Translation and Protein Synthesis: Macrolides

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1. Introduction

The term "macrolide" was originally proposed by R. B. Woodward in 1957 as an abbreviation of macrolactone glycoside antibiotics, a class of natural products composed of macrocyclic lactones to which were attached one or more deoxysugar residues.[1,2] Macrolides are produced as secondary metabolites largely from the actinomycete family of bacteria, organisms that inhabit the soil. The first macrolide discovered was pikromycin in 1950, followed shortly thereafter by erythromycin, the first macrolide introduced for clinical use in human medicine.[3,4] Macrolide antibiotics have been used to treat infections in humans and animals for more than 50 years. Interest in derivatization of erythromycin to improve its properties started in the 1960s and has continued to the present time. A recent chemical derivative of erythromycin, telithromycin, was approved for clinical use in the United States in 2004.

Macrolides can be classified in a number of ways. From a chemical viewpoint they are divided into groups based on the number of atoms in the macrocyclic rings: 12, 14, 16, or larger, as outlined in section 2. Each group is subdivided further on the basis of the general structure of the lactone moiety or sugar substitutions. From a clinical point of view the compounds are described as first-, second-, or third-generation macrolides, as discussed in section 3. The first-generation molecules are the natural products that were introduced as drugs in the 1950s, followed by the semisynthetic second-generation compounds in the 1990s, and the semisynthetic third-generation molecules in the early 2000s.

Macrolides act as antibiotics by binding to ribosomes and consequently blocking protein synthesis. The high affinity to bacterial ribosomes, together with the highly conserved structure of ribosomes across virtually all of the bacterial families, gives macrolides broad-spectrum activity. The mode of

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Gary W. Ashley obtained his S.B. degree in Chemistry from the Massachusetts Institute of Technology. He obtained his Ph.D. degree in Chemistry from the University of California, Berkeley, under the guidance of Paul A. Bartlett. After a postdoctoral fellowship in biochemistry with JoAnne Stubbe at the University of Wisconsin, Madison, he taught chemistry at Northwestern University. In 1993 he returned to California, where he established the chemistry program at Kosan Biosciences, Inc. His interests include bioorganic and natural products chemistry as well as patent law. He was licensed to practice before the U.S. Patent and Trademark Office in 2002 and currently divides his time at Kosan between scientific and legal duties.

action of macrolides will be discussed in section 4 within the framework of recent structural information on macrolide−ribosome interaction. Clinical resistance to macrolides in bacterial pathogens and self-resistance to macrolides in the macrolide-producing actinomycetes have been well characterized and found to share many common mechanisms. Resistance will be discussed in section 5 as a basis for the discovery of novel, more potent compounds.

The aglycone components of macrolides are complex polyketides, partially reduced acyl chains formed from the condensation of thioester-containing precursors in a manner common to all members of this class of molecules. At the genetic level, and corresponding biochemical level, biosynthesis of the polyketide and deoxysugar components of macrolides is now understood well enough to account not only for the structure of macrolides, but also for the structural diversity seen among this family of compounds. In section 6 we will describe our level of understanding of biosynthesis and discuss briefly the changes to the structure of erythromycin and other macrolides produced from manipulation of the genes responsible for their syntheses.

2. Classes of Macrolides

This section is limited to macrolides that have been isolated as natural products. Some are congeners of the parent compound. In general, the congeners are late-pathway intermediates to the final product.

2.1. Twelve-Membered Macrolides

Only two macrolide antibiotics have been identified that contain 12-membered rings: methymycin [**1**] and neomethymycin [**2**]. They differ in the position of a single OH group: C-12 in methymycin vs C-14 in neomethymycin. Both contain the deoxyaminosugar D-desosamine.

2.2. Fourteen-Membered Macrolides

Five compound families have been identified in this class: erythromycin A [**3**] and its B, C, and D congeners [**4**−**6**], pikromycin [**7**] and its 12-deoxy congener narbomycin [**8**], megalomicin A [**9**] and its congeners, oleandomycin [**10**], and lankamycin [**11**]. Erythromycin A is commonly referred to simply as erythromycin. Megalomicin and erythromycin share a common aglycone, 6-deoxyerythronolide B (6-dEB). The aglycone of oleandomycin, 8,8a-deoxyoleandolide, differs by the absence of the methyl group that is present at C-15 in 6-dEB. All 14-membered macrolides except lankamycin contain desosamine at C-5; lankamycin contains the neutral sugar chalcose at that position. The neutral sugar in erythromycin A at C-3 is L-cladinose, the 3″-*O*-methyl derivative of L-mycarose present at C-3 in megalomicin and in erythromycins D and C. Megalomicin also contains a second aminosugar, megosamine, at the 6-OH position. Megalomicin is less potent as an antibiotic than erythromycin A but has antiparasitic activity through its inhibition of vesicular transport between the medial- and trans-Golgi, resulting in the undersialylation of cellular proteins.[5] The C-3 sugars in oleandomycin and lankamycin are L-oleandrose and L-arcanose, respectively. Pikromycin and narbomycin contain only desosamine. The oxygen atom present at C-3 is in the form of the ketone. Pikromycin, discovered in 1950, therefore, is a natural "ketolide", a term first applied in the mid-1990s to describe the new 3-descladinosyl-3-oxo derivatives of clarithromycin (6-*O*-methylerythromycin) that were found to have increased antibacterial potency over erythromycin. Pikromycin, however, has weak antibacterial activity. Erythromycin A has hydroxyl groups at both C-6 and C-12 that are introduced by cytochrome P450-type hydroxylases. Erythromycin congeners

lacking the 6-OH group are weaker antibiotics. Pikromycin lacks the 6-OH group. Oleandomycin and lankamycin also lack the 6-OH group but are hydroxylated (lankamycin) or epoxidated (oleandomycin) at C-8. The presence of the 6- and 12-OH groups in erythromycin A is a major source of instability (Scheme 1). In protic solvents erythromycin A exists as a mixture of the 9-keto form [**3**], the 9,12-hemiketal form [**3a**], and the 6,9-hemiketal form [**3b**]. Under acidic conditions the hemiketal forms dehydrate to form enol ether derivatives [**3c**] and [**3d**], respectively, which further degrade by reaction to form the spiroketal derivative [**3e**]. Further degradation involves acid-catalyzed hydrolysis of the cladinose residue from [**3e**] to form erythralosamine. The keto form [**3**] is the only species to have significant antibacterial activity, whereas enol ether [**3d**] is a potent agonist of the motilin receptor and is the main cause of the gastrointestinal distress associated with erythromycin therapy. The 12-membered macrolide methymycin and the 14-membered macrolide pikromycin are made in the same host, *Streptomyces venezuelae*.[6] With the exception of the additional two carbons in the aglycone component of pikromycin, the two compounds are identical in structure.

2.3. Sixteen-Membered Macrolides

Sixteen-membered macrolides represent the largest group of macrolides. We have subdivided this group into four subgroups on the basis of the structure of the polyketide backbone that forms the macrolactone after release from the corresponding polyketide synthase and before any further modification, e.g., glycosylation, hydroxylation, etc., takes place. Some of these aglycones are inferred from our current understanding of the biochemistry of complex polyketide synthesis, which is described in detail below.

2.3.1. Tylactone Group

The most commercially important member of this group is tylosin [**12**], produced from the bacterium *Streptomyces fradiae* and used in veterinary medicine. Tylosin contains the disaccharide D-mycaminosyl-L-mycarose at C-5 and the monosaccharide D-mycinose at C-23. Tylosin carries the C-20 aldehyde group: oxidation of the 6(*S*)-ethyl side chain of the aglycone of tylosin takes place after macrolactone is formed. Similarly, hydroxylation of the 14(*R*)-methyl side chain (to enable subsequent glycosylation) is a post-polyketide processing step. *S. fradiae* also produces tylosin D [**13**] (formerly named relomycin) in which the aldehyde is reduced to the alcohol. Tylosin D is much less potent than tylosin. Conversion of tylosin to tylosin D is carried out by an adventitious reductase that is not associated with the tylosin biosynthesis gene cluster.[7] Tylosin has undergone extensive chemical derivatization, and the genes for its biosynthesis have been characterized. Another compound in this group includes rosamicin [**14**], which carries only a single sugar, desosamine, at C-5, and the 12,13-epoxide was in human clinical trials but not further developed into a drug. Additional members include cirramycin and the juvenimicins.

2.3.2. Platenolide Group

This represents the largest group of 16-membered macrolides. All members carry the 6(*S*)-CH_3CH_2CHO side chain that is essential for antibiotic potency and the mycaminosyl−mycarose disaccharide at C-5. Platenolide does not have a C-14 methyl side chain and thus offers no possibility of glycosylation on the left side of the macrolactone. Fully elaborated compounds in this group may also contain various acylations at C-3 and at the 4-hydroxyl of mycarose; hence, families rather than single species of molecules are often produced from a single organism. We have subdivided the platenolide-based group on the basis of additional modification to the aglycone moiety. The carbomycin B series contains no further modifications. An example of a compound of this subgroup is niddamycin [**15**]. The carbomycin A series contains the 12,13-epoxide. The leucomycin series is characterized by reduction of the C-9 keto group and includes midecamycin A1 [**16**] and the spiramycins (a series of three congeners) [**17**−**19**]. Midecamycin A1 and spiramycin were commercialized for human use. The spiramycins carry the aminosugar forosamine at C-9 and various acyl groups at C-3 or C″4. Other members of the leucomycin series include the maridomycins, which carry the 12,13-epoxide.

2.3.3. Mycinamicin

This group consists of one series of molecules, the mycinamicins, produced by *Micromonospora griseorubida*. The aglycone contains a 2,3-trans double bond, 4(*R*)-Me, 6(*S*)-Me, 14(*R*)-Me, 15(*S*)-Et. The mycinamicins all contain the sugars desosamine at C-5 and D-mycinose at C-21. The mycinamicins differ from each other in the presence or absence of the 12,-13-epoxide and 14(*S*)-OH group. An example is mycinamicin I [**20**]. Mycinamicins were not developed for human use.

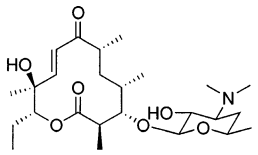
2.3.4. Chalcomycin–Neutramycin Group

The aglycones of chalcomycin and mycinamicin differ by the presence of a methyl group at C16 in the latter compound. Neutramycin differs from chalcomycin [**21**] by the substitution of the C6-methyl group in chalcomycin for a hydrogen atom. All compounds in the group contain D-mycinose at C-20. Chalcomycin has the neutral sugar D-chalcose at C-5, the 12,13-epoxide, and an 8-hydroxyl group.

3. Clinical Uses of Macrolides

3.1. First-Generation Macrolides

The first-generation macrolides developed for clinical use were the natural products erythromycin A, spiramycin, midecamycin A1, leucomycin, and carbomycin. These were isolated as fermentation products and required purification. Specifications of the drugs allowed for small amounts of congeners; spiramycin was a mixture of **17**−**19**. In general, the compounds displayed excellent activity against Gram-positive bacteria and were used initially to treat skin caused by *Staphylococcus aureus* and *Staphylococcus*



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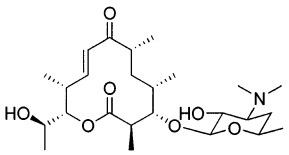

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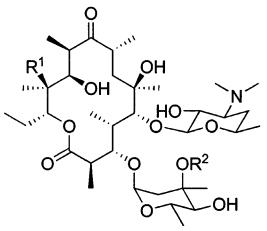
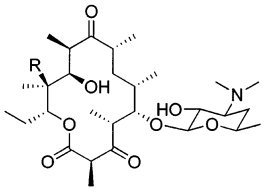
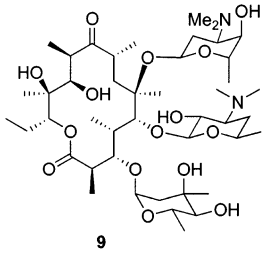


3 R^1 = OH; R^2 = Me
4 R^1 = H; R^2 = Me
5 R^1 = OH; R^2 = H
6 R^1 = H; R^2 = H



7 R = OH
8 R = H



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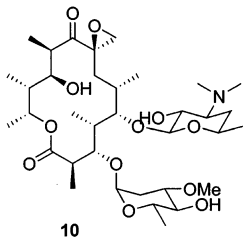

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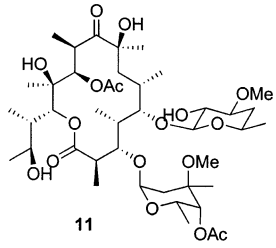

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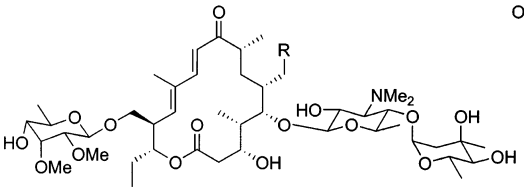
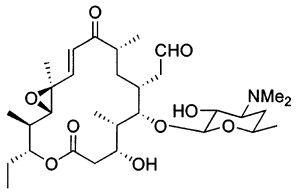


12 R = CHO
13 R = CH_2OH



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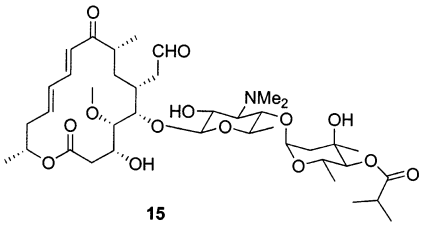

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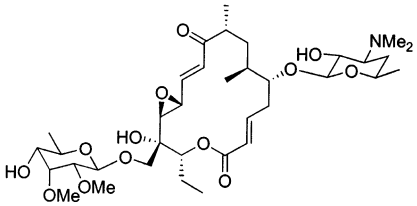

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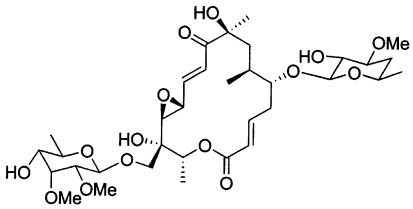

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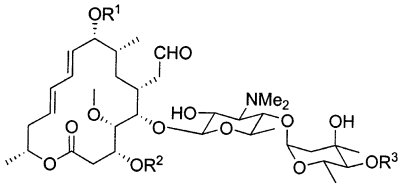
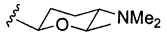
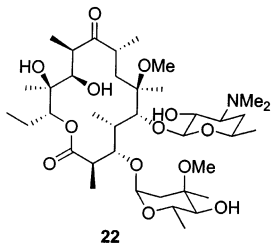


	R^1	R^2	R^3
16	H	$MeCH_2CO$	$MeCH_2CO$
17		H	H
18		MeCO	H
19		$MeCH_2CO$	H



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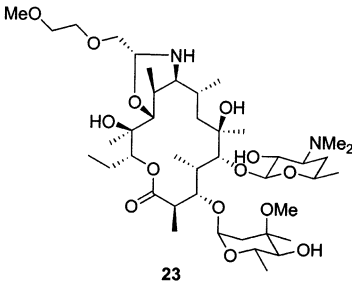

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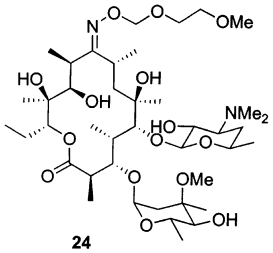

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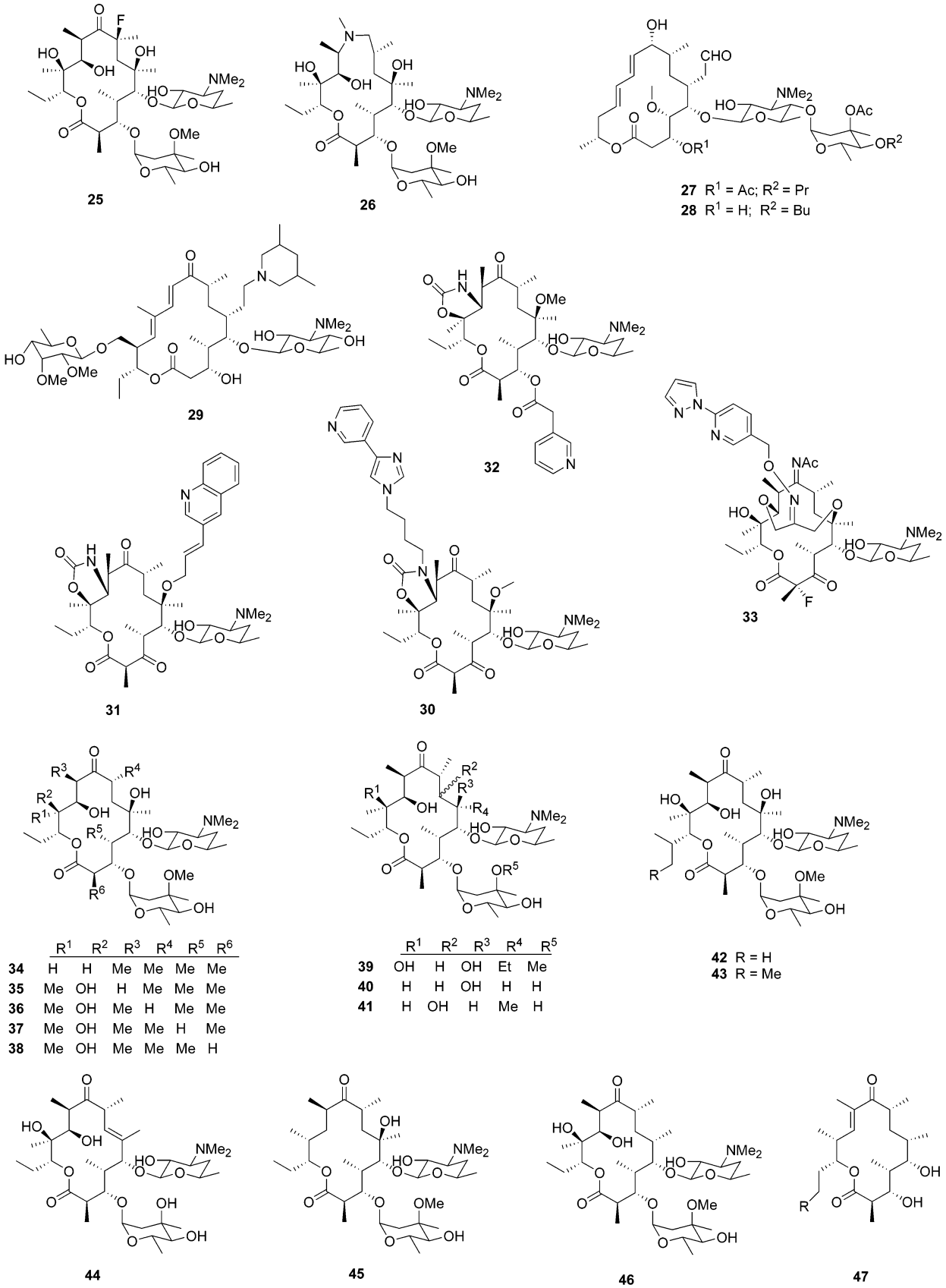
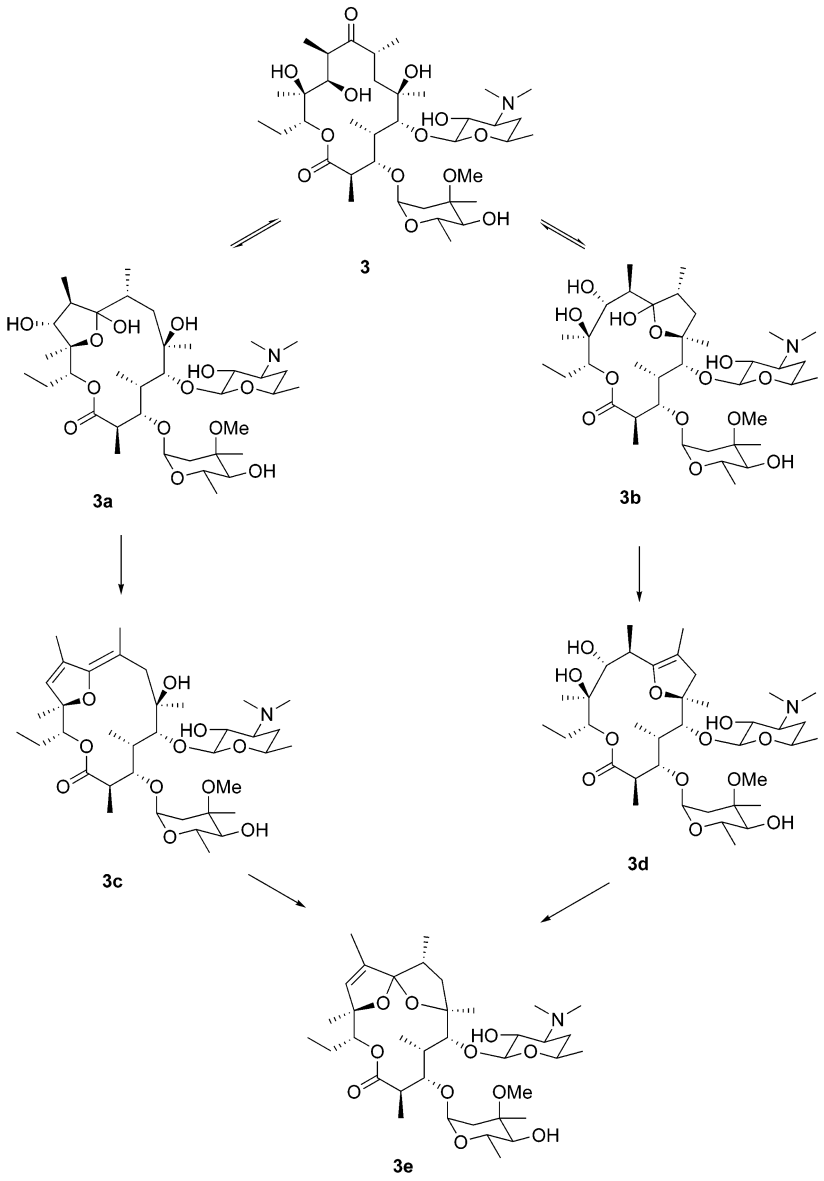


Figure 1. Structures of macrolides and ketolides.

epidermidis and soft tissue infections caused by *S. aureus*. In the 1960s these compounds began to be used to treat upper and lower respiratory infections caused by *Streptococcus pneumoniae* or *Streptococcus pyogenes* and found lesser use against staphylococci. The enterococci are much less susceptible to macrolides. These compounds have also been used for the treatment of Legionnaire's Disease (*Legionella pneumophila*), Lyme Disease (*Borrelia burgdorferi*), syphilis (*Treponema pallidum*), diphtheria (*Corynebacte-*

Scheme 1



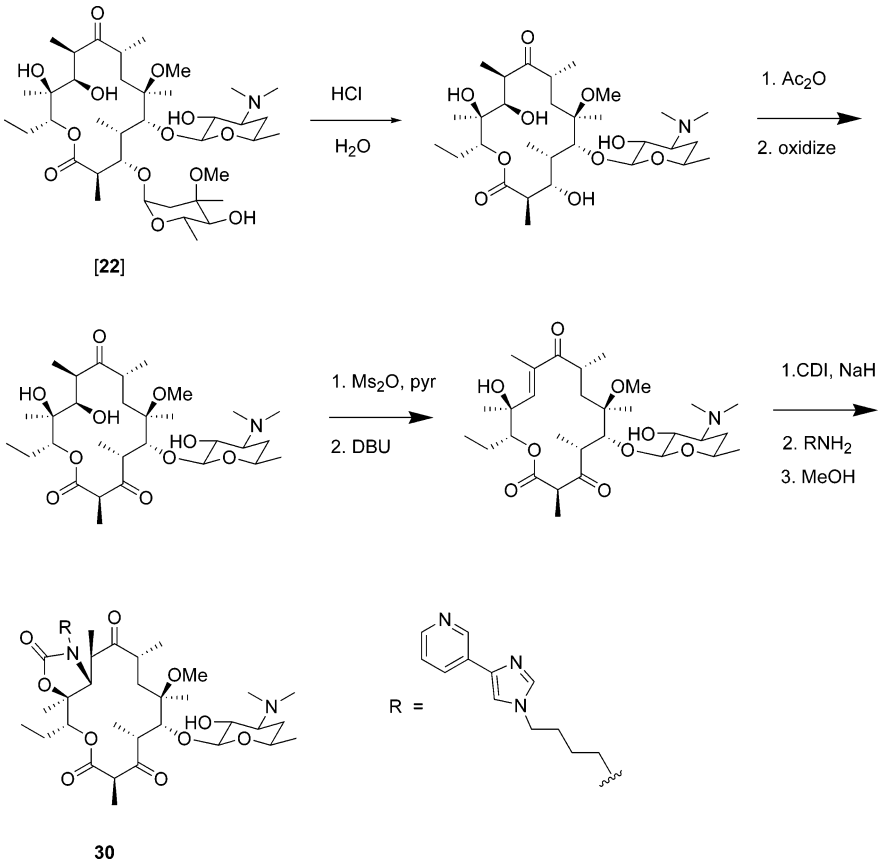
rium diphtheriae), pertussis (*Bordatella pertussis*), and respiratory infections caused by *Moraxella cattarhalis* and *Mycoplasma pneumoniae*. *Chlamydia pneumoniae* is also susceptible to macrolides. Erythromycin has only modest activity against Gram-negative enterobacteria (e.g., *Escherichia coli*, *Klebsiella*) and no activity against *Pseudomonas* strains. Sixteen-membered macrolides are somewhat more potent against Gram negatives. Tylosin was developed to treat respiratory infections in animals, largely caused by the Gram negatives *Pasteurella multocida*, *Mannheimia haemolytica,* and various species of *Haemophilus*. *Haemophilus influenzae*, a common intracellular respiratory pathogen in children, is treatable with macrolides.

The first-generation macrolides proved to be effective and fairly well tolerated. The most prominent side effects of erythromycin were bitter taste and stomach cramps, which was later found to be due to the ability of the 8,9-anhydro-6,9-hemiketal form ([**3e**], Scheme 1) to mimic the effects of the hormone motilin and stimulate gastrointestinal contractions.[8] The most important drawbacks to the use of first-generation macrolides were their short half-life and poor oral bioavailability, prompting the requirement for dosing three or four times a day. Despite these weaknesses, these compounds were used successfully for more than 25 years and were important first-line agents for individuals with respiratory infections who were hypersensitive to penicillin and its derivatives. Because of their relatively low cost of production, they are still used in Latin America, Africa, and some parts of Asia.

3.2. Second-Generation Macrolides

The generally poor bioavailability, acid instability, and unpredictable pharmacokinetics of the first-generation macrolides prompted the search for new derivatives with improved properties. Five derivatives of erythromycin were developed and commercialized: clarithromycin (Biaxin; Abbott) [**22**], dirithromycin (Dynebac; Sanofi) [**23**], roxithromycin (Rulide; Aventis) [**24**], flurithromycin (Pierrel) [**25**], and azithromycin (Zithromax; Pfizer) [**26**]. Miokamycin (Meiji) [**27**] and rokitamycin **[28**] were the only 16-membered second-generation compounds devel-

Scheme 2



oped for human use. Tilmicosin (Elanco) [**29**], a semisynthetic derivative of tylosin, was developed for veterinary use. Clarithromycin and azithromycin are marketed worldwide; dirithromycin, flurithromycin, and roxithromycin have much more limited distribution.

Clarithromycin is prepared from erythromycin A in a short sequence of chemical transformations. The propensity of the 6- and 12-OH groups to form hemiketal derivatives with the 9-carbonyl together with the higher reactivity of the 2′- and 4″-OH groups on the glycosyl residues precludes efficient direct alkylation of the 6-OH group. In a typical synthetic sequence, erythromycin A is converted into the 9-oxime, which is then protected as an oxime ether. The use of acetal groups to protect the oxime has been found to be particularly convenient. Subsequent blocking of the glycosyl hydroxyls, most simply as trimethylsilyl ethers, provides protected derivatives that can be efficiently methylated on the 6-OH under basic conditions. The selectivity for alkylation of the tertiary 6-OH group over the secondary 11-OH or tertiary 12-OH groups is not entirely understood but appears to be related to the unusually high acidity of the 6-OH in erythromycin oxime derivatives. Subsequent hydrolysis of the oxime acetal and trimethylsilyl ethers and deoximation provides clarithromycin. This six-step sequence produces clarithromycin in high yields yet significantly increases the cost of the drug relative to erythromycin.

Azithromycin is prepared from erythromycin A oxime by Beckmann rearrangement, for example, by treatment with a sulfonyl chloride buffered with aqueous sodium bicarbonate. This reaction is dependent upon trapping of the reactive Beckmann intermediate by the 6-OH group rather than solvent water to provide an isolable isoamide, which is subsequently reduced to provide an intermediate ring-expanded azalide. *N*-Methylation completes the synthesis of azithromycin.

The second-generation erythromycin derivatives all contain modifications at C6 or C9, preventing formation of the enol ether [**3e**] and thereby imparting greater resistance to acid-catalyzed inactivation. Clarithromycin is still degraded under acidic conditions to form derivatives analogous to [**3d**] and descladinosyl derivatives, albeit at reduced rates relative to erythromycin A.[9−11] The five analogues each had improved oral bioavailability and extended half-life in plasma, enabling them to be taken orally once (azithromycin) or twice (clarithromycin) a day.[12] These compounds also exhibited enhanced tissue penetration due to their increased lipophilicities over the parent compound erythromycin A and hence were effective for treatment of intracellular pathogens such as *H. influenzae*.[13,14] Although the search for second-generation macrolides was predicated on the desire to discover compounds with expanded spectra and improved activity, the compounds selected did not exhibit improved activity against Gram-positive bacteria, and some, in fact, such as azithromycin, had reduced potency.[15,16] Nevertheless, they were selected for development mainly because of their enhanced pharmacokinetic profiles, in particular the ability to accumulate to high levels in lung tissue. Clarithromycin is also used, generally in combination with

other antibiotics, for the treatment of gastric ulcers caused by *Helicobacter pylori* and for AIDS-related respiratory infections caused by *Mycobacterium avium* complex.

The second-generation 16-membered macrolides miokamycin and rokitamycin did not show enhanced potencies in vitro over their parent compounds, midecamycin A1 or leucomycin A5, but did show improved in vivo potencies in experimental animals. These compounds are not marketed for use in the United States.

3.3. Third-Generation Macrolides: Ketolides

Whereas the search for second-generation macrolides in the 1970s and 1980s was driven by the need for improved stability and pharmacokinetics, the basis for the search for third-generation compounds shifted to macrolide resistance that had arisen suddenly and rapidly in the 1980s and 1990s. A 2001 report indicated that 23% of the *S. pneumoniae* strains in the United States were resistant to macrolides.[17] Macrolide resistance is described in some detail below. The only third-generation macrolide in clinical use as of 2004 is telithromycin (Ketek; Aventis) [**30**], a 14-membered "ketolide" that employs clarithromycin as the starting material. The term "ketolide" is used to indicate the presence of the 3-keto group in place of the L-cladinose present in clarithromycin and other second-generation compounds and was adopted in the early 1990s to describe new, semisynthetic series.[18,19] Removal of the cladinosyl group from erythromycin could be accomplished by acid treatment (after protection of the 9-keto group), but the resulting 3-OH derivative was found to have lost much of its potency. Moreover, oxidation of the 3-OH to the ketone was not practical because of subsequent 3,6-cyclization. Pikromycin, the first natural ketolide, exhibited weak potency. Hence, generation of the potent semisynthetic ketolides awaited the creation of clarithromycin, which occurred in the mid-1980s. Replacement of the L-cladinose moiety with the 3-keto group in clarithromycin rendered the resulting compound a noninducer of MLS_B resistance (described below), but it also exhibited decreased potency, likely through loss of binding interactions to the cladinose group and/or increased flexibility of the macrolactone. Addition of the fused 11,12-cyclic carbamate made the macrolactone more rigid, adding potency against some strains, and addition of the aryl alkyl side chain to the N-11a position compensated for loss of binding interactions to cladinose and imparted 2−10-fold enhanced in-vitro activity against macrolide-susceptible streptococci and staphylococci over clarithromycin. Telithromycin is at least as potent in vitro as clarithromycin against *H. influenzae* and the atypical respiratory pathogens *M. cattharalis, L. pneumophila, M. pneumoniae,* and *C. pneumoniae.*[20] Telithromycin is not as potent as azithromycin against *H. influenzae* but accumulates in lung tissue well enough to be clinically useful against this organism.

Telithromycin is prepared from clarithromycin using a sequence of eight chemical steps (Scheme 2).[21] Acid hydrolysis of clarithromycin provides the 3-descladinosyl derivative, which is protected at the 2′-OH by acetylation with acetic anhydride in the absence of added base. Under such conditions the 2′-OH is unusually reactive toward acylating (but not alkylating or silylating) reagents due to the adjacent dimethylamino functionality. Presumably the amine reacts with the anhydride to form an acylammonium salt, which transfers the acyl group to the 2′-OH. This is suggested by the observation that use of acid halides rather than anhydrides results in formation of *N*-acyl-*N*-demethyl derivatives rather than *O*-acyl derivatives. Subsequent oxidation of the 3-OH to a ketone is followed by introduction of the 11,12-cyclic carbamate according to the method of Baker, using an amine prepared in several steps from 4-(3-pyridyl)imidazole and 4-bromobutylphthalimide. Treatment of the product with methanol results in removal of the 2′-acetate group and production of telithromycin. This rather lengthy sequence starts from clarithromycin, and so is 14 steps removed from erythromycin A. This adds substantially to the cost of the drug, and indeed, telithromycin may represent the economic limit of what is feasible in the antibacterial market.

The most important benefit of telithromycin is its unprecedented in-vitro potency against macrolide-resistant *S. pneumoniae.*[22,23] Resistance to telithromycin in *S. pneumoniae* has not yet been reported over the 2 years that the drug has been in clinical use in Europe. As will be described in more detail below, the two most prominent mechanisms of acquired macrolide resistance are efflux and ribosome methylation. Unlike azithromycin and clarithromycin, telithromycin evades the efflux pumps found in *S. pneumoniae* and *S. pyogenes* and does not induce ribosomal methylation associated with inducible MLS_B resistance in streptococci and staphylococci. However, staphylococcal and *S. pyogenes* strains that carry methylated ribosomes are not susceptible to telithromycin. The differences between *S. pyogenes* and *S. pneumoniae* with regard to ribosomal methylation and telithromycin susceptibility are further discussed below.

A second ketolide, cethromycin (ABT-773; Abbott) [**31**], also designated ABT-773 (developed by Abbott Laboratories and not yet FDA approved), carries the 11,12-cyclic carbamate and the 3-keto group present in telithromycin, but the aryl alkyl side chain is attached in an ether linkage to the 6-hydroxyl group. Synthesis of cethromycin and other 6-*O*-arylalkyl ketolides has been described previously.[24] As with telithromycin, cethromycin is prepared through a lengthy series of chemical transformations. Erythromycin A is converted into the 9-oxime and protected as the 9,2′,4″-tribenzoate. This derivative is allylated on the 6-OH using the *tert*-butyl carbonate of 1-(3-quinolyl)-2-propenol with palladium catalysis. Subsequent deblocking of the oxime and deoximation provides the 9-ketone, which is subjected to 11,12-cyclic carbamate formation in a one-pot, four-step sequence. Subsequent cladinose hydrolysis requires rather forcing conditions, as 4″-*O*-acylated cladinose is rather resistant toward hydrolysis. Oxidation of the resulting 3-OH group provides the 3-ketone. Final

debenzoylation of the 2′-OH provides cethromycin.

Cethromycin has similar potencies as telithromycin against the macrolide-susceptible organisms, does not induce MLS_B resistance, and is active against efflux-mediated resistant and constitutive MLS_B-resistant *S. pneumoniae* and, unlike telithromycin, *S. pyogenes*.

In addition to their activity against macrolide-resistant streptococci, the ketolides also have the unexpected and unprecedented property of bactericidal activity against *S. pneumoniae.* All macrolides exhibit time-dependent (12−24 h after administration), concentration-independent killing of bacteria and are classified as bacteriostatic rather than bactericidal agents. Ketolides, on the other hand, exhibit concentration-dependent killing of *S. pneumoniae* but not *S. pyogenes*, *S. aureus,* or *H. influenzae*.[25] Thus, although the basis is not understood, the ketolides are considered bactericidal for *S. pneumoniae* only. This desirable property may forestall the development of resistance to ketolides in these organisms.

Although some differences between cethromycin and telithromycin in individual pharmacokinetic parameters have been demonstrated, the two compounds are quite comparable overall in efficacy in experimental animals and according to initial reports in humans as well. Telithromycin is administered once per day, albeit at 800 mg dosing; the dosing of cethromycin to humans has not yet been reported. At present, the only significant reported difference between the two compounds is the lack of efficacy in vitro of telithromycin against constitutive MLS_B-based macrolide-resistant *S. pyogenes*.[26]

3.4. Side Effects of Macrolides and Ketolides

Until recently the most significant side effects of macrolides reported have been their ability to induce stomach cramps in some individuals and the bitter aftertaste of some of the compounds. High-level interest is now focused on the occurrence of torsades de pointes upon treatment with macrolides. Torsades de pointes is a rare but potentially fatal ventricular arrhythmia associated with delayed repolarization and prolongation of the QT interval. Interactions between macrolide antibiotics and other drugs that prolong the QT interval have been known to cause torsades de pointes, but recent studies have demonstrated that clarithromycin itself may induce prolongation of the QT interval and may lead directly to ventricular arrhythmia. Azithromycin alone does not appear to have any effect on the QT interval in rats, but reports of QT prolongation associated with azithromycin in combination with other drugs have appeared recently.[27−29] Telithromycin also induces a modest increase in the QT interval, although smaller than that induced by erythromycin or clarithromycin. Subsequent studies and clinical use have also suggested unexpectedly frequent cases of temporary visual disturbances.[30] Concerns were voiced over both potential side effects at the FDA Anti-infective Drugs Advisory Committee hearing on telithromycin in 2001.[31]

4. Mode of Action

4.1. Inhibition of Translation

It has been known since their discovery that macrolides block protein synthesis, but the molecular details of how they arrest translation has been uncovered only very recently. Footprinting experiments and detailed studies of macrolide resistance over a period of more than 20 years indicated that these compounds bind to the 50S component of bacterial ribosomes and make specific interactions with the 23S RNA. Early studies employing biochemical assays of the individual activities associated with the translation process—initiation, peptide bond formation, and translocation—led to the following conclusions: all macrolides bind in the region of domain V of the ribosome in the peptidyltransferase center; carbomycin and other 16-membered macrolides that carried acyl extensions on the mycarose moiety were found to block peptidyltransferase activity (peptide bond formation) by binding the A site and blocking the binding of aminoacyl tRNA; erythromycin and other 14-membered macrolides were found to have no effect on peptidyltransferase activity. Treatment of bacterial cells with macrolides were found to cause accumulation of peptidyl-tRNA, prompting workers to suggest that the primary mechanism of action common to all macrolides was premature ejection of peptidyl tRNA from the ribosomes.[32]

Determination of the nucleotide sequences of ribosomal RNA and proteins enabled identification of the sites on the ribosome with which macrolides interacted. Footprinting experiments (protection of nucleotides in ribosomal RNA from chemical modification due to binding of added compounds to purified ribosomes) demonstrated direct interaction between macrolides and 23S rRNA.[33] All macrolides, ketolides, lincosamides, and streptogramin B protected nucleotides 2058−2062 (in domain V), but tylosin also protected nucleotide A752 (in domain II).[34] Telithromycin and cethromycin also protected A752.[35,36] Erythromycin, on the other hand, protected the domain V region but made A752 more susceptible to chemical modification.[34] These experiments, along with determinations of the sites in the 23S ribosomal RNA that conferred resistance by mutation or enzymatic modification, identified the precise locations on the ribosome where macrolides were bound. Less was known about the atoms on the macrolides themselves that interacted with the ribosomal RNA.

4.2. Macrolide–Ribosome Structural Studies

Solution of the ribosomal structure at atomic resolution with macrolides bound has clarified some of the enigmas that have arisen associated with macrolide action yet has raised new issues as well. X-ray crystal structures of the 50S subunits of ribosomes from both *Haloarcula morismortui* and *Deinococcus radiodurans* in the presence of macrolides, ketolides, or the streptogramins were determined in the laboratories of Tom Steitz and Ada Yonath, respectively.[37−41] The structures of the *Haloarcula* ribosomal subunit with bound macrolides

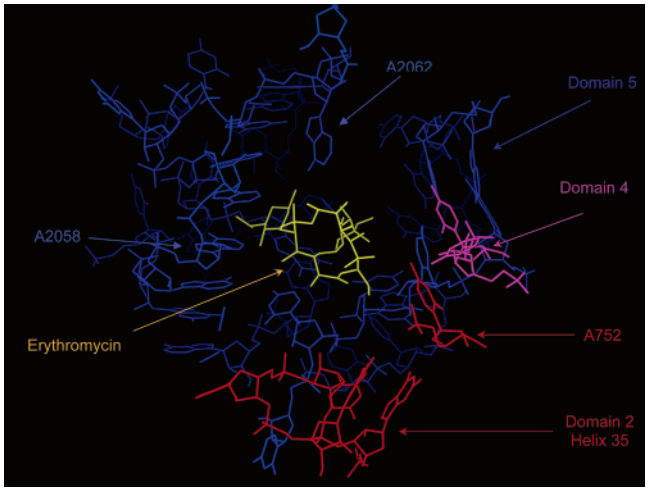


Figure 2. View of erythromycin A bound to the 50S subunit of the *D. radiodurans* ribosome, looking down the peptide exit tunnel toward the peptidyl transferase center. The macrolide binding site is composed of a purine-rich pocket formed by residues from domain V (blue) with contributions from domains II (red) and IV (magenta). Binding of erythromycin blocks peptide formation by closing the peptide exit tunnel some distance from the peptidyl transferase center. Residue A2058 (blue) is critical to binding the desosamine sugar and is the site of methylation in *erm*-based resistance. Residue 752 (red) is protected by ketolide binding.

were obtained even though the *Haloarcula* ribosome is not expected to be macrolide susceptible due to the presence of G rather than A at position 2058 (*E. coli* numbering). As revealed by the structure of erythromycin A bound to the *Deinococcus* subunit (Figure 2), the macrolide binding pocket consists of RNA from domains II, IV, and V, with the majority of the pocket being composed of residues from domain V. There are some stabilizing contributions to the binding pocket from ribosomal proteins L3, L4, L22, and L34, but there appear to be no direct contacts between the macrolide and these ribosomal proteins. The more highly conserved structural region of the macrolides, from C1 to C8, lies against a wall of mostly purine residues from domain V. This interaction with domain V includes tight, specific interactions between the desosamine residue and a binding pocket containing A2058 (Figure 3). The remaining region of erythromycin interacts rather loosely with a pyrimidine-rich side of the tunnel composed of residues from domains II and IV. The dearth of specific contacts (seven H-bonds) between the macrolide and the ribosome make it difficult to rationalize the very high binding affinities observed. Nonetheless, RNA (Figure 4) and ribosomal protein mutations previously known to affect macrolide susceptibility lie within or near this binding pocket, thus adding confidence in the relevance of these crystal structures. The binding pocket lies in the peptide exit tunnel 10−15 Å distal from the peptidyltransferase site; macrolide binding appears to block progression of peptide chain upon contact between the growing peptide chain and the macrolide, which occurs after a small number of elongation steps. This is in agreement with biochemical data showing the formation of very short peptides in the presence of erythromycin. The cladinose residue of erythromycin points along the tunnel toward

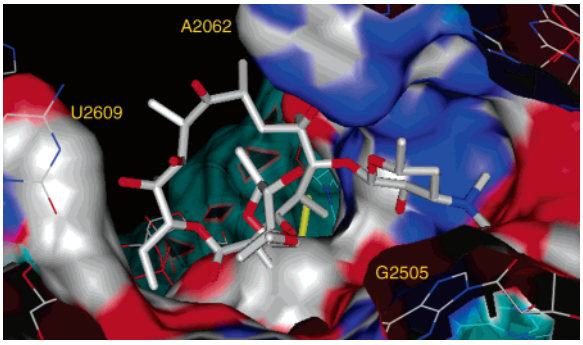


Figure 3. View of the erythromycin binding pocket on the 50S subunit of the *D. radiodurans* ribosome, showing the close interactions with the desosamine residue. Regions of high negative charge are colored red; a primary interaction appears between the phosphate of G2505 (far right) and the desosamine amino group. A2058 lies at the bottom of the pocket in this view.

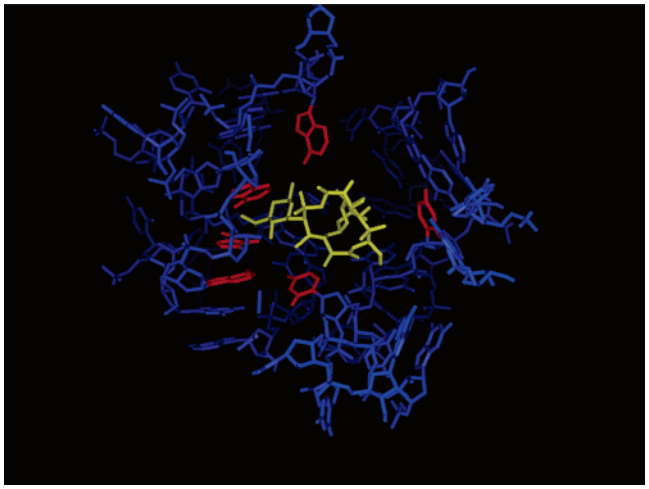


Figure 4. Position of 23S RNA residues at the macrolide binding site (red) where mutation is known to lead to erythromycin (yellow) resistance.

the peptidyltransferase site, in agreement with biochemical experiments, indicating that derivatives of erythromycin acylated on the 4″-OH of the cladinose residue and thus extending further toward the peptidyltransferase site may interfere with peptidyltransferase activity.

While much of the macrolide binding pocket appears loose and rather devoid of specific contacts, quite specific contacts are observed between the desosamine sugar and the RNA in the region of A2058, including a probably crucial charge interaction with the phosphate of G2505 (Figure 3). Not surprisingly, quinupristin, a streptogramin B compound, also makes specific interactions with A2058. As described in more detail in section 5, alterations to A2058 result in macrolide resistance; methylation at N6 of A2058 is a common mode of bacterial resistance to macrolides, lincosamides, and the streptogramin B compounds as is mutation of A2058 to G. Both alterations to A2058 result in loss of specific contacts between A2058 and the 2′-hydroxyl and 3′-dimethylamino groups of desosamine. Similarly, chemical modifications to either the 2′-hydroxyl or the 3′-dimethylamino group of macrolides has been found to greatly reduce or destroy antibacterial activity.

Crystal structures of ketolides bound to these 50S subunits have also been revealing. Removal of the 3-*O*-cladinosyl group in the ketolides results in a dramatic loss in potency that is compensated for by the addition of heteroaryl groups either at the 11-position (telithromycin) or the 6-position (cethromycin). In agreement with ribosomal footprinting experiments, which indicated protection of residue A752 in domain II, the heteroaryl groups of both telithromycin and cethromycin were found to bind to a region of domain II adjacent to the ribosomal binding pocket.[42] In both cases, the position of the macrolactone portion of the ketolides was observed to be slightly shifted relative to that seen for erythromycin A, leading to the suggestion that the ketolides may tolerate some perturbations to the macrolide binding site while compensating for lost interactions by picking up new binding from the heteroaryl groups. However, the observed shift is roughly within the resolution of the structures, and such findings should not be overinterpreted at this stage of refinement. More specific interactions were observed between the telithromycin heteroaryl group and the ribosome than for the cethromycin heteroaryl group; as cethromycin has generally better in-vitro activity against a wide range of organisms, it is clear that such apparently improved ribosomal binding does not necessarily translate to improved antibacterial activity.

The current X-ray crystal structures of macrolides bound to 50S ribosomal subunits offer a snapshot of macrolide action at the ribosome, and it is important to remember that ribosomes are dynamic machines and that the complete picture of macrolide activity is likely to be significantly more complex. Macrolides are known to act during translation, for example, with the actual inhibited complex consisting of a macrolide bound to a complete ribosome having a partially completed peptide in the exit tunnel. There may well be more specific interactions between the macrolide and the complete ribosome−peptide complex than are observed in the current X-ray crystal structures.

The structural studies have led to the conclusion that binding of the macrolide to the ribosome is sufficient to block the progression of peptide synthesis beyond the di- to hexapeptide stage. Hence, binding alone may be sufficient for the antibiotic action of these compounds, and the additional effects of macrolides observed in vitro may not be required for efficacy. On the other hand, the information obtained from the structural work on two ribosomes that are from clinically nonrelevant organisms does not, at this point, provide answers to all effects seen by macrolides on different pathogenic strains or by different macrolides on individual strains. Azithromycin and claithromycin appear to bind in a fashion similar to the ribosome, but azithromycin has better potency against *H. influenzae* and is less potent against *S. pneumoniae* and *S. pyogenes*.[43,44] Telithromycin binds *E. coli* and *S. pneumoniae* ribosomes with $K_d = 2-10$ nM; the K_d of clarithromycin is 30−50 nM, yet the two compounds have equal potencies against *S. aureus* and *S. pneumoniae* in vitro.[45] Finally, the structural studies do not themselves provide any clues as to why the ketolides are bactericidal to *S. pneumoniae* but only bacteriostatic to *S. pyogenes*, *S. aureus,* and *H. influenzae*. Are the differences among the ribosomes from these different organisms sufficient to account for the different effects of these compounds? Is the mode of action of these compounds entirely explained by their binding? It is likely that differences in intracellular accumulation among the various bacteria, rather than differences in ribosomal structure, may account for all or most of the observed differences. Nonetheless, it would be interesting to see the molecular details of interaction of macrolides and ketolides with ribosomes from *E. coli*, *H. influenzae,* and Gram-positive pathogens.

4.3. Inhibition of Ribosome Assembly

Champney and co-workers have shown that macrolides and ketolides inhibit the assembly of the 50S ribosome unit in a number of organisms including *S. aureus*, *S. pneumoniae*, *E. coli,* and *H. influenzae*.[46−51] Assembly of the 30S ribosome was unaffected by these compounds. Using sucrose density gradient sedimentation analysis of ribosomes prepared from cells pulse-labeled with ^{3}H-uridine and chased with an excess of the unlabeled nucleoside, they showed that the addition of macrolides and ketolides promotes accumulation of a 32S particle which degrades upon continued exposure to the drug. Using pulse labeling to analyze translation, they determined that the IC_{50}s for ketolides for arresting translation and inhibiting ribosome assembly in *S. aureus* were the same, ca. 10 nM, the approximate K_d of ketolide−ribosome interactions in vitro. Not surprisingly, inhibition of 50S subunit assembly requires macrolide/ketolide binding. Assembly of the 50S subunit in bacteria takes place unobstructed in cells that carry MLS_B resistance in the presence of macrolides/ketolides, suggesting that these compounds can interact with subribosomal particles. These data also indicate that in the assembly of 50S ribosomes, if methylation of A2058 does take place, it must occur before the assembly of a ribonucleoprotein particle that can interact with macrolides. Champney proposed that macrolide binding to such a particle directly prevents the addition of one or more ribosomal proteins to the maturing particle and leaves segments of the rRNA in the particle exposed to the action of cellular RNases. Whether cessation of ribosome assembly is sufficient to explain the bactericidal effect of ketolides in *S. pneumoniae* remains to be seen.

5. Macrolide Resistance

Resistance to erythromycin was first reported in 1952, the same year erythromycin was introduced into clinical practice, in two strains of *S. aureus*, the first organism targeted by the drug.[52] Resistance also developed in most of the other organisms against which erythromycin and other macrolides were used, but accurate estimates of macrolide resistance in different countries and different locations within

countries have been difficult to determine accurately. Though there is uncertainty about the exact extent of resistance, there is no doubt, however, that that resistance to macrolides is an important basis for clinical failure of macrolide therapy.

Genes associated with macrolide resistance have been found in all the Gram-positive pathogens for which erythromycin and other macrolides were prescribed as well as in strains that were not targeted by macrolides. Resistance genes are present in the microorganisms that produce macrolides. The two most common resistance mechanisms in the bacterial pathogens are (1) reduced binding of the drug due to modification of the bacterial ribosome, either through the acquisition of a "resistance" gene or through mutation in the host, and (2) efflux of macrolides from the bacterial cell, through acquisition of a resistance gene. Less common mechanisms include direct inactivation of the antibiotic itself. Clinical strains have been uncovered that carry more than a single type of resistance. Most of the genes that confer self-resistance in the macrolide-producing actinomyctes are counterparts of the resistance genes found in clinical isolates.

5.1. MLS_B Resistance

Gram-positive cells (and *E. coli*) can acquire a gene that confers high-level resistance to macrolides, lincosamides (e.g., clindamycin), and members of the streptogramin B class of antibiotics (e.g., pristinamycin I).[53] The basis for this type of resistance is either N6-mono- or N6,N6-dimethylation of nucleotide A2058 (*E. coli* numbering) in 23S ribosomal RNA. Genetic, biochemical, and structural data have shown that the MLS_B phenotype is conferred from the overlapping binding of these molecules to domain V making contact with A2058. It is believed, but has yet to be proven conclusively, that either methylation of A2058 changes the structure of the site sufficiently so that macrolides no longer bind or the bulky methyl groups interfere directly with the binding of the drug. The enzyme class was named Erm for erythromycin resistance methylase, and the genes that determine these enzymes were designated *ermA*, *ermB*, *ermC*, etc. At present, 21 classes of *erm* genes, some containing six or more members, have been identified.[54] These proteins are approximately 29 KDa and show very high degrees of sequence conservation. In vitro, Erm-mediated methylation uses 23S rRNA as substrate and does not take place on intact ribosomes or the 50S subunit.[55] The actual substrate for methylation in bacteria has not been determined conclusively. The Erm enzymes do not appear to be specific for their cognate substrates: all Erm enzymes tested use 23S rRNA obtained from many species of bacteria as well as 23S RNA generated by in-vitro transcription. Some of the enzymes, such as ErmN, catalyze only monomethylation, whereas others, such as ErmE and ErmC, catalyze dimethylation, but it is not known whether dimethylation takes place through a concerted two-step process. These latter enzymes can use monomethylated RNA as a substrate.[56] ErmAM (also called ErmB) catalyzes either mono- or dimethylation. Methylation employs *S*-adenosyl-methionine as methyl donor and all enzymes have signature sequences characteristic of *S*-AdoMet binding sites. Structures of ErmAM and ErmC have been solved.[57−59] The *erm* genes have been found on high and low copy plasmids and within transposons, often in association with other antibiotic-resistance genes. They are also found in the chromosomes of macrolide-producing organisms, clustered among the genes for macrolide biosynthesis. The *ermE* gene from the erythromycin-producer *Saccharopolyspora erythraea* has been found in commercial preparations of the drug, causing one to wonder whether resistance in clinical isolates originated from the producing strain and whether it was spread directly from use of the drug.[60−62]

Erm-mediated resistance exists in two forms: inducible and constitutive. In the inducible form resistance and hence ribosome methylation develop only after the macrolide is administered to the cells. In hosts that are constitutively resistant to macrolides, Erm-catalyzed methylation of the ribosomes does not require the presence of macrolides. Both inducible and constitutive MLS_B resistance require an intact coding sequence of the *erm* gene.

5.1.1. Inducible Resistance

The best-studied mechanism of inducible MLS_B resistance involves the *ermC* gene found in *S. aureus* and was based on the initial observations that cells resistant to erythromycin and susceptible to 16-membered macrolides, lincomycins, and pristinamycin I could become resistant to the latter three classes if treated first with small doses of erythromycin.[63] The basis of inducible MLS_B resistance has emerged over the past 30 years and is summarized here.[53] The *ermC*-coding region is preceded by a sequence which encodes a 19-amino acid leader peptide and the two genes, separated by a segment consisting of 81 nucleotides, form an operon. Each gene has its own ribosome binding site (RBS). The mRNA segment corresponding to the leader peptide contains several overlapping inverted repeats and, theoretically, can assume a number of secondary structures, including one in which the ribosome binding site of *ermC* is sequestered, resulting in the inability of the ribosomes to enter the site and translate the mRNA corresponding to the *ermC* gene. Under such conditions the ribosomes would not be methylated and the cells would be susceptible to macrolides. The gene for the leader peptide, however, whose RBS is exposed, is expressed in these cells. In the presence of erythromycin, the model proposes that the mRNA corresponding to the leader peptide undergoes reorganization wherein the RBS of the *ermC* gene is exposed so that it can be translated, producing the methylase that acts to generate methylated ribosomes and thereby conferring resistance to erythromycin and other MLS_B antibiotics. A fascinating model of the induction process has been developed and is reviewed in detail by Weisblum.[53] Briefly summarized, molecules of erythromycin enter the cells, bind to ribosomes engaged in synthesis of the leader peptide, and cause the translation process to stall after the ninth amino acid is introduced into the

nascent peptide, generating the peptide MGIFSIFVI attached to tRNA. The induction model proposed an association among erythromycin, the stalled leader peptide on the ribosome, and the mRNA into a yet to be understood complex that results in the change in the secondary structure of the leader region to permit ribosomes to bind to the RBS and translate the *ermC* gene. A requirement for the stalled leader peptide in the induction process was based on the findings that mutations affecting the leader sequence after Ileu-9 had no effect on inducibility, but mutations resulting in translation termination of the leader before Ileu-9 resulted in noninducibility (failure of erythromycin to confer resistance). Mutations further into leader segment in the region surrounding the RBS, which themselves would destabilize the secondary structure of the mRNA in that region, resulted in constitutive resistance, i.e., expression of *ermC* in the absence of erythromycin. Separation of the leader peptide from the *ermC* gene, or introduction of a nonsense codon at a position corresponding to residue 10, also resulted in noninducibility. Finally, within the first nine residues of the leader peptide, amino acid substitutions of some of the residues did not affect inducibility, but substitutions at other residues resulted in noninducibility. These findings demonstrated that induction required the first nine amino acids of the leader peptide, that the structure of the peptide was important, and that the leader peptide must located cis to the *erm* gene and be interrupted in its translation. Moreover, and most importantly, induction depended upon the presence of the antibiotic with the correct structure—a 14- or 15-membered macrolide that contained the neutral sugar at C-3; 16-membered macrolides and (14-membered)-ketolides are not inducers. The lincosamide celesticetin was later determined to be an inducer.[64] Derivatives of erythromycin that are devoid of antibiotic activity are also not inducers.

Within the current framework of ribosome structure and macrolide binding, it is difficult imagine the role of the macrolide in the induction process. Erythromycin, binding in the polypeptide exit tunnel, could allow the stalling of translation to generate the 9-residue leader peptidyl-tRNA, but other than the tRNA component of the leader peptidyl-tRNA, neither the peptide itself nor erythromycin is in contact with the mRNA, in particular the segment 70 nucleotides downstream that contain the RBS. If erythromycin does make direct contact with the mRNA, it must employ different atoms than those used for binding to rRNA in domain V. The cladinosyl moiety is a likely candidate for such interactions since it is required for induction. On the other hand, it has not been ruled out that the noninducers, such as the ketolides and 16-membered macrolides, cause the ribosome to stall in the leader at a site different from that caused by erythromycin so that the correct "inducer peptide" is not produced. A structure of the "induction complex" at atomic resolution is needed to enable fuller understanding of inducible resistance.

Other examples of inducible MLS_B resistance have been reported. TlrA (also named ErmSF and ErmS) in *Streptomyces fradiae*, the tylosin producer, is an A2058-dimethyltransferase that is induced by tylosin (or a precursor in the biosynthesis pathway) not erythromycin.[64] ErmSV in *Streptomyces viridochromogenes* NRRL 2860 is induced by either tylosin or erythromycin.[65] Interestingly, the *S. fradiae* host also contains two additional 23S rRNA methyltransferases, TlrD (ErmN), an A2058 monomethyltransferase that is induced by tylosin but not erythromycin, and TlrB, a constitutive methyltransferase that acts on G748 in domain II. Methylation by either TlrB or TlrD alone does not confer tylosin resistance; resistance is conferred by the two methylations acting synergistically.[66] Induction of each of these A2058 methyltransferases is believed to occur through a translational attenuation process analogous to that described for ErmC with different structural requirements for the leader peptide and macrolide.

An interesting variation on the mechanism for inducible ErmK-mediated MLS_B resistance in *Bacillus lichenoformis* has been reported. In addition to translational attenuation observed for ErmC production, in the absence of inducer, transcription is halted in the leader region through a *rho*-independent transcription terminator. In the presence of inducer, transcription proceeds through *ermK*.[67]

5.1.2. Constitutive Resistance

In this class the *erm* genes are constitutively expressed in their hosts and thus confer resistance to all MLS_B antibiotics without the need for prior exposure to one or another macrolide. Both MLS_B-inducible (resistant to erythromycin but susceptible to tylosin) and MLS_B-constitutive (resistant to erythromycin and tylosin) strains have been found in clinical isolates of *S. aureus* harboring *ermC*. Most of the isolates in the latter class carry mutations, deletions, or duplications in the leader region that are thought to destabilize the secondary structure and allow expression of the *ermC* gene in the absence of inducer. Mutation from MLS_B inducible to MLS_B constitutive can also be accomplished in the laboratory by simply plating inducible cells in the presence of tylosin and selecting for survivors.[68]

5.1.3. Inducible vs Constitutive

S. aureus cells that carry *erm* genes exhibit either fully MLS_B-inducible or MLS_B-constitutive phenotypes. In inducible strains methylation of ribosomal RNA could not be detected prior to exposure of the cells to erythromycin.[69] Hence, MLS_B-inducible *S. aureus* strains are almost fully susceptible to noninducers such as 16-membered macrolides and ketolides. In clinical isolates of *S. pneumoniae* carrying *ermAM*, a wide range of susceptibility to noninducers has been observed. The degree of resistance (minimum inhibitory concentration) to the noninducing macrolide and ketolides has been correlated with the degree of dimethylation of A2058 in these strains determined before exposure to the drug.[69] Addition of erythromycin to all strains promoted increased resistance to tylosin, resulting from additional dimethylation of A2058. Thus, the high-level resistance of all clinical isolates of *S. pneumoniae* containing *ermAM* to clarithromycin and azithromycin is most

likely due to full induction of the methyltransferase by the drugs resulting in production of fully dimethylated ribosomes. The differential response of the same strains to the noninducers tylosin and telithromycin can also be rationalized. Resistance to tylosin, where seen, is likely due to the constitutive presence of sufficient numbers of dimethylated ribosomes to allow a level of protein synthesis necessary for survival. Susceptibility to telithromycin, on the other hand, may be due to the unique bactericidal effects that take place upon binding of the drug to the unmethylated (or monomethylated) ribosomes present in these hosts.

S. pyogenes strains carrying *ermAM* are much more resistant to telithromycin than their *ermAM*-containing *S. pneumoniae* counterparts but are still very susceptible to cethromycin. It is not yet known whether the difference between the two species is due to the fact that telithromycin (but not cethromycin) can induce *ermAM*-mediated resistance in *S. pyogenes* but not in *S. pneumoniae*. A more compelling explanation would be that the levels of dimethylation are greater in *S. pyogenes* than in *S. pneumoniae* and that dimethylated *S. pyogenes* ribosomes, while refractory to telithromycin binding, can still bind cethromycin with clinical efficacy.

5.2. Efflux

Decreased accumulation due to efflux in a macrolide-resistant isolate was first reported in the 1980s in *S. epidermidis* and in the 1990s in *S. pyogenes* and *S. pneumoniae* and presently accounts for a significant proportion of the macrolide-resistant *S. pneumoniae* strains identified.[70−75] Efflux-mediated resistance is still relatively rare in *S. aureus*. In streptococci, macrolide efflux is mediated by the gene product encoded by *mefA*, the name denoting a group of genes encoding proteins that share >90% identity. MefA confers resistance to 14- and 15-membered macrolides but not 16-membered macrolides, lincosamides, or streptogramin B. Furthermore, Mef-mediated resistance is induced by the presence of clarithromycin and azithromycin but not by 16-membered macrolides. Ketolides are poor inducers of MefA and hence are still very potent antibacterials against streptococci carrying this gene. Since the Mef proteins do not contain recognizable ATP binding sites and resistance to macrolides in *mefA*-containing hosts takes place in the presence of ATP-associated energy uncouplers, Mef-mediated transport of the macrolide is believed to be driven by a proton motive force.

Efflux in staphylococci is mediated by MsrA, a member of the ABC superfamily that employs ATP as the energy source for transport and is thought to work in concert with a membrane-associated host protein to confer resistance. MsrA confers high-level resistance to 14- and 15-membered macrolides and streptogramin B and weak resistance to ketolides, and it does not confer resistance to 16-membered macrolides and lincosamides. MsrA is induced by clarithromycin, azithromycin, and telithromycin but not by streptogramin B, even though the latter compound is a substrate for MsrA-mediated transport. Nucleotide sequencing of the region upstream of *msrA* revealed a leader sequence reminiscent of the leader upstream of *ermC*, suggesting that MsrA is induced by a translational attenuation process.[76] ABC transporters have also been found in some *Streptomyces* hosts that produce macrolides. These genes are located at the edges of their cognate biosynthesis gene cluster and confer resistance to 16- or 14-membered macrolides when expressed in heterologous hosts. Their roles in conferring self-resistance or in export of the macrolide during production are not yet known.

A number of transport systems not specific for macrolides have been identified in Gram-negative bacteria. These tripartite pumps are members of the RND family and are composed of an inner membrane component, which extrudes the macrolide in exchange for a proton, a protein in the outer membrane that may form a gated channel (pore), and a periplasmic protein that links the two membrane-associated efflux proteins. Examples include the MexAB−OprM system in *Pseudomonas aeruginosa*, the AcrAB−TolC system in *E. coli,* and the acrAB−Omp2 system in *H. influenzae*. These systems, encoded in the chromosomes of Gram negatives, are the primary bases for intrinsic resistance to membered macrolides as well as many other compounds including antibiotics such as rifampicin, novobiocin, and tetracycline. In some hosts the RND pump genes are expressed constitutively; in others, a mutation is required RND-mediated resistance.

5.3. Mutations in Ribosomal RNA

Bacteria contain between one and seven copies of the operons that encode the genes for ribosomal RNA. Mutations in domain V encoding resistance to clarithromycin have been reported in clinical isolates of a number of organisms, including *H. pylori*, *S. aureus*, *S. pneumoniae*, *H. influenzae, Neisseria gonorrheae*, *Mycobacterium tuberculosis*, *Mycobacterium avium,* and *Treponenum pallidum*.[77−87] Several patterns of resistance were seen. Deletion of A2058 in *S. pneumoniae* conferred high-level resistance to macrolides and increased the MIC to 4 μg/mL of telithromycin, but it is not clear whether the increase in MIC translates to resistance to the ketolide in a clinical setting. A2058G or A2058T mutations conferred high-level resistance to all three classes of MLS_B antibiotics. A2059G mutations conferred intermediate-level resistance to macrolides and lincosamides but did not confer resistance to streptogramin B. In *N. gonorrheae*, a C2611T mutation (in domain V) was identified. In *H. pylori*, the particular mutation was found in each of the two copies of the *rrl* gene (23S rRNA) present in the chromosome. In *S. aureus*, the mutation was present in a minimum of four of the six *rrl* genes present in the host and in *N. gonorrheae* three of the four *rrl* genes. In *T. pallidum* the A2058G mutation was present in both copies of *rrl*. In no cases did a resistant strain carry more than a single type of mutation, suggesting that each mutation was introduced into a single copy of the *rrl* genes, and through selection in the presence of the drug, the mutant allele replaced all or most of the "wild-type" copies of the gene in the host, likely via a process involving recombination.

Mutations in domain V in *E. coli* strains selected for resistance to macrolides have been mapped to nucleotides, 2058, 2059, and 2612.[88] A U2609C mutation in domain V was found in an *E. coli* strain selected for resistance to telithromycin or cethromycin.[89] Interestingly, this mutation increased slightly the susceptibility of the host to erythromycin and azithromycin.

Mutations in domain II in the vicinity of A748 conferring resistance to macrolides or ketolides have not been reported in clinical isolates, but the U754A mutation in the hairpin 35 segment of domain II was found in an *E. coli* host selected for resistance to telithromycin.[90] A different class of laboratory-selected strains of *E. coli* that exhibited increased resistance to erythromycin was found to carry mutations within a hairpin structure between nucleotides 1198 and 1247 in domain II of the 23S rRNA, close to a segment of the RNA that encodes a pentapeptide (E-peptide) that confers resistance to erythromycin.[91] It is believed that mutation in this region of domain II increases expression of the segment encoding the E-peptide. Interestingly, it was found that the peptide acted only cis on ribosomes carrying the 23S RNA harboring the domain II mutation and conferred resistance only to erythromycin and not ketolides or 16-membered macrolides.[92] By site-directed mutagenesis of the E-peptide coding region, the sequence could be changed to permit the production of different peptides that conferred resistance to ketolides or 16-membered macrolides.[93] Although this mechanism of macrolide resistance in *E. coli* is not clinically relevant at the present time, these findings raise the possibility that short peptides produced from the rRNA as well as segments of the ribosomal RNA itself may play a role in the binding of macrolides to ribosomes to stop translation or, perhaps, to promote expression of an *erm* gene.

5.4. Mutations in Ribosomal Proteins

A number of clinical isolates of *H. influenzae, S. aureus,* and *S. pneumoniae* resistant to macrolides have been characterized to carry mutations in genes for 50S ribosomal proteins L4 or L22.[81,82,87] As described above, these two proteins border the polypeptide exit tunnel. Mutations in *E. coli* conferring resistance to erythromycin were also determined to reside in the genes for L4 and L22 proteins. Ribosomes from L4 mutants exhibited reduced binding of erythromycin, but the L22 mutant ribosomes could still bind drug, indicating that the mutation affected the structure of the tunnel such that binding of macrolide did not block translation.[94−96]

5.5. Enzymatic Inactivation of Macrolides

5.5.1. Hydrolysis of the Macrolactone

Two unrelated genes, *ereA* and *ereB*, each conferring resistance to erythromycin in *E. coli*, were identified on separate plasmids and shown to encode esterases that opened the macrolactones of erythromycin and oleandomycin.[97,98] These genes were subsequently identified in a number of members of other Gram-negative bacteria (*Citrobacter*, *Proteus*, *Klebsiella,* and *Enterobacter* species) as well as in clinical isolates of *S. aureus*.[99,100] Currently, esterase-mediated resistance to erythromycin is rare in *S. aureus* and has yet to be detected in streptococci. These enzymes are specific for 14-membered macrolide substrates. Two streptogramin B hydrolases, VgbA and VgbB, have recently been identified in *S. aureus*.[101] These enzymes do not employ macrolides as substrates.

5.5.2. Phosphorylation

Enzymes that transfer phosphate from ATP to the 2′-OH of erythromycin were originally discovered in *E. coli*. Members of the MphA group employ 14- and 15-membered macrolides as substrates exclusively.[102,103] The MphB enzyme can phosphorylate both 14- and 16-membered macrolides.[104] Macrolide 2′-phosphotransferase activity, related to MphA, was recently detected in two clinical isolates of *P. aeruginosa* from hospital patients in Japan, where macrolides are used for long-term chemotherapy of *P. aeruginosa* panbronchiolitis.[105] A related enzyme, MphC, has also been identified in a clinical isolate of *S. aureus*.[106] Expression of the *mphA* gene in *E. coli* is regulated by an adjacent gene, *mphR*, whose gene product binds to the operator−promoter region of *mphA* and represses transcription. Transcription of *mphA* takes place in the presence of erythromycin, which enters the cell, binds to MphR, and removes it from the operator−promoter.[107] In this system erythromycin is the inducer of (self-)resistance. Although the MphA−MphR resistance system has thus far been found only in *E. coli*, it is reasonable to suggest that it originated in a macrolide-producing bacterium and that a 2′-phosphatase, which would restore antibacterial activity to 2′-phosphoerythromycin, would also be uncovered in a macrolide-producing host.

5.5.3. Glucosylation

Macrolide resistance mediated through 2′-glucosylation has not been reported in a bacterial pathogen but has been found in *Streptomyces antibioticus*, the producer of oleandomycin.[108] Extracts of several other streptomycetes were found to contain activities that transferred the glucose moiety from UDP−glucose to a number of 12-, 14-, and some 16-membered macrolides, suggesting that the resistance gene spread from a macrolide producer.[109−111] In their natural locations in the chromosome the *mgt* genes confer weak resistance to macrolides on their hosts. In *S. antibioticus*, the MGT gene, *oleI*, is accompanied by the gene *oleR*, which encodes an enzyme that removes the glucose residue from 2-glucosyloleandomycin, restoring the antibacterial activity to the compound.[108] Both *oleI and oleR* are located in the oleandomycin biosynthesis cluster. OleI is thought to confer self-resistance to the host while the compound is produced intracellularly, and OleR restores its activity during or prior to transport from the host. It is interesting to note that the oleandomycin biosynthesis cluster does not contain an *erm* gene; hence, the host makes oleandomycin-employing ribosomes that are susceptible to the drug.

6. Biosynthesis of Macrolides

Biosynthesis of macrolides follows discrete biochemical pathways but can be viewed as taking place in three stages: synthesis of the aglycone, synthesis of the sugars and attachment to the aglycone, tailoring steps to produce the completed product. The genes for the biosynthesis of the aglycone and deoxysugars and the genes for the tailoring steps are generally clustered. Genes that confer self-resistance are located within the biosynthesis cluster. Much of our current understanding of macrolide biosynthesis has come from the determination of the nucleotide sequence of the biosynthesis genes in the 1990s. However, the biochemical pathways for erythromycin and tylosin were largely understood well before this period from the analysis of compounds produced in fermentations of mutants blocked at different steps of the synthesis.[112−114] In addition, early feeding experiments indicated that macrolides were produced from acetate, propionate, and butyrate, but the key experiments demonstrating the bioconversion of compounds 5−9 carbons in length with structures representing intermediates in aglycone biosynthesis into the aglycones of erythromycin and tylosin indicated that biosynthesis of the macrolactone takes place through a stepwise process.[115,116]

6.1. Biosynthesis of the Aglycone: Modular Polyketide Synthases

The aglycones of macrolides are complex polyketides that are assembled through successive decarboxylative condensations of small carboxyacyl thioesters (e.g., malonyl CoA, methylmalonyl CoA) in a manner resembling fatty acid biosynthesis. Each step of the synthesis is programmed to determine the acyl unit incorporated into the growing chain (e.g., malonyl CoA, methylmalonyl CoA, etc.) and the degree to which the resulting β-carbonyl generated from the condensation is reduced. In addition, the stereochemistry of the α-side chain (if present) is also programmed. Programming is carried out by the polyketide synthase (PKS) that catalyzes all the steps in assembly of the aglycone. In general, each enzymatic step is conducted by a discrete component of the PKS, and as in fatty acid biosynthesis, all steps take place with the growing acyl chain tethered to the enzyme in a thioester linkage. Macrolide PKSs are large, multifunctional polypeptides that can contain more than 30 enzymatic functions, but the functions associated with a single condensation and β-carbonyl reduction cycle are present in an uninterrupted linear sequence, commonly referred to as a *module*, hence the term modular PKS. Each module is similar in overall organization to type I fatty acid synthases.

The enzymatic functions within each module are called *domains*. The domains are arranged in a linear sequence and separated by interdomain spacer regions. The KS domain, approximately 550 amino acids in length, encodes the β-ketoacyl ACP synthase that catalyzes the condensation between the growing acyl chain (attached in thioester linkage to the Cys-173 residue of the KS) and the extender unit tethered to the ACP domain (acyl carrier protein) through a thioester linkage with the 4-phosphopantotheine prosthetic group.[117] The AT domain, ca. 300 amino acids, encodes the acyltransferase, the component that binds the extender acyl−CoA unit via an ester linkage with the Ser residue in the GHSxG active site, and transfers it to the ACP for condensation with the nascent acyl chain. Each AT domain is selective for the extender unit it binds and transfers to its cognate ACP. Comparisons of the sequences of AT domains showed that malonyl- and methylmalonyl-transferring domains each clustered with members of the same group strongly, indicating structure-determined selectivity.[118]

All modules in macrolide PKSs contain KS, AT, and ACP domains. The remaining domains determine the extent to which the β-carbonyl produced through condensation is reduced. If the KR domain (β-ketoreductase) is absent or mutated, the β-keto group will not be processed further. If the KR is present, the β-keto group is reduced to the hydroxyl. The stereochemistry of the hydroxyl group is determined by the KR domain.[119,120] The KR domains have the GxGxxAxxxA motif for NADPH binding.[121] The DH (dehydratase) domain removes the β-OH group and a proton from the α-carbon to leave an α,β-double bond. It is not known if the DH domains remove 3(*R*)-OH and pro-2(*S*) hydrogen in syn eliminations as observed in fatty acid synthase.[122] All double bonds found in macrolides are trans. The ER (enoylreductase) domain reduces a trans double bond to the β-methylene center. ER domains contain a NADPH binding motif.

All macrolide PKSs contain a TE (thioesterase) domain at the C terminus of the last module that acts to release the polyketide chain from the PKS and cyclize it. These are referred to as TE-I domains. The TE-I domains of the erythromycin and pikromycin PKSs have been crystallized.[123,124] Macrolide biosynthesis clusters also contain a discrete gene encoding a short-chain thioesterase (TE-II) that play a role in macrolide production by removing aberrant intermediates produced from improper decarboxylation of the extender molecule.[125−127]

6.1.1. Erythromycin

The erythromycin PKS, 6-dEB synthase, or DEBS, was the first modular PKS identified through sequencing of the corresponding genes.[128,129] DEBS consists of three proteins though each is thought to exist as a head-to-head dimer in the holoenzyme.[130] 6-dEB is made from the successive condensations of one propionate molecule and six molecules of methylmalonate.

The predicted domain organization of DEBS and biosynthetic intermediates at the end of each cycle of condensation and β-carbonyl reduction is shown in Figure 5. DEBS1 contains the loading module and modules 1 and 2. The AT domain of the loading domain binds propionyl CoA and transfers it to the adjacent ACP [**a**]. All ACP domains of DEBS are phosphopantetheinylated by the phosphopantetheinyltransferase SePptII, whose gene is not found in the erythromycin biosynthesis cluster.[131] The propionyl residue is then transferred to the KS domain of

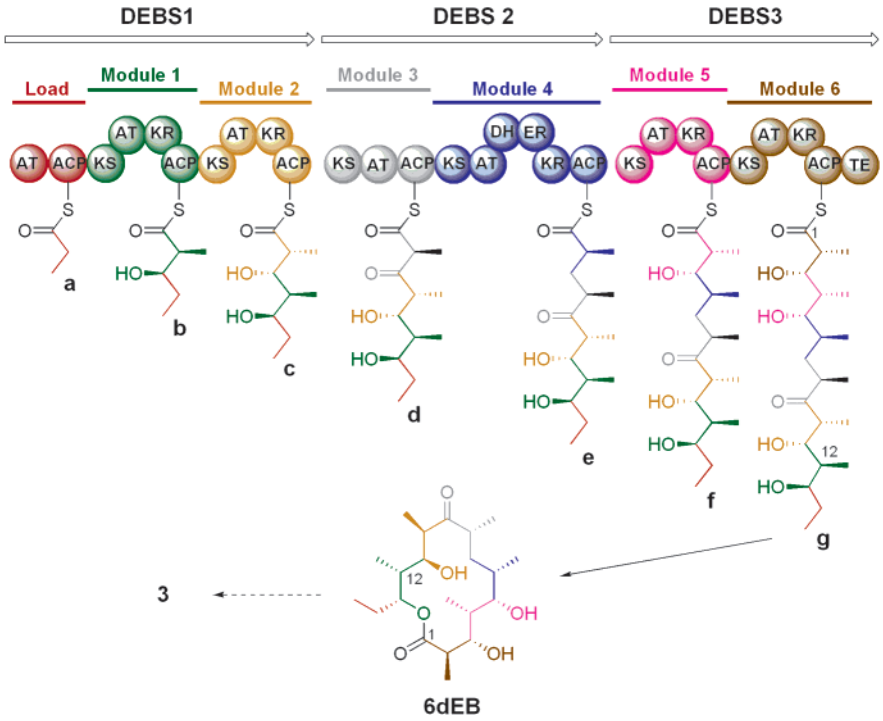


Figure 5. Domain organization of DEBS and structures of proposed intermediates at the end of each condensation cycle. Linear sequences of polypetides are shown as open arrows. Domains are shown as spheres. Color-coding indicates components of the nascent polyketide chain programmed by particular modules. Abbreviations: ACP, acyl carrier protein; AT, acyltransferase; DH, dehydratase; ER, enoylreducase; KR, β-ketoreductase; KS, β-keto acyl−CoA synthase; TE, thioesterase.

module 1. It has been shown that propionyl CoA, and not methylmalonyl CoA, binds directly to the loading domain and that propionyl CoA can bind directly to the KS1 domain in the absence of a loading module, albeit very inefficiently.[132−134] All AT domains of modules 1−6 bind and transfer the 2(*S*) enantiomer of methylmalonyl CoA to their cognate ACPs; hence, epimerization of the α-methyl group produced after the second, fifth, and sixth condensations must take place, but it is not yet known how these epimerizations are controlled by the PKS.[135] After the first condensation, reduction of the β-carbonyl is catalyzed by the KR domain of module 1 to generate the diketide intermediate **b**. As seen in Figure 5 the carbon atoms of the propionyl starter and first extender ultimately become C 11−15 of the completed aglycone. The acyl chain of **b** is transferred to the KS of module 2, and condensation with the methylmalonyl CoA extender on ACP2 generates a triketide whose β-carbonyl is reduced by the KR2 domain [**c**]. Direct evidence for the activities of associated with DEBS 1 comes from the production of the predicted triketide lactone both in vivo and in vitro from a DEBS construct in which the TE domain was moved from the end of module 6 to the C terminus of DEBS 1.[136−139]

The next step requires interpolypeptide transfer of the nacent acyl chain from the ACP2 of DEBS1 to KS3 of DEBS2. Recognition sequences (linker regions) at the ends and beginnings of PKS subunits ensure proper associations to prevent aberrant nascent chain passage.[140,141] Module 3 contains a sequence that resembles a KR domain, but the conserved NADP(H) binding site is not present and, hence, is not functional. The β-carbonyl of the formed tetraketide [**d**] is not further processed and becomes the C-9 keto group in 6-dEB. After the fourth condensation the KR, DH, and ER domains process the β-carbonyl to the methylene [**e**] found at C7 in 6-dEB. After the fifth and sixth condensations only ketoreductions are programmed to take place to produce the OH groups at C-5 and C-3 of 6-dEB. After reduction of the β-carbonyl of the heptaketide, the TE domain acts to release **f** from the PKS and promotes the nucleophilic attack of the C-13 hydroxyl on the C-1 carbanion formed, resulting in the production of the macrolactone. How the PKS is programmed to avoid premature release of the chain prior to the last β-ketoreduction is not yet understood.

The genes that determine DEBS have been expressed in a number of heterologous hosts, including *Streptomyces coelicolor*, *Streptomyces lividans,* and *E. coli.*[142−145] The DEBS proteins have been purified and used to make 6-dEB, intermediates, or derivatives in vitro.[138,139,146,147] The specificities of the various KS domains of DEBS have been examined using *N*-acetylcysteamine thioesters of the syn or anti diastereomers of 2-methyl-3-hydroxyl-containing acyl chains for direct loading onto the KS2, KS5, or KS6 domains for single or multiple chain extensions in vivo or in vitro.[117,148−151] It was found that all three domains utilized only the syn diastereomers and that whereas KS2 and KS5 could use either enantiomer KS6 showed high preference for the (2*S*,3*R*) enantiomer. It should be pointed out that KS5 normally does not utilize a 2-methyl-3-hydroxy-containing substrate for elongation; its substrate is fully reduced at C3.

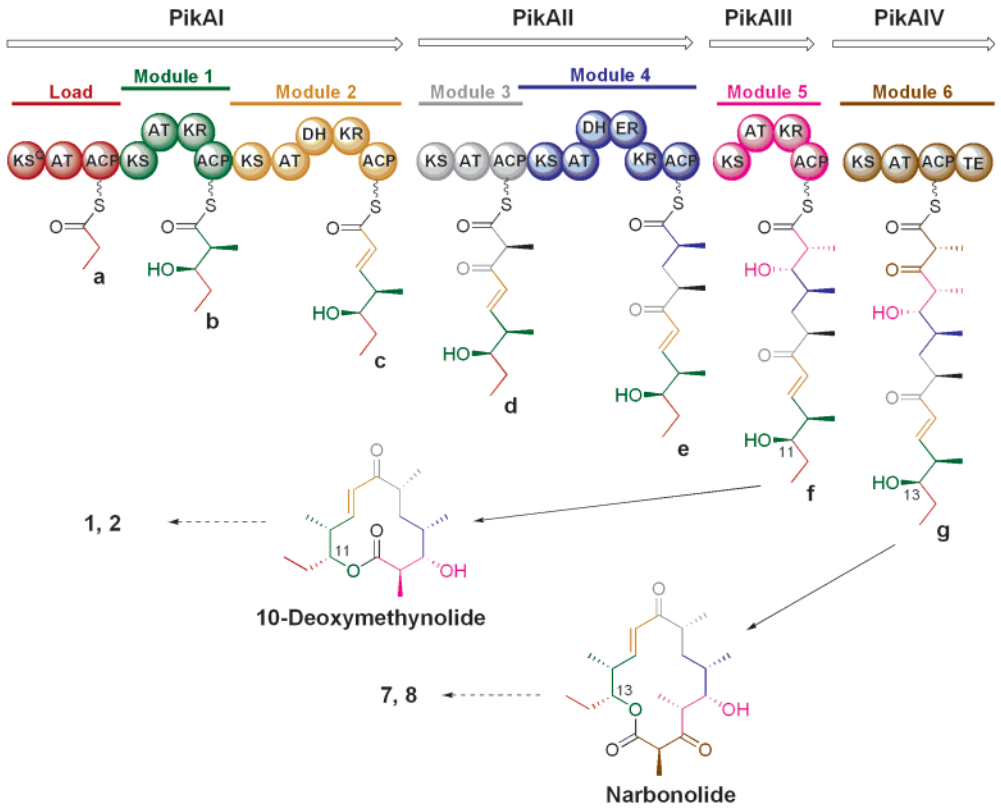


Figure 6. Domain organization of the Pik PKS, and structures of proposed intermediates at the end of the condensation cycle. Polypeptides and domains as in Figure 5. Abbreviations: KS^Q, KS domain carrying Cys173Ala mutation; all others as in Figure 5.

6.1.2. Lankamycin and Oleandomycin

The lankamycin PKS is identical in both module and domain organization the DEBS but differs in amino acid sequence.[152] The only structural difference between the untailored aglycones is the replacement of the 13-ethyl side chain in 6-dEB with the 1(*S*)-methyl-2(*S*)-hydroxypropyl group in the aglycone of lankamycin. The discovery that the lankamycin PKS contained only six modules (and a loading module) suggests that the starter is either 2-methylbutyryl CoA (which is hydroxylated at C-2 after polyketide synthesis) or 2-methyl-3-hydroxybutyryl CoA.

The aglycone of oleandomycin is built from an acetate starter and six molecules of methylmalonyl CoA. The Ole PKS is organized identically to that seen for DEBS with a single difference, a KS^Q domain in the loading module, which is discussed immediately below.

6.1.3. Methymycin and Pikromycin

The aglycones of methymycin and pikromycin differ in structure only with respect to the additional two carbons in the ring of pikromycin. Methymycin is produced from one propionate, one malonate, and four methylmalonate residues. Pikromycin requires an additional methylmalonate. Both compounds are produced in *S. venezuelae* from a single PKS (Figure 6); thus, the nascent chain to the end of the fifth module is the same for both compounds.[153] 10-Deoxymethynolide is released after the fifth condensation and narbonolide after the sixth. The Pik PKS is similar to DEBS in overall organization, with a number of interesting differences. At the N-terminus of the loading module is a domain labeled KS^Q in which the Cys173 residue at the active site is replaced by Gln.[154] This domain, therefore, cannot make a thioester linkage with an acyl chain and hence cannot participate in a condensation reaction. The domain is still capable of the decarboxylation event that is required for chain elongation. Hence, loading modules that carry KS^Q domains use starters that require decarboxylation such as malonyl CoA or methylmalonyl CoA to yield the required acetyl or propionyl moieties found in the side chains of the completed aglycones.[155,156] Reduction of the resulting carbanion is likely conducted by the KS^Q as well. The Pik PKS, therefore, uses methylmalonyl CoA as the starter and decarboxylates it to propionyl−ACP. The Ole PKS uses malonyl CoA as the starter and decarboxylates it to acetyl−CoA. The first, third, and fourth condensations and β-carbonyl-processing events resemble those seen for 6-dEB. The second condensation employs malonyl CoA rather than methylmalonyl CoA as the extender unit, and the β-carbonyl of the triketide is reduced and then dehydrated by the KR and DH domains in module 2. The 2,3-double bond of **c** thus becomes the 8,9- or 10,11-trans double bond of methymycin or pikromycin, respectively. The most interesting differences from DEBS are the events that take place after the fifth condensation. Modules 5 and 6 in the Pik PKS are split into separate polypeptides, PikAIII and PikAIV, respectively. Under conditions that favor the production of methymycin, nascent chain growth terminates after the fifth condensation event to release and cyclizes the acyl chain to produce 10-deoxymethynolide. It has

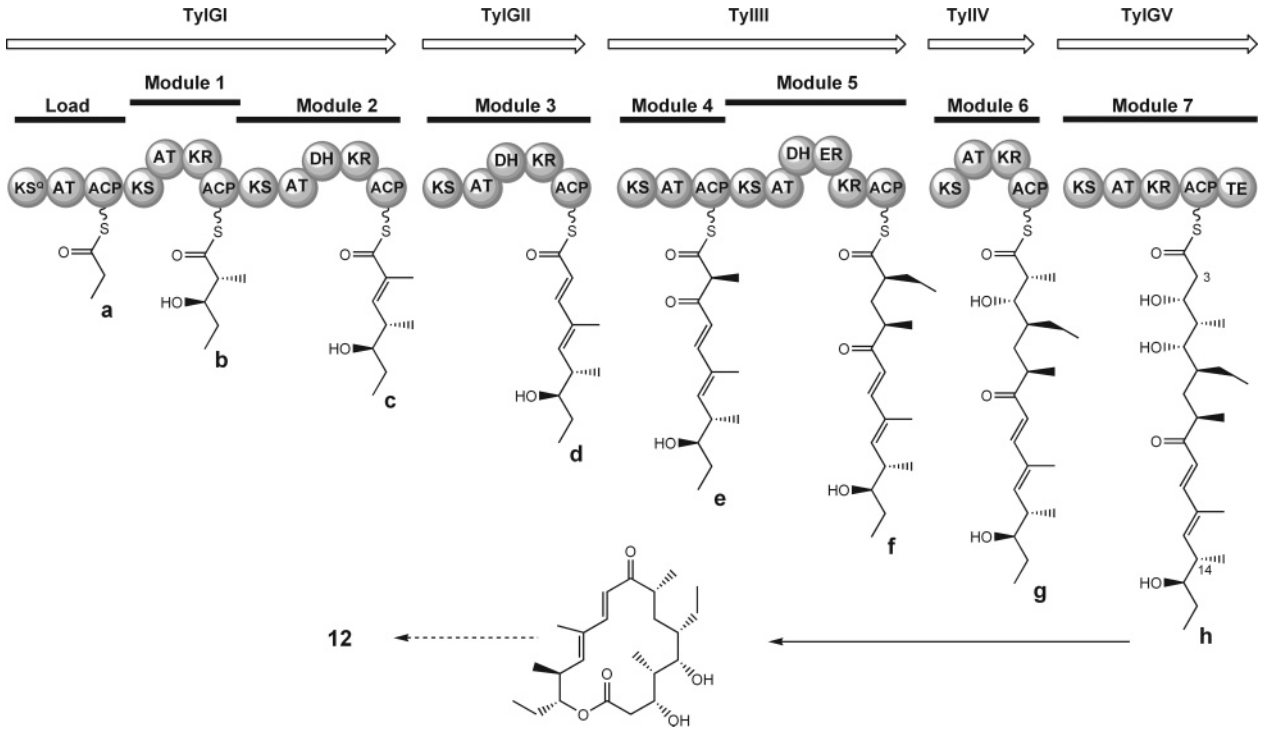


Figure 7. Domain organization of the Tyl PKS, and structures of proposed intermediates at the end of the condensation cycle. Polypeptides, domains, and abbreviations as in Figure 5.

been proposed that this is accomplished by the transfer of intermediate **f** to the ACP of module 6 without chain elongation, a process referred to as "skipping".[157−159] Once attached to ACP6, the adjacent TE domain can release and cyclize the acyl chain. Under conditions that favor 14-membered ring production, normal transfer of intermediate **f** to the KS of module 6 would take place. How the organism regulates the production of one macrolide over another is still not fully understood. The *pik* PKS genes have been expressed, in whole or in part, in heterologous hosts.[144,160] Employing various 2-methyl-3-hydroxypentanoyl-S-NACs, the specificities of KS5 and KS6 of the Pik PKS were shown to be similar to those found for the corresponding DEBS KS domains, although KS5 was found to have high preference for the syn (2*S*,3*R*)-enantiomer.[161,162]

6.1.4. Tylosin

The PKS-encoding genes from at least one member of each of the four groups of 16-membered macrolides have been sequenced. All contain seven modules and are organized as shown in Figure 7 for the tylosin PKS.[163] The PKS is composed of five polypeptides: polypeptide I—load, modules 1 and 2; II—module 3; III—modules 4 and 5; IV—module 6; V—module 7. The aglycone tylactone is made from the precursors malonyl CoA, methylmalonyl CoA, and ethylmalonyl CoA. The presence of the KS^Q domain in the loading module suggests that the starter is methylmalonyl CoA, which is decarboxylated to propionyl-S-ACP. The first, second, fourth, and sixth condensations employ methylmalonate extenders; the third and seventh use malonyl CoA. The fourth extension uses ethylmalonyl CoA, which is produced in the cell through the 2-carboxylation of butyryl CoA. Butyryl CoA may be produced from the degradation of fatty acids or through a single round of fatty acid synthesis from acetyl−CoA. A gene for crotonyl CoA reductase, which catalyzes conversion of crotonyl CoA to butyryl CoA, is present in the tylosin biosynthesis cluster.[164] The specificities of the KS domains of the Tyl PKS have not been examined; thus, it remains to be determined whether the KS2 domain, which is normally presented with the *anti*-2-methyl-3-hydroxypentanoyl-S-ACP, has preference for one enantiomer over the other or whether the syn diastereomer can also be extended.

6.1.5. Platenolide

The predicted domain organization and biosynthetic intermediates of platenolide synthase, which has been sequenced from the spiramycin and niddamycin producers, is shown in Figure 8.[165,166] The domains are identical to that of the tylosin PKS with two exceptions: the ATs of the loading module and module 2 transfer malonyl CoA rather than methylmalonyl CoA; the AT of module 6 transfers methoxylmalonate−thioester rather than methylmalonyl CoA. In the platenolide cases it is not known whether the thioester moiety of methoxymalonate is CoA, but it is thought that methoxymalonyl−ACP is the precursor employed for biosynthesis of the complex polyketides ansimitocin and ascomycin.[167,168]

6.1.6. Chalcomycin

The PKS of chalcomycin is shown in Figure 9. Although chalcomycin contains a 2,3-trans double bond, the Chm PKS does not contain the required KR and DH domains in module 7 to catalyze its formation.[169] A gene that could encode a ketoreductase was identified 3 kb downstream of the PKS, but a DH gene was not found. Expression of the *chm* PKS

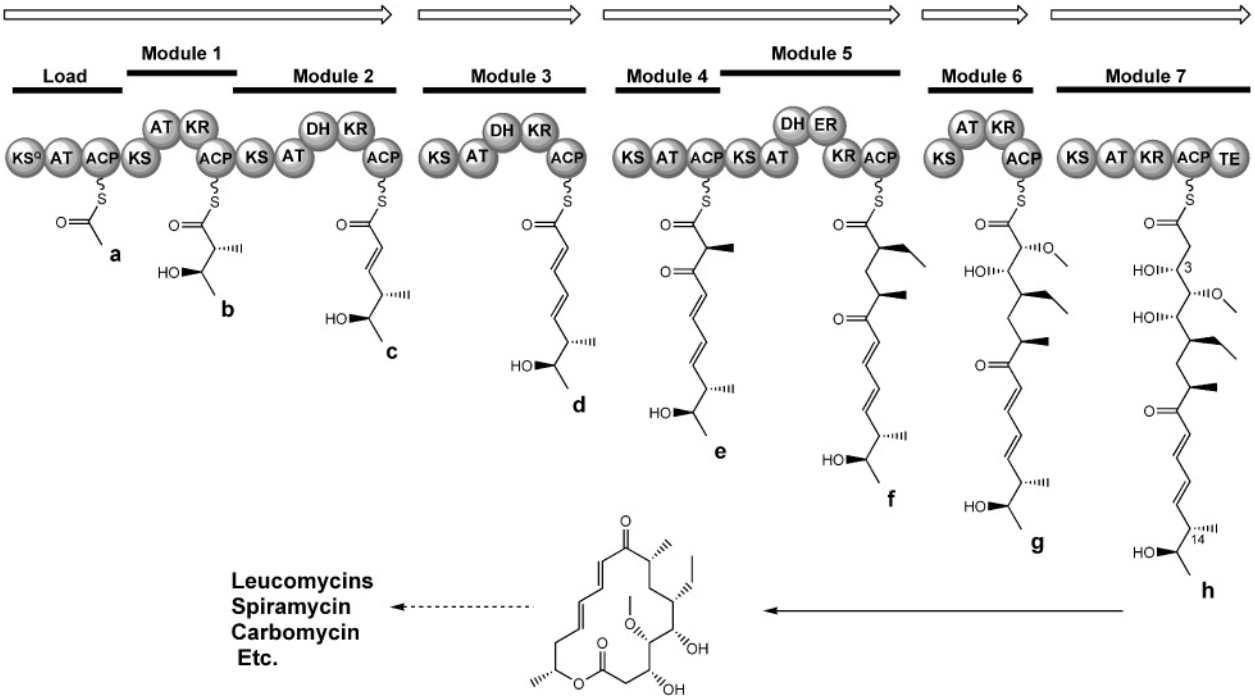


Figure 8. Domain organization of the platenolide PKS, and structures of proposed intermediates at the end of the condensation cycle. Polypeptides, domains, and abbreviations as in Figure 5.

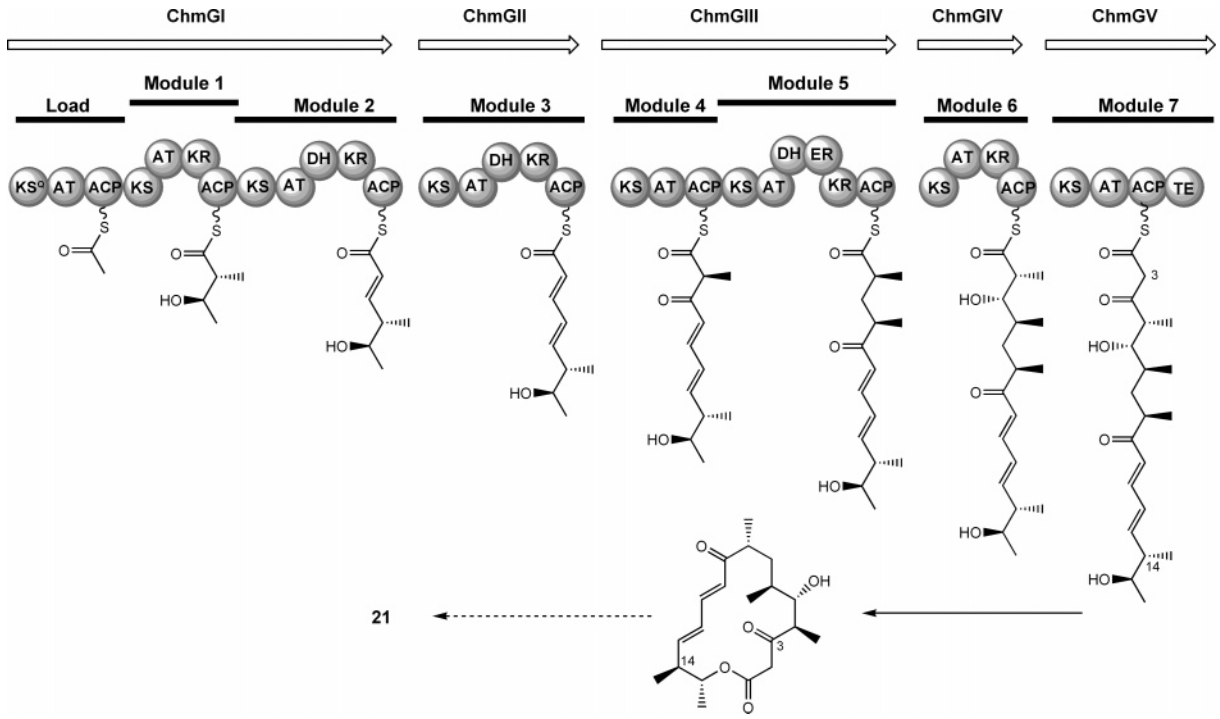


Figure 9. Domain organization of the Chm PKS, and structures of proposed intermediates at the end of the condensation cycle. Polypeptides, domains, and abbreviations as in Figure 5.

genes in an *S. fradiae* host that had been deleted of the *tyl* PKS genes resulted in the production of the predicted macrolactone containing a 3-keto group (chalconolide) but which contained mycaminose at C-5, indicating that the mycaminosyltransferase used for tylosin production could utilize chalconolide as well.[169,170] The basis for the introduction of the 2,3-double bond in chalcomycin is not yet understood. In contrast, the seventh module of the mycinamicin PKS contains the KR and DH domains, which indicates formation of the double bond on the nascent polyketide.[152]

6.2. Biosynthesis of Deoxysugars

Genes for the biosynthesis of the deoxysugar components of macrolides have been identified in the erythromycin, pikromycin, tylosin, megalomicin, chalcomycin, oleandomycin, and lankamycin clusters.[152,153,164,169,171−178] Verification of the pathways have come from (a) transfer of the genes to a

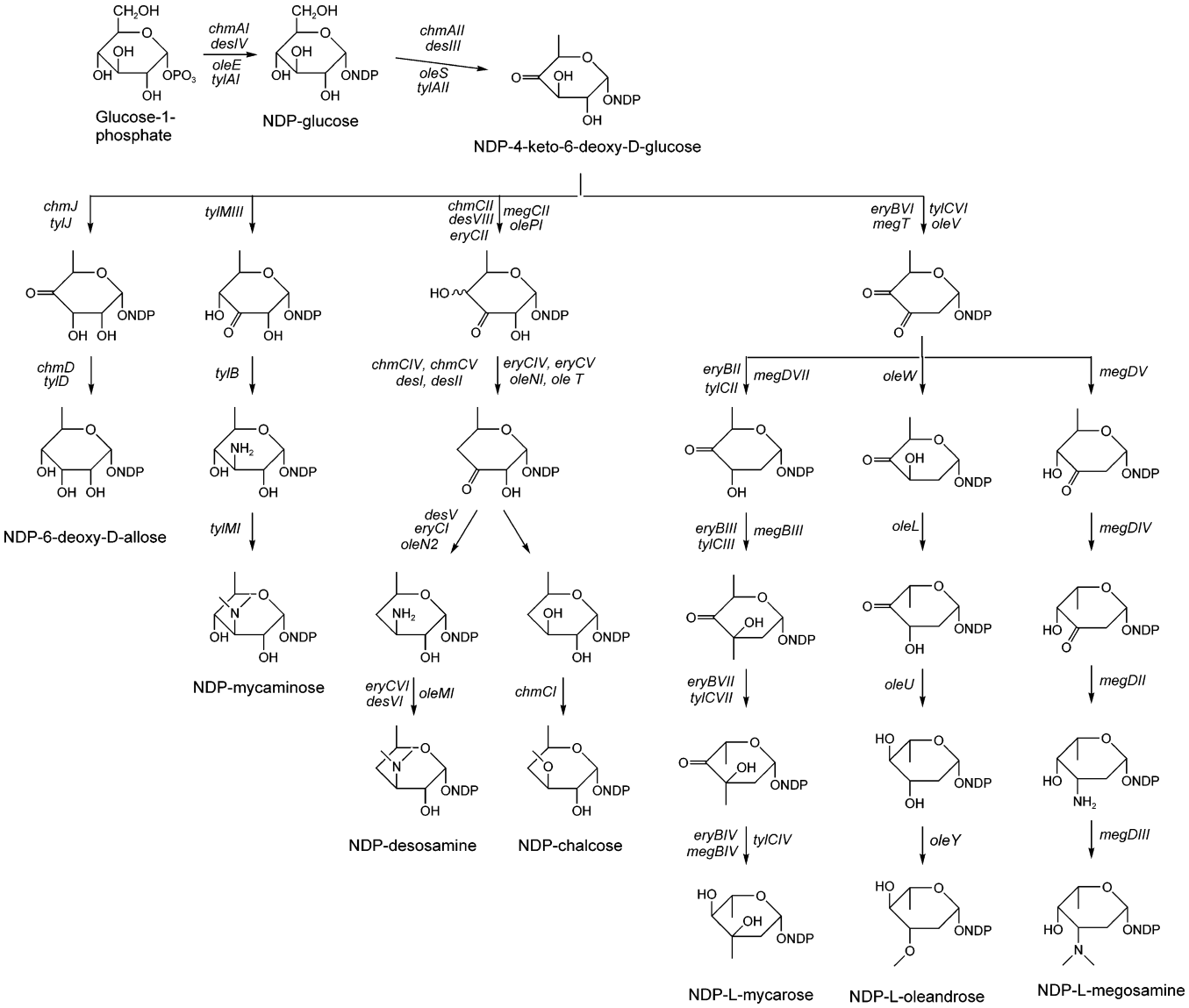


Figure 10. Composite biochemical pathways of deoxysugar biosynthesis in macrolide-producing strains. Proposed enzymes for given steps are shown.

heterologous host and production of a macrolide containing the corresponding deoxysugar, in some cases a novel macrolactone−sugar combination, or (b) loss of synthesis of the sugar component or change of the structure of the sugar through the introduction of a mutation in the corresponding genes.[172,174,179−183] A compilation of the proposed pathways of seven deoxysugars present in macrolides is shown in Figure 10 along with the proposed genes involved in the particular steps from the corresponding antibiotic-producing strains. The proposed pathway for the synthesis of L-arcanose, the neutral sugar of lankamycin, is not shown. Genes from different organisms involved in a particular step of a pathway, e.g., *eryBIV*, *tylM*, show highest similarity scores to each other of all matches in the sequence databases and are assigned the given step on the basis of proposed function. Because they have not been determined experimentally, the absolute order of reactions for pathways involving more than two steps are not certain. The nucleotide carrier thymidine diphosphate has been identified only for the deoxysugars of tylosin, erythromycin, and oleandomycin; hence, Figure 10 shows the generic NDP as the carrier. All deoxysugars are made from the common intermediate 4-keto-6-deoxyglucose, which is itself made in two steps from glucose-1-phosphate. Genes believed to determine the enzymes for these steps have been found in all of the macrolide biosynthesis gene clusters examined except erythromycin, which uses the enzymes involved in the synthesis of the deoxysugars of the cell wall.[184]

6.3. Post-Polyketide Modification

Following their synthesis, the aglycones are modified through glycosylation, oxidation, reduction, and acylation. The deoxysugars also may be modified. Each macrolide has an order sequence of reactions to assemble the final compound, but it is often the case that various steps may be substituted or bypassed.

6.3.1. Erythromycin and Megalomicin

Pathways for the formation of erythromycin and megalomicin from the aglycone 6-dEB in *S. erythraea*

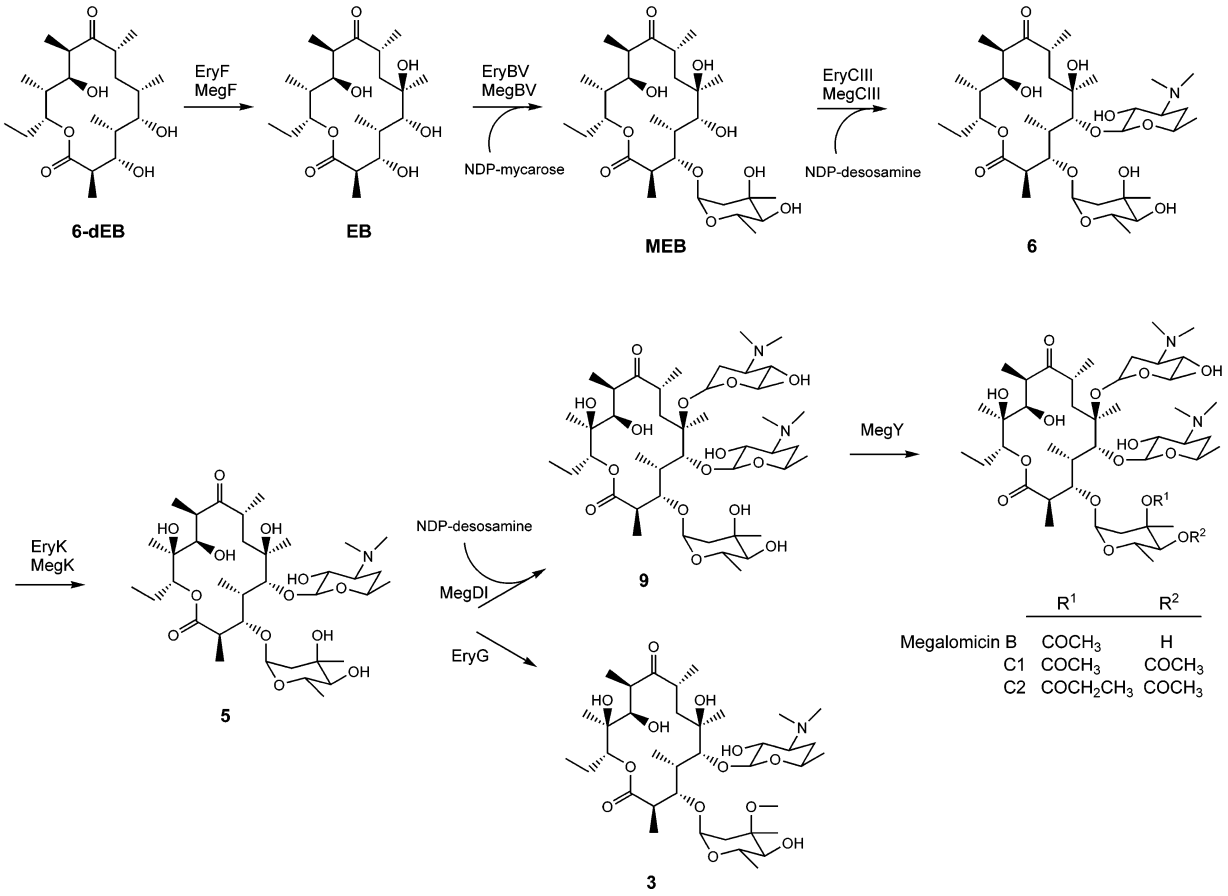


Figure 11. Biochemical pathways of erythromycin and megalomicin biosyntheses. Proposed enzymes for given steps are shown.

and *Micromonospora megalomicea,* respectively, are shown in Figure 11.[177] The erythromycin pathway was determined from the identification of compounds produced in mutants blocked in different steps of the pathway.[185] The aglycone is hydroxylated at C-6 by the product of the *eryF* or *megF* gene to produce erythronolide B (EB), which is then glycosylated at the C-3 OH with NDP-L-mycarose to produce 3-α-mycarosyl EB (MEB) by the mycarosyltransferases EryBV or MegBV. MEB is glycosylated at the C-5 OH with NDP-desosamine by the desosaminyltransferases EryCIII or MegCIII to yield erythromycin D.[186] In *S. erythraea* the 6-hydroxylation step can be bypassed in strains defective in EryF and 6-deoxyerythromycin is formed.[187] Hydroxylation of erythromycin D at C12 by EryK or MegK produces erythromycin C, the last common intermediate in the pathways of erythromycin A and megolamicin. In *S. erythraea* the 3″-OH of the mycarosyl residue is methylated by EryG, converting the residue to L-cladinose and the compound to erythromycin A. EryG-mediated methylation of erythromycin D can also take place to produce the side product erythromycin, but this compound is only poorly converted to erythromycin A by EryK.[188−190] In *M. megalomicea* erythromycin C is glycosylated at the C-6 OH with NDP-megosamine by MegDI to produce megalomicin A. The 3‴- and 4‴-OH groups of the mycarose residue can be acylated with acetate or propionate in various combinations to produce megalomicins B, C1, and C2. Acylations are thought to be catalyzed by MegY.[177] The cytochrome P450 hydroxylase EryF has been crystallized.[191,192]

6.3.2. Methymycin and Pikromycin

The pathways from 10-deoxymethynolide and narbonolide to methymycin and pikromycin, respectively, are shown in Figure 12. Each is converted to its final product in two steps: glycosylation at C-5 or C-3 with desosamine catalyzed by DesII followed by hydroxylation by PikC (also called PicK) to produce the final compound.[174,193−195] It should be noted that PikC utilizes both YC-17 and narbomycin, two different size macrolides as substrates, and produces two different products from YC-17.[196]

6.3.3. Tylosin

The pathway for the formation of tylosin is shown in Figure 13 and has been formulated from identification of the compounds produced in mutants blocked at various steps.[113] Unlike erythromycin, glycosylation at C-5 precedes the first oxidation step that produces the C20 aldehyde. This is followed by a second oxidation to add the hydroxyl at C-23 for subsequent glycosylation. The next step is the addition of D-allose to the 23-OH to produce the diglycoside, followed by addition of L-mycarose to the mycaminose moiety. Glycosylation of OMT by D-allose can be bypassed in *tylD*, *tylJ*, or *tylN* mutants to produce the compound desmycinosyltylosin (DMT),

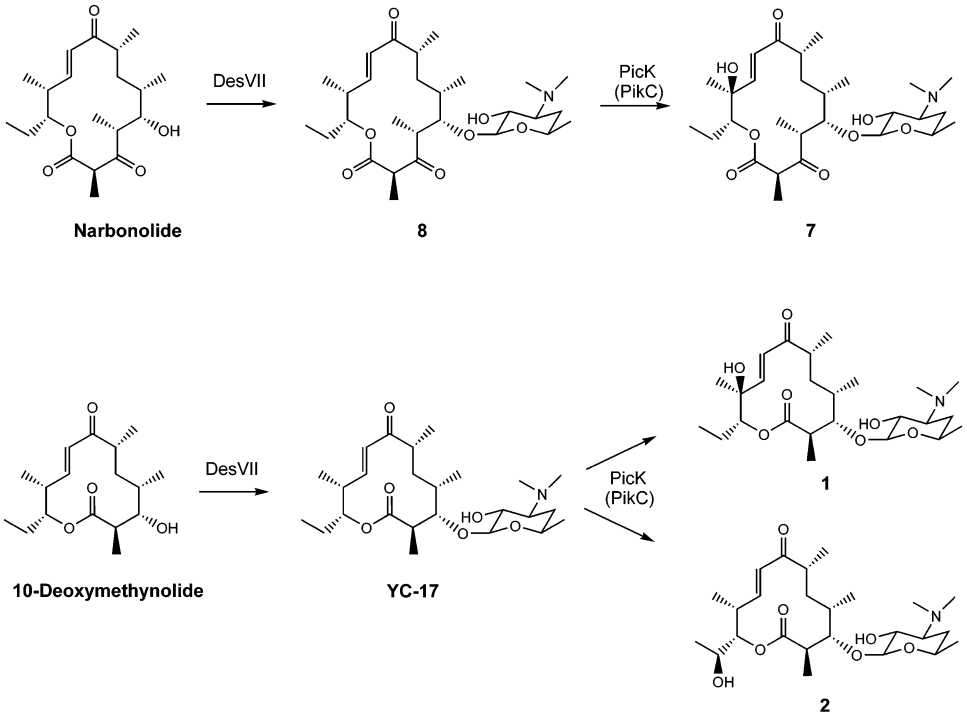


Figure 12. Biochemical pathways of pikromycin and methymycin biosyntheses. Proposed enzymes for given steps are shown.

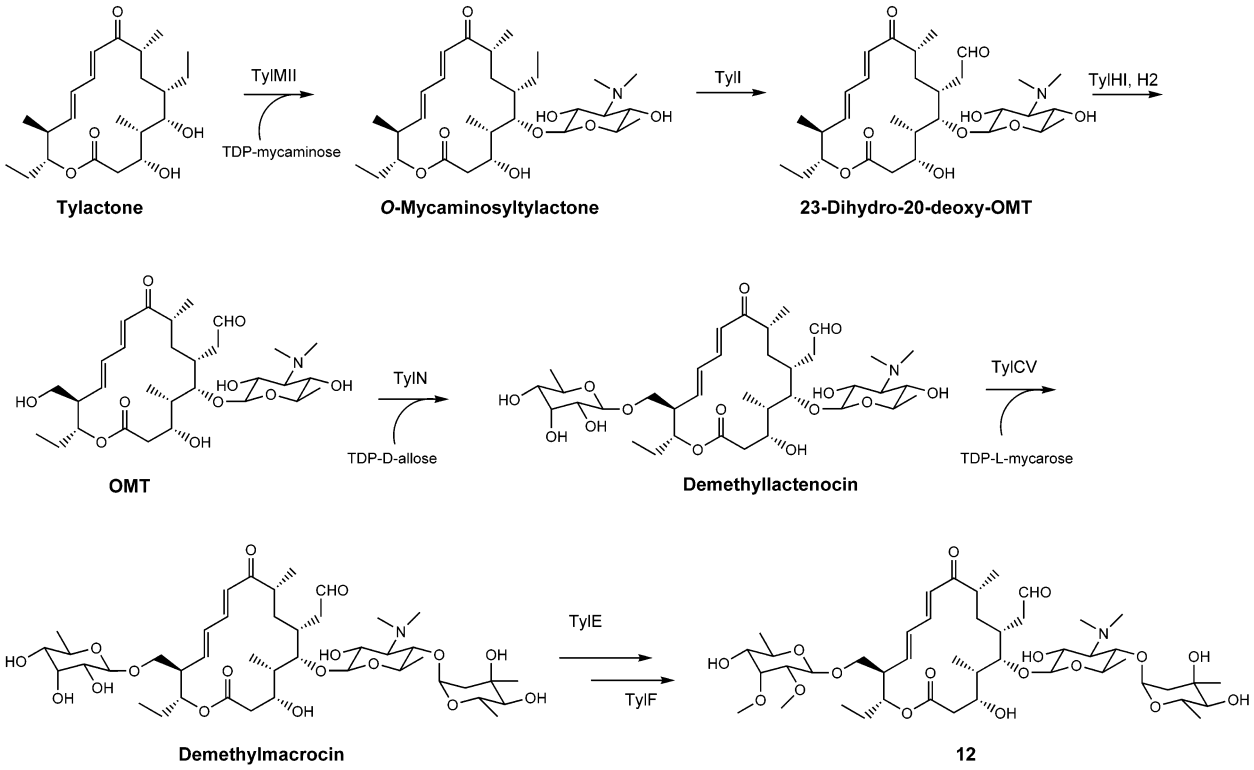


Figure 13. Biochemical pathways of tylosin biosynthesis. Proposed enzymes for given steps are shown.

OMT containing the mycarose residue, as the end product of the pathway.[197,198]

6.3.4. Other Macrolides

The biosynthesis of oleandomycin [**10**] follows a pathway similar to that described for erythromycin.[199−201] Three subsequent post-polyketide modifications take place after biosynthesis of the aglycone in the following order: oxidation mediated by the P450-enzyme OleP to produce the aglycone containing the 8,8a-epoxide, attachment of the neutral sugar L-oleandose at C-3, attachment of desosamine to C-5. Because the biosynthetic products have not been identified, the pathway to lankamycin is less clear. The pathway from the aglycone requires hydroxylations at C8 and C12, glycosylations employing an

NDP-neutral sugar at C3 and NDP-chalcose at C5, and acetylation of the 11- and 4″- hydroxyls. The order of these reactions has not been established. It is not known if the neutral sugar that is attached at C-3 is L-arcanose or L-olivose, which is converted to L-arcanose through 4-*O*-methylation after the sugar(s) is attached to the aglycone, as in the case of erythromycin. Furthermore, as described in section 6.1.2, if the starter for the synthesis of the aglycone is 2-methylbutyryl CoA, hydroxylation of C15 would also be required to complete the synthesis of lankamycin.

The full complement of genes for the biosynthesis of any platenolide-based macrolide has not been reported; thus, little is known about the pathways of synthesis of these compounds beyond the point of the aglycones. In cases where reduction of the C-9 carbonyl takes place, a post-polyketide reductase is thought to be involved. Complete sets of genes for the biosynthesis of chalcomycin and mycinamicin have been reported.[169,202] For chalcomycin, the order of reactions (8-hydroxylation, 12,13-epoxidation, and glycosylations at C-20, following C-20 hydroxylation, and at C-5) has not been established. The order of reactions from the aglycone of mycinamicin to final products is not known, but it has been established that a cytochrome P450 enzyme catalyzes both the 12,13-epoxidation and 14-hydroxylation steps as the final steps in the synthesis of mycinamicins.[203]

6.4. Regulation of Macrolide Biosynthesis

Though macrolides are considered to be secondary metabolites, little is known of how their biosynthesis is controlled to initiate toward the end of the logarithmic phase of growth and to stay on through the stationary phase. Specific regulatory genes that regulate expression of the PKS and other macrolide biosynthesis genes have been identified and studied in the pikromycin and tylosin biosynthesis clusters. The gene *pikD*, present in the *pik* cluster, encodes a DNA binding protein that is required for the expression of the pik PKS and desosamine genes but not for expression of pikC.[204] Inactivation of PikD leads to loss of pikromycin and methymycin production. Tylosin biosynthesis appears to be regulated in a cascade fashion.[205] The gene *tylP* appears to encode a repressor that represses expression of *tyQ*, a transcriptional activator.[206] Repression is relieved by the presence of a yet to be identified γ-butyrolactone, similar to the A factor that regulates production of streptomycin in *Streptomyces griseus*.[207] TylQ is a transcriptional repressor of *tylR*, global regulator required for tylosin biosynthesis, and a transcriptional activator of *tylS*, which encodes a tylosin pathway specific activator and is classified as a member of the SARP (*Streptomyces* antibiotic regulatory proteins) family.[208,209] TylS also appears to regulate *tylR*.[210] In addition, it has been found that intermediates in the pathway beyond tylactone which contain the deoxysugar mycaminose stimulate production of tylactone, but the mechanism of this regulation is not yet understood.[211]

7. New Macrolides and Ketolides

7.1. Chemistry

Erythromycin derivatives wherein the 3-*O*-cladinosyl moiety has been replaced with an acyl functionality, termed "acylides", have been reported.[212] Of particular interest is the 3-*O*-(4-nitrophenyl)acetyl derivative of clarithromycin, TEA0777, which shows potent activity not only against macrolide-susceptible and efflux-resistant *S. pneumoniae*, but also against inducible-MLS_B-resistant *S. aureus* as well. Recent efforts have led to TEA0929 [**32**], which shows good in-vitro activity against macrolide-susceptible and MLS_B-inducible *S. aureus* and *S. pneumoniae* and against *H. influenzae* and also shows in-vivo activity equivalent to clarithromycin.[213] Ketolides bridged across the 6-O and 11-O positions, such as EP-13417 [**33**], have been found to possess high in-vitro and in-vivo activity against typical respiratory pathogens.[214]

7.2. Genetic Engineering

Following the discovery of modular macrolide PKSs, efforts commenced to alter the specificities and activities of the domains for the purpose of changing the structure of the corresponding aglycone. This was enabled by the development of genetic tools for streptomycetes that permitted DNA to be introduced into the macrolide producers and recombination to be selected. Hence, to create desired changes to the structure of aglycones, the following has been accomplished: to reduce the extent of β-carbonyl reduction, KR, DH, or ER domains have been inactivated through mutation (or deletion); to increase the extent of reduction, these domains have been introduced into modules where not present originally; to change the extender unit incorporated into the nascent polyketide chain, AT domains have been exchanged. These exchanges have been performed in the macrolide producers or in hosts into which the PKS genes were introduced, such as *E. coli* or *S. coelicolor*.

Replacement of DEBS AT1 or AT2, AT3, AT5, and AT6 with a malonyl-transferring AT domain in *S. erythraea* or in strains of *S. coelicolor* or *S. lividans* that carried the DEBS genes resulted in the production of the expected erythromycin analogues: AT1−12-desmethylerythromycin B [**34**]; AT2−10-desmethylerythromycin A [**35**] and 10-desmethylerythromycin B, AT3−8-desmethylerythromycin A [**36**]; AT5−4-desmethylerythromycin A [**37**]; AT6−2-desmethylerythromycin A [**38**].[170,215−217] In the AT1 exchange 12-desmethylerythromycin A was not produced, indicating that the EryK hydroxylase could not use 12-desmethylerythromycin D as a substrate, but the EryG methyltransferase utilized the intermediate to some extent (see Figure 11). In the other cases the A congener was found but was not the most predominant form of the product. In addition, **38** was detected after an uncharacterized (and unrecovered) segment of DNA from the oleandomycin producer was introduced into a strain of *S. erythraea* that carried a mutation in the DEBS PKS.[218] Although the basis of the production of **38** has not been determined,

sequencing of the host used for introduction of the DNA revealed an in-frame deletion of AT6 (Katz, L. et al. Unpublished results). It is likely that the incoming DNA carried a malonyl-transferring AT domain that acted *trans* to provide malonyl-AT function to module 6. trans AT domains have recently been discovered in nonmacrolide modular PKSs.[219,220]

Replacement of the AT4 domain of DEBS with an ethylmalonyl-transferring domain resulted in production of 6-desmethyl-6-ethylerythromycin A [**39**], but the host required the addition of a *ccr* gene encoding crotonyl CoA reductase.[221] In the absence of *ccr*, the host containing the exchanged AT domain produced a small amount of erythromycin. Replacement of AT4 in DEBS in *S. erythraea* with a malonyl-transferring AT was done at Biotica Technology, Ltd., and Kosan Biosciences, Inc., with different results. Using the soil isolate NRRL 2338 and a malonyl-transferring AT from the rapamycin PKS, the Biotica group found that the engineered strain produced 6-desmethylerythromycin D [**40**], indicating that both EryK and EryG could not utilize **40** as substrate.[222] The Kosan approach employed introducing two site-specific mutations into DEBS AT4 to alter the specificity of the domain. In the *S. lividans* host carrying the altered DEBS, the expected 6-desmethylerythronolide B was produced.[223] When the same mutations were introduced into an industrially optimized *S. erythraea* host, the strain produced a small amount of 6-desmethyl-6-deoxy-7-hydroxyerythromycin D [**41**].[224] The production of a D congener in the Kosan strain coincides with the findings at Biotica. The finding of a 7-OH group in **40** is difficult to explain. Either the EryF hydroxylase had changed its specificity to hydroxylate the substrate at C7 rather than C6 in the Kosan host or the host contains an adventitious enzyme that hydroxylates 6-dEB at C7 to produce a compound that cannot be hydroxylated by EryF.

Exchanges of the loading module have also been reported. Exchange of the loading AT domain of DEBS with the loading AT domain from the avermectin PKS in *S. erythraea* resulted in the production of 14-methylerythromycin A [**42**] and 14-ethylerythromycin A [**43**] along with their B and D congeners.[225]

Changes were also introduced into the reductive domains of DEBS to produce novel compounds. Two examples of such changes in *S. erythraea* that produced fully elaborated molecules include the inactivation of the ER4 domain to produce $^{\Delta}$6,7-anhydroerythromycin C [**44**] and the replacement of the KR2 domain with a DH4/ER4/KR4 domain from the rapamycin PKS to produce 11-deoxyerythromycin [**45**].[170,226] Multiple changes in DEBS have been done employing DEBS genes that had been engineered to contain unique restriction sites at the edges of the various domains.[217] These compounds were produced in *S. lividans* that carried the modified DEBS genes; hence, the compounds were not elaborated beyond the aglycone.

Hybrid PKSs carrying at least one module of two different PKSs have also been made. The loading domain of the spiramycin PKS (Figure 8) was replaced with the loading domain of the Tyl PKS in a *Streptomyces ambofaciens* host that carried a deletion of the spiramycin sugar biosynthesis genes. The resulting compound was the expected 15-methylplatenolide.[227] DEBS-Pik, DEBS-Ole, and Tyl-Pik PKS hybrids yielding predicted compounds have also been reported.[144,228]

Of the dozen or so fully elaborated novel macrolides produced by PKS genetic engineering, most retained some measure of bioactivity but none showed enhanced potencies over their parent compounds. The only example of an engineered compound that showed improved properties was 6-deoxyerythromycin [**46**], produced by targeted disruption of the *eryF* gene in *S. erythraea*.[187] The compound was less potent than erythromycin in vitro but showed improved in-vivo activity in experimental infections due to enhanced acid stability.[187]

The most promising new molecules originate from a combination of genetics, chemistry, and fermentation development. Jacobsen et al. demonstrated that an *S. coelicolor* strain carrying DEBS that contained a C173A replacement (KS1null) could be fed SNAC diketides in which the C5 methyl group could be replaced with a number of substitutions (Figure 14: [**47**]) including H atoms and phenyl rings to produce 6-dEB analogues that contained the corresponding substitutions at C13 [**48**].[117,148,149,229] These novel aglycones could be converted into erythromycin analogues [**49**] after purification and feeding to an S. *erythraea* strain carrying a mutation in the one of the DEBS genes (e.g., KS1 null host). This technology was employed by Kosan in collaboration with J & J Pharmaceutical Research Institute to produce a number of novel 6-*O*-arylalkyl ketolides [**50**−**55**]. Preliminary studies reported that a number of these compounds displayed in-vitro and in-vivo activities comparable to telithromycin or cethromycin.[230,231]

8. Conclusions

The advancements in the isolation and crystallization of ribosomes have allowed a fuller understanding of how macrolides and ketolides exert their antibiotic effects. Whereas it was formerly thought that these compounds block a specific event during the initiation or elongation cycle of protein synthesis, it is currently believed that their binding in the exit tunnel is sufficient to prevent elongation of the nascent polypeptide chain. It is not yet known if the efficacy of a compound is directly related to its strength of binding. The ketolides, which bind to domains V and II of 23S rRNA and so may bind more tightly to ribosomes, may become preferred as antibiotics over macrolides, which only bind in domain V. The current limitations of telithromycin, the only currently approved ketolide, is its modest activity against *H. influenzae*, prompting the need for administration of 800 mg/day and the lack of efficacy against MLS_B-resistant *S. pyogenes* and constitutive MLS_B-resistant *S. aureus*. Ribosome binding studies have shed light on the basis of macrolide resistance, but they do not as yet enable an understanding of why, in cases of

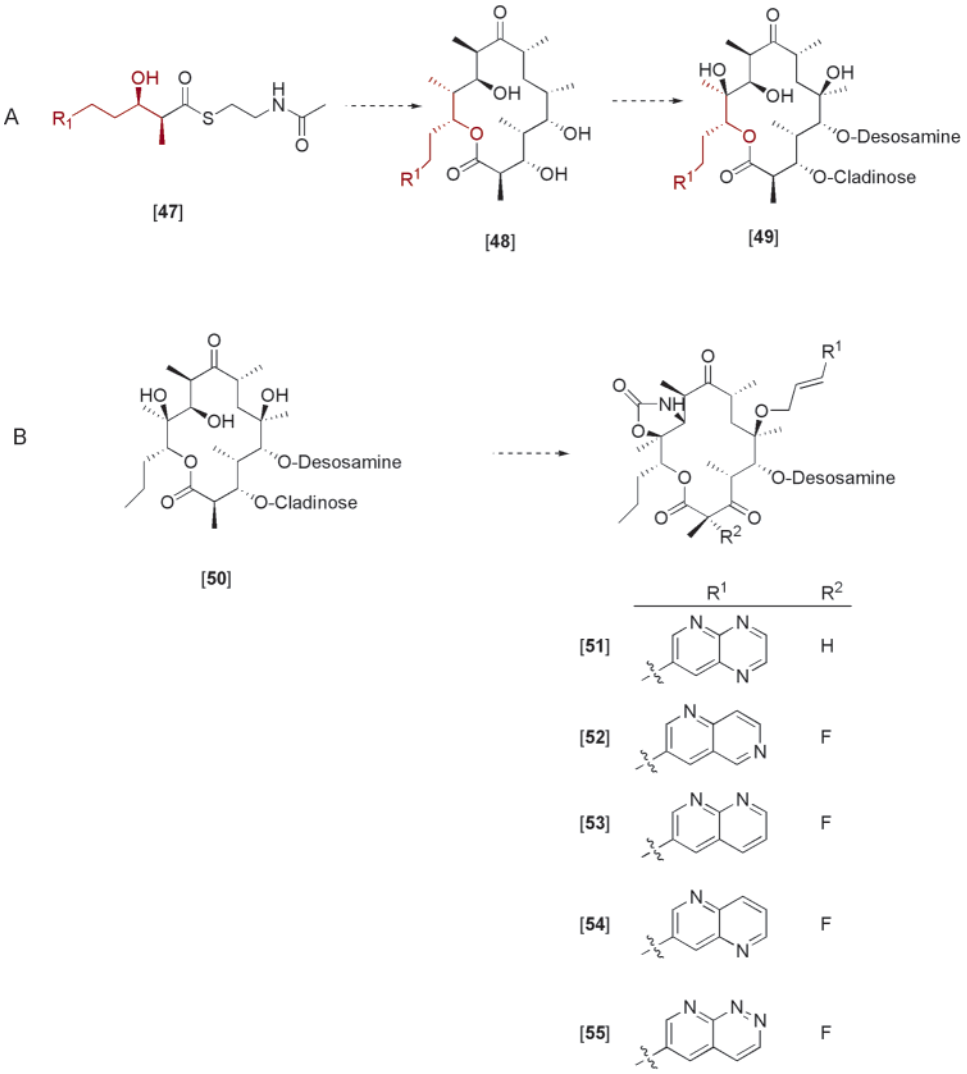


Figure 14. Schemes showing production of 15-R erythromycin analogues. (A) Pathway to produce 15-R erythromycin. **47** is fed to *S. coelicolor* DEBS (KS1null) to produce **48**, which is fed to *S. erythraea* KS1null to produce **49**. (B) Production of 15-methyl ketolides. **50** is produced using scheme A and converted to **51**−**55** as described in the text.

macrolide-resistant strains, the ketolides are effective as antibiotics against some but not effective against others. The contribution of the effect on 50S ribosome assembly by macrolides to the overall bacteriostatic or bactericidal activities of these molecules also requires further clarification. The ability to manipulate PKSs provided great promise initially that novel macrolides could be made, including ones that could not be obtained by conventional chemical modification, and which would contain enhanced properties. Other than the small number of compounds made by the feeding of short-chain thioesters to a genetically engineered host, as only a first step in a three-part process, the few fully elaborated novel macrolides produced by genetic engineering have not yet fulfilled the original promise. It is still too early to tell whether this avenue of discovery will prove effective. The findings that many of engineered PKSs either do not produce the expected compounds or do so at levels too low to be useful indicate that greater understanding of the biochemical details of polyketide biosynthesis is required before full exploitation of their chemical potential can be realized.

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