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Decoding and Engineering Tetracycline Biosynthesis

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Abstract

Tetracyclines have been important agents in combating infectious disease since their discovery in the mid-twentieth century. Following widespread use, tetracycline resistance mechanisms emerged and continue to create a need for new derivatives that are active against resistant bacterial strains. Semisynthesis has led to second and third generation tetracycline derivatives with enhanced antibiotic activity and pharmacological properties. Recent advancement in understanding of the tetracycline biosynthetic pathway may open the door to broaden the range of tetracycline derivatives and afford analogs that are difficult to access by synthetic chemistry.

Introduction

Tetracyclines are members of the polyketide family of natural products and include a number of important pharmaceutical compounds. This group is characterized by their tetracyclic ring structure, the richly substituted A ring, and a heavily oxidized periphery that includes a C11, C12 and C11a keto-enol configuration. The latter feature allows tetracycline to chelate divalent cations and bind to the 30S ribosome subunit (Brodersen et al., 2000; Chopra and Roberts, 2001). Several well-known tetracyclines, including both naturally produced and semisynthetically derived, are shown in Figure 1. Chlorotetracycline was the first of these compounds to be discovered. It was first reported by Benjamin Duggar in 1948 as an isolate from *Streptomyces aureofaciens* and was named aureomycin due to its yellow hue (Duggar, 1948). The discovery of oxytetracycline, produced by *Streptomyces rimosus*, followed shortly thereafter by A.C. Findlay in 1950 (Finlay et al., 1950). The importance of tetracyclines was immediately recognized due to their effectiveness against both gram-negative and gram-positive pathogens, and low toxicity in animals. Tetracyclines were the first major class of therapeutics to earn this distinction of “broad-spectrum antibiotic” (Chopra et al., 1992). This contributed to their widespread production and use in both human and animal medicine in the decades following their discovery. As a result, this subsequently led to emergence of resistance mechanisms and decreased effectiveness of tetracyclines as front line antibiotics.(Levy et al., 1999; Levy et al., 1989). Currently there are 40 known tetracycline resistance genes(Roberts, 2005).

The severity of bacterial resistance created an urgent need to develop new tetracycline derivatives capable of evading these resistance mechanisms. To achieve this end, there was a large push in the period around 1970–1980 to create semisynthetic variants of naturally occurring therapeutics. This period was termed by some as the “golden age of antibiotic medicinal chemistry”, where naturally occurring scaffolds were modified to achieve compounds with improved pharmacological properties (Wright, 2007). These semisynthetic variants represent the second generation tetracyclines. The most clinically valuable of these are minocycline and doxycycline, which are produced from 7-chloro-6-demethyltetracycline

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and oxytetracycline, respectively(Martell and Boothe, 1967). These analogs are more lipophilic than their parent compounds, which resulted in more efficient uptake into the cell. This feature was key in overcoming efflux resistance mechanism by lowering the net efflux from the cell (McMurry et al., 1982). In contrast, tetracycline and minocycline were both ineffective when exposed to resistance determinants of the ribosomal protection class (Clermont et al., 1997).

After a long gap in new tetracycline development, the glycylcyclines or so called third generation tetracycline analogs were developed recently. The sole successful member, tigecycline, was approved by the FDA in 2005 (Agwuh and MacGowan, 2006; Sum and Petersen, 1999; Sum et al., 2006). These compounds have modifications at position C-9 on the minocycline scaffold, which lead to evasion of both efflux and ribosomal protection mechanisms of resistance (Bergeron et al., 1996), making it a viable choice against tetracycline resistant infections. Another third generation derivative currently in Phase II clinical trials is PTK-0796 from Paratek Pharmaceuticals (Butler, 2008). This compound also contains a C-9 substitution and is a broad-spectrum antibiotic, including activities against multi-drug resistant MRSA and vancomycin resistant enterococci. The development of these compounds demonstrates that the tetracycline scaffold remains a valuable starting point for further drug discovery.

In addition to the well-documented antibacterial properties of tetracyclines, some tetracycline derivatives have also been investigated for anticancer properties. Antimicrobial compounds such as oxytetracycline and doxycycline were found to also have an inhibitory effect toward human matrix metalloproteinases(Gu et al., 2001; Lokeshwar et al., 2001). These enzymes are involved in the degradation of the extracellular matrix and have implications in inflammatory disorders and cancer. Doxycycline hyclate is being used as a treatment for periodontis under the brand name Periostat® as it inhibits the enzymes that break down gum tissue (Golub et al., 1998). A family of chemically modified tetracyclines (CMTs) has been developed that lack the C-4 dimethylamino group from tetracycline and doxycycline, and thus lack antibiotic activity, but have improved activity against matrix metalloproteins.

Therefore, the tetracycline family has yielded a large number of biosynthetically and chemically modified variants, several of which have found uses as valuable pharmaceutical compounds. A large majority of these variants are semisynthetic derivatives. Total synthesis has been achieved for oxytetracycline in 1979 (Muxfeldt et al., 1979). More recently the Myers group has developed a facile method for synthesizing tetracycline and related pentacycline compounds (Charest et al., 2005). However, biosynthetic/fermentative approaches remain the most cost effective methods for large-scale production of tetracyclines (Khosla and Tang, 2005). Additionally, with increased knowledge of tetracycline gene clusters and biosynthetic pathways, engineered biosynthesis has emerged as a new tool to generate analogs that may be inaccessible using synthetic chemistry alone. Investigation of other clinically relevant type II polyketide biosynthetic pathways has led to a number of novel bioactive compounds (Lombó et al., 2006; Madduri et al., 1998; Méndez and Salas, 2001). The clinical importance of tetracyclines and their range of biological activity provide powerful motivation to utilize metabolic engineering towards the goal of developing superior tetracycline analogs.

Tetracycline biosynthesis

Rational engineering of secondary metabolites begins with a thorough understanding of the biosynthetic pathway responsible for their production. Since the initial discovery of oxytetracycline and chlorotetracycline, researchers have manipulated the tetracycline

producers using the tools available to elucidate their biosynthetic origins. Much of the early knowledge about the biosynthetic pathway was investigated by McCormick et al in the 1960s through a series of blocked mutants and substrate feeding experiments (McCormick and Jensen, 1965; McCormick et al., 1968; McCormick et al., 1963a; McCormick et al., 1962; McCormick et al., 1963b). They identified several key intermediates, including 6-methylpretetramid and anhydrotetracycline (ATC), and were able to deduce valuable information about the sequence of events leading to the production of tetracycline even though the genes themselves were not known. For example, substrate feeding experiments using mutants blocked in early tetracycline biosynthesis showed that feeding of pretetramid and 6-methylpretetramid could restore tetracycline biosynthesis, however, feeding of C4-dimethylamino-pretetramid to these substrates did not restore tetracycline biosynthesis. This indicated that the dimethylaminonaphthacenes were not intermediates in the pathway and several tailoring steps stood between the pretetramid precursor and the addition of the dimethylamino group. (McCormick et al., 1963a). In another experiment with labeled substrate feeding they showed that 5a, 6-ATC can be converted to tetracycline, demonstrating that this bioconversion takes place at a late stage in the synthesis. The different substrate 5a, 6-anhydro-12a-deoxytetracycline however, was converted to 12a-deoxytetracycline, demonstrating that the 12a-hydroxylation is a key modification step that occurs prior to the C-4 amidation(McCormick et al., 1962). The next fundamental insight into tetracycline biosynthesis was accomplished in a feeding experiment using ^{13}C-labeled acetate. Thomas and Williams verified the polyketide origin of the tetracycline backbone and supported the idea of a malonamyl-CoA as the origin of the C-2 amide (Thomas and Williams, 1983a; Thomas and Williams, 1983b).

The first heterologous expression of genes required for tetracycline biosynthesis was carried out by Binnie et al. This work demonstrated that all of the genes required for oxytetracycline synthesis were located within a *S. rimosus* chromosomal DNA fragment flanked by *otrA* and *otrB* (Binnie et al., 1989). This was in contrast to previous gene mapping experiments that suggested oxytetracycline biosynthetic genes reside on two separate regions on the *S. rimosus* genome (Rhodes et al., 1981). The Butler group also located the gene *otcC (or oxyS*) responsible for ATC oxygenase activity using a reverse genetics approach, and made the first attempt to rationally engineer improvements in oxytetracycline production through manipulation of gene copy number(Binnie et al., 1989).

Each of these efforts made great strides in understanding the tetracycline biosynthetic pathway. The next big leap in understanding was enabled by sequencing of the gene clusters associated with chlorotetracycline (Ryan, 1999) and oxytetracycline biosynthesis (Hunter, 2002; Kim et al., 1994; Zhang et al., 2006a). The respective gene clusters are shown here for comparison in Figure 2. The annotation for the oxytetracycline cluster reflects that of Zhang et al. as published in 2006 (Zhang et al., 2006a). As the chlorotetracycline gene cluster has not been fully annotated, genes are identified here by their role in the biosynthetic pathway. As expected, there is a large degree of similarity between the two clusters, and all enzymes shown so far to be essential to oxytetracycline biosynthesis have homologs in the chlorotetracycline gene cluster. Several additional genes are included in the chlorotetracycline cluster, including those encoding additional regulatory proteins and the C-7 halogenase. We will focus on the oxytetracycline biosynthetic pathway in this review.

The function of several genes in the oxytetracycline pathway has been shown through genetic knockouts in *S. rimosus* (Peric-Concha et al., 2005; Petkovic et al., 1999) or reconstitution in heterologous *Streptomyces* strains. The most comprehensive study of gene function has been a systematic reconstitution of the oxytetracycline biosynthetic pathway in a heterologous host carried out by Tang and coworkers (Zhang et al., 2006a; Zhang et al., 2008; Zhang et al., 2006b; Zhang et al., 2007a; Zhang et al., 2007b). By using *Streptomyces*

coelicolor strain CH999 (McDaniel et al., 1993), the minimal set of genes required to produce key intermediates in the pathway such as the amidated decaketide backbone, 6-methylpretetramid and ATC have been determined and are shown in Figure 3.

As with all type II polyketide biosynthesis, the oxytetracycline pathway contains a minimal PKS consisting of ketosynthase (KS), chain length factor (CLF), and acyl-carrier protein (ACP)(Hertweck, 2007). In the oxytetracycline biosynthetic gene cluster, these proteins have been named OxyA, OxyB and OxyC, respectively(Zhang et al., 2006a). Together, these enzymes are responsible for the iterative condensation of malonyl-CoA to yield the poly-β-ketone backbone of tetracycline. Initiation of polyketide synthesis is proposed to involve an amide containing starter unit, mostly likely in the form of malonamyl-CoA or malonamyl-ACP. The malonamyl starter unit is reflected in the tetracycline compounds as the C-2 amide. Evidence supporting this proposal initially came from labeled substrate feeding experiments(Thomas and Williams, 1983b). Later observations of shunt products containing the amide group further supported the notion of an amide starter unit (Peric-Concha et al., 2005; Petkovic et al., 1999). Recently, Tang and coworkers showed that OxyD, which is an amidotransferase homolog, was responsible for the incorporation of this amide unit and termed the set of genes OxyA through OxyD the “extended minimal PKS”. They showed that these set of four enzymes expressed together was sufficient to produce an amidated decaketide polyketide backbone, which spontaneously cyclizes via C11-C16 regioselectivity to yield WJ85 (Zhang et al., 2006b). When OxyJ, the C-9 ketoreductase, was added to the extended minimal PKS, a C-9 reduced amidated polyketide WJ35 was biosynthesized (Zhang et al., 2006a). The isoquinolone part of WJ35 is formed through the spontaneous cyclization between carbons C13–C18. Neither WJ35 nor WJ85 exhibits the C-7 to C-12 connectivity of the D-ring of oxytetracycline, indicating the need for additional enzymes to direct the regioselectivity of the cyclization. Although these two compounds confirmed the role of OxyD, it remains unknown the exact reaction catalyzed by OxyD, as well as the exact starter unit. These insights will require in vitro biochemical investigation of OxyD properties.

Sequential cyclization of the tetracyclic scaffold is the next important step in tetracycline biosynthesis. These reactions are typically performed by dedicated cyclases (Hertweck, 2007). Surprisingly, only two cyclases, OxyK and OxyN, are required to cyclize all four rings during tetracycline biosynthesis, as shown by Zhang et al through heterologous reconstitution in CH999 (Zhang et al., 2007a). OxyK was first identified by Petkovic et al. (Petkovic et al., 1999) and confirmed by Zhang et al.(Zhang et al., 2007b) as the enzyme responsible for D-ring cyclization through C-7/C-12 connectivity. Expression of OxyABCDJKN resulted in the cyclization of all four rings to yield pretetramid, which was oxidized in vivo to yield WJ83. When OxyD was removed from the construct, the major product was the tricyclic anthracene carboxylic acid and was not cyclized in the fourth ring. This indicated an important role for the amide starter unit in directing the spontaneous cyclization of ring A. Subsequently, addition of the C-methyltransferase OxyF to OxyABCDJKN yielded the key intermediate 6-methylpretetramid, confirming the sequence of these early biosynthetic steps.

Recently, the late stage tailoring steps leading to the production of anhydrotetracycline (ATC) have been elucidated. Again using the heterologous host/vector pair, it was shown that starting from 6-methylpretetramid, the enzymes OxyL, OxyQ and OxyT catalyze the C-12a hydroxylation, C-4 amidation, and C-4 *N*,*N*-dimethylation steps, respectively, to afford ATC (Zhang et al., 2008). An in vitro reaction of OxyL with 6-methylpretetramid as a substrate showed that this single enzyme is responsible for the double hydroxylation (C12a and C4) of ring A, resulting in the unstable product 4-keto-ATC. Addition of OxyQ to the host producing 4-keto-ATC (OxyABCDJKNFL) led to the reductive amination of C-4 and

synthesis of 4-amino-ATC, confirming the function of OxyQ. In vitro addition of OxyT to 4-amino-ATC resulted in a *N*, *N*-dimethylation modification as confirmed by the isolation of ATC. Finally, in vivo expression of the entire construct OxyABCDJKNFLQT proved this to be the minimal set of enzymes required to produce ATC in heterologous host CH999 (Zhang et al., 2008).

The final steps converting ATC to oxytetracycline require two oxygenations and a dehydration step. OxyS (OtcC) has been shown to be responsible for the oxygenation of ATC (Binnie et al., 1989). In vitro studies of the homologous gene in chlorotetracycline biosynthesis showed that purified enzyme could convert ATC to 5a,11a-dehydrotetracycline (DHTC) (Binnie et al., 1989; Vancurová et al., 1988). A second oxygenation reaction is needed in the oxytetracycline pathway to add the C-5 hydroxyl group resulting in 5a, 11a-dehydrooxytetracycline(DHOTC). The enzyme responsible for this specific tailoring step is unknown. The final reduction step converting 5a, 11a-DHOTC to oxytetracycline is still not entirely clear. Nakono et al. reported a tetracycline dehydrogenase gene from *S. aureofaciens* external to the CTC gene cluster was capable of performing this last step (Nakano et al., 2004). This was shown through the identification of a mutant deficient in the reduction step, and subsequent restoration of function by integrating TchA into the host genome. A homologous ORF was identified in the *S. rimosus* genome, but its function has not been verified. The most up to date oxytetracycline biosynthetic pathway is shown in Figure 3. A number of steps in the pathway are unresolved and further genetic and biochemical studies are needed to complete our understanding of tetracycline biosynthesis.

Manipulation of Oxytetracycline Pathway

Throughout the recent studies of the tetracycline pathways, several new compounds/shunt products have been generated and the substrate specificities of several enzymes in the pathway have been examined. The first such study was the expression of the oxytetracycline minimal PKS in heterologous host CH999. OxyABC were expressed along with the *act* C-9 ketoreductase and resulted in an acetate primed decaketide product previously identified as RM20b (Fu et al., 1994). This was significant as it demonstrated the relaxed starter unit specificity of oxytetracycline minimal PKS, as well as its functional interaction with a heterologous tailoring enzyme *act* KR (Fu et al., 1994). This prompted Zhang et al. to inquire if the amidotransferase OxyD would be able to introduce an amidated starter unit to heterologous minimal PKSs to generate amidated polyketides of different sizes. When OxyD, OxyC and *act* KR were coexpressed with the *act* KS-CLF and the *tcm* KS-CLF in engineered strain CH999, however, no amidated polyketides were produced (Zhang et al., 2006a).

The Hunter group constructed *S. rimosus* knockouts strains lacking *oxyK* (Petkovic et al., 1999) and *oxyS* (Peric-Concha et al., 2005). Surprisingly, both strains produced new polyketides with truncated carbon backbones. Although these results revealed the functions of OxyK and OxyS, they also demonstrated the inherent unpredictability of genetic manipulation of the oxytetracycline gene cluster in the native host. Several new products and shunt products (see previous section) were also formed through the systematic reconstitution of oxytetracycline pathway enzymes by the Tang group. Though many are interesting for their novel cyclization and structure features, the most useful from a metabolic engineering standpoint are the intermediates that can be used as building blocks for the generation of tetracycline analogs. These include 6-methylpretetramid, 4-amino-ATC, and ATC. 6-desmethyl-ATC was also produced through rational engineering of a heterologous construct lacking the *oxyF* gene (Zhang et al., 2008). While compounds lacking the C-6 methyl were previously produced by blocked-mutant studies, this is the first rational production of 6-desmethyl-ATC in a heterologous host. This also confirmed the

relaxed specificity of the downstream tailoring enzymes toward this position. The ability to reliably produce intermediates and variants in the tetracycline pathway will undoubtedly lead to engineered biosynthesis of other tetracycline derived compounds.

In addition to producing building blocks for tetracycline biosynthesis, the unique elements of the oxytetracycline biosynthetic pathway have also been exploited for the production of other chemical moieties. In a 2007 paper, Zhang and coworkers reported the utilization of OxyD as a new mechanism for benzopyrone formation (Zhang et al., 2007b). This mechanism involves the displacement of the amide starter unit by the nucleophilic phenol group, which is in contrast to the nucleophilic attack of the free amide on a ketone carbonyl. Once this mechanism was identified, they set out to rationally biosynthesize an analog of HMG-CoA reductase inhibitor pannorin via an amidated backbone. This is an example of how genes unique to the tetracycline pathway can serve as enzymatic tools towards creating other classes of compounds.

Metabolic Engineering of Tetracycline Biosynthesis

As the naturally occurring tetracyclines have been of such great commercial importance over the past 60 years, there had been much effort dedicated to strain improvement and titer optimization. However, most of the strain improvement was performed in industrial settings, and little has been made available to the scientific community. Nearly all strain improvement efforts rely on mutagenesis techniques, which is a black-box approach that provided few, if any genotypic information. Studies on the optimization of the use of NTG for mutagenesis of *S. rimosus* were detailed by Delić et al in 1970 (Delic et al., 1970). The randomly introduced mutations can be beneficial in a number of ways, not only through increased production but also give rise to strain phenotypes that display improved fermentation properties. In one instance, genetic recombination was used to produce a strain that resulted in no foaming in the fermentation medium, as well as increased oxytetracycline production (Mindlin et al., 1961). In several studies phage resistant mutants have been found which can be of great benefit in industrial fermentation (Vesligaj et al., 1981).

Some of the naturally occurring variation in oxytetracycline production levels has been attributed to the genetic instability of the oxytetracycline biosynthetic cluster. Genetic instability is commonly observed among *Streptomyces* species and in the case of the oxytetracycline gene cluster, is likely due in part to its location on the end of the linear chromosome of *S. rimosus*. The gene cluster is located approximately 600 kb from one end of the chromosome (Petkovic et al., 2006). These instability variants were classified into three groups based on oxytetracycline self-resistance (Gravius et al., 1993). Class I (99%) showed unchanged resistance from the parent strain, but differed in a wide variety of morphologies including oxytetracycline production. Class II (1%) showed increased sensitivity to oxytetracycline, and class III (.1%) showed increased resistance to oxytetracycline and higher titer of oxytetracycline. Class II mutants were shown to have deletion of the oxytetracycline cluster through a fusion of two inverted copies of the chromosome both lacking the end with the antibiotic biosynthetic genes. Class III mutants, on the other hand resulted from increased copies of the oxytetracycline biosynthetic genes. Though class III mutants themselves can then revert to class I variants, this illustrates the possibility of using amplified DNA sequences to increase tetracycline production (Petkovic et al., 2006). The Butler group made the first attempt to use a gene dosing approach to increasing oxytetracycline production in *S. rimosus* (Butler et al., 1990). Here they constructed a low copy plasmid containing the oxytetracycline biosynthetic pathway between *otrA* and *otrB*. Transforming this plasmid into strains with lower productivity than the donor strain resulted in increased production of oxytetracycline in the low producing strains, however, production in excess of the parent strain was not achieved.

Other approaches have focused on genes external to the tetracycline biosynthetic pathway. One example is the overexpression of the alpha-amylase gene in *S. rimosus*. This was motivated by the observation that high alpha-amylase activity was observed during oxytetracycline production(Yang and Wang, 1999). They report an increase of amylase activity of up to two-fold and an increase of oxytetracycline production of up to 2.5 fold as a result of overexpression of this gene (Cheng et al., 2000). The underlying biochemical reasons for this increase remain unknown.

Another interesting strategy for increased antibiotic production is the ribosomal engineering technique outlined by Ochi (Ochi, 2007). In this method, the rRNA is the target for mutation rather than the pathway genes themselves. One mutation in the S12 rRNA resulted in preferential transcription of secondary metabolite regulation genes such as ActII-ORF4 and RedD in *S. coelicolor*. These SARP (Streptomyces Antibiotic Regulatory Protein) genes are directly involved in the up-regulation of specific biosynthetic genes cluster and are the terminal activators in the regulatory signaling cascade(Bibb, 2005). This concept was demonstrated in a recent example by Chen and coworkers. The production of another type II polyketide, fredericamycin, was increased by a factor of 5.6 in the wild-type host by overexpression of the SARP FdmR1(Chen et al., 2008). Both the oxytetracycline and chlorotetracycline pathways include SARP-like regulatory proteins. Their respective influences on tetracycline production and regulation have yet to be investigated.

Other Tetracycline Natural Products

There are other tetracycline natural products, which have been more recently identified, with very interesting structural features and bioactivity (Figure 4). Though they have not yet achieved the clinical and commercial success of the classic tetracyclines discussed here, a detailed understanding of their biosynthetic pathways may add to the enzyme toolbox that one can use to construct and diversify new tetracycline molecules. SF2575, identified by Hatsu and coworkers, is an intriguing tetracycline compound containing a salicylic acid substitution at C4 and a C-deoxysugar at C-8(Hatsu et al., 1992a;Hatsu et al., 1992b). The core of the molecule is also methylated at numerous hydroxyl positions. While this compound showed antibiotic activity only against gram-positive bacteria, it was demonstrated to have potent antitumor activity against P388 leukemia cells in mice (Hatsu et al., 1992b). Around the same time, another group reported isolation of dactylocyclines, from *Dactylosporangium Sp*. SC14051(Tymiak et al., 1993). In addition to their novel structure and glycosylation patterns, they showed antibiotic activity against tetracycline resistant gram-positive bacteria. Cervimycin C is also an aromatic polyketide antibiotic with a tetracycline-like core, and also contains extensive decoration and modification by deoxysugars. It was reported to be very effective against vancomycin-resistant *enterococci* and has a different mode of action from that of traditional tetracycline antibiotics, making it an attractive antibiotic target (Herold et al., 2004). The Hertwerk group has begun biosynthetic investigation of this compound, starting with the origin of the rare dimethylmalonyl group (Herold et al., 2004). Finally, the structurally related compound viridicatumtoxin is produced by fungal species *Penicillium viridicatum* (Raju et al., 1982). While it is structurally similar to the tetracycline family, the polyketide backbone exhibits a distinct folding mechanism (type F) compared to the *Streptomyces* tetracyclines (type S) (Thomas, 2001). Further genetic studies with these gene clusters would provide a fascinating comparison to known tetracycline pathways.

Concluding Remarks

The unique structural features of tetracyclines provide exciting opportunities for synthetic chemists and biomolecular engineers alike. The recent knowledge gained from sequencing

of the tetracycline producing gene clusters has afforded the biosynthetic blueprints to these pathways. At the same time, the systematic investigation and rational engineering of the oxytetracycline biosynthesis have provided the first glimpse into the combinatorial potential of these polyketide pathways. Metabolic engineering and engineered biosynthesis, combined with chemical synthesis, will play valuable roles in the discovery and production of future generations of tetracyclines.

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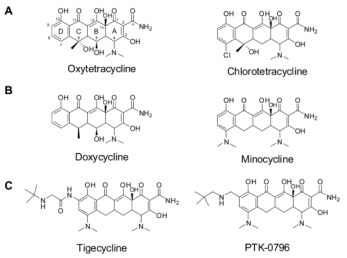

Figure 1.
A) Natural product tetracyclines; B) Second-generation semisynthetic tetracycline antibiotics; C) Third generation tetracycline antibiotics based on minocycline.

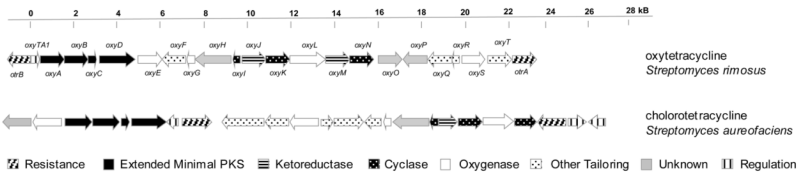

Figure 2.
Organization of biosynthetic gene cluster responsibly for the synthesis of oxytetracycline (top) and chlorotetracycline (bottom).

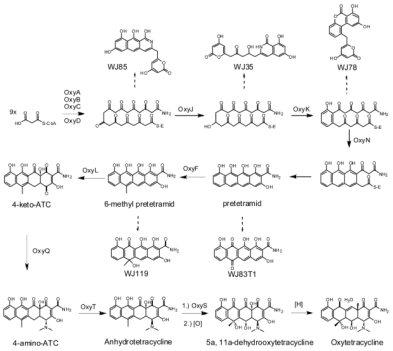

Figure 3.
Oxytetracycline biosynthetic pathway. Dashed arrows indicate shunt products formed at each step in the absence of the remaining enzymes.

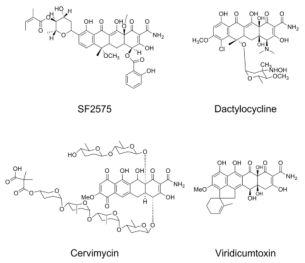

Figure 4.
Additional natural products containing the tetracycline scaffold.