1-^{13}C]propionate[64], as shown in Fig. 14. The labeling pattern of [1-^{13}C]propionate demonstrates that herbimycin might be derived from m-C_7N unit *via* shikimate pathway for a benzoquinone moiety and from a glycolate (or glycerate) for C-11/C-12, two acetates for C-3/C-4 and C-5/C-6, four propionates and four O-methyls from methionine in the ansa-chain moiety.
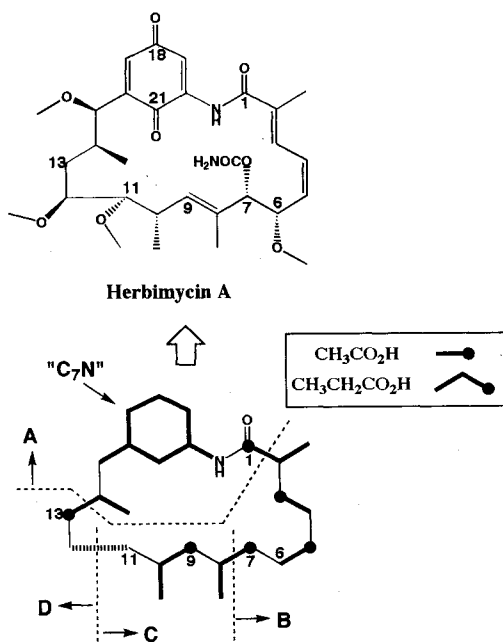
C. Irumamycin

A new macrolide antibiotic, irumamycin[65] which is produced by *Streptomyces subflavus* inhibits strongly the growth of the phytopathogenic fungi. The structure elucidation of irumamycin was successfully assigned from the feeding experiments using ^{13}C doubly labeled acetate and propionate, in addition to chemical degradation[66]. Feeding experiments of [1-^{13}C]acetate and [1-^{13}C]propionate showed a high level of enrichment for five carbon signals and for eight signals, respectively, indicating that the aglycone consists of five acetate and eight propionate units, as shown in Fig. 15. Each ^{13}C NMR spectrum of

irumamycin enriched with [1,2-^{13}C$_2$]acetate and [1,2-^{13}C$_2$]propionate exhibited additional satellites based on intermolecular and intramolecular ^{13}C-^{13}C coupling for each of the carbon signals which appeared as doublet. The ^{13}C-^{13}C decoupling experiments of these satellite peaks deduced a 20-membered macrocyclic structure containing a 6-membered ring hemiketal. Appearance of a long range ^{13}C-^{13}C coupling ($^3J_{cc} = 11.3$ Hz) between C-24 and C-26 of ethyl ketone supported the exsistence of α,β-epoxy-α',β'-ethyl ketone moiety in irumamycin. The proposed structure was also evidenced from the structural feature of an ozonolysis products of diacetylirumamycin. In general, the carbon skeleton of polyketides produced by actinomycetes originates from malonate (and/or acetate) as the biosynthetic origin for C_2 unit and methylmalonate (and/or propionate) for C_3 unit in polyketide chain. On the other hand, fungal polyketides add extra carbons from the methyl group of methionine. This is especially evident when the labeling patterns of fungal metabolite such as phomenoic acid[67] which is produced by *Phoma lingam* are compared with those of irumamycin, as illustrated in Fig. 16.

During the biosynthetic studies of irumamycin, its aglycones, irumanolides I and II were found from a mutant of an irumamycin-producing strain obtained by NTG treatment[68]. Both aglycones were microbially converted to irumamycin under the presence of a polyketide biosynthetic inhibitor, cerulenin[69] during the fermentation of the parent strain. This implies that both aglycones are converted to irumamycin *via* an epoxidation and glycosidation.

D. Phthoramycin

In the course of screening program for cellulose biosynthesis inhibitor, phthoramycin[70] was found in the

Fig. 14. The structure of herbimycin A and the proposed biosynthetic building unit.

Herbimycin A

Fig. 15. The structure elucidation of irumamycin by incorporation pattern of [1,2-^{13}C$_2$]acetate and [1,2-^{13}C$_2$]propionate.

Irumamycin
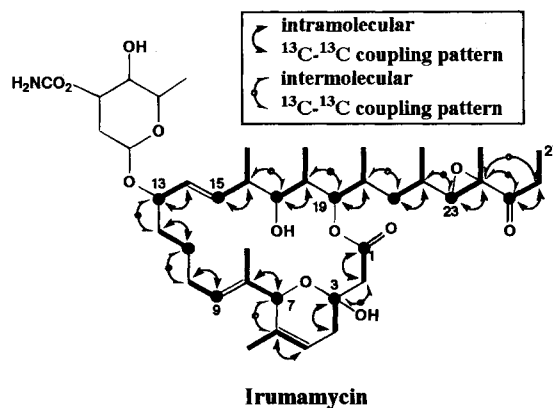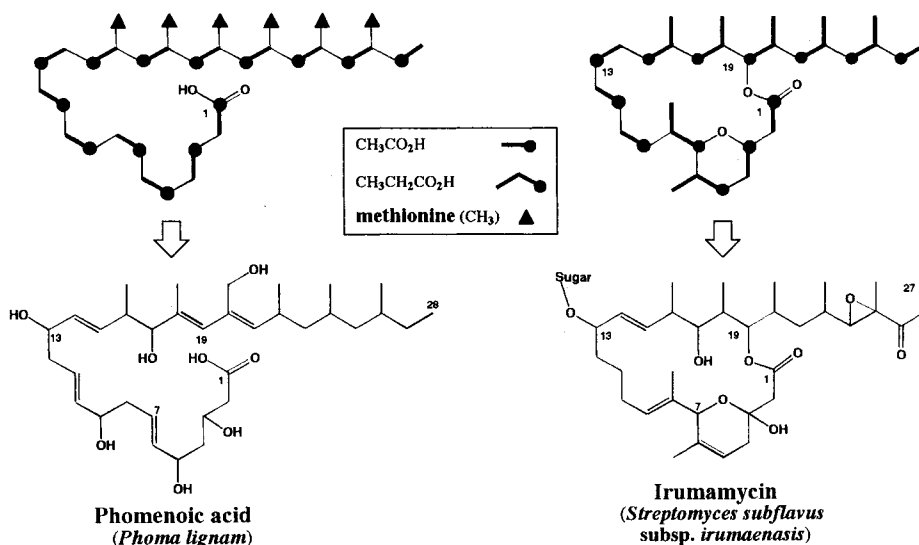
Fig. 16. Comparison of the biosynthetic building units of irumamycin and phomenoic acid.

Phomenoic acid
(Phoma lignam)

Irumamycin
(Streptomyces subflavus
subsp. irumaenasis)

cultured broth of *Streptomyces* sp. WK-1875. The antibiotic shows the inhibitory activity against filamentous fungi such as the plant pathogen *Phytophthora parasitica* and also herbicidal activity. The antibiotic is structurally similar to the aglycone of cytovaricin[71]. Phthoramycin enriched with ^{13}C was derived to the pentaacetate to simplify the chemical shift assignment of each carbon signal. Each feeding experiment of [1-^{13}C]acetate and [1-^{13}C]propionate showed the enrichment for nine carbon signals and six ones, respectively, indicating that the carbon skeleton of phthoramycin is built up biosynthetically from nine acetates, six propionates and four carbon atoms (C-32, C-33, C-34 and C-35), as illustrated in Fig. 17. 2D-INADEQUATE of phthoramycin labeled with [1,2-[13]C$_2$]acetate revealed clearly the C-C connectivity for nine pairs of intramolecular acetate units. The connectivity between acetate and propionate units was successfuly clarified from ^{1}H-^{1}H and ^{13}C-^{1}H COSY and LSPD experiments. Thus, a 22-membered macrocyclic structure with C$_{11}$ alkylated side chain at 21-position and C$_4$ unit at 16-position on the lactone ring was proposed for phthoramycin[72]. The feeding of [1-^{13}C]isobutyrate revealed a high level of enrichment at C-32 together with that of two carbons which correspond to carboxyl carbons of acetate and propionate metabolized from ^{13}C enriched isobutyrate. An extremely high incorporation of sodium [1-^{13}C]isocaproate into C-15 implies that isocaproate is intact precursor. Although there are some reports that partially elaborate polyketides (C$_6$ and C$_8$ fatty acids) were

Fig. 17. The structure elucidation of phthoramycin by incorporation pattern of ^{13}C enriched compounds.

Phthoramycin
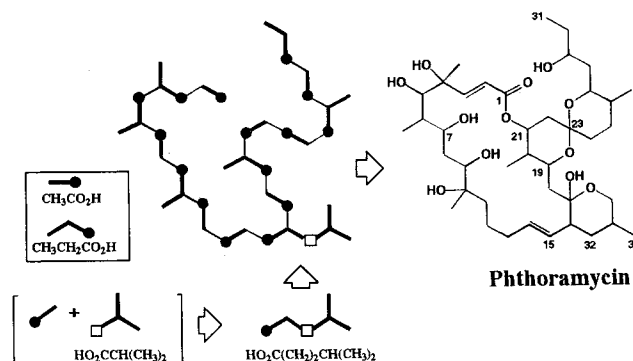
incorporated into polyketides molecule[73], it seems to be the first finding in the biosynthesis of microbial metabolites that isocaproate was incorporated as an intact precursor into the middle position on the polyketide chain.

E. Aurantinin

Although many antibiotics with polyketide skeleton from actinomycetes and fungi have been found, one from eubacteria are extremely less. A novel antimicrobial polyketide, aurantinins A and B[74] were found from *Bacillus aurantinus*. Aurantinin B includes a novel sugar, 6-deoxy-β-ribohexo-pyranos-3-ulose in the aglycone. The feeding experiments of [1-^{13}C]acetate showed a strong enrichment for 16 carbon signals. On the other hand, 17 carbon signals including two methyl carbons were enriched by feeding of [2-^{13}C]acetate, suggesting

that the polyketide chain of aurantinin consists of 16 malonate units. The ^{13}C spectrum of component B enriched with [1,2-^{13}C$_2$]acetate exhibited additional satellite peaks for all carbon signals except for methyl carbons arising from methionine as a biosynthetic precursor and carbons from sugar moiety. The observation of intra- and intermolecular ^{13}C-^{13}C coupling patterns of acetate units in the 2D-IN-ADEQUATE spectrum permitted derivation of partial structures (A), (B) and (C), as depicted in Fig. 18. The C-C connectivity among partial structures was evidenced from the NMR analysis of dihydroaurantinin B obtained by NaBH$_4$ reduction of aurantinin B and a dimethyl ester of the dicarboxylic acid derivative obtained by alkaline hydrolysis followed by methylation with diazomethane. The feeding of L-[^{13}C-methyl]methionine revealed a high level of incorporation for five methyl carbons (C-32, C-35, C-36, C-37 and C-38). The location of the methyl groups in the polyketide chain is reasonably assigned by appearance of a doublet signal for each carbon atom attached directly to five ^{13}C-enriched methyl carbons. Thus, aurantinin B[75] possesses a novel structure involving an acid anhydride moiety and a novel sugar, in addition to a conjugated polyketide skeleton consisting of four rings. Aurantinin seems to be formed biosynthetically from two polyketide chains. A long chain originates from eleven acetate units, three C$_1$ units arising from methionine, two C$_1$ units at C-5 and C-7 from an acetate via decarboxylation and one C$_1$ unit at C-1 from an acetate via demethylation. A short chain consists of

four acetate units in which a succinate might be a starter unit from the labeling "tail to tail condensation" of two acetate units, and two C$_1$ units from methionine. Aurantinin biosynthesis give us the following three biosynthetic characteristics: 1) the propionate pathway in eubacteria does not function, 2) the C$_1$ unit located in a head of an acetate unit in a polyketide chain arises from the methyl of an acetate unit, 3) the C$_1$ unit located in the tail of an acetate unit common to biosynthetic pathways in fungal metabolites arises from methionine, but not from a methylmalonate. The occurrence of a methyl group from acetate via decarboxylation seems to be common to the biosynthetic pathway of the secondary metabolites from eubacteria. The biosynthetic characteristics have been found in myxopyronin A$_1$[76], myxothiazol[77], and myxovirescin A[78] from myxobacteria, and pseudomonic acid[79] from Pseudomonas. As shown in Fig. 19, methyl groups located at C-2 of an acetate unit in a polyketide chain of pseudomonic acid come from the methyl group of methionine and the methyl attached at C-1 of an acetate unit, from a methyl group of acetate. ZIMMERMAN et al.[80] and WILSON et al.[81] have reported the structure determination of unusual macrolide antibiotics, difficidin and oxydifficidin produced by eubacteria, Bacillus subtilis. Taking the findings from aurantinin biosynthesis into consideration, the tentative building units consisting of 13 acetate units, three methionines, and two C$_1$ units arising from acetate can be speculated for biosynthetic origin of difficidin.

F. Xanthoquinodin

Xanthoqinodins[82], A$_1$, A$_2$, A$_3$, B$_1$, and B$_2$ are novel anticoccidial antibiotics produced by Humicola sp. FO-888. Xanthoquinodins possess a unique heterodimer consisting of xanthone and anthraquinone moieties in which the structures are very similar to beticolins[83] and cebetins[84], reported as fungal toxins. However, thus far, the biosynthesis of this class of compounds has not been clarified. The structures of xanthoquinodins were clarified from biosynthetic labeling patterns of ^{13}C enriched precursors, in addition to extensive NMR spectroscopic techniques[85], as shown in Fig. 20. The feedings of [1-^{13}C] and [2-^{13}C]acetate indicated the enrichment of the 14 carbons and 16 ones, respectively. Component A$_1$ labeled with [1,2-^{13}C$_2$]acetate revealed 12 pairs of doublet signals (C-2/C-15, C-3/C-4, C-5/C-6, C-7/C-8, C-12/C-15', C-1'/C-14', C-2'/C-3', C-4'/C-16', C-6'/C-7', C-8'/C-9', C-10'/C-11', and C-12'/C-13'), two uncoupled signals (C-16 and C-5'), and three pairs of signals comprised of two doublets (C-10/C-9 and C-11, C-14/C-9

Fig. 18.   The structure elucidation of aurantinin B by incorporation pattern of ^{13}C enriched compounds.
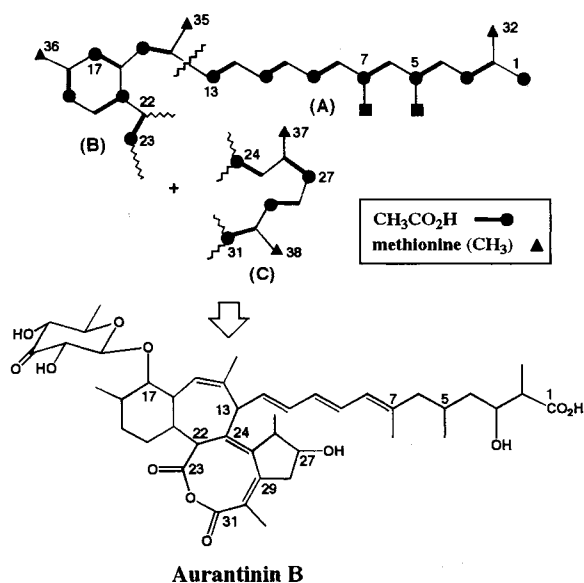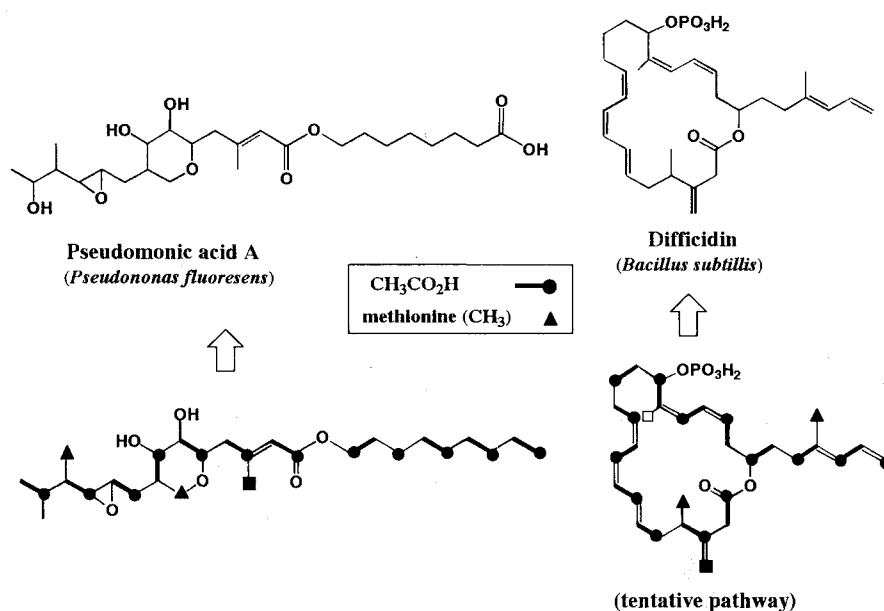

CH$_3$CO$_2$H ⟶ ●
methionine (CH$_3$) ▲

Aurantinin B

Fig. 19. The biosynthetic building units of pseudomonic acid and difficidin.

Pseudomonic acid A
(*Pseudononas fluoresens*)

CH₃CO₂H   ●
methionine (CH₃)   ▲

Difficidin
(*Bacillus subtillis*)

(tentative pathway)

and C-13, and C-9/C-10 and C-14). The C-9 signal observed as a doublet happend to have the same coupling constant to both C-10 and C-14. The NMR data from the labeling patterns including HMBC and NOE experiments deduced a heterodimer of xanthone and anthraquinone monomers derived from octaketide for A_1. The biosynthetic process of A_1 would initiate from two molecules of octaketide. One of two anthraquinones derived from two octaketides *via* decarboxylation of the tail of an acetate unit is subjected to oxidative cleavage at the B ring to form a xanthone skeleton. Appearance of two pairs of doublet-doublet signal (C-9/C-10 and C-14, C-10/C-9 and C-11, and C-14/C-9 and C-13) in A_1 labeled with $[1,2-^{13}C_2]$acetate can be explained as a result of free rotation at the bond between C-8 and C-9. A mixture of two rotational isomers of the oxidative intermediate is connected at the two sites of anthraquinone to form the skeleton of A_1, as shown in Fig. 21. Extensive NMR analyses of each component established that both two components of A_1 and A_2, and B_1 and B_2 have the same general structure. Component A_3 possesses a γ-lactone ring which seems to be originated from A_1. It seems to be reasonable that the cleavage between O-1 and C-2 of components A_1 and A_2 and recombination of C-2 with O-10 would produce components B_1 and B_2. The relative configurations at C-2, C-3, C-11' and C-14' in components A_1, A_2, B_1, and B_2 were assigned by NMR analyses including NOE measurements and studying the mechanism of inter-

Fig. 20. The structures of xanthoquinodins A_1, A_2, A_3, B_1, and B_2.

Xanthoquinodin A_1 Xanthoquinodin B_1

Xanthoquinodin A_2 Xanthoquinodin B_2
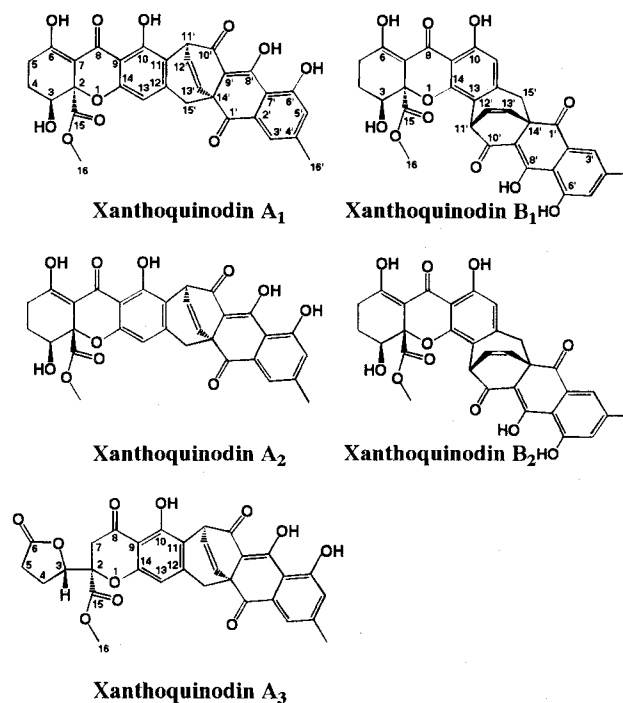
Xanthoquinodin A_3

conversion on heat treatment among components[85]. Xanthoquinodins are the first heterodimers of octaketide-derived xanthone and anthraquinone monomers connected in an "end-to-body".

Fig. 21. The biosynthetic pathway of xanthoquinodin A_1.
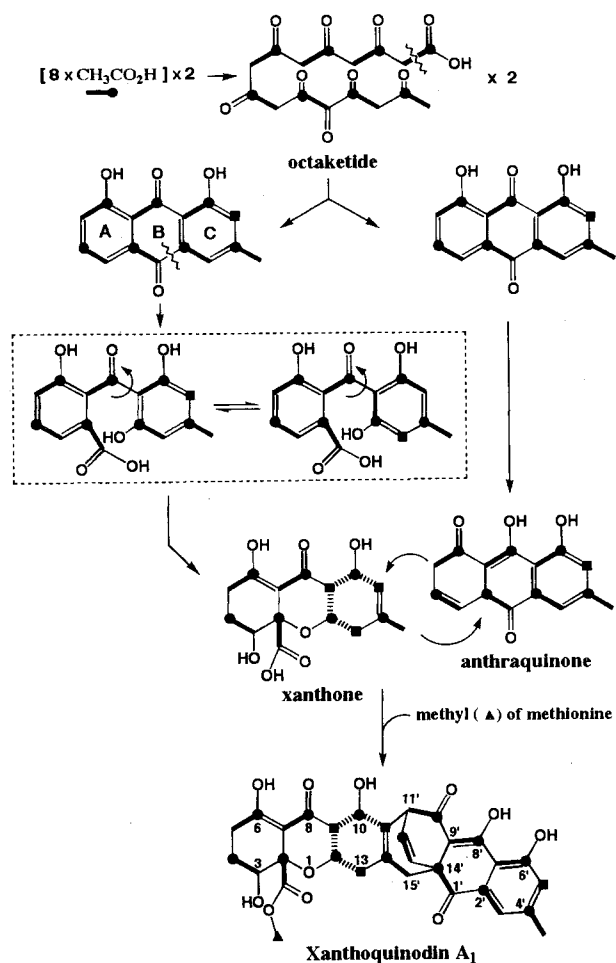
[8 × CH₃CO₂H] × 2 →

octaketide

anthraquinone

xanthone

methyl (▲) of methionine

Xanthoquinodin A_1

III. Biosynthesis and Stereochemistry of Macrolide Antibiotics and Production of New Hybrid Macrolides and Polyketide

Macrolide antibiotics are classified as 12-, 14-, or 16-membered ring macrolides which contain amino sugar and/or neutral sugar moieties, according to the size of the macrocyclic lactone ring of the aglycone[86~88]. These macrolides have occupied a large position in antibiotics, because of their excellent antibacterial activities, particularly against Gram-positive bacteria and *Mycoplasma*. Among macrolide antibiotics, 14-membered macrolides, erythromycin and oleandomycin, 16-membered ones, leucomycin (josamycin), spiramycin, midecamycin, and tylosin, and their derivatives have been used in medical and veterinary fields. To date, macrolides antibiotics have been a focus of the world's attention in organic chemistry and biochemistry owing to the structural and biological characteristics. Biosynthetic origin of the carbon skeleton of the aglycone, the stereochemical biosynthetic pathway and the regulation

are described in this chapter. This review also deals with the production of new hybrid macrolides by a microbial transformation and a polyketide by gene manipuration.

A. Biosynthesis of the Aglycone Moiety in 16-Membered Macrolides, Leucomycins and Tylosin

In the biosynthetic origin of the carbon skeleton of the lactone ring, magnamycin A has been studied intensively using ^{14}C-precursor by groups of WOODWARD et al.[89], ACHENBACH et al.[90], and SRINIVASAN and SRINIVASAN[91]. Improvement of ^{13}C NMR spectroscopy and the propagation of ^{13}C enriched compounds have resulted in a great contribution for studying the biosynthesis of macrolides[92~95]. The biosynthetic origin for the aglycone moiety of leucomycin A_3 was established from the feeding experiments of [1-^{13}C] and [2-^{13}C]acetate, [1-^{13}C]propionate, and [1-^{13}C]butyrate, as shown in Fig. 22. Indeed, a high level of incorporation by the feeding of [1-^{13}C]butyrate and [4-^{13}C]2-ethylmalonate is related to the finding that an aglycone, platenolide containing a methyl group in place of the formyl group was isolated from a mutant of a platenomycin-producing strain. It is interesting findings that three carbons at positions 7, 8 and 19 corresponding to a propionate unit, were weakly enriched by the feeding of [2-^{13}C]acetate but not by [1-^{13}C]acetate. This means that the propionate pathway through the tricarboxylic acid cycle *via* succinyl-CoA and methylmalonyl-CoA is active in a leucomycin-producing microorganism. Evidence for the biosynthetic origin of the C_2 unit at C-3 and C-4 was obtained finally from the enrichment at C-4 by the feeding of [2-^{13}C]glycerol[96], indicating that glycolate metabolized from glycerol may be a direct precursor for the C_2 unit.

Similarly, it has been clarified that the aglycon moiety of tylosin derives from two acetate units, five propionate units, and one butyrate unit, as shown in Fig. 23. An interesting thing is a high level of incorporation by the feeding of [1-^{13}C]butyrate not only at C-5 but also at carbons, 3, 7, 11, 13, and 15 which should be derived from the carboxyl carbon of propionate. This labeling pattern indicates obviously that butyrate are metabolized into propionyl-CoA or methylmalonyl-CoA *via* succinate involving an oxidation and isomerization[97]. Further, the feeding experiment of [1,3,1'-$^{13}C_3$]2-ethylmalonic acid showed an additional enrichment at carbons, 3, 4, 11, 12, 13, 14, 15, and 16 accompanied with a doublet signal arising from ^{13}C-^{13}C coupling, in addition to enrichment at carbons 5 and 19, indicating the metabolic pathway of [1,3,1'-$^{13}C_3$]2-ethylmalonate to methylmalonate *via*
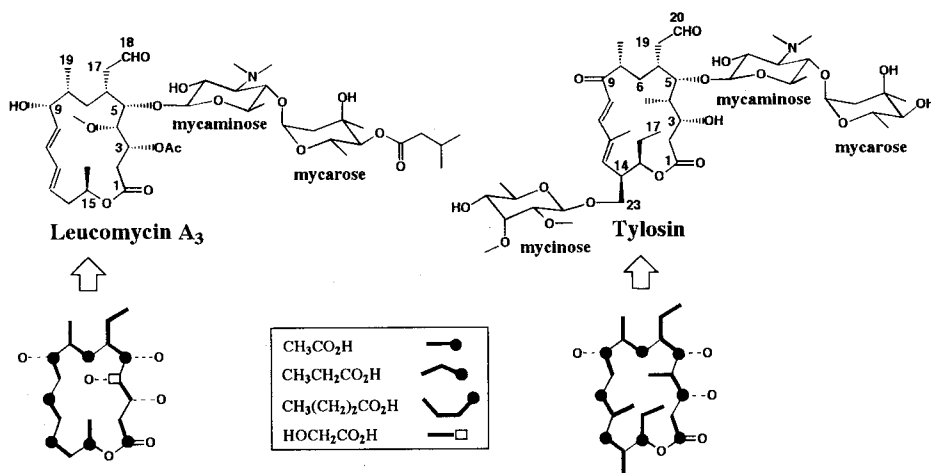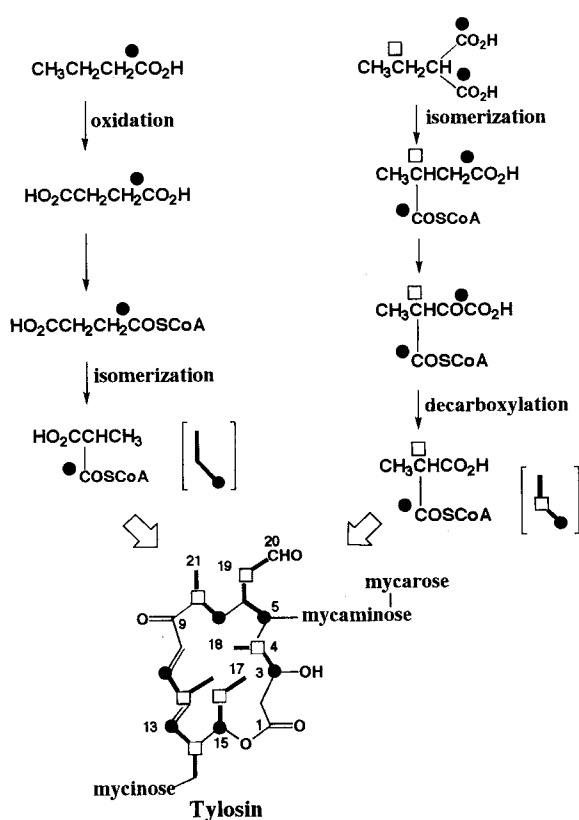
Fig. 22. The structures of leucomycin A_3 and tylosin and their biosynthetic building units of the aglycones.

Leucomycin A_3 Tylosin

Fig. 23. Proposed pathway of butyric acid and ethyl-malonic acid into methylmalonyl-CoA or propionyl-CoA in a tylosin-producing organism.
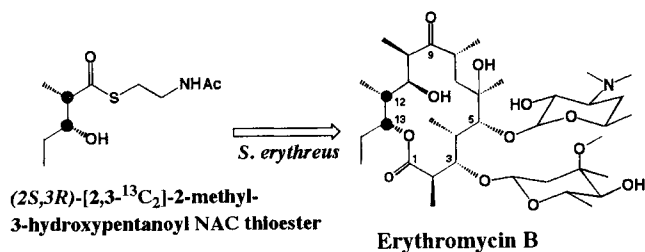

Tylosin

methylmalonyl-CoA mutase, as shown in Fig. 23. Thus, macrolide biosynthesis gave us that trace experiment of ^{13}C atom by feeding of ^{13}C enriched compounds are useful tools to know the existence of new metabolic pathways of lower organic acids in microorganism.

B. The Intact Incorporation of a Chain-elongation Intermediate into Macrolides

After certification regarding to the biosynthetic origin of the lactone skeleton, particular interestings are a mechanism of growing polyketide chain in macrolide biosynthesis. To clarify the condensation mechanism CANE et al.[98,99] and HUTCHINSON et al.[100] have reported the incorporation of partially elaborated intermediate, 2-methyl-3-hydroxypentanoic acids labeled specifically with ^{13}C which are a condensate of methylmalonyl-CoA with propionyl-CoA into erythromycin B and tylactone which is an aglycone of tylosin. As a results, no significant enrichments or ^{13}C-^{13}C couplings could be detected by ^{13}C NMR, indicating that the precursor had most likely been degradated to a mixture of [1-^{13}C] and [2-^{13}C]propionate during incorporation into the macrolides. However, the conversion of above (2S,3R)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoic acid into the corresponding N-acetylcysteamine (NAC) thioester recognized the enrichment of carbon signals and coupled doublets at C-12 and C-13 in erythromycin B, as shown in Fig. 24. Especially, incorporation of the NAC thioesters, putative intermediates of polyketide chain assembly into erythromycin B provides a biosynthetic evidence for the processive nature of reduced polyketide chain elongation. CANE et al.[101] have applied the systematic analysis of the chain-elongation process by stepwise incorporation to a 12-membered macrolide, methymycin. The aglycone of methymycin is formed by condensation of a propionyl thioester starter unit with an equivalent of (2R)-methylmalonyl CoA. The feeding experiment of a diketide intermediate, (2S,3R)-[2,3-$^{13}C_2$]-2-methyl-3-hydroxypentanoyl NAC thioester to

Fig. 24. Intact incorporation of (2S,3R)-[2,3-^{13}C$_2$]-2-meth-yl-3-hydroxypentanoyl NAC thioester into erythromycin B.

(2S,3R)-[2,3-^{13}C$_2$]-2-methyl-
3-hydroxypentanoyl NAC thioester

Erythromycin B

Fig. 25. Intact incorporation of (2S,3R)-[2,3-^{13}C$_2$]-2-methyl-3-hydroxypentanoyl NAC thioester, (2S,3R)-[3-^{2}H,3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester, and (4R,5R)-[2,3-^{13}C$_2$]-4-methyl-5-hydroxyheptanoyl NAC thioester into methymycin.

Propionate
Acetate

S. venezuelae
SC 2366

Methymycin

desosamine

(2S,3R)-[2,3-^{13}C$_2$]-2-methyl-
3-hydroxypentanoyl NAC thioester

O—desosamine

(2S,3R)-[3-^{2}H, 3-^{13}C]-2-methyl-
3-hydroxypentanoyl NAC thioester

O—desosamine

(4R,5R)-[2,3-^{13}C$_2$]-4-methyl-5-
hydroxyheptanoyl NAC thioester

O—desosamine

methymycin revealed as expected the enhanced and coupled doublets for C-10 and C-11 in the lactone ring. Furthermore, the successful incorporation at C-8 and C-9 of a triketide intermediate, (4R,5R)-[2,3-^{13}C$_2$]-4-methyl-5-hydroxyheptanoyl NAC thioester is fully consistent with the proposed intermediacy, presumably generated from (2S,3R)-[2,3-^{13}C$_2$]-2-methyl-3-hydroxy-pentanoyl NAC thioester by a sequence of malonyl condensation, keto reduction, and dehydration[101], as illustrated in Fig. 25. A similar intact incorporation of a variety of chain-elongation intermediates, (2S,3R)-[3-^{2}H, 3-^{13}C]-2-methyl-3-hydroxypentanoyl NAC thioester, (2E,4R,5R)-[2,3-^{13}C$_2$]-2,4-dimethyl-5-hydroxyhep-tenoyl NAC thioester, and (2E,4E,6R,7R)-[2,3-^{13}C$_2$]-4,6-dimethyl-7-hydroxy-2,4-nonadienoic acid NAC thioester, into nargenicin, an unusual macrolide of Nocardia argentinesis, has been reported by CANE et al.[102,103].

KINOSHITA et al.[104] isolated partially assembled fragments of protomycinolide IV, an aglycone of mycinamicin, from the fermentation broth of a mycinamicin-producing strain, Micromonospora griseorubida, as shown in Fig. 26. These fragments can be rationalized as a stepwise series of intermediates in the processive assembly of the aglycone. KINOSHITA et al. proposed a biosynthetic pathway to protomycinolide IV, based on the isolation of a fully assembled seco acid[105] and two novel macrocyclic lactones[106] from the mutant strain of M. griseorubida. Particular interesting things from a biosynthetic point of view are the diversity of the substituents at 4-, 6-, and 15-positions on the aglycone among mycinamicins VII, VIII, and IX, as indicated by arrows in Fig. 27. The difference for the building units among these components demonstrates that the polyketide synthase shows a degree of tolerance and will substitute malonyl CoA for methylmalonyl CoA at various positions on the aglycone during the polyketide chain elongation. Intact incorporation of polyketide chain intermediates and isolation of polyketide fragments
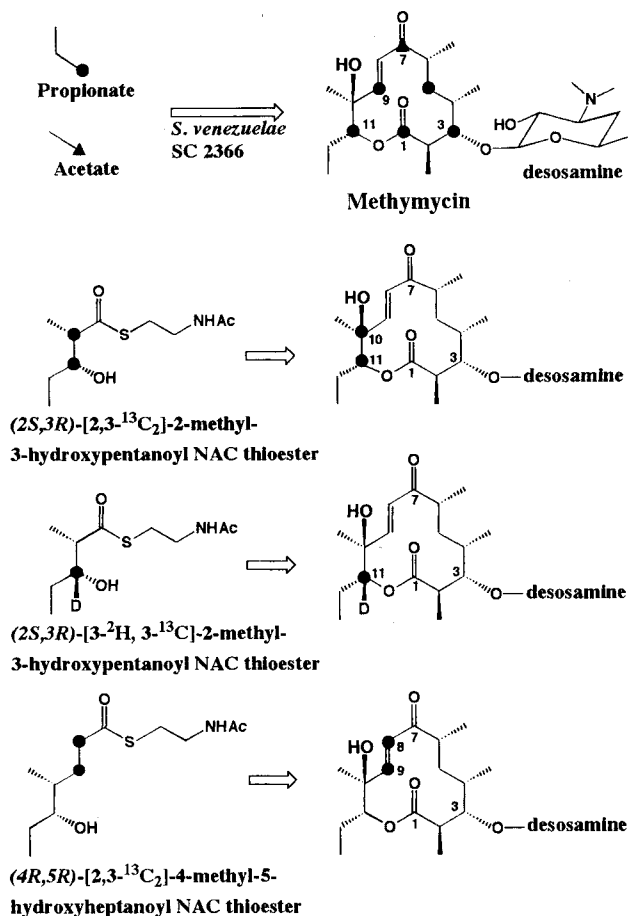
from fermentation broth assume particular significance in light of the important discoveries in molecular genetics regarding to the organization of the polyketide synthase responsible for individual steps of polyketide chain elongation and functional group modification.

C. Biosynthesis of 16-Membered Macrolides after the Formation of the Lactone Ring

After the formation of the aglycone skeleton, the biosynthetic sequence for macrolide antibiotics was investigated by introduction of two methods using a blocked mutant and microbial transformation using cerulenin[69]. FURUMAI et al.[107,108] applied 'cosynthesis' deviced by McCORMIC and DELIC to the biosynthetic studies of the leucomycin-family macrolide, platenomycin. They obtained many blocked mutants of a platenomycin-producing strain, Streptomyces platensis subsp. malvinus and tested them for their capability for cosynthesis. In order to the clarify the biosynthetic

Fig. 26. Proposed biosynthetic pathway of protomycinolide IV, an aglycone of mycinamicin.
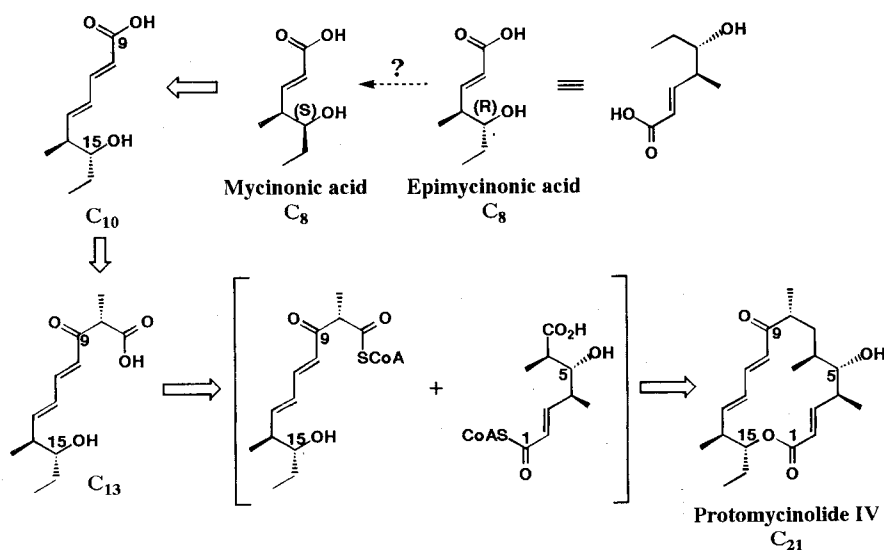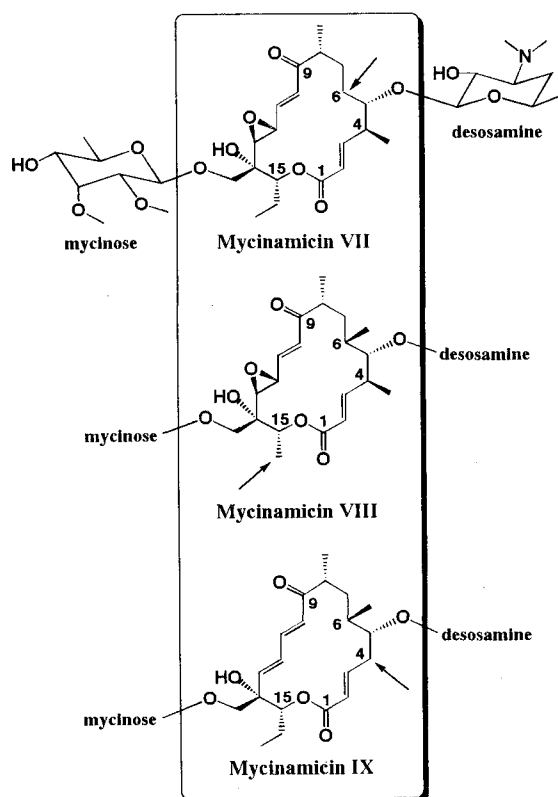

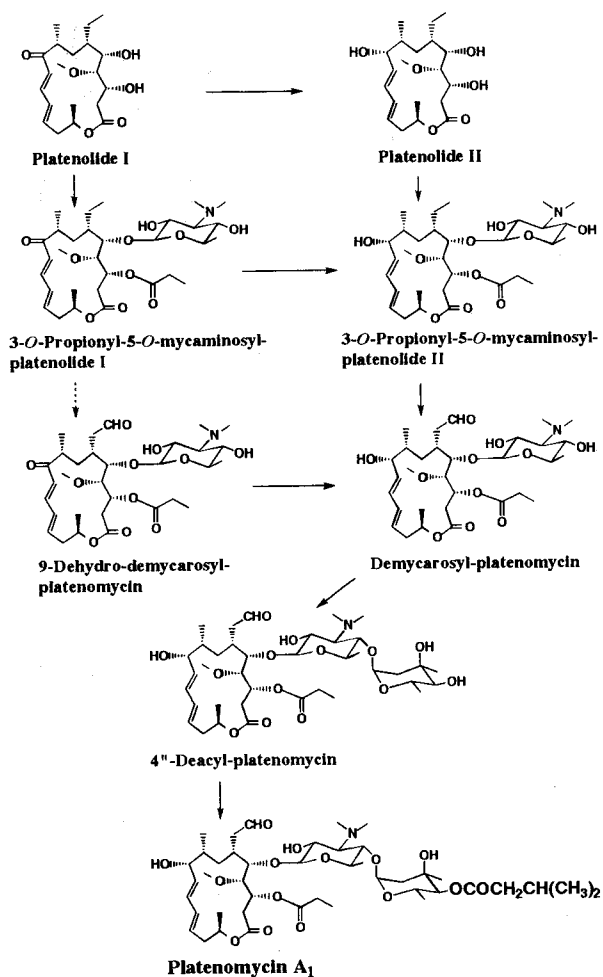Fig. 27. The structures of mycinamicins, VII, VIII, and IX.

pathway of platenomycin, biosynthetic correlation of the intermediates isolated from the cultured broth of the mutants was made by using growing cultures or the washed mycelia of blocked mutants. These feeding experiments showed that platenomycin is biosynthesized

from the aglycone, platenolide I, via demycarosyl-platenomycin and 4″-deacyl-platenomycin, as indicated in Fig. 28.

On the other hand, in place of the use of a blocked mutant, ŌMURA et al.[109,110] applied cerulenin to the study of the biosynthetic sequence in each step such as oxidation, reduction, acylation and glycosidation after the formation of the aglycone moiety of tylosin and spiramycin. Cerulenin is a specific inhibitor of condensing enzyme involved in the condensation step between acetyl-ACP and malonyl-ACP in fatty acid biosynthesis[69]. The antibiotic inhibits also the biosynthesis of polyketides such as leucomycin, tetracycline, and 6-methylsalicylic acid. Under the conditions when cerulenin inhibits the formation of the aglycone moiety, the bioconversion of the lactone moiety of tylosin to the final product, was examined by feeding the compounds derived chemically from parent macrolides to the culture medium of macrolide producing strains. The correlation between the sequence of aldehyde formation on the lactone ring and the order of glycosidation of the neutral sugars, mycarose and mycinose during the course of tylosin biosynthesis is summarized in Path A of Fig. 29.

BALTZ et al.[111,112] isolated a series of mutants blocked in specific tylosin biosynthetic steps and proposed its probable whole pathway (Path B). They demonstrated a pronounced correlation between increased tylosin production and increased expression of macrocin O-methyltransferase which catalyzes the terminal conversion of macrocin to tylosin in many of the mutants.

Fig. 28.   The biosynthetic pathway of platenomycin A₁.

Platenolide I

Platenolide II

3-O-Propionyl-5-O-mycaminosyl-
platenolide I

3-O-Propionyl-5-O-mycaminosyl-
platenolide II

9-Dehydro-demycarosyl-
platenomycin

Demycarosyl-platenomycin

4″-Deacyl-platenomycin

Platenomycin A₁

D. Stereochemistry of Tylosin and Its Related Compounds by Microbial Conversion

The majority of 14- and 16-membered macrolides possesses various functional groups with the structural and stereochemical regularity at the definite position on the lactone ring. W. D. CELMER[113,114] has proposed a predictive model of configurational uniformity among macrolides, based on the configurational assignment of oleandomycin and biogenetic consideration. After that, this model was applied to 16-membered macrolides such as leucomycin A_3, magnamycin B and tylosin, and the configuration at C-14 and C-15 in tylosin has been proposed to be R and S, respectively, as illustrated in Fig. 30. Comparative NMR and CD analyses of tylosin and the derivatives with the aglycone derived from leucomycin A_3 whose absolute configuration has been determined by X-ray crystallographic analysis[115] revealed the 3R, 4S, 5S, 6R and 8R-configurations for tylosin. However, the configurations at C-14 and C-15 of tylosin have not been specified. For configurational assignment of all

asymmetric centers of tylosin, a biosynthetic intermediate, protylonolide assigned by X-ray crystallographic analysis[116,117] was transformed microbially to tylosin under the inhibition of the production of tylosin aglycone in the presence of cerulenin. Retention with the configuration of the protylonolide during the microbial transformation to tylosin was evidenced from comparative CD analysis between protylonolide and 5-O-mycaminosyl-protylonolide which was derived from the transformed tylosin. As a result, the configuration for the aglycone moiety of tylosin was assigned to be 3R, 4S, 5S, 6R, 8R, 14S and 15R[117]. Therefore, 14R-configuration of tylosin proposed by CELMER was revised to be 14S. After that, the stereochemistry of 16-membered macrolides such as rosaramicin[118], acumycin[119], and mycinamicin[120] which possess an asymmetric center at C-14 was confirmed to possess 14S-configuration by X-ray crystallographic analysis. Furthermore, it has been clarified that an antibiotic, M-4365 G[121] also possesses the same configuration with the aglycone of tylosin from the microbial transformation of protylonolide to M-4365 G_1 with retention of the configuration[122].

E. Production of Hybrid Macrolides by Microbial Transformation

The isolation of aglycone from a mutant may serve not only for the biosynthetic studies described above, but also as a starting material for the construction of new macrolide antibiotics. MAEZAWA et al.[123] has applied to the production of new macrolide by a microbial glycosidation of narbonolide which is an aglycone of a 14-membered macrolide, narbomycin, with mycaminose finding in only 16-membered macrolides. 5-O-Mycaminosyl-narbonolide was obtained successfully by the feeding experiments of narbonolide to the fermentation broth of a platenomycin-producing strain. Attempt of the glycosidation of platenolide with desosamine involving in only 14-membered macrolides affords also a new hybrid macrolide, 5-O-desosaminyl platenolide[124], as shown in Fig. 31. Both new hybrid macrolides possess antibacterial activity against Gram-positive bacteria.

ŌMURA et al.[125] attempted the microbial production of new hybrid macrolides using a biosynthetic inhibitor of polyketide, cerulenin. By means of so called "hybrid biosynthesis", the biologically inactive tylosin aglycone was converted into novel antibacterial macrolide, chimeramycin by a spiramycin-producing strain Sm. ambofaciens KA-448 in the presence of cerulenin. Novel hybrid macrolides, chimeramycins A and B, in which spiramycin constituted sugars, mycaminosyl-mycarose

Fig. 29. The biosynthetic pathways of tylosin and macrocin.
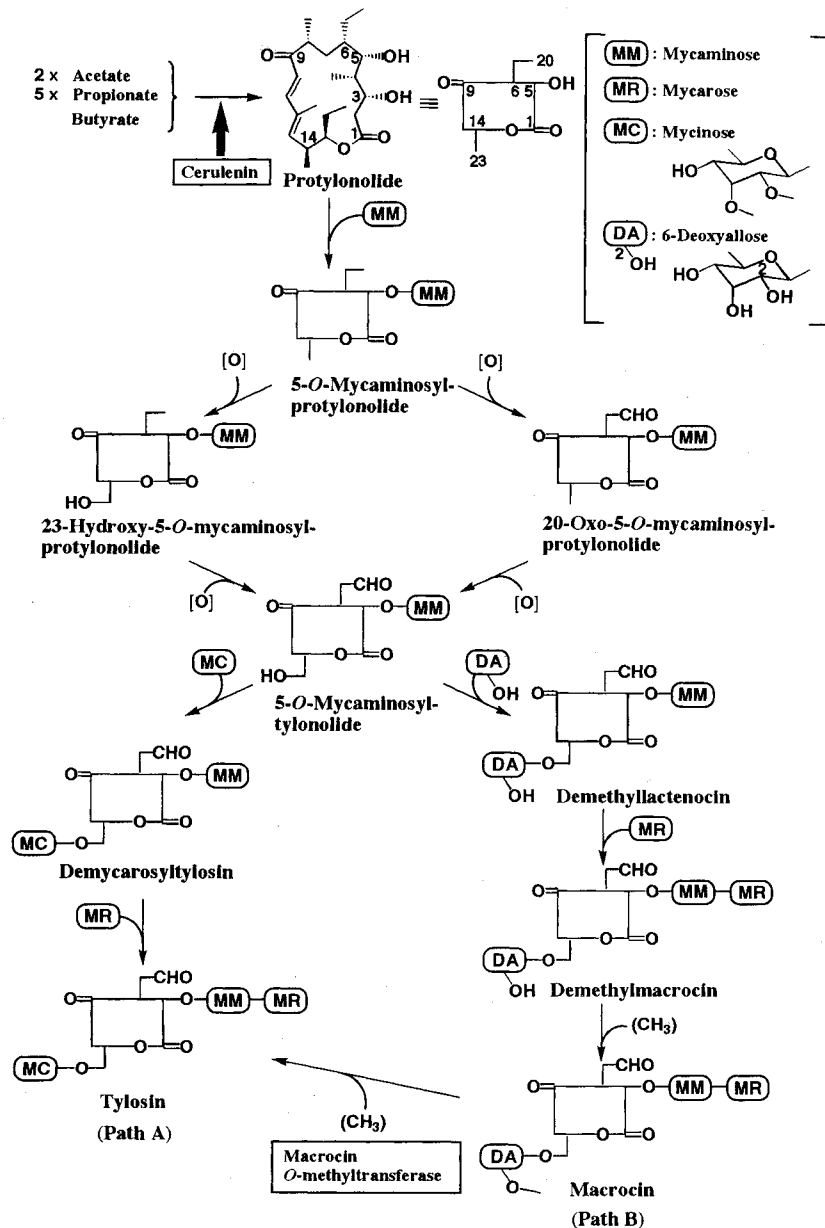
and forosamine were bonded glycosidically at C-5, and C-9 of protylonolide, respectively, were successfully produced by the method, as shown in Fig. 32. Chimeramycins exhibited *in vitro* antimicrobial activity as that of tylosin and spiramycin I. Demycarosyl-chimeramycins obtained by an acidic hydrolysis of chimeramycins were stronger activity than that of parent antibiotics. A hybrid biosynthesis using cerulenin would be a useful method for the production of new bioactive compounds for secondary metabolites which consist of polyketide skeleton containing sugars, amino acids, and fragments arised *via* a shikimate pathway.

F.   Production of a New Hybrid Antibiotic, Mederrhodin by Genetic Manipulation

Recent advances in understanding the biosynthesis of bacterial polyketide have led to the concept of polyketide synthase which can utilize different starter and chain-extender units and can process the poly-β-ketone intermediates in a certain manner[126~128]. HUCHINSON et al.[129~133] have vigorously investigated enzymatic synthesis of a bacterial aromatic polyketide, tetra-cenomycin biosynthesis in *Streptomyces* from acetyl and malonyl coenzyme A and demonstrated the similarity of biochemical connection between the biosynthesis of bacterial fatty acids and polyketides by isolation and

Fig. 30.   Configurational model of macrolide antibiotics.

Model   Oleandomycin   Leucomycin A₃   Acumycin   Tylosin
(a)   Tylosin
(b)
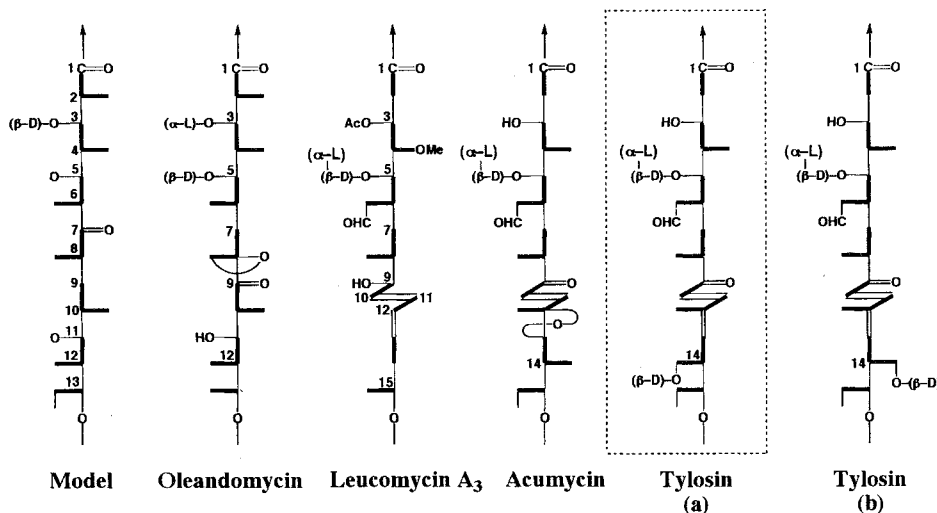
(a) Celmer model (1966)
(b) Correct configurational model of tylosin
(1980)

Fig. 31.   Production of new hybrid macrolides, 5-O-my-caminosyl-narbonolide and 5-O-desosaminyl-platenolide.
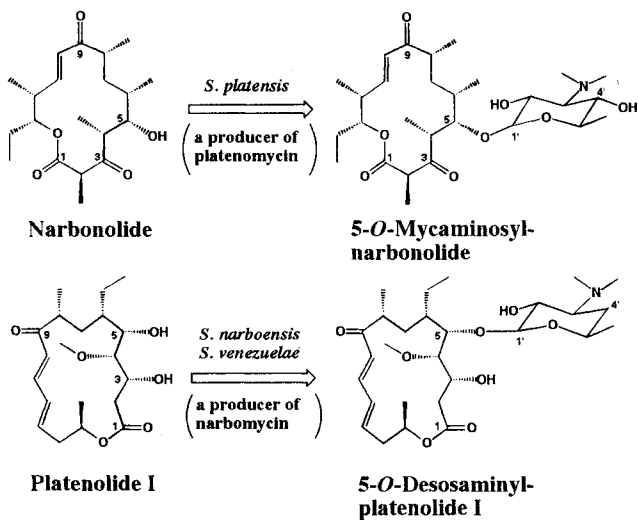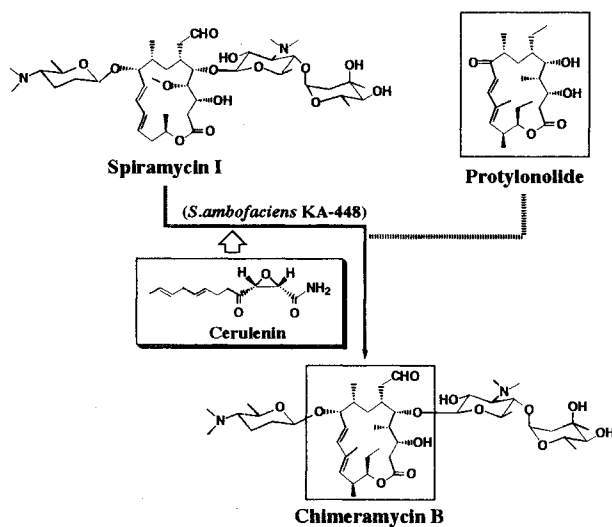

Fig. 32.   Production of a new hybrid macrolide, chimera-mycin B.

purification of acyl carrier protein acyltransferase from bacteria.

Interest in the biosynthesis of natural products has risen recently, because of advances in the genetic understanding of polyketide metabolism and the prospect of manufacturing new polyketide-derived drugs with recombinant microorganisms. HOPWOOD et al.[134], planned the development of this method by genetic manipulation and reported the first case of the production of new hybrid antibiotics by genetically engineered strains, as shown in Fig. 33. Whole genes for actinorhodin

biosynthesis of Streptomyces coelicolor A3 had been already isolated and the position of genes corresponding to each biosynthetic step on the cloned DNA fragment had been also determined[135]. Then, it was tried to introduce the whole or a part of fragments of actinorhodin biosynthetic genes into other Streptomyces strains which produced actinorhodin-related antibiotics. Since most of Streptomyces strains possessed the restriction system, it was difficult to introduce recombinant plasmids, however, transformants were obtained from two kinds of strains, kalafungin producer Streptomyces tanashiensis

Fig. 33.   Production of a new hybrid antibiotics, mederrhodin
A by genetic manipulation.

Actinorhodin
(*S. coelicolor* A3)

Medermycin
(*Streptomyces* sp. AM-7161)

Hydroxylase
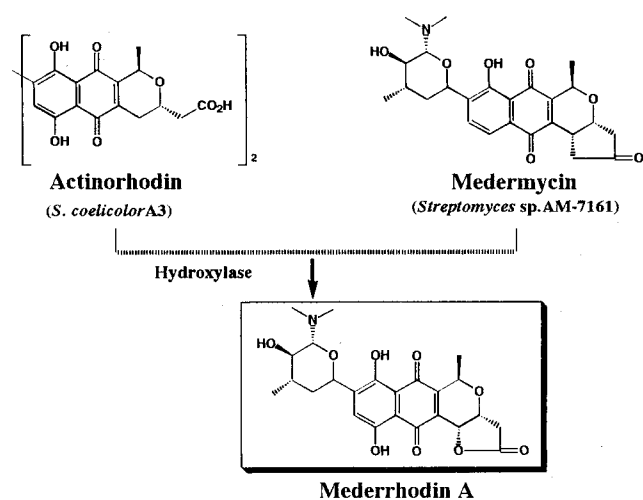
Mederrhodin A

and medermycin producer *Streptomyces* sp. AM 7161, but not those of kalafungin producer. New antibiotics, mederrhodins A and B were produced by the transformants carrying pIJ 2301, 2315 or 2316 which contained a part of actinorhodin biosynthtic genes. On the other hand, the transformant introduced pIJ 2303 including whole genes for actinorhdin biosynthesis did not produce mederrhodins, however, produced actinorhodin and medermycin. The biosynthesis and productivity of mederrhodins by transformants were relatively high and quite stable. All recombinant plasmids (pIJ 2301, 2315 and 2316) contained the transcriptional unit of *act* V and the inserted DNA fragment would be mentioned as follow: 1) medermycin was synthesized by biosynthetic genes of *Streptomyces* sp. AM 7161. 2) the hydroxyl residue was substituted at C-6 position of the naphthoquinone skeleton by hydroxylase which is a gene product of an inserted DNA fragment (act V) of pIJ 2315. Antimicrobial activity of mederrhodin A was similar to that of medermycin. In a future, knowledges from the breeding of the mederrhodin's producer will give many informations to molecular breeding of other antibiotic-producing organisms.

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